Nucleic Acids Structure and Gene Expression

1

European Molecular Biology Network: EMBnet

Reinhard Doelz, Biocomputing, Biozentrum der Universitaet,
CH 4056 Basel , Klingelberstrasse 70
eMail: embnet@comp.bioz.unibas.ch

Sequence databases in Biology are growing exponentially. Besides the need of large sites keeping the data, the number of customers is permanently increasing. EMBnet takes care of this data distribution. Access to the Swiss EMBnet Node and its data are provided.
Network access plays a key role in utilizing remote resources. However, both synchronous and asynchronous access require tools which are currently non-standard in molecular biology computing. Research is needed to optimize the methods. HASSLE (Hierarchical Access System for Sequence Libraries in Europe) is the first implementation of a user-transparent access tool in molecular biology. It features tools for both clients and information providers to permit accounting and/or prioritization on various levels. HASSLE focuses on the network aspect of the molecular biology computing and assumes that it is possible to have database applications available as remote "services" (programs , program packages or utilities) which can be started by a simple command script after a suitable feed of datafiles.

2

Cloning of the *C. elegans* telomeres

Ch. Wicky, H. Tobler and F. Müller, Institute of Zoology, University of Fribourg, Pérolles, CH-1700 Fribourg

Telomeres are specialized structures conferring stability to chromosomes by protecting them from degradation, recombination and by allowing complete replication of the ends. In the nematode *C. elegans*, they are among the few missing parts of the almost complete physical map, which is one of the major reasons for cloning and analyzing them. In a first step, by doing *Bal* 31 digestion experiments, we have shown that about 4 to 9 kb of the simple repeated sequence TTAGGC are located at the end of the *C. elegans* chromosomes. This sequence, also present at the *A. lumbricoides* telomeres, shows similarities to the telomeric sequences found in all organisms analyzed so far, including some lower eukaryotes, plants and vertebrates.

The TTAGGC sequences are not only located at the end of the *C. elegans* chromosomes, but also at internal chromosomal sites. Different cloning approaches (e.g. YAC deficiency cloning) failed to discriminate between telomeres and internally repeated TTAGGC sequences. To solve this problem, we have constructed a λ Zap library from specially prepared chromosomal long *C. elegans* DNA, which yielded several positive clones.

3

STRUCTURE OF SUPERCOILED DNA IN VITRO AND IN VIVO. TIGHT SUPERCOILING: A POSSIBLE NOVEL FUNCTIONAL STATE OF DNA.

Bednar, J., Furrer, P., Stasiak, A. and **Dubochet, J.,** Laboratoire d'Analyse Ultrastructurale, Université de Lausanne, CH-1015 Lausanne-Dorigny.

A cryo-electron microscopy approach to study the shape of supercoiled DNA molecules freely suspended in vitrified buffer was combined with Monte Carlo simulations. We investigated the influence of the neutralization of intersegmental electrostatic repulsion on the writhe of supercoiled DNA molecules. We provide experimental evidence that the decrease of the effective diameter of DNA causes a higher fraction of the linking number deficit to be absorbed by the writhe. We show that under ionic conditions typical for in vitro reactions with DNA, supercoiled DNA adopts a so called tight interwound structure. In this structure the two opposing segments of interwound superhelix are in direct longitudinal contact with each other. It is likely that under in vivo conditions where the charge of DNA is greatly neutralized, supercoiled DNA also adopts this tight form. We propose that properties of the tight form (direct longitudinal contacts of two DNA helices) can have important consequences for cis acting regulatory mechanisms like transcriptional and recombinational enhancement.

4

Imaging of RAP1-DNA complexes by TEM and STM

Müller T.¶, Gilson E.§, Gasser S.§, Sogo J ¶ and Gross H.¶
¶Inst. Cell Biol., ETHZ, CH-8093 Zürich; §ISREC, CH-1066 Epalinges

The Repressor-Activator Protein (RAP1) of *Saccharomyces cerevisiae* is involved in both the activation and repression of numerous genes involved in mating type control, glycolysis and translation. In addition, RAP1 binds in a sequence specific manner to the repetitive sequence found at the ends of yeast telomeres. It has been shown biochemically that RAP1 appears to bend DNA and creates a permanganate-sensitive distortion in the double helix. Electron microscopic analysis confirms that RAP1 binds to DNA at its consensus sequence. Because of the disadvantages of low-angle shadowing ("self shadowing"), analysis of Protein-DNA interactions by electron microscopy is limited mainly to length measurements. We therefore have begun to use freeze-dried, Pt/Ir/C-coated RAP1-DNA complexes for analysis by STM. The protein-DNA complex is clearly visualized, and the proposed RAP1-dependent bend in the DNA structure could be confirmed.

5

EARLY BIOCHEMICAL CHANGES ASSOCIATED WITH ADAPTIVE RESPONSE

Kleczkowska, H.E. and Althaus, F.R.
University of Zürich - Tierspital, Institute of Pharmacology and Toxicology, Winterthurerstrasse 260, CH-8057 Zürich

Low doses of radiation or genotoxic chemicals reduce cytogenetic damage in cells upon subsequent treatment with larger doses of these agents, a phenomenon known as the adaptive response. We were able to detect distinct biochemical changes in quiescent human keratinocytes (HaCaT cell line) adapted to a very low concentration of the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). One hour adaptation of the cells with 2.5 nM MNNG decreased the levels of constitutive DNA strand breaks, NAD and poly(ADP-ribose). Subsequent challenging of HaCaT cells with 2.5 μM MNNG reduced the amount of DNA strand breaks and NAD levels, and increased poly(ADP-ribose) levels, as compared to cells exposed to the challenging treatment alone. The response was dependent on the length of the challenge treatment and was abolished by 2 mM benzamide (an inhibitor of poly(ADP-ribosyl)ation). This is the first demonstration of early biochemical events accompanying the adaptive response of human cells to an alkylating agent.

6

INDUCTION AND REMOVAL OF CROSSLINKS IN rRNA GENES IN CELLS FROM PATIENTS WITH FANCONI'S ANEMIA

Rey, J.P., Scott, R., Müller, Hj., Humangenetic, Departement Research Kantonsspital, CH-4031 Basel

Fanconi's anemia (FA) is an autosomal recessive disease associated with chromosomal instability and a predisposition to cancer. A defect in DNA repair appears to be involved, however, studies concerning the ability of FA cells to repair DNA damage have led to conflicting results. To study DNA repair, we treated control and FA cells in culture with mitomycin C (MMC) which results in DNA-interstrand crosslinks that can be quantified before and after repair. Quantification was performed at the level of the actively transcribed rRNA genes using a denaturation-renaturation gel electrophoresis procedure. It was apparent for all cell lines studied that MMC-crosslinks were induced rapidly and that their level remained unchanged after 12 hours, indicating that MMC lesions are more stable in the genome than previously reported. After 48 hours, repair efficiencies varied greatly in the two control cell lines suggesting heterogeneity in crosslink repair of rRNA genes. In two FA cell lines, crosslink repair was not detectable over 48 hours supporting the idea that FA cells have an impaired DNA-crosslink repair mechanism.

7

EFFECT OF DNA DAMAGE ON THE NUMBERS AND SIZE DISTRIBUTIONS OF ADP-RIBOSE POLYMERS IN VIVO.

Malanga, M. and Althaus, F.R.
University of Zürich - Tierspital, Institute of Pharmacology and Toxicology, Winterthurerstrasse 260, CH-8057 Zürich.

Poly(ADP-ribosyl)ation is a common cellular response to DNA damage resulting from a variety of genotoxic agents. We have analyzed the consequences of cell exposure to different DNA damaging compounds on poly(ADP-ribose) metabolism in cultured human keratinocytes, namely, N-methyl-N'-nitro-N-nitrosoguanidine and hydrogen peroxide. Their effect on the cellular poly (ADP-ribosyl)ation system was very similar, although the two carcinogens differ in several aspects, including the kind of damage they inflict on DNA, the kinetics of DNA damage and repair and the combination of enzymes involved in the repair process. In both cases, a transient increase in poly(ADP-ribose) content was observed, accompanied by a decrease in NAD levels. The newly-synthesized ADP-ribose polymers exhibited a continuous size distribution ranging from short to very long and branched molecules. This polymer pattern was highly conserved and independent of 1) the number of DNA strand breaks, 2) the type of DNA lesions, and 3) the extent of stimulation of poly(ADP-ribose) metabolism. Thus, a specific, highly conserved size distribution of ADP-ribose polymers seems to be an intrinsic property of the mechanism by which the poly(ADP-ribosyl)ation system operates, and this is independent of the nature or magnitude of the triggering stimulus.

8

CORRECTION OF THE DNA REPAIR DEFECT IN CELLS FROM XERODERMA PIGMENTOSUM COMPLEMENTATION GROUP G

Scherly, D.[1], Nouspikel, T.[1], Corlet, J.[1], Bairoch, A.[2] and Clarkson, S.G.[1],
Départements de Génétique et Microbiologie[1], et Biochimie Médicale[2],
Centre Médical Universitaire, CH-1211 Genève 4.

Xeroderma pigmentosum is a rare autosomal recessive disorder characterized by hypersensitivity to ultraviolet light and predisposition to skin cancer. Seven genetic complementation groups (XP-A to XP-G) are thought to be due to mutations in distinct genes from the nucleotide excision repair pathway. We have isolated frog and human cDNAs that encode proteins resembling RAD2, a yeast protein involved in this pathway. The human protein expressed from an EBV-based vector is able to reduce to normal levels the UV sensitivity of lymphoblastoid cells from XP group G but not C. The ~ 4 kb XPGC mRNA is present in normal amounts in XP-G cells, suggesting that the defect may be due to a small deletion, insertion or point mutation in a normally active allele.

9

DNA DAMAGE RECOGNITION: A POSSIBLE FUNCTION FOR THE YEAST RAD3 HELICASE DURING NUCLEOTIDE EXCISION REPAIR (NER)

Naegeli, H., Bardwell, L. and Friedberg, E.C., Department of Pathology, The University of Texas Southwestern Medical Center at Dallas, Dallas Tx 75235

NER is a genetically and biochemically complex DNA repair pathway which processes a wide diversity of DNA lesions. In *S. cerevisiae RAD3* is one of at least six genes that are required for early events in NER, including damage recognition and damage-specific incision of DNA. The *RAD3* gene (and presumably its human homolog *ERCC2*) encodes a DNA-dependent ATPase and DNA helicase. We have demonstrated that the Rad3 DNA helicase activity is profoundly inhibited by DNA damage caused by UV radiation, by small or bulky DNA adducts, and by sites of base loss. In all cases the inhibition of helicase activity is strictly DNA strand-specific. The Rad3 helicase is not inhibited by drugs that bind to DNA non-covalently, suggesting that Rad3 protein is exclusively sensitive to the chemistry of DNA. Using filter binding and competition assays we have also shown that Rad3 protein forms stable complexes with damaged DNA presumably at sites of covalent base lesions. Based on these results we propose that the DNA helicase activity of Rad3 protein may serve to strand-specifically target sites of base damage for further processing by the NER machinery.

10

Genetic mobility of the chloroplast ribosomal intron of *Chlamydomonas reinhardtii* requires the intron-encoded DNA endonuclease I-*Cre*I

Dürrenberger,F., Thompson,A.J.*, Herrin D.L.* and Rochaix,J.-D.
Depts de Biologie Moléculaire et de Biologie Végétale, Univérsité de Genève, Genève. *University of Texas, Austin, USA.

The group I intron located within the chloroplast 23S ribosomal RNA genes of the green unicellular algae *Chlamydomonas reinhardtii* has been shown to have the ability to move to an intronless version of the 23S rRNA gene (23S cDNA). This intron encodes a DNA endonuclease (I-*Cre*I) which specifically recognizes and cleaves the 23S cDNA close to the intron-insertion site. Deletion of the I-*Cre*I gene from the chloroplast DNA showed that the I-*Cre*I endonuclease is required for the *in vivo* mobility of this intron. The I-*Cre*I$^-$ strain was crossed to a wild-type strain such that the modified chloroplast DNA (I-*Cre*I$^-$, 23S cDNA) was inherited by all the progeny. Shortly after fusion of the cells, double-stranded DNA breaks were detected *in vivo*, close to the intron-insertion site. Analysis of the progeny of these crosses revealed that the ribosomal intron invades with high frequency (~70%) the intronless allele of the 23S rRNA gene, thus demonstrating the genetic mobility of this intron.

11

REGULATION OF V(D)J RECOMBINATION BY PROTEIN KINASES AND TRANSCRIPTION FACTORS

Döbbeling, U., Hobi, R., Berchtold, M.W. and Kuenzle, C.C.
Institute of Veterinary Biochemistry, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich.

Rearrangement of immunoglobulin genes by V(D)J recombination normally occurs only in pre B and pre T-cells, but experimentally also in cells which have been stably transfected with the recombination activating genes rag-1 and rag-2. Using the transfected fibroblast cell line L4 we show that V(D)J recombination is stimulated by the protein kinase A (PKA) pathway and the transcription factor Oct2A. PKA probably exerts its effect on V(D)J recombination through the transcription factor CREB, as CREB A133S a mutant which cannot transactivate but binds well to DNA blocks efficiently the stimulation of V(D)J recombination by the cotransfected catalytic subunit of PKA.
V(D)J recombination is repressed by TPA an activator of protein kinase C. This effect is probably mediated by the transcription factors AP-1 and jun B. Thapsigargin that increases the intracellular Ca 2+ concentration also reduces the rate of V(D)J recombination. TPA and thapsigargin overcome the stimulating effect of the PKA activators Br-cAMP and caffeine, but not that of Oct 2A.

12

DISRUPTION OF A PHOTOSYNTHETIC NUCLEAR ENCODED GENE IN *CHLAMYDOMONAS REINHARDTII*

FARAH J.& ROCHAIX J.-D.
University of Geneva, Department of molecular and plant biology, 30 quai E. Ansermet 1211, Genève 4

In plants, the nuclear *PsaF* gene encodes the PSI-F subunit of the photosystem I (PSI) complex. This subunit is located on the luminal side of the thylakoids and although its precise role is not known, it is generally accepted that it functions as a docking protein for plastocyanin, an electron carrier protein. The *PsaF* gene has been cloned from the green alga *C. reinhardtii* and we have devised a strategy to disrupt its resident counterpart by using a mutated version of it. Briefly, a mutated copy of the cloned *PsaF* gene was cut by a restriction endonuclease to create a small gap in the middle of the coding sequence. The gene was then cotransformed along with the *ASL* gene (conferring arginine prototrophy) in the double mutant alga strain *cw15* (cell wall less) ; *arg7A* (arg. auxotroph), by vortexing the cells in the presence of glass beads and DNA. Disrupted *PsaF* mutants were detected by their characteristic PSI$^-$ fluorescence phenotype. We are currently analysing several such transformed mutants for the targetted disruption of *PsaF* at the DNA level.

13

BINDING OF IHF TO SMALL REPETITIVE ELEMENTS OF THE E.COLI GENOME

Boccard, F. and Prentki, P., Département de Biologie Moléculaire, Université de Genève, CH-1211 Genève 4

The Integration Host Factor (IHF) of *E.coli* is a small histone-like protein which bends the DNA sharply upon binding to its specific sites. Most *ihf* sites found so far in the *E.coli* chromosome were located in promoter regions. We have recently observed that *ihf* sites can also be found at the 3' end of a large number of *E.coli* genes. A particularly interesting class of such sites lies within "REP" elements, which are small, abundant, highly conserved repetitive sequences usually found at the 3' end of genes. We are now characterizing the functional significance of this interaction.

14

CLONING OF MOUSE DNA POLYMERASE δ (pol δ)

G. Cullmann, M.W. Berchtold, and U. Hübscher. Institute of Pharmacology and Biochemistry, University of Zürich, Winterthurer Strasse 190, CH-8057 Zürich, Switzerland

Cloning and sequencing of the yeast *S.cerevisiae* pol δ (A. Boulet et al. EMBO J. 8:1849 (1989)) allowed the definition of homology boxes that are present only in herpesviral DNA polymerases and *S.cerevisiae* pol δ (δ-like boxes). In order to clone a mammalian pol δ from a mouse cDNA library we performed PCR using one primer corresponding to one of the δ-like boxes and another one corresponding to the DNA polymerase homology box I highly conserved in the α-type polymerases. The resulting PCR product had the expected size of 1300bp. A subclone of the PCR product was used to screen the cDNA library. The missing 3' and 5' ends were amplified by PCR using internal pol δ primers and an oligo dT primer and a primer located on the cDNA vector, respectively. The open reading frame is 1105 amino acids long and the calculated molecular weight is 124 kDa. The amino acid sequence identity to *S.cerevisiae* pol δ is about 49%. Homologies to the meanwhile known sequences of *S.pombe* pol δ (Pignede G. et al., J.Mol.Biol. 222: 209 (1991)) and *P.falciparum* pol δ (Ridley G.R. et al., NAR 19(24): 6731 (1991)) are in the same range. Amino acid sequence identity to the also sequenced pol δ's of man (Chung D.W. et al., PNAS 88: 11197 (1991); Yang C.-L. et al., NAR 20(4): 735 (1992))and calf (Zhang J. et al., Biochemistry 30: 11742 (1991)) is about 90%. A polyclonal antibody induced with a synthetic peptide of 30 amino acids derived from the mouse sequence and highly homologous to calf and man reacts specifically with calf thymus pol δ. The pol δ gene is a single copy gene in mouse. A mouse cDNA-probe was used to map the human pol δ-gene to chromosome 19q13-14. Further characterization is in progress.

15

DETECTION OF SLIPPAGE DNA REPLICATION IN A EUCARYOTIC MODEL SYSTEM

Martin Hug; Sandro Rusconi; *Institut für Molekularbiologie II der Universität, UZ Irchel, Winterthurerstrasse 190, 8057 Zürich, Switzerland*

Monotonous aminoacid repeats can have a strong influence on the activity of the host protein and are often encoded by triplet nucleotides whose number is subject to marked polymorphism. Slippage during DNA replication has been proposed among the mechanisms which could explain the remarkable incidence of length polymorphism. Slippage replication of repeated motifs has been documented so far only *in vitro* or in bacterial systems. In order to assess the extent of such phenomena in a eucaryotic system we have constructed plasmids containing different triplet nucleotide repeats of progressive length along with an SV40 origin of replication. In a typical assay, plasmids are transfected into HeLa cells together with an SV40 T-antigen expression vector. After two days low MW DNA is harvested and digested with DpnI to eliminate unreplicated material. The digested DNA is transformed into competent bacteria which can be grown in a pool. The DNA prepared from the transformants serves either for another transfection cycle or is analyzed by endlabelling of a fragment spanning the repeated motif. The endlabelled fragment is analyzed in high resolution gels. The results suggest that slippage replication occurs in both eucaryotically and procaryotically replicating systems although the extent may be different, and that the phenomenon is dependent on both sequence composition and length as reported with *in vitro* systems. We speculate that slippage replication mechanisms may play an important role important during cell ageing.

16

DNA unwinding activity of replication protein A

Anthi Georgaki, Bettina Strack, Vladimir Podust and Ulrich Hübscher
Department of Pharmacology and Biochemistry, University of Zürich
Winterthurerstrasse 190, CH-8057 Zürich, Switzerland.

Replication protein A (RP-A) is a heterotrimeric single-stranded DNA-binding protein involved in cellular DNA replication and DNA repair. Calf thymus RP-A was purified to apparent homogeneity by a four- column procedure. We give evidence that DNA unwinding can be carried out by RP-A and that this unwinding is independent of magnesium and ATP, two essential cofactors for *bona fide* DNA helicase activity. RP-A can unwind up to at least 350 basepairs and appears to be required in stoichiometric amounts. Four different monoclonal antibodies against RP-A (αSSB70A, -70B, -70C, and -34A, Kenny et al., JBC 265, 7693, 1990) were tested for interference with DNA unwinding activity. The 70C antibody blocked unwinding by RP-A suggestive that this activity is located on the large subunit. Since the 70 kDa subunit interact with the initiator protein SV40 Tantigen as well as with the initiating DNA polymerase α, this activity might have a possible unwinding function in initiation of DNA replication.

17

The activity of the G1 specific DSC-1 like transcription factor of *S. pombe* is cell cycle regulated.

Alexandre Reymond and Viesturs Simanis, Unité de Recherches sur le cycle cellulaire, ISREC, 1066 Epalinges.

In the yeasts *S. pombe* and *S. cerevisiae*, a number of genes required for progression through S-phase are regulated at the level of transcription, peaking in late G1 phase. Their promoters contain one or more MCB related sequences (MluI Cell Cycle Box) which bind a factor DSC-1 (DNA Synthesis Control). A homologous factor (DSC1-like) has also been identified in the distantly related yeast *S.pombe*. The product of the start gene *cdc10* is a component of this factor in *S. pombe*. In *S. cerevisiae* the level of activity of the DSC1 factor during the cell cycle has been subject of contradictory reports. We present results showing that the *S. pombe* DSC1-like factor binds to the promoter of the *cdc22* gene coding for the large subunit of ribonucleotide reductase in a cell cycle dependent manner. The binding activity disappears as a cell exits from M phase and reappears in late G1 just before S-phase, as cells progress through the START control and become committed to the mitotic cell cycle with respect to other fates. Interestingly the gene coding for ribonucleotide reductase is regulated at its expression level in many eukaryotes and may be a rate limiting step for entering into S-phase. It is not yet clear if a DSC1-like activity is present in all those organisms, but budding and fission yeasts are widely divergent evolutionarily so that systems conserved in both organisms are frequently widespread in nature. We will also present results showing which part of the $p85^{cdc10}$ protein are important for the DSC1-like activity.

18

Replication fork proteins: calf thymus DNA polymerases and auxiliary proteins and their possible roles.

Vladimir Podust, Anthi Georgaki and Ulrich Hübscher
Department of Pharmacology and Biochemistry, University Zürich-Irchel, CH-8057 Zürich, Switzerland

DNA polymerases (pol) α, β, δ, ε and the three auxiliary proteins proliferating cell nuclear antigen (PCNA), replication protein A (RP-A) and replication factor C (RF-C) were purified to apparent homogeneity from calf thymus. RF-C could form a specific complex on a 3'-OH primer terminus in the presence of PCNA and ATP. This complex made the pol δ and ε to holoenzyme forms, which were able to replicate efficiently RP-A (or SSB)-covered singly-DNA primed M13 DNA. Holoenzyme complexes of pol δ and pol ε could be isolated by gel filtration suggesting that both pol's interact specifically with the auxiliary proteins. While pol δ holoenzyme was more efficient in elongation of singly-primed M13 DNA, pol ε holoenzyme could utilize better the DNA synthesized by pol α-primase on ss-DNA. The activities of four pol's were tested on a gapped substrate produced by hybrydization of two oligonucleotides to ss-DNA template leaving the gap of an Okazaki fragment size (230 nt). Pol's α, β, ε and pol ε holoenzyme completely filled the gap, while pol δ holoenzyme synthesized the DNA chains up to 30 nucleotides longer than the gap size suggesting strand displacement synthesis. Based on our data we favour the hypothesis that pol ε is the second lagging strand polymerase.

19

Properties of replication proteins encoded by the geminivirus Tomato Golden Mosaic Virus

Pia Thömmes and Ken W. Buck,
Dept. Biology, Imperial College, Prince Consort Road, London SW7 2BB

Tomato Golden Mosaic Virus (TGMV) is a member of the geminiviruses, which contain a bipartite single-stranded DNA genome. Of the two DNA components A and B only the former is required for virus replication in single cells. DNA A comprises four open reading frames, which are transcribed clockwise (AR1) or anticlockwise (AL1, 2 and 3).While AR1 encodes the coat protein of the virus, it has been shown, that AL1 is essential for virus replication and AL2 is needed for the production of single-stranded DNA. To study the functional properties of the proteins encoded by AL1, 2 and 3 we have cloned the genes in the expression vector pGEMEX and overexpressed the proteins in *E. coli*. AL1 was purified to apparent homogeneity as a polypeptide of 40 kD, which during the preparation can be degraded to 27 kD. By using the purified AL1 protein we can show by various different techniques that it binds specifically to the 200 bp region of the presumed origin of replication of the TGMV DNA. This binding is stronger to single-stranded than to double-stranded DNA. By *in vitro* transcription and translation experiments we are able to show that AL1, 2 and 3 polypeptides can be derived as separate translation products from a single mRNA.

20

A LOCUS THAT REGULATES COPY NUMBER IN THE REPLICATION ORIGIN OF PLASMID PSC101.

Danielle MANEN, Guixian XIA, Lucien CARO. Dept. Biologie moléculaire, Sciences II, Université de Genève, CH-1211 Genève 4

The pSC101 origin encodes a protein essential for replication, RepA, and contains a cluster of directly repeated sequences RS1, RS2, and RS3 and two palindromes, IR2 and IR1, partially homologous to the direct repeats and overlapping the *repA* promoter. We found that RepA binds to directly repeated sequences and initiate replication in monomeric form, but that it binds to inversely repeated sequences and autoregulates its own transcription in dimeric form (Manen *et al.* PNAS **89**: 8923-8927, 1992). Here, we study in more details the influence on the regulation of the plasmid copy number of a region which comprises the IR1 inverted repeated sequences and straddles the -35 region of the *repA* promoter. We show that the integrity of IR1 greatly influences pSC101 copy number. IR1 is separated from the nearest repeated sequence, RS3, by approximately four turns of the DNA helix. Copy number is preserved if this distance is increased by one whole turn but not if it is increased by a fraction of a turn. These results suggest interactions between RepA dimers binding at IR1 and RepA monomers binding at the RS sequences in the initiation of replication.

21

CRYO-ELECTRON MICROSCOPY; A METHOD OF CHOICE TO STUDY THE 3-D ORGANIZATION OF CHROMATIN FIBERS.

Dubochet, J., Bednar, J., Furrer, P., Stasiak, A., Laboratoire d'Analyse Ultrastructurale, Université de Lausanne, CH-1015 Lausanne-Dorigny, **Woodcock, C.,** Dept. Biology, Univ. of Massachusetts, Amherst, MA, USA. **Paduwat, N.** and **Beard, P.** ISREC, CH-1066 Epalinges.

Using cryo-electron microscopy we studied the structure of chromatin freely suspended in a thin layer of vitrified solution. We utilized purified minichromosomes of SV40 in low salt buffers for the observations. Under these conditions there are no internucleosomal interactions and the path of DNA between the nucleosomal cores can be followed throughout the circular minichromosome. In this structure the DNA path is uniquely determined by wrapping around the nucleosome cores and by the length and number of helical turns of the linkers. When linkers are approximated as straight segments the angle between consecutive linkers should be uniquely determined by the primary structure of the nucleosome core or chromatosome, while the torsion angle for any three consecutive linkers is determined by the twist of the middle linker. By comparing the observed images with computer simulations of chromatin structure in which the effect of variable linker length and linker entry/exit angle are incorporated we are able to test and verify various models of the arrangement of nucleosomes and linker DNA in the folding of chromatin.

22

FINE STRUCTURAL MORPHOMETRICAL AND CYTOCHEMICAL ANALYSES OF THE CELL NUCLEUS DURING HIBERNATION

Malatesta, M.°,², Zancanaro, C.°,³, Vogel, P.* and Fakan, S.°. °Center of Electron Microscopy, and *Institute of Zoology and Animal Ecology, University of Lausanne, ²Institute of Histology and Laboratory Analyses, University of Urbino, ³Institute of Human Anatomy and Histology,University of Verona

Modifications of the cell nucleus during rapid and intensive changes in cell activity of the brown adipose tissue (BAT) and liver from Muscardinus avellanarius were studied. Morphometric analyses of nuclear and nucleoplasmic surface as well as of perichromatin granule frequency, carried out on ultrathin sections, indicated significant tendency for all parameters to rank according to the sequence hibernating > arousing > euthermic. Moreover, ultrastructural cytochemistry allowed us to describe and characterize unusual nuclear structures such as coiled body-like constituents, amorphous bodies and bundles of nucleoplasmic fibrils in hibernating animals. Finally, a series of specific antibodies recognizing different nuclear proteins and ribonucleoproteins were used as probes, in order to better approach possible functional role of the above structural constituents.

23

CRYOFIXATION AND CRYOSUBSTITUTION OPEN NEW PATHWAYS INTO THE INVESTIGATION OF THE MORPHO-FUNCTIONAL ASPECTS OF THE CELL NUCLEUS

von Schack, M.L. and Fakan, S., Centre de Microscopie Electronique de l'Université, CH-1005 Lausanne

Cryofixation followed by freeze-substitution in an organic solvent on biological materials has been used as an alternative to conventional chemical fixation for ultrastructural studies. Mouse cell culture line and liver tissue were cryofixed without the use of any cryoprotectant by impact freezing onto a cooled copper mirror, or frozen under high pressure. They were then substituted in pure acetone without any chemical fixative and embedded in different acrylic or epoxy resins. Fine structural studies on the cell nuclei comparing different cryofixation and embedding techniques showed a nicely preserved morphology similar to conventionally prepared material but sometimes with more detail regarding e.g. the nuclear membrane or nuclear RNP constituents. For larger tissue samples, high pressure freezing gave better structural preservation than slam freezing. Considering that no chemical fixatives were used, these results provide excellent morphology and are very promising for further cytochemical and immunocytochemical applications.

24

ADP-RIBOSE POLYMER BINDING TO PROTEINS ON NITROCELLULOSE P. L. Panzeter, B. Zweifel, M. Malanga, S. H. Waser, M.C. Richard, and F. R. Althaus *University of Zürich, Institute of Pharmacology and Biochemistry, CH-8057 Zürich*

Polymers of ADP-ribose are formed in the nuclei of eukaryotes in response to DNA damage. These polymers are covalently attached to specific proteins and can be >200 ADP-ribose residues in size. We have recently discovered that the polymers themselves noncovalently alter histone function by providing polynucleotidic binding sites as an alternative to DNA. Since these polymers structurally represent polynucleotides in nuclei, noncovalent interactions with non-histone proteins may also be possible. To rapidly screen nuclear lysates for poly(ADP-ribose)-binding proteins, we have modified the Western blot technique to test for polymer binding to proteins transferred to nitrocellulose. A comparison of rat liver, keratinocyte, and yeast nuclear lysates and a *Xenopus* egg extract revealed that histones from all species bound to ADP-ribose polymers. In addition, other polymer-binding proteins were detected in rat liver and keratinocyte lysates which have yet to be identified. We also used this technique to determine which histone domains are responsible for histone binding to poly(ADP-ribose). Interestingly, only the respective histone regions which would be accessible for macromolecular interactions in a chromatosomal configuration showed significant affinity for polymers of ADP-ribose, thus suggesting a role for poly(ADP-ribosyl)ation in chromatin relaxation during DNA repair.

25

Stimulation of Topoisomerase II and Restriction Enzyme Cleavage of Chromatin by Distamycin: A Model for Chromatin Opening

Emmanuel Käs, Leonora Poljak, Yasuhisa Adachi and Ulrich Laemmli
Department of Molecular Biology, University of Geneva
30, Quai Ernest-Ansermet, CH-1211 - Geneva 4, Switzerland

Histone H1 preferentially and cooperatively binds scaffold-associated regions (SARs) *in vitro* via specific interactions with the numerous short A+T-rich tracts (A-tracts) contained in these sequences. Selective titration of A-tracts by the oligopeptide distamycin abolishes this interaction and results in a redistribution of H1. Similarly, treatment of intact cells and isolated nuclei with distamycin specifically enhances cleavage of inter-nucleosomal linkers of SARs by topoisomerase II and restriction enzymes. The increased accessibility of these linkers is thought to result from the unfolding (or opening) of the chromatin fiber and to be due to a reduced occupancy by histone H1. Chromatin extraction and H1 assembly experiments support this view. We discuss a model whereby open, H1-depleted chromatin regions may be generated by titration of A-tracts by putative distamycin analogues ("D-proteins"); this local opening may spread to adjacent regions assuming highly cooperative H1-H1 interactions in chromatin. Several properties of a candidate D-protein are described in the accompanying poster (K. Zhao, E. Käs and U.K. Laemmli).

26

Expression of histone H1 in yeast *Saccharomyces cerevisiae*

Caroline Linder und Fritz Thoma. Institut für Zellbiologie, ETH-Hönggerberg, CH-8093 Zürich.

In order to test the structural and functional properties of histone H1 *in vivo*, the sea urchin histone H1 was expressed from the inducible GAL1 promoter in a 2μ vector (strain YCL7). H1 copurifyies with crude NP-40 washed nuclei in roughly stoichiometric amounts compared with core histones. Fractionation of soluble chromatin on sucrose gradients showed that fractions containing DNA and core histones also contained H1. No soluble H1 was found. We conclude that H1 was bound to chromatin. However, no obvious change in the nucleosome spacing was observed.
Expression of H1 inhibits growth of YCL7 on plates and in liquid media. Furthermore, incorporation of ^{3}H-uracil into RNA was reduced, suggesting that H1 might repress transcription by binding to chromatin.

27

CHROMOSOME INACTIVATION BY A ZINC-FINGER PROTEIN

F.Cléard, F.Pâques, C.Seum, C.Tonka, M.Matsarskaia, S. Amaldi, A. Spierer, D. Pauli, and P. Spierer. Département de Zoologie et Biologie animale de l'Université. 154 route de Malagnou, 1224 Genève

Position-effect variegation is the inactivation in some cells of a gene translocated next to heterochromatin. The number of copies of the Drosophila gene *Suvar(3)7* is a dose-limiting factor in this phenomenon, and seems from its sequence to encode a large protein with six widely spaced zinc-fingers. This novel arrangement of zinc-fingers could help in packaging the chromatin fibre into heterochromatin, and could reflect a novel method of controlling the expression from DNA domains. The transcript and protein distribution have been determined and show a prevalent maternal contribution. An homolog of the gene was isolated and sequenced in *D. teissieri*. The sequence is notably divergent (70% identity) for a species very close to D. melanogaster, but the fingers, their spacing and other motives are well conserved.

28

TRANSCRIPTION DEPENDENT STRUCTURAL TRANSITIONS IN YEAST CHROMATIN

Cavalli, G., and Thoma, F., Institut für Zellbiologie, ETH-Hönggerberg, CH-8093 Zürich.

We have studied the fate of nucleosomes during transcription by RNA polymerase II and the process of chromatin refolding after gene repression in yeast. In the repressed state, an artificial gene, called GAL-URARIB, showed clearly positioned nucleosomes on the promoter and the 5' transcribed region. When the gene was transcribed positioning was lost, but a residual nucleosomal repeat was observed. Psoralen cross-linking analysis confirmed that nucleosomes are not lost upon transcription. We conclude that the active chromatin of GAL-URARIB is nucleosomal, but nucleosomes might be unfolded or rearranged. When chromatin was analysed ten minutes after gene repression by shifting cells from galactose to glucose media, nucleosomes were repositioned to a large extent and complete restoration of positioning was observed after 2.5 hours. Thus, nucleosome refolding and repositioning is a rapid process and seems not to require histone synthesis or DNA replication. The data were confirmed by analysis of the chromosomal GAL1 and GAL10 genes.

29

REGULATION OF STABLE GENE REPRESSION AT THE LEVEL OF HIGHER ORDER CHROMATIN ORGANIZATION

Paro, R.,Franke, A.,Messmer, S.,Moehrle, A.,Orlando, V. and Zink, D. ZMBH, University of Heidelberg, INF 282, D-6900 Heidelberg

Pattern formation is generated by a spatially and temporally controlled expression of regulatory genes (i.e.homeotic genes). Early established patterns need to be faithfully maintained through development. In *Drosophila* the genes of the Polycomb group (Pc-G) are part of such a cellular memory mechanism, by keeping developmental regulators permanently and stably repressed in appropriate domains. We have evidence that the products of the Pc-G exert their repressory function by changing the higher order chromatin structure into a heterochromatin-like state. Proteins of the Pc-G form multimeric complexes, associated with particular target genes. So far we have found four members of the Pc-G (Pc, ph, Psc and Pcl) to be part of the complex. We are testing whether the complexes change the local chromatin organization by compaction and/or by compartmentalization within the nucleus. The Polycomb (Pc) protein contains the highly conserved chromo domain. We find that a particular modification of the domain is necessary for building the Pc-G complexes. Using in vivo cross-linking methods we have been able to monitor the DNA sequences on the homeotic BX-C interacting with the Pc-G complexes. The finding of homologous Pc-G proteins in other systems suggest that the stable maintenance of gene repression imprinted into the higher order chromatin organization is a general mechanism of gene regulation.

30

PURIFIED CO-FACTOR PROTEINS INTERACT WITH CTF/NF-I AND MEDIATE TRANSCRIPTIONAL ACTIVATION.

Dusserre,Y and Mermod, N., Institut de Biologie animale, Université de Lausanne, CH-1015 Lausanne.

To dissect the mechanisms of gene transcription control by human CTF/NF-I DNA binding factors, purified full length or truncated proteins were covalently coupled to a chromatography resin, which was then used to fractionate cell nuclear extracts by protein affinity chromatography. Two polypeptides which specifically interact with CTF/NF-I species were identified. In vitro reconstitution experiments indicated that these polypeptide co-factors are involved in CTF/NF-I mediated induction of transcription. For instance, these cofactors restore regulation when added to a depleted extract, they mediate histone H1 antirepression, and they alleviate squelching by an excess of activator. Functional and physical interaction of these purified cofactors with activators and with the basal transcription machinery will be discussed.

31

TFII-I : CLONING AND STRUCTURE-FUNCTION ANALYSES OF AN INITIATOR-BINDING BASAL TRANSCRIPTION INITIATION FACTOR

Martinez, E., Roy, A.L., Gregor, P., Carruthers, C., Gutjahr, T. and Roeder, R.G., Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, 1230 York Ave., New York, N.Y. 10021, USA.

TFII-I is a novel basal transcription initiation factor that interacts with the initiator element(s) in the core promoter of several cellular and viral RNA polymerase II(Pol II) - transcribed genes and can functionally substitute for TFIIA in an in vitro transcription system reconstituted with purified basal transcription initiation factors and Pol II. Unlike the other basal initiation factors, TFII-I also interacts with the upstream USF site found in the Adenovirus major late promoter. In order to further characterize the function of TFII-I, the human TFII-I cDNA has been cloned. TFII-I mRNA has been detected in all tissues analyzed, suggesting that the protein is ubiquitously expressed. TFII-I is a proline-rich protein containing 6 tandemly repeated domains and has the potential to form repeated α-helices and turns in alternation. Recombinant TFII-I wild-type and mutant proteins have been produced and are currently been tested functionally. The results of these functional analyses will be presented.

32

CHARACTERIZATION OF MAMMALIAN ACTIVATION DOMAINS

Institut für Molekularbiologie II der Universität Zürich, CH 8057 Zürich
Katja Seipel, Hans-Peter Gerber, Oleg Georgiev, Licen Xu, Kostadin Gramatikov and Walter Schaffner

We have previously characterized activation domains of known transcription factors according to their ability to activate transcription in cell culture (in vivo) from proximal (promoter) and/or remote (enhancer) positions (Seipel et al.,EMBO J.1992;11No.13). We also tested the same chimeric proteins produced in mammalian cells in cell-free extracts (in vitro). Activation domains rich in acidic residues stimulated transcription in vivo from remote and close by position, but failed to work in vitro. By contrast, glutamine-rich activation domains stimulated transcription in vitro, while in vivo they were unable to stimulate transcription from remote positions and dependent on the presence of an enhancer to work from proximal positions. Proline-rich domains exhibited intermediate features: they did not stimulate transcription in vitro, while they were weak activators in vivo from both proximal and remote positions. We also tested the chimeric proteins produced in E.coli in frog oocytes.
Furthermore we found that N-terminal subdomains of the human TATA binding protein (TBP) functioned like the "proximal" activation domain of Oct-1, while the C-terminal domain of RNA polymerase II functioned like the VP16 activation domain. These findings imply that TBP and RNA pol II CTD are directly involved in promoter and enhancer function, respectively.

33

CHARACTERIZATION OF THE ACTIVATION DOMAINS OF THE TRANSCRIPTION FACTOR OCT-2A

E. Friedl and P. Matthias
Friedrich Miescher-Institut, 4002 Basel

The octamer transcription factor Oct-2a is mostly expressed in lymphoid cells and belongs to the POU domain protein family.
In previous work two major transcriptional activation domains at the N-terminus and the C-terminus were identified by testing N- and C-terminal deletions of Oct-2a in transcriptional activation assays done in heterologous cells (HeLa). The N-terminal activation domain is rich in glutamine whereas the C-terminus contains a high amount of proline, serine and threonine residues.
At the moment it is not clear which role the C-terminal region plays in transcriptional activation and which amino acids are involved. In addition it is also unknown whether the N- and C-terminal activation domains are interchangeable or interdependent.
To further address these open questions and to find out whether duplication or multimerization of single activation domains create a more potent activator, we have constructed expression vectors containing different parts of the Oct-2a activation domains and the DNA binding domain of the yeast transcription factor GAL4 in varying arrangements.
These chimeric proteins were tested in transient transfection assays performed in HeLa cells as well as in the B cell lines X63Ag8 and Namalwa to see whether there is a B cell-specific factor involved in transcriptional activation by Oct-2a.

34

MECHANISMS OF SHORT- AND LONG-RANGE COOPERATION IN TRANSCRIPTION

Tiziano Tallone, Franca Baggi, Astrid Rüfli, Sandro Rusconi; *Institut für Molekularbiologie II der Universität, UZ Irchel, Winterthurerstrasse 190, 8057 Zürich, Switzerland*

In general enhancer and promoter regions are composed of clustered binding sites for transcription factors whose geometrical arrangement appears to be important in determining the degree of cooperation or interference among the binding factors. We have systematically studied the influence of site arrangement in model promoters by constructing different reporter genes with a system which allows quantitative measurement of the transcription rate. Our model clusters consist of glucocorticoid receptor response elements (GRE) intermingled with other sites (e.g. Sp1 or Octamer). The spacing between elements has been varied in different constructs and the clusters have been placed either in proximity of the TATA-box (promoter position) or at a 2 kb distance from a minimal promoter (enhancer position). Care has been taken to avoid cryptic GREs generated by bacterial *dam* methylation. The results confirm and extend our previous observations that the full length GR has different geometry optima than the carboxy-truncated GR, and further suggest that it is possible to "bridge" the cooperation among GREs by appropriately placing intervening binding sites for unrelated factors. We are testing whether enhancers can be composed of binding sites for "true" enhancer activating factors (like GR) along with sites for rather "enhancer inert" factors (like Sp1) which albeit can function as key intermediates for local cooperation required for maximum enhancer activity.

35

MAPPING THE MOUSE OESTROGEN RECEPTOR (MOR) DOMAINS RESPONSIBLE FOR THE SYNERGISM WITH THE PROLINE-RICH DOMAIN OF THE *HUMAN* CTF/NF-1.

Mahfoudi, A.[1], Roulet, E.[1], Parker, M.G.[2] and Wahli, W.[1]
1.Inst. de Biol. Animale, Uni Lausanne,CH-1015 Dorigny,2.Mol. End. Lab., Imperial center research fund, Lincoln's Inn field, London WC2A 3PX,UK.

The oestrogen receptor is made of two activation functions AF-1 in the N-terminal part of the protein, which is constitutively active, and AF-2, C-terminally located, which is ligand dependant. Cotransfection experiments in *Hela* cells using the wild type MOR together with the *human* CTF/NF-1 Proline-rich domain fused to the Gal4 DNA binding domain resulted in a synergistic transcriptional activation.Deletion of AF-2 decreases significantly the synergistic transcriptional activation whereas removal of AF-1 produces a strickingly higher synergism when compared to the wild type MOR receptor. Moreover, synergistic activation increases gradually when the MOR is progresssively deleted in AF-1. These results suggest that AF-2 is mainly responsible for the synergism observed with the CTF/NF-1 Proline-rich domain and that AF-1 could act rather as an inhibitor of this interaction. The use of deletion and point mutants in the AF-2 domain will help us to define the part of AF-2, which is implicated in the MOR/CTF/NF-1 synergistic transcriptional activation.

36

ACTIVATOR TRAP - CLONING OF TRANSCRIPTIONAL ACTIVATION DOMAINS BY EXPRESSION SELECTION IN MAMMALIAN CELLS

Matthias Gstaiger, Oleg Georgiev and Walter Schaffner
Institut für Molekularbiologie II der Universität Zürich, CH 8057 Zürich

The conventional assay for transcriptional activation domains is based on testing the activity of fusion proteins, consisting of a DNA binding domain and different segments of the protein to be analyzed. We have developed a new and fast method, which allows selection in mammalian cells and subsequent cloning of transcriptional activation domains from a mixture of DNA segments. The DNA to be analyzed is fragmented by sonication and fused to the GAL4 binding domain (aa 1-93) in an expression vector containing the SV40 origin of replication. These randomly generated pools of fusion proteins are transfected into monkey cells, which are stably transformed with the SV40 T-antigen gene under the control of a promoter with five copies of the GAL4 binding site. Replication of the transfected plasmids occurs only in cells expressing a transactivating fusion protein, which in turn stimulates T-antigen expression. Replicated plasmids are selected by DpnI digest, individual clones are isolated and finally analyzed by sequencing. Using this selection scheme, we have isolated from a large excess of unrelated DNA the activation domains of the herpesvirus VP16 and pseudorabies IE proteins.

37

The Mapping of RNA polymerase on Mammalian Genes in Cells and Nuclei. Jovan Mirkovitch* and James E. Darnell, Jr. The Rockefeller University, USA. *Present address: ISREC, 1066 Epalinges

The assembly of an RNA polymerase II initiation complex at a promoter is associated with the melting of the DNA template. Using the specific single-stranded modifying reagent $KMnO_4$ and a new genomic sequencing technique, we have explored the melted regions of specific genes in genomic DNA of whole cells or of isolated nuclei. We have demonstrated for the first time *in vivo* the melting in the promoter proximal transcribed region associated with the presence of RNA polymerase II complexes. An interferon-inducible gene exhibited $KMnO_4$ sensitivity downstream of the RNA initiation site in interferon treated cells when the gene was actively transcribed, but not in untreated cells where the gene was not transcribed. The extent of $KMnO_4$ modification was proportional to transcription levels. The $KMnO_4$ sensitivity was retained when nuclei where isolated from induced cells, but was lost if the engaged polymerases were further allowed to elongate the nascent RNA chains. The sensitivity to $KMnO_4$ in isolated nuclei was retained if the run-on incubation was performed in the presence of a-amanitin which blocks progress of engaged polymerases. A similar analysis identified an open sequence of only about 30 bases just downstream of the start site of the transthyretin (TTR) gene in nuclei isolated from mouse liver, a tissue where TTR is actively transcribed. This abrupt boundary of $KMnO_4$ sensitivity, which was removed by allowing engaged polymerases to elongate RNA chains suggests that most polymerases paused at about position +20. The possibility of mapping at the nucleotide level the position of actively transcribing RNA polymerases in whole cells or isolated nuclei opens new prospects in the study of transcription initiation and elongation.

38

INFLUENCE OF MONOTONOUS AMINOACID REPEATS ON THE ACTIVITY OF TRANSCRIPTION FACTORS.

Stefan Wieland; Rainer Lanz; Martin Hug; Manuela Höfferer; Sandro Rusconi; *Institut für Molekularbiologie II der Universität, UZ Irchel, Winterthurerstrasse 190, 8057 Zürich, Switzerland*

In the amino terminal portion of the rat glucocorticoid receptor (GR) cDNA there is a stretch of CAG triplets which encodes a string of Gln residues. This repeat is not phylogenetically conserved and is probably not very relevant for the function of the GR itself. Nevertheless, these repeats are frequently found in the coding portion of many transcription factors and it is conceivable that in some cases they may have acquired a significant role in the function of the host factor. Database searches suggest that the translational reading frame is subject to some constraints in natural sequences. We can show that in the rat GR there are different effects depending on the frame by which the repeated $(CAG)_{19}$ segment is translated (Poly-Gln, Poly-Ser or Poly-Ala). The N-terminal GR segment encoding poly-Gln, poly-Ser or Poly-Ala has been linked to other transcription factors (Octamer, GAL4 and GAL4-VP16 chimeras). In these recombinant factors we consistently observed that Poly Alanine exerts the strongest intracistronic dominant negative effect. However, inter-cistronic dominance (trans dominance) is observed only when Poly-Ala bearing GR is used to challenge wild type GR and has not been so far observed in the chimeric trans-activators. We are extending our studies to other types of repeats, such as the Poly-(GlnAla) motif found in some yeast and Drosophila regulatory factors.

39

FUNCTIONAL ROLE OF REGULATORY FACTOR RFX-1 ON THE HEPATITIS B VIRUS ENHANCER

Siegrist, C.A., Reith, W. and Mach, B.,Department of Genetics and Microbiology, CMU, 1211 Geneva 4

Demonstration that regulatory factor RFX-1 is identical to nuclear factor EF-C (Reith et al, see accompanying abstract) allowed to study the functional role of this transcription factor on the hepatitis B virus enhancer. First, B cells transfectants with the RFX-1 binding site of the HLA-DRA promoter replaced by the inverted repeats of HBV EF-C sites upstream of a CAT reporter gene were shown to conserve the RFX-1 dependant gamma-interferon inducibility of the HLA-DRA promoter. Second, cotransfection of HBV-CAT with RFX-1 in hepatic cells resulted in a dose dependant transactivation of the viral enhancer.Last, RFX-1 antisense oligonucleotides were added to hepatic cells stably transfected by a plasmid coding for the HBV surface envelope under control of its own promoter-enhancer. RFX-1 antisense oligomers were shown to inhibit the synthesis of the HBV envelope measured by HBsAg ELISA in the culture supernatant. These data demonstrate a new function for regulatory factor RFX-1.

40

A LEUCINE-ZIPPER TRANSCRIPTION FACTOR INVOLVED IN VASCULAR-SPECIFIC EXPRESSION IN TOBACCO

Daniel Heierli and Beat Keller, Swiss Federal Research Station for Agronomy, 8046 Zürich

The gene for the cell wall glycine-rich protein GRP 1.8 of French bean is specifically expressed in young xylem elements and phloem fibers. A 205 bp promoter fragment of the GRP 1.8 gene directed vascular-specific expression of a reporter gene in transgenic tobacco. Deletions of the promoter to bp -186 resulted in loss of specific gene expression: Vascular expression was lower in transgenic plants with this deletion but the gene was also expressed in the cortex and other non-vascular tissue. Nuclear extracts from tobacco and tomato cell cultures and leaves were found to contain a protein binding close to the specificity element defined by the in vivo experiments. This protein binding site was 28 bp in length. A gene encoding the binding protein was cloned by screening a cDNA expression library of tomato. It encodes a basic leucine zipper (bZip) transcription factor. The 28 bp specificity element was able to activate the heterologuos 35S CaMV specifically in vascular cells.

41

CLONING OF AN HLA CLASS II TRANSACTIVATOR DEFECTIVE IN A FORM OF PRIMARY IMMUNODEFICIENCY BY GENETIC COMPLEMENTATION

Viktor Steimle and Bernard Mach
Department of Genetics and Microbiology, CMU, 1211 Geneva 4

HLA class II deficiency is a regulatory defect affecting all HLA class II genes. At least three different complementation groups have been identified by cell fusion experiments. We present here the first identification of a gene which is defective in one complementation group.

For the genetic complementation we constructed a eucaryotic cDNA expression system based on plasmid vectors carrying the EBV origin of replication. Size selected cDNA libraries of HLA class II positive cells were transfected into the class II negative mutant Burkitt lymphoma cell line RJ2.25. Transfected RJ2.25 cells were selected for re-expression of HLA DR molecules. Plasmid pools rescued from several independent sortings displayed the same preferential insert type. Retransfection of this CIITA ("class II transactivator") cDNA into RJ2.25 cells restored the expression of all HLA class II types (DR, DQ, DP) to wild type levels. Analysis of the CIITA gene in RJ2.25 revealed an internal deletion spanning 1.8 kb of cDNA which leads to the absence of a full length CIITA messenger in RJ2.25. CIITA is a new gene without homology to known genes. Preliminary results indicate a crucial role for CIITA in HLA class II expression.

42

MOLECULAR BASIS OF REPRESSION OF MHC CLASS II GENE EXPRESSION IN PLASMOCYTES

P. Silacci, V. Steimle, M. Hadam*, W. Reith and B. Mach.
Department of Genetics and Microbiology, University of Geneva Medical School and Hannover Medical School*

One of the issues in B cell ontogeny is the extinction of MHC class II expression upon maturation of B cells into plasmocytes. The use of HLA-DRA promoter-CAT constructs transfected into a human plasmacytomas cell line indicate a block at the level of transcription. This block is mediated by a cis-acting element present in the first 150 bp of this promoter. Cell fusion experiments show that this block is dominant. A novel MHC class II transactivator, CII-TA (see accompanying abstract by Steimle and Mach) was studied in the context of this repression of MHC class II gene expression. When B cells mature into plasmocytes, we observe a concomitant extinction of the expression of the CII-TA transactivator. We therefore propose that a dominant repression of expression of the MHC class II transactivator CII-TA is responsible for the MHC class II-negative phenotype of plasmocytes.

43

Multiple mRNAs encoding novel isoforms of transcription factor AP-2 are differentially expressed during mouse embryogenesis

Pascal Meier, Marieke Koedood, Michael Buchert, and Pamela J. Mitchell
Institute for Molecular Biology II, Uni. Zürich-Irchel

AP-2 is a sequence-specific DNA binding protein that regulates transcription of selected target genes in vertebrate organisms. During mouse embryogenesis AP-2 is expressed in neural crest cells and in a region-specific manner in the developing nervous system, face, epidermis, limbs and nephric system[1]. We used RT-PCR and RACE techniques to obtain full length AP-2 cDNAs from 11.5 dpc mouse embryo mRNA. 3' primers were derived from our previously published mouse partial AP-2 cDNA (λ22) and 5' primers were either AP-2 specific from exon 1 genomic sequences or nonspecific RACE primers. Multiple RT-PCR and RACE products were cloned and sequenced, and RNase protection analysis was used to compare the relative abundances of the obtained cDNAs during embryogenesis. These experiments yielded several unexpected results. First, we found that AP-2 is encoded by multiple mRNA isoforms. One isoform encodes AP-2 protein that is 98.6% identical to human AP-2 from HeLa cells; other isoforms encode proteins that share exons comprising the C-terminal DNA binding domain but differ in exon usage at the N-terminus of AP-2. In total embryo RNA, one of the mRNA isoforms increases 10-fold in abundance between 11.5 and 12.5 dpc, while another decreases 3-fold between 10.5 and 11.5 dpc. We have used RNA in situ hybridization to analyze the expression patterns of the different isoforms during embryogenesis.

[1] Mitchell, et al (1991). Genes Dev.5:105-119.

44

DIFFERENTIAL STABILITY OF AP1 PROTEINS IN HUMAN KERATINOCYTE AND FIBROBLAST CELLS: ROLE IN THE CELL TYPE SPECIFIC EXPRESSION OF HUMAN PAPILLOMAVIRUS TYPE 18 GENES?

E.A. Offord, P.O. Chappuis and P. Beard.
ISREC, 1066 Epalinges, Switzerland.

Human papillomaviruses (HPV) infect keratinocyte but not fibroblast cells. Several factors, including AP1 (Jun/Fos), contribute to the cell-type specific transcription of HPV genes. Here, we show that AP1 levels are low in early passage human fibroblast extracts. In contrast, human keratinocyte extracts contain high levels of AP1. In vivo, Jun and Fos are relatively stable in human keratinocyte cells after serum induction whereas they are much less stable in human fibroblasts. Thus we think that the low level of AP1 found in fibroblasts is due to its rapid breakdown. In vitro, AP1 is degraded by a proteinase in fibroblast extracts. The proteinase is blocked by an inhibitor of cysteine proteinases but not serine proteinases. Furthermore, the activity of a cathepsin B-like cysteine proteinase is elevated in these human fibroblast extracts relative to other cell types. Interestingly, when the fibroblast cells are kept in culture for more than 20 passages they lose this proteinase activity and the amount of AP1 increases to a level similar to that of the keratinocytes. We conclude that the quantitative difference in AP1 proteins between human keratinocytes and early passage fibroblasts is due to the more rapid breakdown of AP1 proteins in the fibroblasts. This may be catalyzed *in vivo* by a cathepsin B-like proteinase.

45

CLONING AND EXPRESSION OF *XENOPUS* cDNAs HOMOLOGOUS TO THE CTF/NF-1 GENE FAMILY.

ROULET, E., ARMENTERO, M-T., MERMOD, N. and WAHLI, W., Institut de Biologie Animale, Université de Lausanne, CH-1015 Dorigny.

Four distinct cDNAs, xCTF1, xNFX1, xNFX2, xNFX3 were isolated from a *Xenopus* cell line library.

Sequence analyses revealed that all these cDNAs have a conserved N-terminal binding domain and variable C-terminal domains: xCTF1 is the homologue of the *human* hCTF1 subtype whereas the three distinct xNFXs share homology with the NF/X subtype and are more likely derived from a common precursor by alternatively spliced mRNAs.

In vitro expression and DNA binding experiments show that the activity of the identical N-terminal DNA binding domain is negatively regulated by a novel type of C-terminal domain present only in the NFX proteins. This negative active C-terminal domain does not prevent the DNA binding of heterodimers made of xNFX and xCTF1.

However cotransfection experiments performed in *Schneider* cells show that all these four proteins could activate transcription even as full length products although they are very weak DNA binding proteins. Using a serie of deletion mutants we were able to confirm *in vivo* the localisation of this NFX " binding inhibitory region" together with the domains responsible for transcriptional activation.

46

FUNCTION OF THE NOVEL FAMILY OF RFX DNA BINDING PROTEINS

Reith, W., Durand, B., Ucla, C., Siegrist, C.A. and Mach, B., Department of Genetics and Microbiology, CMU, 1211 Geneva 4

RFX1, RFX2 and RFX3 are members of a novel family of DNA binding proteins binding to the X box motif of MHC class II gene promoters. RFX proteins show over 90% homology in their DNA binding and dimerization domains but are divergent elsewhere. They have identical DNA binding specificity and can bind as both homo- and heterodimers. RFX1 and RFX3 are expressed constitutively in most cells and tissues while expression of RFX2 is more restricted and variable in level. RFX2 and RFX3 are expressed as two alternatively spliced variants. We have now shown that RFX1 is identical to a nuclear complex called either EF-C (an HBV enhancer binding complex) or MDBP (a site-specific methylation-dependent DNA binding complex). This has allowed us to show that 1) RFX1 is a transactivator of the HBV enhancer as well as of MHC class II genes, and 2) RFX factors bind to certain sites only when these contain methylated CpG dinucleotides. This observation suggests that RFX proteins may also mediate the inhibitory effect of cytosine methylation on gene expression.

47

Structural features of mammalian CpG islands

Institut für Molekularbiologie II der Universität Zürich, 8057 Zürich
Koichi Matsuo and Walter Schaffner

While the CpG dinucleotide is poorly represented in bulk mammalian DNA, there are interspersed sequences of a peculiar composition every 20-100 kb, so-called CpG islands, originally discovered by Adrian Bird. CpG islands are DNA segments of 0.5-2 kb length, with a high (G+C) content and high CpG density, often overlapping with the promoter-leader region of genes. Most CpG islands remain unmethylated at all stages of differentiation. The most remarkable exception is found in female mammals where the CpG islands of the inactivated X chromosome are cytosine-methylated. Aberrant methylation of a CpG island is also correlated with a form of mental retardation (fragile X syndrome). We have analyzed by computer the CpG islands of a number of corresponding genes in mouse and man, including CpG islands of housekeeping and cell type-specific genes from many chromosomal locations, but avoiding members of repeated gene families. We extend the findings of Aissani and Bernardi (Gene 106, 1991,173-183; 185-195) and establish that the human CpG islands are generally longer and contain a higher CpG density than mouse CpG islands. It seems that the human CpG islands are the "better" CpG islands, which should allow the associated genes to be more stably maintained either in the active or in the repressed state, depending on CpG methylation. We also propose a mechanism for promoter methylation associated with fragile X syndrome.

48

Vincent Ossipow, Philippe Fonjallaz and Ueli Schibler.
Department of Molecular Biology University of Geneva Switzerland.

Members of the C/EBP gene family produce multiple proteins by different translation initiations.

Two genes encoding liver enriched transcription factors, lap and c/ebp, produce multiple polypeptides by differential initiation of translation at two in-phase AUGs. Lap has been shown to produce LAP, a 36 Kd transcriptional activator protein and LIP, a 21 Kd transcritional inhibitor protein (Descombes P. and U.S. Cell 67 569 1991). Likewise, the c/ebp gene produces a 43 Kd transcriptional activator protein as well as a 30 Kd protein that activates transcription poorly or not at all. The different translation of two proteins from one LAP or C/EBP mRNA is conserved among vertebrates, including Xenopus, chicken, mouse, rat and man. LAP, LIP, C/EBP 43 and C/EBP 30 all accumulate to higher level in adult as compared to newborn rats. However, while the ratio of the two C/EBP proteins remains constant, the LAP/LIP ratio increases about five fold during this time period. As shown by immunostaining of tissue sections and western blot analysis of nuclear proteins, the developmental increase of LAP and C/EBP levels is due to both an increase in LAP - C/EBP expressing cells and a higher accumulation of these proteins in expressing cells. As LAP and C/EBP are maximally expressed in terminally differentiated, non-dividing hepatocytes, we examined whether these proteins may themselves be involved in the proliferation arrest of parenchymal liver cells. In contrast to differentiating adipocytes in which such a function has been documented for C/EBP (Umek, R. et al 1991 Sciences 251 288), neither C/EBP nor LAP appear to be sufficient for hepatic growth arrest.

49

PCR Amplification of POU-box Genes from the Developing Rat Nervous System.

Philippe Douville and Martin E. Schwab, Brain Research Institute, University of Zurich, August-Forel-Str. 1, 8029 Zürich

A variety of POU-box transcription factor genes have been identified in the mammalian nervous system and several of these have been shown to have crucial roles in cellular differentiation. We sought to assess the composition and to identify novel POU-box genes in developing rat CNS and differentiating oligodendrocytes. Our approach was to PCR amplify POU-box genes from postnatal day 0 (P0) rat spinal cord and P16 rat oligodendrocytes with degenerate primers from both the POU-specific and POU-homeodomains. We sequenced a sample of 23 clones from the PCR amplification of the oligodendrocyte template and identified a previously known POU-box, SCIP. No other POU-box genes were found in oligodendrocytes. Sequence information from a sample of 37 clones of the spinal cord template showed amplification of several POU-box genes including Oct-2 (51% of sample), Brn-4 (22%), Brn-3 (16%), and SCIP (2.5%). Some of these clones showed nucleic acid differences which could result in amino acid substitutions. We are currently assessing these differences using several assays to see if they represent novel POU-box gene products.

50

CLONING AND CHARACTERISATION OF A NEW POU PROTEIN cDNA

Schubart, D., Abbey, NL., Hoffman, M. and Matthias P.
Friedrich Miescher-Institut, 4002 Basel

To identify cDNAs coding for new POU proteins we designed degenerate amplimers for PCR amplification of DNA segments encoding POU domains. We were particularly interested in identifying new T cell- or brain-specific POU proteins. We prepared cDNA from activated Jurkat T-cells and used it as template for PCR reactions. The resulting PCR products were cloned and analysed by sequencing. This approach allowed us to identify a new clone having a very high homology to rat Brn-3 (a POU protein for which only a small part of the POU domain was known so far). Until now expression of that gene had not been reported in T cells. By screening a Jurkat cDNA library we then isolated overlapping clones covering most of the cDNA. We are presently further characterising these clones and a functional analysis will be presented.

51

CLONING AND CHARACTERIZATION OF THE NOVEL POU-DOMAIN PROTEIN BDOM130

Wey, E., Lyons, G.E.* and Schäfer, B.W., Department of Pediatrics, University of Zürich, Steinwiesstr. 75, CH-8032 Zürich and
* Departement of Anatomy, University of Wisconsin, USA.

Indications that other factors than those belonging to the HLH family (MyoD, Myogenin, Myf5, MRF4) might be involved in vertebrate myogenesis made us search for homeobox-containing proteins in human muscle tissues.
Using degenerated primers several homeobox-containing sequences belonging to the POU-domain family were found. One of them, called BDOM130, which shows a unique linker sequence was further analyzed. Its 3'end and at least part of the 5'end have been cloned using the RACE protocoll. We now have a cDNA that contains an open reading frame of 906 bp giving rise to a protein product with an apparent weight of 40 kD. The expression pattern of BDOM130 in adult human tissues and during mouse development has been studied by Northern blot analysis and in situ hybridization, respectively.
The bacterially produced fusion protein GST-BDOM130 binds the octamer motif (ATTTGCAT) to which all POU-domain proteins show some affinity. We therefore conclude to have found a new member of the POU-domain family constituting a novel subclass.
Currently we are investigating the influence of BDOM130 on the transcription of reporter gene constructs.

52

Characterization of POU genes in zebrafish embryos

Giselbert Hauptmann, Philipp Spaniol, Caroline Bornmann and Thomas Gerster

Abt. Zellbiologie, Biozentrum der Univ. Basel, 4056 Basel

We have identified by PCR and cDNA cloning five POU genes expressed during early embryogenesis of zebrafish. Four of these genes show extended homology to the *brn-1* class of POU genes identified before in mammals. Northern blot analysis and *in situ* hybridizations indicate that the expression of these genes begins shortly after gastrulation in the neural tube. In the 24 hour old embryo various structures in the brain and in the spinal cord express these genes. We are currently investigating how the expression patterns of the four *brn*-1-like genes differ from each other and how far they overlap.
A fifth gene analyzed contains a POU domain that forms the prototype for a novel subclass of these DNA binding domains. This particular gene seems to be expressed already during the first cleavages in addition to a large maternal RNA pool. In early gastrulae the transcript is predominantly distributed in a ring around the equator of the egg. Later during gastrulation the expression domain is narrowing to two strong stripes and the posterior half of the rostrocaudal axis. After 24 hours the transcript can only be found in the tip of the tail. The early expression and the spatial arrangement of the transcript strongly argue that this POU gene is involved in early developmental decisions determining the body plan of the zebrafish.

53

Cloned transcription factor MTF-1 activates the mouse metallothionein I promoter

Freddy Radtke, Rainer Heuchel, Oleg Georgiev, Martin Hergersberg, Christopher Hovens, Enrico Brugnera,*Marisa Gariglio, *Zlatko Dembic and Walter Schaffner

Universität Zürich, Institut für Molekularbiologie II, Winterthurerstr. 190, CH-8057 Zürich, Switzerland.
*F. Hoffmann-La Roche AG, PRTB, Grenzacherstrasse, CH-4002 Basel, Switzerland.

Metallothioneins (MTs) are small cysteine-rich proteins whose structure is conserved from fungi to man. MTs strongly bind heavy metals, notably zinc, copper and cadmium. Upon cellular exposure to heavy metal and other adverse treatments, MT gene transcription is strongly enhanced. Metal induction is mediated by several copies of a 15 bp consensus sequence (metal-responsive-element = MRE) present in the promoter region of MT genes. We and others have demonstrated the presence of an MRE-binding factor in HeLa cell nuclear extracts. This factor, termed MTF-1 (MRE-binding transcription factor) is inactivated/reactivated by zinc withdrawal/zinc addition. Here we report the cloning of the cDNA of mouse MTF-1, a 72.5 kD protein. MTF-1 contains 6 zinc fingers (C2H2) and separate transcriptional activation domains with high contents of acidic and proline residues. Ectopic expression of MTF-1 in primate or rodent cells strongly enhances transcription of a reporter gene that is driven by 4 consensus MREd sites, or by the complete mouse MT-I promoter, even at normal zinc levels. The concentration of the factor is unchanged by metal treatment, which is in accordance with previous findings that transcriptional induction does not depend on de novo protein synthesis.

54

ANALYSIS OF NEURON-SPECIFIC $GABA_A$-RECEPTOR GENE PROMOTERS IN TRANSGENIC MICE AND IN VITRO

Motejlek K., Leitgeb S., Rülicke T., Häuselmann R. and B. Lüscher
Abteilung für Molekulare Neurobiologie, Pharmakologisches Institut der Universität Zürich, Gloriastrasse 32, CH-8006 Zürich

Gene promoters driving differential neuron-specific expression of three γ-aminobutyric $acid_A$ ($GABA_A$) receptor subunits have been isolated and mapped. Analysis of the α1 gene indicates that >80% of its mRNA is transcribed from a single promoter that features a TATA box. The 5' end of the γ2 subunit gene is A/T-rich and the major start site also has a TATA-like element. In contrast, the 5' end of the δ subunit gene shows paradoxically all the features known to be typical of so-called "house-keeping" genes: a cluster of initiation sites in the center of a CpG-rich island, a prototype initiator sequence and no TATA box. Thus, $GABA_A$ receptor genes show significant divergence in their promoter structure. Nevertheless, comparison of the 5' flanking sequences of these three $GABA_A$ receptor genes revealed a novel conserved sequence element. A neuron-specific DNA-binding protein has been identified, that binds to this element and may be important for cell type-specific expression of $GABA_A$ receptor genes. Experiments to further characterize and purify this protein are under way. To determine functional elements for neuron type-specific expression we have generated several transgenic mouse lines carrying a lacZ gene driven by δ subunit gene sequences. 5' flanking sequences allow faithful neuron-specifc expression in hippocampus, thalamic nuclei, and in the cerebral cortex, but not in the cerebellar granule cells. Data on transgenic mice designed to test the function(s) of downstream sequences will be presented.

55

MOLECULAR ORGANIZATION OF THE RAT GLIA-DERIVED NEXIN PROMOTER

Ernø H., Küry P. and Monard D.
Friedrich Miescher-Institute, Basle, Switzerland.

Through analysis of rat genomic clones the three first exons and the promoter of rat glia derived nexin (GDN) were identified. The promoter sequence and the first exon were found to be extremely GC-rich thus localizing the 5' part of the rat GDN gene within a GC-island. A TATA box like sequence (TGATAAA) but no CAAT box was found. Primer extension and RNase protection assays identified one transcriptional start site.
A 1600 bp putative promoter fragment cloned in a reporter plasmid was shown to induce firefly luciferase expression after transient transfection in COS-7 cells and several other cell-lines. GDN gene expression in C6 glioma cells and Rat-1 fibroblasts was found to be regulated by a number of GC binding transcription factors, among which are Sp1 and a family of early growth response transcription factors (NGFI-A, Krox 20, Egr3, WT1 and NGFI-C) which have been shown to be important regulators of gene expression during development. A negative regulator was found to bind to the GDN promoter sequence between -377 and -238. This factor recognizes the E box consensus site (CAXXTG). In cultures of primary Schwann cells GDN mRNA increases with time while the amount of the negative regulator decreases. This suggests that this factor is regulating GDN expression in Schwann cells.

56

IDENTIFICATION AND CHARACTERISATION OF THREE NOVEL PROMOTERS IN THE HPV-18 GENOME

Stéphane Karlen and Peter Beard. Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges, Switzerland.

Most studies on the regulation of gene expression in human papillomaviruses have focussed on the promoter for the early genes E6 and E7 located in the long control region (LCR). We are interested in knowing whether or not there are other promoters outside the LCR. By *in vitro* transcription and primer extension analysis we found promoter activities near the start of the E2 gene and at the end of the L2 ORF in the genome of HPV-18. These promoters are active in HeLa cells as shown by CAT assay. Co-transfection experiments with a BPV-1 E2-expressing vector and reporter plasmids containing HPV-18 DNA fragments with the promoter activities showed that the promoter located within the E2 ORF is transactivated by E2 even in the absence of binding sites for the E2 protein. Transactivation was also observed when a plasmid expressing only the transactivation domain and not the DNA-binding domain of E2 was used. This suggests that E2 mediates transactivation through binding to another factor. Sp1 being a potential target for E2, we investigated the role of a putative Sp1 site (AGGTGG) in the promoter activity. Deletion of this site completely abolished transcription. Bandshift analysis showed a weak binding of Sp1 to the AGGTGG site. These results were in agreement with the model in which the E2 protein is targeted to the promoter region by physical interaction with Sp1

57

MULTIPLE NUCLEAR FACTORS REGULATE THE ACTIVITY OF THE CHICKEN α1 AND α2 (VI) COLLAGEN PROMOTERS

T. E. Willimann and B. Trueb
Laboratorium für Biochemie I, ETH Zürich, Switzerland

The promoters of the α1(VI) and α2 (VI) collagen genes exhibit features characteristic of housekeeping genes and of proto-oncogenes: They have a high G/C content and lack a typical TATAA box. Transcription is initiated at multiple start sites. Previously, we have analyzed the α1 and α2 promoter by footprinting analyses and found multiple DNA elements interacting with nuclear factors.
Now we have further characterized the two promoters by gel retardation assays. The α1 promoter contains three binding sites for nuclear proteins showing the following pattern: AP1 - Sp1 - Sp1. The second Sp1 site is located just upstream of the major transcription initiation site. The α2 promoter exhibits four binding sites for nuclear factors: Sp1 - Sp1 - X - Sp1 (X stands for a novel, not further characterized factor binding specifically to a DNA element of the α2 promoter). Again, the last Sp1 site is located just upstream of the major transcription initiation site.
To analyze the biological relevance of these factors we constructed artificial mini-promoters utilizing the DNA elements of the α1 and α2 promoter recognized by transcription factors. These constructs were tested for their promoter activity in transient expression vector assays. We found that the promoter constructs had an activity comparable to that of the corresponding promoter fragments.

58

ANALYSIS OF THE FACTORS BINDING TO THE PYRIMIDINE-RICH MOTIF OF SOME IMMUNOGLOBULIN PROMOTERS

Schwarzenbach, H., Newell, J. and Matthias P.
Friedrich Miescher-Institut, 4002 Basel

Some immunoglobulin variable region promoters contain, in addition to the highly conserved octamer motif, a pyrimidine-rich sequence: -CTTCCTTA-, or a variation of it. This motif has previously been shown to be important for full promoter activity, in particular for those promoters which have only an imperfect octamer site. We have analysed the factors binding specifically to this pyrimidine-rich motif. By electrophoretic mobility shift assays with extracts from B cells at least two sets of complexes can be detected. One represents the binding of a B-cell specific factor of the ETS family. The other is due to the binding of a ubiquitous, but lymphoid cells-enriched factor. The interplay between these factors and octamer factors is being studied and a functional analysis will be presented.

59

FACTORS REGULATING IL-2 TRANSCRIPTION IN T HELPER CELLS

Mouzaki, A.. and Rungger, D., Division de Hématologie, Hôpital Cantonal Universitaire, 1211 Genève 4 et Station de Zoologie expérimentale, Université de Genève, 1224 Chêne-Bougeries

The trans-active factors regulating IL-2 expression have been analyzed by bandshift assays and functional transcription studies in the *Xenopus* oocyte. In human primary T lymphocytes, IL-2 regulation turns out to be strikingly different from the models elaborated using immortalized T cell lines: T cells in different states of maturity were isolated through immuno-labeling of their surface antigens and cell sorting on FACS. In resting naive T helper cells, the IL-2 gene is repressed by a silencer binding to the Pu-rich promoter elements. Upon first antigenic stimulation, an activator appears, displaces the silencer, and de-represses IL-2 transcription. Post-stimulated, rested memory cells do not acquire a silencer, but their activator looses its functional activity and is trans-located to the cytoplasm. New stimulation of such memory cells leads again to nuclear targeting of the activator and to its reactivation without requiring *de novo* protein synthesis. By contrast, immortalized tumor cells, such as Jurkat, have no silencer and their activator (NF-AT) is not capable of de-repressing genes arrested with silencer from primary T cells. Tumor cells thus may not be used as a model to study IL-2 regulation *in vivo*.

60

REGULATION OF CELL-SPECIFIC AND cAMP-DEPENDENT STEROID 17α – HYDROXYLASE (CYP17) GENE EXPRESSION

Zanger U.M., Momoi K., and Waterman M.R.*, Dept. of Pharmacology, Biocenter, CH-4056 Basel, and *Dept. of Biochemistry, Vanderbilt University, Nashville TN, USA.

Steroid 17α-hydroxylase cytochrome P450, the product of the CYP17 gene, is required for steroid hormone biosynthesis in the adrenal cortex and the gonads. CYP17 gene expression is restricted to these steroidogenic tissues and regulated by pituitary peptide hormones via cAMP. Transcription of the bovine CYP17 gene involves at least two distinct cAMP-responsive sequence elements (CRS) which are located in its 5'-flanking sequence (Lund et al., JBC 265:3304).
Our studies on the distal element CRS1 show that it activates reporter gene transcription only in steroidogenic cells suggesting that it may play a dual role, namely in cAMP-inducible as well as in cell-specific gene transcription. By *in vitro* transcription and other evidence we demonstrate that it interacts with a factor which is unrelated to the cAMP response element (CRE) binding protein CREB. We are presently isolating CRS1 binding proteins from bovine adrenal cortex. The proximal element CRS2 apparently interacts with several different factors one of which is only detectable in steroidogenic cell-types. Mutational analysis is being performed to elucidate their sites of interaction as well as their functional significance for cAMP-dependent and cell-specific transcription.

61

FUNCTIONAL AND STRUCTURAL ROLE OF CONSERVED HYDROPHOBIC RESIDUES IN THE ZINC FINGER REGION OF THE GLUCOCORTICOID RECEPTOR

Mauro Gola; Rainer B. Lanz and Sandro Rusconi; *Institut für Molekularbiologie II der Universität UZ Irchel, Winterthurerstrasse 190, 8057 Zürich, Switzerland*

One of the Ile residues in the second zinc finger is highly conserved among the members of the steroid receptor superfamily. We have analyzed the effect of substitution by every possible aminoacid at the corresponding position of the rat glucocorticoid receptor (rGR map position 484). The results indicate that only hydrophobic aminoacids can functionally substitute for Ile484. Interestingly, (and opposite to wild type GR and other mutations within the zinc finger region) the mutant Cys484 fails to transactivate when tested as a small GR fragment 407-556 but is permissive for transactivation when extended to include the entire N-terminus. Furthermore, the transactivation of the Cys-substitution of Ile484 is similar to the corresponding GR fragment lacking the putative transactivation domain *tau-2* located C-terminally to the second zinc finger. Mobility shift and in vivo trans-footprint assays confirmed the DNA binding properties of the mutants. Crystallographic analysis by Luisi (et al., Nature 352) of the DBD has revealed a hydrophobic core involving also the ultra conserved residue Cys500. Mutation of this residue to Ala yields in a fully functional receptor while its substitution by Phe destroys receptor functions. We have tried to construct compensatory mutants by simultaneous substitution of Cys500 by Phe and Phe464 by Ala.

62

REGULATION OF THE MOUSE MAMMARY TUMOR VIRUS PROMOTER BY UBIQUITOUS AND TISSUE-SPECIFIC FACTORS

C. Cavin and E. Buetti, ISREC, 1066 Epalinges

We investigated the role of a tandem binding site for the ubiquitous oct-1/NF-III factor, located upstream of the TATA box and adjacent to a CTF/NF-I site in the MMTV promoter. In mouse L cells stably transfected with a mutant of both oct-1 sites we observed a 50-fold reduction of the base level of transcription; the glucocorticoid-stimulated level was unaffected. Binding of NF-I and oct-1 on wild-type and mutant DNA was studied by DNAse I footprinting with nuclear extracts and oligonucleotide competition experiments. We conclude that in L cells oct-1 is mainly involved in basal promoter activity, and NF-1 in glucocorticoid induction. In the distal promoter region, we analyzed DNA-binding nuclear proteins from mouse tissues (spleen, permissive for MMTV expression, and liver, non-permissive) by DNAse I footprinting, methylation interference, gel retardation and oligonucleotide competition, and UV-crosslinking. 5' of the glucocorticoid receptor binding site, a sequence (DRa, -189 to -206) bound tissue-specific factors. Next to it, a sequence (DRc, -206 to -223) bound a protein present with different abundance in the tissues. We are presently testing in transfection assays if these sequences can modify the level of transcription from the MMTV promoter.

63

POSITIVE RESPONSE TO RU486 BY THE GLUCOCORTICOID RECEPTOR

Rainer B. Lanz and Sandro Rusconi; *Institut für Molekularbiologie II der Universität UZI, Winterthurerstrasse 190, 8057 Zürich, Switzerland.*

We have studied the behavior of several mutations in the glucocorticoid receptor (GR) by expressing GR-cDNAs along with appropriate reporter genes in mammalian cells. Here we report experiments involving mutations in the hormone binding domain (HBD) of the receptor. Others have shown that mutagenesis in the most carboxy terminal portion of the GR can abolish ligand dependent transcriptional activation without apparent effect on steroid or DNA binding (Danielian et al., 1992). On the other hand, a progesterone receptor (PR) deletion mutant lacking the carboxy terminal 42 aa has been reported to be not responsive to progesterone, but efficiently activated by the potent antiprogestin RU486 (Vegeto et al., 1992). We reconstructed these carboxy terminal mutations by either substitution (GR aa 770, 771) or by deletion of the C-terminal 29 aa of the HBD and tested them for response to agonist/antagonist and for trans-dominance. We found that the substitution mutants have a differential response to dexamethasone/RU486, while the truncated form failed to react in the way anticipated from work with PR. Moreover, deletion of two residues -which are conserved in the GR/PR/MR/AR-class of the steroid receptor superfamily-, generated a mutant which can respond positively to RU486, but not to the agonist. None of these GR mutants appears to have trans-dominant negative properties. Further C-terminal deletions have been made and tested for responsiveness to RU486.

64

STEROID BINDING DOMAINS AS REGULATORY CASSETTES

Louvion, J.-F., Ghika A., Fankhauser, C., P. and Picard, D.
Département de Biologie Cellulaire, Université de Genève, CH-1211 Genève 4

The hormone binding domain (HBD) of steroid receptors is an autonomous regulatory cassette which can subject activities of heterologous proteins to hormonal control probably as an effect of HSP90 binding to this portion of steroid receptors in the absence of ligand. We now show that heterologous proteins can also be regulated by HBDs in yeast. We fused the estrogen receptor HBD to a GAL4-VP16 activator and found that transcriptional activity of the chimera is completely estrogen dependent. This provides an efficient tool to express proteins of interest in yeast in a regulated fashion. We are currently exploring the possible role of HSP82 (HSP90 yeast homologue) in this regulation.
The unliganded HBD acts as a negative regulator of various steroid receptor functions including nuclear localization signals (NLS). While the HBD of the glucocorticoid receptor (GR) contains a NLS, the HBD of the mineralocorticoid receptor (MR) does not. To further define this hormone-regulated function, the NLS activity of a set of chimeric GR-MR HBDs, fused to ß-galactosidase as a tag, was tested.

65

FATTY ACIDS AND RETINOIDS REGULATE LIPID METABOLISM THROUGH ACTIVATION OF PPAR/RXR HETERODIMERS

Keller,H.[1], Mahfoudi,A.[1],Dreyer,C.[2],Medin,J.[3],Ozato,K.[3] and Wahli,W.[1],1.Inst. de Biol. animale,Uni Lausanne,CH-1015 Lausanne,2.Max Planck Inst.für Entwicklungsbiol.,D-7400Tübingen 3. Lab. of Mol. Growth Reg.,NIH, Bethesda,MD,20892,USA.

The nuclear hormone receptors PPARs (peroxisome proliferator-activated receptors) control the peroxisomal β-oxidation of fatty acids by induction of the acyl-CoA oxidase gene which encodes the rate limiting enzyme of the pathway. Gel retardation and cotransfection assays revealed that PPARα heterodimerizes with retinoid X receptor β (RXRβ) and that the two receptors cooperate for the activation of the acyl-CoA oxidase gene promoter. The strongest stimulation of this promoter was obtained when both receptors were exposed simultaneously to their cognate activators. Furthermore, we show that natural fatty acids, and especially polyunsaturated fatty acids, activate PPARs as potently as does the hypolipidemic drug Wy 14'643, the most effective activator known so far. Moreover, we discovered that the synthetic arachidonic acid analogue 5,8,11,14-eicosatetraynoic acid (ETYA) is 100 times more effective in the activation of PPARα than Wy 14'643. In conclusion, our data demonstrate a convergence of the PPAR and RXR signalling pathways in the regulation of the peroxisomal fatty acid β-oxidation system by fatty acids and retinoids.

66

ROLE OF THE ARNT PROTEIN IN THE AH (DIOXIN) RECEPTOR MEDIATED ACTIVATION OF *CYP1A1* TRANSCRIPTION

Probst,M.R., Reisz-Porszasz,S., and Hankinson,O., Laboratory of Biomedical and Environmental Sciences, UCLA, Los Angeles, CA 90024

The Ah receptor as a bHLH transcription factor binds a variety of environmentally important carcinogens such as chlorinated polycyclic aromatic hydrocarbons (eg. TCDD) as well as polychlorinated biphenyls (PCBs) and activates transcription of several genes involved in xenobiotic metabolism, such as cytochrome P450 *CYP1A1* and *CYP1A2*, as well as glucuronyl-transferase (GT) and glutathion-S-transferase (GST). The unliganded receptor is generally found in cytosol as a complex of the ligand binding subunit (LBS), heat shock protein 90 (HSP90) and other as yet unidentified proteins. After ligand binding, a dimeric complex of LBS and the Ah receptor nuclear translocator (ARNT) protein translocates into the nucleus and binds to cis-acting regulatory sequences, termed Xenobiotic Responsive Elements (XRE), which control transcription of the *CYP1A1* gene by the liganded Ah-receptor. We investigated the role of ARNT in mediating CYP1A1 transcription in the mouse hepatoma cell line Hepa-1 and provide evidence for a TCDD dependent dimerization of ARNT with LBS after release of HSP90, nuclear translocation and direct interaction of both subunits of the heterodimer with the 6 bp XRE core sequence. Functional domains within the ARNT molecule are presently being investigated by means of site directed mutagenesis.

Hoffman et al. (1991) Science **252**, 954-958
Reyes et al. (1992) Science **256**, 1193-1195
Burbach et al. (1992) PNAS **89**, 8185-8189

67

STAINING OF EARLY ECDYSTEROID INDUCIBLE PUFFS WITH ANTI-ECDYSTEROID RECEPTOR ANTIBODIES IN CHIRONOMUS

Ines S. Wegmann, Markus O. Imhof, and Markus Lezzi
Institut für Zellbiologie, ETH Hönggerberg, CH-8093 Zürich

We have cloned a cDNA sequence of Chironomus coding for a protein homologous to the Drosophila ecdysteroid receptor. Its N-terminal domain, the hinge domain, and the hormone binding domain were separately overexpressed in E. coli. The resulting polypeptides were injected into rabbits after gel purification for antisera production.
An analysis of the antisera by Western blotting revealed the presence of antibodies specific for each of the protein domains. On Western blots of protein extracts of Chironomus tissue culture cells, several protein bands were detected. One band of an approximate molecular weight of 65 kDa was detected with each of the three antisera. This corresponds with the size of the protein predicted by the amino acid composition.
Using these sera, immunohistochemistry of squash preparations of Chironomus prepupal salivary gland polytene chromosomes was performed. About ten bands showing strong immunofluorescent signals, including the known early ecdysteroid inducible puff sites I-18C and IV-2B, were detected. These results agree with the notion that the protein against which the antisera were raised represents an ecdysteroid receptor.

68

CHARACTERISATION OF VACCINIA VIRUS EXPRESSED XENOPUS FTZ-F1

Hihi, A., Keller, H.-J., Dreyer, C. and Wahli, W.
Institut de Biologie Animale,Université de Lausanne,
CH-1015 LAUSANNE
Max Planck Institut für Entwicklungsbiologie,
D-7400 TÜBINGEN

A cDNA has been isolated which codes for the Xenopus homolog of the Drosophila FTZ-F1 protein (dFTZ-F1) and the Mouse ELP protein.FTZ-F1 and ELP are members of the nuclear hormone receptor superfamily.The Xenopus FTZ-F1 (xFTZ-F1) was over-produced using the Vaccinia Virus expression system, and production of xFTZ-F1 was assayed by gel retardation analysis.As probe we used the dFTZ-F1 response element (Ftz-RE) which is located in the Zebra element of the Fushi-tarazu (FTZ) gene promoter, and contains the core AGGTCG sequence.A specific bandshift was only observed with extracts of cells infected with the recombinant virus containing the xFTZ-F1 cDNA, and we did not detect binding to an Estrogen Response Element,containg a core AGGTCA sequence.We observed a less efficient binding to a newly described Hormone Receptor Response Element(RE) containing the AGGTCA sequence,the Peroxisome Proliferator Activated Receptor RE .Finally the results suggest that the recombinant Vaccinia virus produces the xFTZ-F1 protein which is able to bind to the Ftz-RE.

69

Molecular analysis of ecdysteroid-regulated gene expression in the insect *Chironomus tentans*.

Vögtli, M., Imhof, M.O., and Lezzi M.
Institute for Cell Biology, ETH-Hönggerberg, CH-8093 Zürich

The steroid hormone 20-OH-ecdysone triggers coordinate changes in *Chironomus* larval development that result in metamorphosis to the adult insect. In an effort to understand the molecular mechanisms of this regulatory network we set out to clone candidate genes that specify proteins of the steroid hormone receptor superfamily. A cDNA library that was generated from mRNA isolated from a developmental stage of larvae (prepupae) of a high ecdysteroid titre was screened with an oligonucleotide probe specific for a conserved region encoding part of the zinc finger DNA binding domain of steroid hormone receptors. Beside a clone carrying the coding sequences for a putative ecdysone receptor (cEcR; M. O. Imhof, Ph.D. thesis ETH No. 9847) several other clones that appear to specify members of the superfamily were detected. Amino acid sequence comparison of the identified ORFs suggests that these clones code for the *Chironomus* homologs of the *Drosophila* ultraspiracle (usp), E75, and DHR3. While the temporal developmental profile for the expression of all three *Drosophila* genes closely parallels that for the ecdysteroid titre, *E75* and *DHR3* were additionally mapped to loci of ecdysteroid-inducible puffs in polytene chromosomes. The developmental expression pattern, the response to ecdysteroids, and the chromosomal localization of the various cloned *Chironomus* genes are discussed.

70

Zygotic transcription in *Ascaris lumbricoides*

A. Spicher, H. Tobler and F. Mueller, Institute of Zoology, University of Fribourg, Pérolles, CH-1700 Fribourg

The onset of zygotic transcription is an interesting and challenging problem in developmental biology. Organisms differ in the timing and abruptness of this process. In sea urchins, both pronuclei may already transcribe before fusion, and the zygotic nuclei are clearly transcriptionaly active. Transcription in mammals, ascidians, gastropods and molluscs, however, only starts within one or two cell divisions after fertilization. *Xenopus* and *Drosophila*, in contrast, appear to use maternal gene products until the mid-blastula transition, when a marked increase of zygotic transcription occurs. Here we describe a gene (Fert-1) of the nematode *Ascaris lumbricoides* whose transcription begins just after the end of meiosis of the female pronucleus. Fert-1 generates different polyA+ and polyA- transcripts. One of the polyA+ transcript is 560nt long, composed of two exons and transspliced at its 5'end. In contrast to the early zygotic activity of Fert-1, general zygotic transcription is switched on only at the 4-cell stage embryo.

71

THE LA PROTEIN AND RNA POLYMERASE III TRANSCRIPTION TERMINATION: A RE-EVALUATION

Lin-Marq, N. and Clarkson, S.G., Département de Génétique et Microbiologie, Centre Médical Universitaire, CH-1211 Genève 4.

A 47 kd human autoantigen called the La protein transiently binds to the 3' end of nascent RNA polymerase III (Pol III) transcripts and has been implicated in their synthesis, termination and release. Using cloned reagents from *Xenopus laevis* (La cDNAs, proteins, and polyclonal antibodies), we have examined the effects of La depletion of cell-free extracts on the stability of pre-synthesized RNA, and on the efficiency of Pol III transcription under conditions that distinguish single from multiple rounds of synthesis. The results demonstrate that La is able to protect nascent Pol III transcripts from 3' exonucleolytic attack, that it can slow the rate of normal 3' processing of tRNA precursors, and they suggest that it is not required either to complete the synthesis of the primary Pol III transcripts or to act as a release factor.

72

MYOGENIC DIFFERENTIATION IN XENOPUS EMBRYOS

Reynaud, S., Zhang,H. and Spohr, G., Departement de biologie cellulaire, Sciences III, 30 Quai Ernest-Ansermet, CH-1211 Geneve 4

The myogenic helix loop helix transcription factors MyoD, Myogenin,Myf5 and Mrf4 are capable in tissue culture of converting fibroblasts to muscle cells. During Xenopus embryonic development MyoD and Myf5 begin to be expressed at st.10 whereas myogenin transcripts have not been detected yet. To study the function of myogenin in Xenopus we have isolated a genomic clone possibly encoding myogenin. Promoter and transcription analysis are now in progress to determine at what stage in development this gene is activated.
Alpha 3-actin is expressed in Xenopus skeletal muscle of embryos and adults. A minimal alpha3-actin promoter of 190 nucleotides, containing 3-CArG boxes is turned on between st.14 and st.19, a longer promoter containing 1200 nucleotides, or the 190 minimal promoter fused to four E-boxes are already turned on at st.14.

73

Common Sequence Elements on Milk Protein Promoters Confer Coordinate Regulation upon Hormonal Induction

Standke, G., Groner, B., Friedrich Miescher-Institut, CH-4002 Basel

Differentiated mammary epithelial cells express high levels of milk proteins. 90% of the total milk protein comprises almost six tissue specific proteins, the caseins (α, β, γ); α-lactalbumin; β-lactoglobulin and whey acidic protein (1). Induced by lactogenic hormones cell cultures of mammary epithelial cells, HC11, express milk proteins in a transcriptional regulated manner (2). The milk protein genes are highly divergent except for a few consensus regions in the promoter regions of the genes.
It is reasonable to assume that the milk protein genes are subjected to common regulatory mechanisms and that these regulatory signals are conferred by elements of the promoter region. These promoter regions are recognized by nuclear proteins from mammary epithelial cells. Biochemical and functional studies are carried out to identify the role of common sequence elements and their interaction with individual transcription factors in the hormone, tissue and differentiation specific regulation of milk protein gene transcription.

(1)Clark,A.J., 1992, J.Cell.Biochem.49, 121-127
(2)Schmitt-Ney,M et al., 1992 Proc.Natl.Acad.Sci.89,3130-3134

74

INTRACELLULAR Ca^{2+} AND THE REGULATION OF *c-fos* AND *zif*268 EARLY RESPONSE GENE EXPRESSION IN HL-60 CELLS.

Werlen, G., Belin, D., Conne, B., Roche, E., Lew, D. P. and Prentki, M., Faculty of Medicine, University of Geneva, CH-1211 Geneva 4.

Various Ca^{2+} agonists, including growth factors and Ca^{2+}-ionophores cause rapid induction of early response genes such as *c-fos* and *zif*268. To gain more direct insight into the action of Ca^{2+} on transcriptional regulation, we have developed an intact cell model in which cytosolic free Ca^{2+} concentration can be measured and fixed at any level for various times in parallel with the assessment of early gene expression. Using fura-2 loaded HL-60 cells, we have observed maximal *c-fos* and *zif*268 mRNA accumulation at ~200 nM Ca^{2+}. In addition, full transcript accumulation of both genes measured at 30 min was obtained with a Ca^{2+} perturbation of one minute. Run-on transcription assays indicated a small effect of Ca^{2+} on transcriptional initiation and a pronounced Ca^{2+}-modulated relief of a block to transcriptional elongation in intron 1 of *c-fos*. This extreme sensitivity to Ca^{2+} in term of both time and dose of Ca^{2+} required for full gene induction, demonstrates that Ca^{2+} is a major regulator in its own right of early response gene expression. The results in addition confirm the presence of an intragenic Ca^{2+} response element in the *c-fos* gene. We are currently investigating the signal transduction pathway implicated in the Ca^{2+}-regulated *c-fos* gene expression. Preliminary experiments indicate that the C-kinase and the Ca^{2+}/calmodulin pathways do not mediate the action of Ca^{2+} on *c-fos*.

75

THE TRANSCRIPTION OF THE DELAYED EARLY GENE T1 IS REGULATED BY FOS AND C-MYC

Thomas Trüb, Markus B. Kalousek, Erika Fröhli and Roman Klemenz, Division of Cancer Research, Department of Pathology, Schmelzbergstr. 12, CH-8091 Zurich, Switzerland

The T1 mRNA was initially found after expression of the oncoproteins Ras or Mos in mouse fibroblasts (NIH 3T3). Transcription of T1 can also be stimulated by serum growth factors. Peak levels of T1 mRNA are reached within 4 to 6 hrs following serum stimulation. The induction depends on protein synthesis.
We identified an 80 bp long sequence, 3.5 kb upstream of the transcription initiation site, which mediates stimulation of T1 expression. It contains one TRE (TTAGTCA) and two E-Boxes (CACATG). This 80 bp fragment was cloned in front of a TK-minimal-promoter CAT-construct (pBLcat2). The 80 bp fragment strongly stimulates transcription in transient transfection assays. Point mutations were introduced into the TRE or into one or both E-boxes. All mutated forms showed a significantly decreased activation of transcription in transient transfection assays.
We stably transfected NIH 3T3 with an expression plasmid directing the synthesis of a fusion protein consisting of FosB and the estrogen receptor. In these cells T1 expression can be stimulated by the addition of estrogen.
To test the effect of c-Myc on the T1 promoter, we stably transfected NIH 3T3 cells with expression plasmids coding for a fusion protein consisting of the estrogen receptor fused either to c-Myc (tranfectants are called NME) or fused to a dominant negative mutant of c-Myc (NMΔE). Addition of estrogen stimulates T1 transcription in NME and represses the expression of T1 in NMΔE.

76

LI-FRAUMENI SYNDROME AND TP53 GERMLINE MUTATIONS

Scott, R., Mary, J.-L., Weber, W., Spycher, M. and Müller Hj.
Humangenetik, Departement Forschung, Kantonsspital Basel, CH 4051

There exist a wide spectrum of diseases encompassing soft tissue sarcoma, brain cancer, adrenocortical carcinoma and leukaemia which segregate in rare families. This association of neoplasias initially described by Li and Fraumeni in 1969 is also atypical as the onset of malignancy occurs at an unusually young age. Recently, the underlying genetic mutation giving rise to this association of neoplasias has been shown to be mutations in the tumour suppressor gene, TP53. The usual function of tumour suppressor genes is to control normal cell proliferation, however, when this control is disrupted (for instance, by carcinogens or radiation) the development of neoplasias appears to be inevitable. We identify a family, where two brothers have developed different types of malignancy yet carry the same germline mutation. The identification of germline p53 mutations has important implications for the handling of patients belonging to the Li-Fraumeni syndrome. Due to the different approaches towards handling the two patients who carry the same germline p53 mutation, very different and unpredictable outcomes may occur.

77

CHARACTERISATION OF P53 DELETIONS IN COLON CARCINOMAS.

Billotte, J., Vautravers, P. and Shaw, P., Institut universitaire de pathologie, division d'oncologie expérimentale, 1011 Lausanne.

P53 mutations are one of the most common genetic lesions appearing in virtually every type of human cancer. Both alleles of p53 are altered in 80% of colon carcinomas, one allele being usually mutated (missense point mutation) while the otherone is completely deleted. Such observations suggest that mutations in the p53 gene are recessive to the wild-type allele. The extent and mechanism of deletion has not yet been characterized. We have established a detailed restriction map of the 17p chromosomal region encompassing the p53 gene. This was accomplished by analyzing a YAC (Yeast Artificial Chromosome) containing the p53 gene. This clone contains a human insert of 620 Kb. Lambda DASH II was used to subclone the human sequences, and these subclones were used to screen the presence of microsatellites surrounding the p53 gene. In an average of 80% of the cases the microsatellites are heterozygotes and offer therefore a very powerful tool to screen primary tumors for deletions. Analysis of the p53 chromosomal region will be presented and discussed. The presence and extent of deletions of p53 locus in colon carcinomas will be discussed in view of experiments using microsatellites analysis, as well as results obtained with more traditional probes derived from the p53 chromosomal region.

78

A Cluster of Six S100 Genes on Human Chromosome 1q21: Identification of Two Novel Genes Coding for the Calcium Binding Proteins S100D and S100E

[1]D. Engelkamp, [1]B.W. Schäfer, [2]M.G. Mattei, [3]P. Erne and [1]C.W. Heizmann.
[1]Abteilung für Klinische Chemie, Kinderspital, Universität Zürich, Steinwiesstrasse 75, CH-8032 Zürich; [2]Hôpital d'enfants, F-13385 Marseille; [3]Abteilung für Kardiologie, Kantonsspital, CH-6000 Luzern

The human genome contains large regions that are highly structured. Sequence related members of multigene families are often found in a clustered organization. Here we describe a new gene cluster composed of genes coding for calcium binding proteins of the S100 family. The linkage of six genes was established by pulse field gel electrophoresis, and a contigous DNA sequence of 15 kb contains the full coding regions of four different S100 genes. This is the tightest gene cluster discovered so far. Two novel S100 genes are located within the cluster which both exhibit unique structural features when compared to other S100 genes. S100E is cysteine-rich, whereas S100D contains a long hydrophobic N-terminal tail. The gene cluster was assigned to chromosome 1q21, one of the bands showing rearrangements in neoplasms at high frequency. The deregulated expression of some S100 genes in the cluster during tumor progression suggests that chromosomal abnormalities may influence the expression of S100 genes in late stages of cancer, particularly associated with the formation of metastases.

79

STRUCTURAL ORGANIZATION AND CHROMOSOMAL LOCALIZATION OF THE HUMAN CALMODULIN GENE FAMILY

Rhyner, J.A., Wicki, R., Ottiger, M.[1], Foletti, D., Koller, M.[2], Berchtold, M.W.[3], Hameister, H.[4] and Strehler, E.E.[5], Lab. for Biochemistry, ETH Zurich; [1]Inst. for Molec. Biol. & Biophys., ETH Zurich; [2]Catalys AG, 8304 Wallisellen; [3]Inst. for Pharmacol. & Biochem., Univ. of Zurich; [4]Div. of Clin. Genetics, Univ. of Ulm, FRG; [5]Dept. of Biochem. & Molec. Biol., Mayo Clinic, Rochester, MN, USA.

The human calmodulin (CaM) gene family consists of three members coding for an identical protein. In addition, at least one intronless gene for a CaM-like protein (CLP) and numerous pseudogenes exist in the human genome. The complete structures of all human CaM genes have been elucidated by a combination of genomic screening and walking techniques as well as by direct PCR-based amplification of genomic DNA. All intron locations are precisely conserved in the human genes; in contrast, the rat CaMII gene appears to have lost intron 3. The hCaMII gene can be distinguished from all other CaM genes by its over 50% larger size (due mainly to an expanded intron 2). The putative promoter regions are highly divergent in the three CaM genes indicating separate mechanisms of regulation. The genes for CaMI, II, III and for CLP are widely dispersed in the genome; they have been mapped to human chromosomes 14, 2, 19 and 10, respectively, by a combination of Southern blotting, PCR and *in situ* hybridization methodology.

80

HIGH-FREQUENCY DISRUPTION OF PORPHOBILINOGEN DEAMINASE GENE IN EMBRYONIC STEM CELLS BY HOMOLOGOUS RECOMBINATION

Lindberg R L P, Grandchamp B, Porcher C, Ledermann B, Bürki K, and Urs A Meyer, Dept of Pharmacology, Biocentre, University of Basel, CH-4056 Basel

Porphobilinogen deaminase (PBGD) is the third enzyme in the pathway of heme biosynthesis. A defect in PBGD causes acute intermittent porphyria (AIP). The main clinical manifestation in AIP is a neurological syndrome, usually precipitated by compounds that induce cytochrome P-450. The pathogenesis of nervous system dysfunction is still unclear. The human and mouse PBGD structures are very similar (90% homology). The mouse therefore potentially could serve as an useful animal model to study human porphyria. We used homologous recombination techniques to disrupt the murine PBGD gene in embryonic stem cells.

The targeting gene construct has the neomycin gene as a positive selection marker and the HSVTK gene (herpes simplex virus thymidine kinase) as a negative selection marker. ES cells were derived from the C57BL/6 mouse strain. Initially targeting constructs of non-isogenic DNA (C3H mouse strain) were evaluated but no homologous recombination events occurred (5000 clones were screened). Using isogenic DNA, a high frequency of homologous recombination (1/20) was observed. Presently five clones carrying the disrupted PBGD allele are being injected into blastocysts to generate partially PBGD-deficient (porphyric) mice. We are also evaluating promoterless neomycin gene constructs in the assumption that the endogenous PBGD-promoter might direct neomycin gene expression in the homologous recombinant.

81

MOLECULAR DEFECT IN ERYTHROPOIETIC PROTOPORPHYRIA WITH TERMINAL LIVER FAILURE

Schneider-Yin, X., Hungerecker, G., Schäfer, B., Burg, G. and Minder, E. I. Institute of Clinical Chemistry, University Hospital, CH-8091 Zurich

Human erythropoietic protoporphyria (EPP) is an inherited metabolic disorder and characterized biochemically by accumulation of protoporphyrin as a result of deficiency in ferrochelatase activity. A common clinical symptom is photosensitivity. However, in rare instance, EPP patients develop terminal liver failure.
We report here the molecular defect in a EPP patient who had severe liver disease and was treated by liver transplantation. Sequencing of ferrochelatase cDNAs from this patient revealed a single point mutation, C to T at nucleotide 185. This nonsense mutation converted glutamine to a stop codon near the amino-terminus of the enzyme, Q-59-stop codon. The patient was heterozygous since a normal ferrochelatase sequence was also identified besides the mutated one. We investigated two other EPP patients without liver involvement. The molecular defect in the patient with liver failure was different from the mutations in those two patients and in 3 published EPP cases, who were all lack of hepatic complication.
We conclude that EPP is heterogenous on molecular level since 6 different mutations have been identified so far. Further studies are to be done to investigate if there is any correlation between the molecular defect and the severity of the disease, as it has been established in cystic fibrosis.
Supported by Swiss NSF grant no. 32-34015.92 and by Hartmann-Müller Foundation.

82

TAQ ERROR VS mtDNA MICROHETEROPLASMY IN CLONED POLYMERASE CHAIN REACTION PRODUCTS.

Mariaux, J.°*, Parsons, T. J.*, and Braun, M. J.*
° Institut de Zoologie, Université de Neuchâtel, CH-2007 Neuchatel
* Laboratory of Molecular Systematics, MSC, Smithsonian Institution, Washington, DC 20560. USA.

The high level of occurrence of point mutations in plasmid-cloned PCR products from the mitochondrial cytochrome B gene of several birds led us to ask what proportion of these mutations was due to misincorporation by the Taq polymerase during the PCR process versus heterogeneity in the DNA template. We therefore cloned and sequenced 20 copies of a 0.7kb CytB sequence amplified from a single clone in order to estimate the Taq polymerase error rate. We found a significantly lower level of point mutations, yielding an empirical rate of 2.2 $x10^{-5}$ errors per nucleotide polymerized by the Taq polymerase. We concluded that up to 74% of the original variation observed was due to heteroplasmy of the mtDNA template. This microheteroplasmy may arise by somatic mutation due to a lower fidelity of the mtDNA polymerase or because of a high number of duplications of the mitochondrial genome. These results show that the sequencing of cloned mtDNA can reveal a significant amount of variation which is not phylogenetically informative and which could have a significant effect in certain types of studies.

83

MALIGNANT HYPERPHENYLALANINEMIA CAUSED BY MUTATIONS IN A HUMAN BIOSYNTHETIC GENE FOR TETRAHYDROBIOPTERIN

Bürgisser, D., Leimbacher, W., Redweik, U., Heizmann, C.W. and Thöny, B., Division of Clinical Chemistry, Department of Pediatrics, University of Zürich, CH-8032 Zürich

Tetrahydrobiopterin (BH_4) is the essential cofactor for the hepatic phenylalanine hydroxylase. A cofactor deficiency causes malignant hyperphenylalaninemia, an autosomal recessive disease. BH_4 is synthesized by at least three consecutive enzymatic steps, and most of the patients suffering from BH_4 deficiency have been shown to bear a defect in the second enzymatic step, the 6-pyruvoyltetrahydropterin synthase (PTPS). As a first step towards the understanding of the molecular nature of the deficiencies, we have cloned the human liver cDNA encoding the PTPS. The coding region predicts a polypeptide of 145 amino acids with a 82% identity when compared to the rat liver enzyme. Expression of the cDNA in *E. coli* yielded the active enzyme. Fibroblasts originating from three different patients exhibiting a enzymatic deficiency in PTPS activity were the source for total RNA isolation and subsequent cDNA production. PCR amplification and DNA sequence analysis of the PTPS cDNA yielded three different types of mutations in these patients: (1) a C to A and an A to G transition, causing a change in Arg_{25} to Gln, and Tyr_{128} to Cys, respectively; (2) a triplet deletion resulting in Val_{57} deletion; and (3) a deletion of 23 nucleotides causing a truncated protein of 55 amino acids due to a frame shift mutation. Investigations are under way to confirm whether these alterations are the cause for the reduced enzymatic activity, and whether the mutations are homozygous in the different patients.
Rapid isolation of highly purified enzyme was achieved by overexpressing PTPS in *E. coli* as a fusion protein with MalE. Subsequent affinity chromatography purification followed by enzymatic cleavage and size fractionation to separate the PTPS from the fusion partner yielded the enzyme in mg quantities. This will allow us to investigate the biophysical and structural properties of the protein.

84

ORNITHINE TRANSCARBAMYLASE DEFICIENCY: FOUR NEW CASES OF THE ARG277TRP MUTATION.

Oppliger, E., Wermuth, B. and *Liechti-Gallati, S, Chemisches Zentrallabor, Inselspital and *Institut für Rechtsmedizin, Universität Bern, CH-3010 Bern

Ornithine transcarbamylase (OTC) deficiency, the most common inborn error of the urea cycle, shows X-linked inheritance with frequent new mutations. Symptoms range from lethal neonatal hyperammonemia to cases with mild clinical symptoms. Recently, Hata and coworkers reported a C to T mutation in the OTC gene leading to the substitution of Trp for Arg in two Japanese boys with mild hyperammonemia (Hum. Genet. 87, 28-32, 1991). Using the polymerase chain reaction followed by sequence analysis, we screened 19 male patients with OTC deficiency for the presence of the C to T base exchange. The mutation was present in four apparently unrelated patients from Austria, Italy (2) and Turkey. Liver biopsy was performed on three patients. In agreement with the findings from the Japanese patients, two liver samples exhibited residual enzyme activities of about 20% of control values and higher enzyme activity at pH 9.5 than at the usual pH optimum of 7.7. OTC activity in the liver sample of the third patient was 6% of control values and no shift in pH optimum was detectable.

85

MOLECULAR ANALYSIS OF CHRONIC GRANULOMATOUS DISEASES: CHARACTERIZATION OF AN UNUSUAL SPLICING DEFECT IN THE GP91-PHOX mRNA

J.-P. Bourquin, F. Berthet, R.A. Seger, and J.P. Hossle, Children's University Hospital, Div. of Immunology/ Haematology, Zurich

Chronic granulomatous diseases (CGD) are rare immunodeficiencies characterized by a predisposition to recurrent bacterial and fungal infections. The underlying cause are defects in phagocytic NADPH oxidase, leading to absence or malfunction of microbicidal killing. Close to 70% of all CGD patients suffers from the X-linked form of the disease resulting from mutations in the gene encoding the gp91-phox subunit of the flavocytochrome b558, the terminal redox center of the oxidase. Gp91-phox mRNAs from two related patients (uncle and nephew) were analyzed by RNA PCR. Using overlapping primer pairs sequence parts in between primers at sequence positions 179 and 816 respectively were shown to be missing in both patients. PCR using these two primers detected two shortened products in both boys, while no products with normal length were detectable. Amplifications of both mothers' mRNAs revealed a mixture of normal and truncated PCR products. Sequencing of the PCR products showed the deletion breakpoints to correspond to intron/exon boundaries of exons 5 and 6. Both patients have mixed populations of truncated gp91-phox trancripts lacking either exons 5 and 6 or only 6. Genomic PCRs are currently performed to find out about the mutation at the genomic level that caused this unusual type of splicing defect.

86

Genetic correction by viral transfection of a cystic fibrosis pancreatic adenocarcinoma cell line modifies interleukin-1α stimulated interleukin-6 production and activities of free radical scavenging enzymes

Y. Cao, W. Liu, S.P. Inaebnit, R.A. Frizzell*, P. Birrer and U.N. Wiesmann, Dept. of Pediatrics, University of Berne, Switzerland and *Dept of Physiology and Biophysics, University of Alabama, Birmingham, USA

Genetic correction of inherited genetic defects by viral genomic vectors in cultured cells has raised hope for patients. We have compared gene corrected cystic fibrosis (CF) pancreatic adenocarcinoma cells expressing chloride channel regulating protein (P+) with uncorrected CF (P-) and non CF (C) pancreatic carcinoma cells for interleukin-1α (IL-1) induced interleukin-6 (IL-6) production and O_2^- radical scavenging enzymes. In P- and C cells no response to stimulation with IL-1 was noted while gene corrected P+ cells showed greatly increased IL-6 production (+1000-2000%). Glutathione-disulfide reductase was 300%, glutathione-S-transferase was 25% and glutathione peroxidase was slightly increased above control cells. Cu/Zn-Superoxide dismutase activities and reduced glutathione were same in all cultures. Inhibition of cell growth with mitomycin C had no effect on the observed cellular characteristics.
We conclude that complementation of defective gene products after retrovirus mediated gene transfer to the genome with random insertion of genes may possibly modify yet other biological characteristics of cells and may lead to unexpected and possibly undesired effects in vitro and in vivo.

87

PHOSPHATASE INHIBITORS MODULATE THE EXPRESSION OF E-SELECTIN GENE IN HUMAN ENDOTHELIAL CELLS.

P. Ghersa, R. Hooft van Huijsduijnen, J. Whelan, Y. Cambet and J.F. Delamarter, GLAXO Institute for Molecular Biology, Chemin des Aulx 14, 1228 Plans-les-Ouates, Geneva, Switzerland

E-selectin is a membrane protein specifically expressed on activated endothelial cells. Protein phosphorylation has been shown to play a key role in E-selectin gene activation. Here we show that dephosphorylation of specific factors is also implicated in E-selectin gene regulation.
Okadaic Acid, an inhibitor of Protein Phosphatases 1(PP1) and 2A (PP2A), increases the levels of E-selectin mRNA induced by cytokine. We have previously shown that E-selectin mRNA is superinducible by inhibition of protein synthesis. However, the addition of Okadaic Acid blocks the superinduction of E-Selectin mRNA.
We show that activation of NFkB is increased by Okadaic Acid and that in endothelial cells transfected with vectors carrying E-selectin promoter/CAT reporter gene sequences, CAT activity is also enhanced by Okadaic Acid . On the contrary, using Calyculin A, a more potent inhibitor of PP1 , different results are observed.
The opposite effects obtained using two phosphatase inhibitors suggest that different phosphatases might be implicated during the induction and down-modulation of the E-Selectin gene.

88

The response to xenobiotics of cultured rat hepatocytes is affected by physiological oxygen tension.

P. Maier, B. Saad and H.P. Schawalder, Institute of Toxicology, Swiss Federal Institute of Technology and University of Zürich, CH-8603 Schwerzenbach,.

The role of oxygen tension in the site specific toxicity of xenobiotics within liver lobules was analyzed in male rat hepatocytes cultured under periportal- (incubator 13%O_2) or perivenous-like (4%O_2) oxygen tension. Cells were exposed to phenobarbital (PB) or to 3-methylcholanthrene (3-MC) from day 1 to 4 or 6 to 9. The inducibility of 6 different P450 isoenzymes was analyzed with ELISA using monoclonal antibodies. Enzyme activities were determined in cell homogenates (ethoy-resorufin-O-deethylase) or in situ (testosterone hydroxylation). In untreated cultures, oxygen tension did not affect xenobiotic metabolism. After exposure to PB or 3-MC, a dose dependent pattern of alterations was detectable similar to that found in vivo. CYP1A1/2 and CYP2C6 were more strongly inducible in 13% O_2 compared to 4% O_2 cultures and CYP3A in 4%O_2 cultures only. Similar differences were found in enzyme activities. It is concluded that oxygen tension in rat hepatocyte cultures affect gene expression during the cellular adaptive response.

89

PRODUCTION AND ANALYSIS OF TRANSGENIC MICE WITH ECTOPIC EXPRESSION OF RAT PARVALBUMIN

Bustos Castillo,M.*; Celio,M.R.*; Rülicke,T.†;Mahrer,D.†;Berger,M.# and Berchtold,M.#
Institut d'Histologie et d'Embryologie générale,Université. Fribourg*.Biologisches Zentrallabor,Universität Zürich†.
Institut für Veterinärbiochemie, Universität Zürich-Irchel

In order to study the physiological significance of parvalbumin, we produced transgenic mice overexpressing this Ca^{2+}-binding-protein. Several hybrid constructs containing tissue-specific (neuron-specific enolase), or general promoters (SV 40 and metallothionein), coupled to the rat parvalbumin coding sequence were used. One transgenic line containing the human metallothionein IIB promoter was analyzed by RNA-PCR with oligonucleotides specific for ectopic parvalbumin transcripts as well as by immunohistochemistry using a polyclonal antibody. Both methods revealed expression of the transgene in several tissues, such as liver, brain, kidney, pancreas and organs of the male genital tract.
A detailed analysis of both the constitutive and heavy metal induced expression of this protein in cellular subpopulations, and on its subcellular distribution, will be presented.

90

UPA EXPRESSION IN TRANSFORMED MURINE ENDOTHELIAL CELLS

Besser, D., Ballmer-Hofer, K., Wagner, E.F. and Nagamine, Y., Friedrich Miescher-Institut, PO Box 2543, CH-4002 Basel

The urokinase-type plasminogen activator (uPA) is an extracellular protease which converts plasminogen to plasmin, a protease involved in many biological processes. uPA is highly expressed in different transformed murine endothelial cell lines, namely five mT-transformed cell lines (e.g. eEnd2) and a cell line F2 derived from a UV light-induced tumour. Another SV40-transformed endothelial cell line, TME, expresses only low levels of uPA. The difference of uPA expression between these cells, as shown in a protein activity assay, is correlated to the uPA mRNA concentrations. The uPA mRNA levels in these cells are 80:40:1 for eEnd2, F2 and TME, respectively. To determine the mechanisms underlying the uPA mRNA up-regulation, we investigated both transcriptional and posttranscriptional events. The stability of uPA mRNA was modest in eEnd2 ($T_{1/2}$ = 15h) and TME ($T_{1/2}$ = 9h) but very high in F2 ($T_{1/2}$ >> 24h). The template activity of the uPA gene in eEnd2, F2 and TME is 8:3:1. These results suggest that transcriptional events are more important for the different uPA expression in these cells although the influence of the mRNA stability can not be excluded, especially for the uPA regulation in F2 cells. Functional analysis of the uPA promoter suggests that a PEA3 site at -2.4kb is essential for uPA expression in eEnd2 and F2.

91

REGULATION OF ERYTHROPOIETIN GENE EXPRESSION

Arnold, N., Straumann, U., Aschkenasy, S., Marti, H., Bauer, C., Physiologisches Institut der Universität Zürich, Winterthurer Str. 190, Ch-8057 Zürich

Erythropoietin (EPO) is the most important regulator of the maturation of erythrocyte progenitor cells in mammals. It has been shown that EPO is mainly expressed in fetal liver and adult kidney in response to the physiological stimulus, hypoxia. However, recent studies on transgenic and normal mice showed low level EPO gene expression in other tissues as lung and spleen. The physiological role of EPO synthesis in these organs remains unknown. To study the regulation of oxygen dependent and tissue specific EPO gene expression, we have established an RT-PCR specific for either murine or human EPO-mRNA. Using *in vitro* transcripts from EPO genes with small deletions the amount of EPO-mRNA in different tissues and cell lines can be quantified. Preliminary data suggests that kidney and liver are the only relevant organs for EPO gene expression. This finding is in agreement with data of EPO gene expression in humans. In a second approach we attempt to establish an EPO producing kidney cell line to study tissue specific expression *in vitro*. To immortalize the EPO expressing cell population, isolated kidney cells have been transfected with different EPO/SV40-T-antigen fusion constructs. These cells as well as hepatoma cell lines serve as an *in vitro* model system to analyse the functions of regulatory elements of the EPO gene. We are currently analysing EPO gene expression of these manipulated cells.

92

GLUCOSE REGULATES ACETYL CoA CARBOXYLASE GENE EXPRESSION IN THE PANCREATIC ß-CELL LINE INS-1

Brun T., Roche E., Kim K.-H., and Prentki M. Faculté de Médecine, Université de Genève, CH-1211 Genève 4.

Nutrients must be metabolized by the pancreatic ß-cell to cause insulin release. We have proposed that malonyl-CoA serves as a metabolic coupling factor when ß-cells are stimulated with nutrient secretagogues (Prentki et al. JBC. 267:5802, 1992). The formation of malonyl-CoA is catalised by acetyl-CoA carboxylase (ACC). We have investigated the long-term regulation of ACC by nutrients using the ß-cell line INS-1. Glucose caused a marked (20 fold) accumulation of ACC mRNA. The threshold glucose concentration was 5mM; half maximal and maximal effects occured at 15 and 20mM, respectively. The lag time of the induction was 3h and maximal transcript accumulation occured at 24h glucose stimulation. ACC mRNA induction was associated with ACC protein accumulation. The action of the sugar is most likely transcriptional since glucose'did not change the stability of the ACC transcript. Non-metabolizable glucose analogues mimicked the action of glucose. The result suggest that glucose is a major physiological regulator of the ACC gene and that in contrast to its action on insulin release, the sugar does not need to be metabolized to induce the ACC gene.

93

A newly formed somatic telomere in *Ascaris lumbricoides* has no influence on the transcription of a nearby located gene

Y-J. Huang , H. Tobler and F. Müller, Institute of Zoology, University of Fribourg, Pérolles, CH-1700 Fribourg

Telomeric position effects were first demonstrated in *Saccharomyces cervisiae*: if a gene is placed near a telomere, its transcription is reversibly repressed. This effect can be eliminated or alleviated through cellular trans- and cis-mechanisms. Moreover, telomeric effects on the transcription of different genes exist in other organisms as well. Preliminary data indicated that a gene encoding a putative GTP-binding protein is located within only 10kb of a *de novo* formed somatic telomere in *Ascaris lumbricoides*. This gene provided us with a good opportunity to see whether the newly formed telomeres of somatic chromosomes during the process of chromatin diminution exert a telomeric position effect on nearby located genes. Our recent experiments show that this gene is expressed in all investigated stages before and after the elimination process, suggesting that the addition of the new telomere has no influence on the transcription of this particular gene. Does the gene simply escape from the position effect or are there more complicated mechanisms involved in this phenomenon? To answer this question, we are currently searching for additional genes being located in the chromosomal breakage region. Their expression pattern before and after chromatin diminution will be compared.

94

The primary structure of four ribosomal proteins in *Ascaris lumbricoides*

V. Bernard, A. Etter, T. Nilsen*, H. Tobler, and F. Müller,
Institute of Zoology, University of Fribourg, Pérolles, CH-1700 Fribourg
*Case Western Reserve University, 2119 Abington Rd., Cleveland, OH 44106 (USA)

Chromatin diminution takes place in the early development of the nematode *Ascaris lumbricoides*. The eliminated material contains mainly satellite DNA, but also a gene encoding a ribosomal protein (S19) (Etter et al., 1991, Proc. Natl. Acad. Sci. USA, **88**, 1593-96).

We looked for other ribosomal protein genes. An *Ascaris* cDNA library was screened and four cDNAs (4, 61, 66, and 69) were cloned. Their sequences were found to have homologies with ribosomal proteins from other species, including yeast, chicken, rat, mouse and human. In Southern hybridizations, S19 is eliminated in the genomic larval DNA, but cDNAs 4, 61, 66, and 69 hybridize to both oocyte and larval genomic DNA. These results show that the genes coding for 4, 61, 66 and 69 are not eliminated during early development of *Ascaris*. Northern blots on germ-line and somatic RNA revealed that the four ribosomal proteins are transcribed in all tissues analysed. Ribosomal proteins are known to have housekeeping functions and the expression of their genes are coordinately regulated. Current experiments are in progress to clone the genes of these four non-eliminated ribosomal proteins, to test by *in vitro* transcription the housekeeping functions of the five promoters, and to prove that chromatin diminution is an alternative way to silence housekeeping promoters.

95

REGULATION OF THIAMINE BIOSYNTHESIS IN *SCHIZOSACCHAROMYCES POMBE*

A. Zurlinden, H. Fankhauser, A.M. Schweingruber, L. Edenharter, C. Steinmann and M.E. Schweingruber

Institute for General Microbiology, University of Bern, Baltzerstrasse 4, 3012 Bern

The thiamine molecule consists of a pyrimidine and a thiazole moiety. The two halfs are synthezised in separate, still unknown pathways and condense to thiamine monophosphate. We defined structural genes responsible for thiazole (*thi2*) and pyrimidine (*thi3*) synthesis and for the condensation reaction (*thi4*). The expression of these 3 genes is strongly repressed by thiamine. The regulatory mutants *tnr1*, *tnr2*, *tnr3* and *thi1*, which affect the expression of thiamine repressible acid phosphatase (*pho4*) also affect the 3 structural genes. All *tnr* mutants are derepressed for the genes *thi2*, *thi3* and *thi4*. *Thi1* mutants are repressed for *thi2*, *thi3* and *thi4*. These results suggest that the thiamine biosynthetic pathway is under complex regulation.

96

Moss transformation with potato *phy*A gene

Bisztray, Gy.[1], Huges, J.[2], Schaefer, D[1], Gätz, C.[3] and Zryd, J-P.[1];
[1] Laboratoire de Phytogénétique Cellulaire, Uni Lausanne, [2] Frei Universitate, Berlin, [3] IGF Berlin

The potato type A phytochrome gene, in both sense and antisense orientations, driven by the constitutive 35S promoter of the CaMV, was cloned into the binary vector Bin19. Purified plasmid DNA was introduced into protoplasts of the mosses (haploid lower green plants) *Physcomitrella patens* and *Ceratodon purpureus* , using the PEG mediated direct DNA uptake method. High numbers ($5x10^3$) of resistant (G-418) clones were obtained. During subsequent transfers most of them lost the resistance. From the 99 stable *Physcomitrella* clones transformed with the sense construct, 41 were investigated. A range of similar phenotypes were observed including; a reduction in plant height, delayed senescence, loss of apical dominance. The dwarf gametophores had expanded leaves under light. Upon decreasing the red/far red ratio these phenotypes gradually disappeared. Those plants which were transformed with the antisense construct or with the plasmids without the phytochrome gene were identical to the wild type.

97

A novel peroxidase mRNA is rapidly up-regulated during turion formation in the water plant *Spirodela polyrrhiza*

Kateřina Chaloupková and Cheryl Smart
Institute of Plant Sciences, ETH Zürich, CH-8092 Zürich

The aquatic plant *Spirodela polyrrhiza*, can be induced to form dormant buds (turions) by both environmental (cold) and chemical (abscisic acid, ABA) triggers. By differential screening of a cDNA library we have isolated a full-length cDNA for an ABA-induced peroxidase (*tur4*). The transcript level is increased within 2 hours of ABA treatment and is also induced by low temperature. The low temperature induction of *tur4* is reversible by cytokinin (which inhibits ABA- and cold-induced turion formation). The deduced sequence of *tur4* has a maximal 48% identity with those of previously published plant peroxidase sequences, and thus represents a new peroxidase family. A signal peptide is found at the amino-terminal end of the peroxidase, which suggests its association with ABA-induced processes in the cell wall.

98

The Patterns of Gene Expression in the Shoot Apical Meristem of Tomato

Andrew Fleming, Therese Mandel, Isabelle Roth, and Cris Kuhlemeier
Institute of Plant Physiology, Bern University, CH-3013 Bern

The shoot apical meristem plays a vital role in plant development. Not only is it the ultimate source of all the cells of the aerial part of the plant, the cell divisions that occur in this area are organised so as to define the initial steps in leaf and stem morphogenesis. Various models of meristem structure and function have been proposed identifying distinct cellular compartments within the apex. Using *in situ* hybridisation, we have carried out an analysis of the expression patterns of a number of genes obtained from a meristem cDNA library. This analysis has revealed a variety of patterns within the apical meristem.which, when put into the context of the proposed models, necessitates a re-interpretation of how the apical meristem is organised at the cellular level.

99

ISOLATION AND SEQUENCING OF VARIOUS CLONES ENCODING THE SOYBEAN MALATE SYNTHASE

Guex, N., Richter, H., Henry, H., and Widmer, F. Institut de Biologie et de Physiologie Végétales de l'Université, CH-1015 Lausanne.

Polyclonal antibodies raised against malate synthase (MS; EC 4.1.3.2) were used for immunoscreening a λ-ZAP II expression library constructed from 3-day old dark germinated soybean cotyledons (*Glycine max.* L.) Ten independent clones were purified and partially or totally sequenced. All of them proved to be MS. Eight showed approximately the same size (1.8 kb) on a 1% agarose gel electrophoresis, whereas one was shorter (1.6 kb) and one considerably longer (*ca* 3 kb). The hypothesis of a chimeric cDNA can be excluded since the size of the insert was determined after digestion of the plasmid with the same restriction enzyme used to clone the cDNA fragments and that a single insert of 3 kb was detected. As the ORF of MS is about 1.7 kb and knowing that splicing in plants is less efficient compared to the animal system, the 3 kb clone could reflect an unprocessed mRNA. While partial sequencing data on the 5' end identifies MS, the 3' end is totally different, which suggests an uncommonly long 3' untranslated region with possibly interesting regulatory aspects. Further studies are necessary to identify the real nature of this 3 kb clone.

100

STRUCTURE AND EXPRESSION OF A SOYBEAN NUCLEAR GENE FOR THE CHLOROPLAST EF-G PROTEIN

Hernandez Torres J., Marc-Martin S. Spielmann A. and Stutz E. Laboratoire de Biochimie végétale, Université de Neuchâtel

Chloroplasts have their own set of translation elongation factors (EF-G and EF-Tu) which in higher plants are nucleus encoded. A partial soybean genomic library (3 to 4 kb EcorI fragments, lambda gt10) was screened with a 200 bp DNA probe of the N-terminal part of the pea EF-G gene (gift of Dr. C. Breitenberger, Ohio State U.) to the end of retrieving the nuclear gene(s) for the chloroplast specific EF-G which so far has never been isolated. We isolated and sequenced a clone (3.6 kb insert) containing the entire coding part for the chloroplast EF-G. The gene is split having two introns in the core part. A third intron separates the putative transit peptide from the core part. There are two EF-G genes per haploid genome as verfied by Southern experiments and cDNA analysis. We currently study the question of EF-G expression during leaf development trying to understand the role of elongation factors in the regulation of chloroplast protein synthesis (see Maurer F. et al, this poster session).

101

AT LEAST TWO ISOENZYMES OF ISOCITRATE LYASE COEXIST IN 3-DAY OLD SOYBEAN COTYLEDONS.

Guex, N., Widmer, F., and Richter, H., Institut de Biologie et de Physiologie Végétales de l'Université, CH-1015 Lausanne.

Polyclonal antibodies raised against isocitrate lyase (ICL; EC 4.1.3.1) were used for immunoscreening a λ-ZAP II expression library constructed from 3-day old dark germinated soybean cotyledons (*Glycine max.* L.). Four independent clones were purified, revealing that at least two isoenzymes of ICL coexist in cotyledons at this stage of development. The cDNA clones are nearly full length and show 95% identity in the coding region and 80% identity in the 3' untranslated region. While most differences between the two isoforms are found to be silent, 11 amino acids are different (including one gap of one amino acid), and determine slightly different pIs for the two isoenzymes. While the isoenzyme containing the gap is more similar to all plant ICL sequences reported so far in databases, the other one appears more unique (or not yet identified in other plant species).

102

CONTROL OF EF-TU GENE EXPRESSION IN SOYBEAN

Maurer F., Spielmann A. Bonny, C. and Stutz E. Laboratoire de Biochimie végétale, Université de Neuchâtel

Chloroplast development and function is tightly controlled by the nucleus. According to several reports the translation elongation step may be one of the control points and the expression of the respective genes in the nucleus could be crucially involved. We have identified an EF-Tu gene family (two sub-families with two members each) and so far sequenced two complete genes including the upstream parts with promoter elements. The promoter region of one gene is studied in detail. Upstream sequences of defined length (deletions) were linked to the GUS-reporter gene. Promoter activity is tested in transformed tobaco. We are currently testing gene activities at various developmental stages (F1 generation). We try to define cis -elements required for control; we test possible differential expression of members of the EF-Tu gene family; we correlate gene expression with the elongation factor activity within chloroplasts. These studies are complemented by the experiments of Hernandez Torres (see this poster session).

103

MOLECULAR-BIOLOGY IN THE HABITAT: GLYCOLYTIC GENE EXPRESSION IN A WETLAND PLANT UNDER NATURAL CONDITIONS

Marcel Bucher and Cris Kuhlemeier, Institute of Plant Physiology, Altenbergrain 21, 3013 Berne

We previously showed that three glycolytic marker genes are coordinately expressed in leaf and rhizome of the amphibious plant Acorus calamus in the laboratory during extremely long periods of anoxia. Here we investigated how the same genes were expressed under natural conditions in the lake. We observed marked differences to our laboratory experiments. In the leaves high mRNA steady-state levels of pyruvate decarboxylase and alcohol dehydrogenase, the two enzymes in alcoholic fermentation, coincided with leaf submergence (hypoxia) in winter and fall. In the permanently flooded rhizome, transcript levels of the same genes rapidly increased with the onset of growth in March. The mRNA levels of fructose 1,6 bisphosphate aldolase, an enzyme in the glycolytic stem, were high in the leaves throughout the year. In the rhizome aldolase transcript levels correlated with leaf submergence and with the generation of young rhizome in summer. The results indicate that under natural conditions multiple interacting factors differentially regulate glycolytic gene expression in leaf and rhizome and that oxygen deprivation and cold may be two of them.

104

ANATOMY AND PROCESSING OF THE *psb*D-*psb*C GENE TRANSCRIPT OF *EUGLENA GRACILIS*

Spielmann A., Orsat B., Marc-Martin S. and Stutz, E. Laboratoire de Biochimie végétale, Université de Neuchâtel

The chloroplast genes *psb*D and *psb*C code for PSII reaction center proteins of 39 anf 51 kb, respectively. The two genes together extend over better than 20 kb containing 22 introns. The 3'terminal part of *psb*D overlaps with the 5'terminal part of *psb*C, and the last intron of *psb*D corresponds to the first intron of *psb*C. Exon 2 of *psb*C is part of the 3'tail of the *psb*D mRNA. The final processed *psb*D and *psb*C mRNAs are 1.4 and 1.5 kb, i.e. a common primary transcript including the entire *psb*D and *psb*C gene must undergo multiple splicing and processing steps, including alternate splicing in the tail region. The *psb*C exons 2 and 3 are separated by a gap of 4.1 kb. In previous EM studies no intron was seen in this gap region while, e.g. each of the 11 introns was visible in the *psb*D region . This could mean that two smaller precursor RNAs exist and exon 2 and 3 of *psb*C are trans-spliced. Experiments are under way to settle this question.

105

Mutational analysis of histone RNA 3' processing in vitro

Adrian Streit, Tom Wittop Koning, Daniel Albrecht, Carmen Spycher, Reto Kohler, André Furger and Daniel Schümperli, Zoologisches Institut, Abteilung für Entwicklungsbiologie, Baltzerstr. 4, CH-3012 Bern.

Histone mRNA 3' ends are formed by a unique endonucleolytic cleavage resulting in poly(A)$^-$ mRNAs with a highly conserved 3'-terminal hairpin loop structure. Mutations of this structure do not abolish processing but have variable quantitative effects depending on the gene and on the biochemical environment (extract) used. In contrast, mutations abolishing the potential base-pairing between a downstream pre-mRNA spacer element and the 5' end of U7 RNA present in U7 small nuclear ribonucleoproteins (snRNPs) completely inhibit processing. We have observed that this base-pairing potential extends further than previously recognised in either direction. In particular, the region surrounding the actual processing site could interact with a U7 RNA segment involved in binding of structural proteins of the U7 snRNP. Mutations in this region of the pre-mRNA do not abolish processing but have variable quantitative effects and sometimes affect the specificity of cleavage. We are currently determining which parts of the pre-mRNA are involved in bona fide and productive base-pairing with U7 RNA by further mutagenesis and by psoralen photo-crosslinking experiments. The finding that a mutation in the spacer element which increases the base-pairing potential with U7 RNA has no effect on processing by itself but can rescue the partial processing deficiency of a stem-loop mutation suggests that the hairpin (and factors binding to it) may serve to stabilise the pre-mRNA:U7 interaction.

106

PURIFICATION AND CHARACTERIZATION OF SPLICING FACTORS SF3a AND SF3b

Reto Brosi, Karsten Gröning, Diana Blank* and Angela Krämer; Département de Biologie Cellulaire, Université de Genève, CH-1211 Genève; *Biozentrum, Universität Basel, CH-4056 Basel.

The splicing of introns from nuclear pre-mRNA occurs in large complexes (spliceosomes) which are assembled by transacting factors (snRNPs and proteins) in a stepwise fashion. The first ATP-dependent intermediate in spliceosome assembly is pre-splicing complex A. We have previously shown that at least three protein factors (SF1, SF3 and U2AF) are required for its formation in addition to U1 and U2 snRNPs.
We have separated SF3 into two activities, both of which are essential for the formation of complex A. Purified SF3a consists of three polypeptides of 60, 66 and 120 kDa and interacts with U2 snRNP in the presence of SF3b. The 60-kDa subunit of SF3a crossreacts with an antibody directed against the yeast splicing factor PRP9 that is essential for the incorporation of U2 snRNP into the spliceosome. Moreover, a cDNA encoding the 60 kDa-subunit of SF3a shows homology to PRP9. In addition, a human homologue of PRP9 has recently been detected as a U2 snRNP-specific polypeptide of 60 kDa. Taken together, these data support the hypothesis that SF3a represents a loosely associated subunit of U2snRNP which is essential for the U2snRNP/pre-mRNA interaction. Since SF3b by itself binds to U2 snRNP and is required for the subsequent association of SF3a, it is possible that it represents other U2 snRNP-specific polypeptides.

107

THE ATYPICAL Sm BINDING SITE OF U7 RNA CAUSES INEFFICIENT snRNA ASSEMBLY BUT IS REQUIRED FOR U7 snRNA FUNCTION

Branko Stefanovic, Christian Grimm and Daniel Schümperli, Abteilung für Entwicklungsbiologie, Zoologisches Institut, Universität Bern, Baltzerstrasse 4, CH-3012 Bern.

The U7 RNAs from mouse, human, Xenopus and sea urchin have an atypical Sm binding site: AAUUUGUCU. In transient expression studies in Hela cells the mouse U7 RNA accumulates to 3-4 fold lower level than the U1 RNA. When its Sm binding site is mutated to the canonical sequence: AAUUUUUGG (U7 Sm CAN), nuclear accumulation and anti-Sm precipitability reaches that of U1 RNA. Transcription of the mouse U7 and U1 gene is equally efficient in Hela cells, thus the atypical Sm site must be responsible for poor assembly of the U7 snRNP, resulting in degradation of the free RNA. We have developed a functional assay for the U7 snRNP based on injection of the mouse gene into Xenopus oocytes. We show that the mouse U7 RNA is functional in Xenopus oocytes. However, the U7 Sm CAN RNA is assembled into a nonfunctional particle, although the particle does accumulate at 3-4 fold higher amount in the nucleus. The U7 Sm CAN RNP is inactive regardless of the amount of RNA transcribed from the injected gene, excluding a possibility that overexpression of the RNA titrates out a limiting assembly factor(s). UV crosslinking experiments have revealed a difference in the pattern of proteins that can be crosslinked to U7 RNA and U7 Sm CAN RNA. This suggests that positioning, conformation or compositon of proteins complexed with the U7 Sm CAN has been altered, rendering the particle inactive.

108

PURIFICATION AND cDNA CLONING OF SPLICING FACTOR SF1

Silke Backes and Angela Krämer; Département de Biologie Cellulaire, Université de Genève, CH-1211 Genève.

We are investigating the splicing of introns from nuclear pre-mRNA with components fractionated from HeLa cell nuclear extracts. At least four protein factors (SF1, SF3a, SF3b and U2AF) and U1 and U2 snRNPs are required at the onset of the reaction for the assembly of a pre-splicing complex. SF1 was purified to homogeneity. It consists of a single poly–peptide of 75 kDa which is completely heat-resistant. Tryptic peptides of SF1 were sequenced and degenerate oligonucleotides were used to screen a HeLa cDNA library. Thus far, we obtained a partial cDNA that encodes an ORF of 369 amino acids containing nine of the twelve SF1 peptides sequenced. No significant homology to proteins in current data bases was found. Northern blot analysis with HeLa poly A$^+$ RNA or RNA from different human cell lines revealed two major mRNAs of 3.0 and 3.5 kb.

The exact function of SF1 during pre-splicing complex assembly is still unclear. Neither in vitro assays nor the derived amino acid sequence revealed intrinsic activities (RNA binding, ATPase, RNA helicase, RNA annealing) that may play auxiliary roles during the splicing reaction. In a search for characteristic protein motifs a putative leucine-zipper and several possible phosphorylation sites were found within the SF1 sequence. Taken together, our results suggest that SF1 does not interact with the pre-mRNA substrate directly, but may associate with other components of the splicing machinery by protein-protein contacts.

109

Cell cycle-related changes in histone RNA 3' processing

Irmgard Haussmann, Renu Mital, Jean-Claude Schaer* and Daniel Schümperli, Abteilung für Entwicklungsbiologie, Baltzerstr. 4, CH-3012 Bern and * Pathologisches Institut, Murtenstr. 31, CH-3010 Bern

Histone mRNA is generated by a unique 3' processing reaction which is regulated during the cell cycle and in response to changes in cell proliferation. U7 small nuclear ribonucleoprotein (snRNP) participates in this reaction by basepairing of the U7 RNA 5' end with a conserved 3' spacer element of histone pre-mRNA. Additional trans-acting factors involved in the reaction are a heat labile factor (HLF) and a factor binding to the hairpin present at the 3' end of mature histone mRNA (HBF). It was previously shown that growth-arrested cells are deficient in HLF and the accessibility of the U7 snRNA 5' end to micrococcal nuclease (MN) undergoes a marked change, although these two events were reported for different cellular systems.
We are currently analysing several parameters of histone RNA processing in a variety of cellular systems. Cells to be analysed include a *ts* mouse mastocytoma cell cycle mutant (21-Tb), mouse fibroblasts that can be arrested by serum starvation (C127), and Chinese hamster ovary cells synchronised by mitotic selection. In time course experiments, we are assaying histone RNA 3' processing, the separate activities of HLF and HBF, the abundance and mobility on native gels of U7 snRNPs, snRNP structural proteins that can be UV-crosslinked to U7 snRNA and the accessibility of the U7 RNA 5' end to MN. Preliminary results with G1-arrested 21-Tb cells indicate that HLF and HBF activities are lost at an early stage, whereas structural changes in the U7 snRNP occur with more prolonged proliferation arrest.

110

Nuclear PRP20 protein is required for mRNA transport

David C. Amberg, Martin Fleischmann*, Igor Stagljar*, Charles N. Cole and Markus Aebi*; Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755, USA and *Institut für Molekularbiologie I, Universität Zürich, Hönggerberg, CH-8093 Zürich, Switzerland

The yeast PRP20 protein is highly homologous in structure and function to the RCC1 protein of higher eukaryotes. The RCC1 protein is involved in the regulation of the onset of mitosis, whereas the PRP20 protein was shown to be required for accurate and efficient mRNA metabolism. Here we report the isolation of new temperature-sensitive alleles of the *PRP20* locus. Most of the mutations affect conserved amino acid residues in the C-terminal region of the PRP20 protein. The first observable phenotype in mutant *prp20* cells when shifted from permissive to non-permissive temperature is a loss of nuclear PRP20 protein. Concomitantly, an accumulation of polyA$^+$ RNA in the nucleus is observed. The temperature-sensitive *RCC1* allele in the mutant hamster cell line tsBN2 leads to a similar accumulation of mRNA in the nucleus.

111

INTRACELLULAR TRANSPORT OF INFLUENZA VIRUS RIBONUCLEOPROTEINS

Iris Kemler and Ari Helenius, *Department of Cell Biology, Yale University Medical School, New Haven, CT 06510, USA*

Influenza virus, after binding to the cell surface, is internalized and delivered to endosomes. The low pH of this compartment has two effects on the virus. First, the interior of the virus particle is acidified, which results in the dissociation of the M1 protein from the viral ribonucleoprotein (RNP). Second, the viral hemagglutinin (HA) protein undergoes a conformational change, resulting in the fusion of the viral envelope with the endosomal membrane and release of the RNPs into the cytoplasm. Since transcription and replication of the viral genome occur in the nucleus, the RNPs have to enter this compartment. Here we show that purified RNPs, free of M1 protein, microinjected into the cytoplasm of CHO or MDCK cells efficiently migrate to the nucleus. These RNPs were prepared from virions under neutral (pH 7.0) or acidic (pH 5.5) conditions. Although both RNP preparations accumulated in the nucleus, only the pH 7.0 RNPs were able to transcribe and replicate the viral genome. At 4°C the injected RNPs remained in the cytoplasm, showing that nuclear transport of influenza vRNPs is an active process. To investigate the role of the viral M1 protein in the export of new RNPs from the nucleus, we used the temperature sensitive mutant ts51. This mutant has a defect in the M1 protein, which accumulates in the nucleus. RNPs, however, are found in the cytoplasm, indicating that M1 does not have to exit the nucleus together with the RNPs.

112

A ribosomal protein is located in the germ-line specific material of the chromatin diminuting parasite *Ascaris lumbricoides*

A. Etter, V. Bernard, M. Geiger, M. Keiser, Y. Galley, H. Tobler and F. Müller, Institute of Zoology, University of Fribourg, Pérolles, CH-1700 Fribourg

Chromatin diminution in the nematode *A. lumbricoides* leads to the formation of somatic cells containing less DNA than cells of the germ-line. We have identified a gene which is cis- and trans-spliced and which is located in the germ-line specific material. The following evidences indicate that this gene, called ALEP-1 (*Ascaris lumbricoides* eliminated protein 1), codes for a ribosomal protein. The predicted translation product shows significant homologies to proteins which are considered to be part of the small subunit of ribosomes in different species. In agreement with this finding, antibodies against ALEP-1 demonstrate that the protein is associated with the small subunit of the *A. lumbricoides* ribosome under salt conditions which are thought to be diagnostic for ribosomal proteins. The fact that this gene is included in the eliminated portion of the genome indicates a difference in somatic and germ-line ribosomes and may suggest an implication in the regulation of translation. Furthermore, the elimination process itself may be used by the organism to specifically shut down transcription of the ALEP-1 gene in cells which are not capable to do so by normal cellular mechanisms designed for gene regulation.

113

NITRIC OXIDE ACTIVATES IRON REGULATORY FACTOR IN MURINE MACROPHAGES

Hirling, H., Kühn, L.C., Wietzerbin, J. and Drapier, J.-C., U 196 INSERM, Institut Curie, Section de Biologie, 26, rue d'Ulm, 75005 Paris, France and ISREC, Genetics Unit, Epalinges, Switzerland

The synthesis of nitric oxide (NO) from L-arginine by mammalian cells is a pathway involved in many biological functions including macrophage cytotoxicity. Previous studies have shown that one target of NO-action are iron-sulfur-dependent enzymes like mitochondrial aconitase. Furthermore, in murine macrophages, which produce NO in response to stimulation with IFNγ, nitrosyl-iron can be detected supporting the idea that NO can interact with [Fe-S]-clusters. Iron regulatory factor (IRF), an RNA-binding protein modulating the expression of different proteins involved in iron metabolism, has sequence homology to mitochondrial aconitase. Recently it could be shown that IRF by itself possesses aconitase activity which is dependent on the presence of a [4Fe-4S]-cluster. Here we found that in the murine macrophage cell line RAW 264.7 the RNA-binding activity of IRF is activated after stimulation with IFNγ, due to the production of NO. IRF is also activated when incubated with the molsidomine derivative SIN-1, which spontaneously liberates NO.

114

TWO NUCLEAR ENCODED FUNCTIONS ARE SPECIFICALLY REQUIRED FOR TRANSLATION OF THE CHLOROPLAST *psbC* MESSAGE IN *CHLAMYDOMONAS*

Zerges, W. and Rochaix, J-D., Départment de Biologie Moléculaire, Université de Genève, 30 Quai Ernest-Ansermet, 1211, Genève 4

In *Chlamydomonas*, the synthesis of the 43 kd photosystem II reaction core polypeptide P6, encoded by the chloroplast *psbC* gene, is blocked by two nuclear mutations, F34 and F64. P6 synthesis was measured during 15 min pulse labeling experiments and, therefore, *F34* and *F64* were proposed to be required for translation of the *psbC* mRNA. To test this hypothesis, we have transformed the chloroplast genome with a chimeric reporter gene having the 5′ non-translated leader of *psbC* fused to the coding sequence of *aadA*, an *E. coli* gene which confers resistance to spectinomycin and streptomycin when expressed in the chloroplast. Our results from these *in vivo* expression studies and from *in vitro* RNA-protein binding experiments will be presented.

115

RECOMBINANT IRON REGULATORY FACTOR: MODULATION OF RNA BINDING BY A [4Fe-4S]-CLUSTER

Hirling, H., Emery-Goodmann, A., Kaldy, P. and Kühn, L., C., ISREC, Genetics Unit, Epalinges, Switzerland

Iron regulatory factor (IRF) modulates post-transcriptionally the expression of transferrin receptor, ferritin and erythroid aminolevulinate synthase by iron-dependent binding to a conserved RNA stem-loop structure, the iron-responsive element (IRE). The amino acid sequence of IRF shows similarity to an iron-sulfur protein, the mitochondrial aconitase. This homology includes residues of the active center with the cysteines holding a [4Fe-4S]-cluster. We expressed the protein from a full-length human cDNA in the baculovirus expression system. Recombinant IRF, when expressed in the absence or presence of iron, was exclusively active either in IRE-binding (apo-IRF) or as an aconitase ([4Fe-4S]-IRF), respectively. The apo-IRF could be converted *in vitro* to an aconitase active protein which is no longer IRE-binding. This supports the idea that the conversion between apo-IRF and [4Fe-4S]-IRF determines the RNA-binding activity in post-transcriptional gene regulation. This is supported by a site-directed point-mutant (cys 437 → ser 437) at the site of Fe-S-cluster attachment in IRF, which is constitutive in RNA-binding.

116

IDENTIFICATION OF A SECOND RNA BINDING PROTEIN WITH SPECIFICITY FOR IRON-RESONSIVE ELEMENTS

Beric R. Henderson, Christian Seiser and Lukas C. Kuhn, Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges s/Lausanne

Iron regulatory factor (IRF) is a cytoplasmic RNA-binding protein involved in regulating iron homeostasis. The IRF controls expression of ferritin and transferrin receptor mRNA post-transcriptionally, via specific binding to stem-loop iron-responsive elements (IREs) located in the untranslated regions of these mRNAs. We have observed that formation of a second RNA-protein complex in rodent cell extracts is (like IRF) regulated by iron levels. This faster migrating complex represents a specific interaction between the IRE and an as yet undefined iron-regulated protein, that is distinct from IRF, as concluded from the following evidence: a) cross-linking and V8 protease digestion reveals different peptide patterns for the two RNA-protein complexes, b) antisera raised against two different IRF peptides immunoprecipitate only the IRF bandshift complex, c) IRF could be separated from the second IRE-binding protein by ion-exchange chromatography, and only the fraction containing IRF reacted with the antibodies in Western blots, and d) the affinity of the faster migrating bandshift complex for the IRE is similar to that of IRF, as demonstrated by competition assays with related RNA stem-loop structures.

117

TRANSLATION INITIATION AND PLANT DEVELOPMENT

Owttrim, G., Brander, K., Mandel, T., Lutziger, I., and Kuhlemeier. C., Pflanzenphysiologisches Institut, Universität Bern, CH-3013 Bern

Eukaryotic translation initiation factor 4A (eIF-4A) is an RNA-dependent ATPase which functions as an RNA helicase, removing secondary structure from the 5' UTR region of mRNA during translation initiation in eukaryotic systems. eIF-4A is also the prototype member of a large gene family, the D-E-A-D box family, whose members function in a diverse range of processes including RNA splicing, cell growth, and cell development. We have characterized two divergent eIF-4A gene families in tobacco, NeIF-4A2 and NeIF-4A3, which are coordinately expressed in all tobacco organs. The NeIF-4A2 gene family consists of at least eight members which are expressed in leaves. An anti-plant eIF-4A antibody detects numerous eIF-4A isoforms whose presence changes both quantitatively and qualitatively in various organs. Furthermore at least one eIF-4A isoform is chloroplastically localized. Promoter analysis indicates that one NeIF-4A2 family member is expressed only in mature pollen. These results suggest that the expression of specific eIF-4A genes may be developmentally regulated and possibly even regulating in plants. Transgenic plants which either over-(sense) or under-(anti-sense) express NeIF-4A2 and NeIF-4A3 are currently being produced to further analyze eIF-4A function *in vivo*.

118

TRANSLATION INITIATION FACTOR EIF-4A AND ITS ENVIRONMENT IN YEAST

Buser Peter, Coppolecchia Raffaella, Fischli Andrea, Hänsel Anja, Stotz Agathe and Linder Patrick
Dept. of Microbiology, Biozentrum, CH-4056 Basel

The eukaryotic translation initiation factor eIF-4A is an RNA dependent ATPase which, together with eIF-4B, unwinds ds RNA in vitro. In higher eukaryotic cells the initiation factor 4A can also be found in the cap binding complex eIF-4F, i.e. together with the cap binding protein eIF-4E and a high molecular weight protein of 220kDa. In the yeast *S. cerevisiae* two genes, *TIF1* and *TIF2*, code for exactly the same 4A protein. We have also isolated the *S. pombe* genes encoding eIF-4A. To understand better the function of eIF-4A we carried out a mutational analysis of the *TIF* genes from *S.c.* genes encoding this factor. We have used some of these mutations to isolate suppressors in order to find genes coding for proteins interacting with eIF-4A. In this analysis we have isolated several intragenic suppressors, which restore eIF-4A function. We also have isolated gene dosage dependent suppressors using a wild type library on a multicopy plasmid. These suppressors are currently under investigation.

119

IN VITRO AND IN VIVO CHARACTERIZATION OF TOBACCO TRANSLATION INITIATION FACTOR eIF-5A

Chamot, D. and Kuhlemeier, C., Pflanzenphysiologisches Institut der Universität Bern, CH-3013 Bern

Translation initiation factor eIF-5A is a protein involved in the formation of the first peptide bond of protein synthesis. eIF-5A is also the only known protein to contain the unique post-translationally modified amino acid, hypusine, which is derived from the polyamine spermidine. In order to study the role of eIF-5A and its hypusine modification in plants, we have characterized in detail 3 members of a multigene family representing eIF-5A in tobacco. At least 2 of the genes (NeIF-5A1 and 2) are differentially expressed in various tissues at the mRNA level. Transgenic plants expressing sense or antisense constructs using NeIF-5A1 and NeIF-5A2 are being used to study the possible differential functions of these 2 genes. NeIF-5A3, a genomic clone, is virtually identical to the NeIF-5A1 cDNA. Transgenic plants expressing NeIF-5A3 promoter-GUS fusions reveal tissue specificity for this third gene member. IEF western blots also reveal a differential expression pattern of NeIF-5A polypeptides between tissues. Taken together, these data suggest that specific NeIF-5A isoforms perform different functions. The possible roles of different NeIF-5A isoforms are being investigated further in terms of the translational regulation of tissue-specific processes in tobacco.

539 (received after deadline)

Silencing of Class I Chitinase Expression in Transgenic *Nicotiana sylvestris* plants. Christian Kunz and Frederick Meins, Jr, The Friedrich Miescher Institute, Box 2543 4002 Basel

Transgenes introduced into plants can interact with homologous host genes leading to a decreased expression of both genes. We found a similar phenomenon in *N. sylvestris* containing a tobacco class I chitinase transgene. This effect, called "silencing," only occurs in plants homozygous for the transgene and is developmentally regulated. Although all young seedlings showed the high-chitinase phenotype, three patterns of expression were observed in mature plants: most plants showed uniformly high or low chitinase levels in different leaves. A few plants showed position-dependent expression which varied in different leaves. The incidence of silencing is also environmentally regulated. Approx. 20-40% of plantlets germinated from seed in closed culture vessels showed the silent phenotype at maturity in the greenhouse, whereas, none of the plants germinated and raised to maturity in the greenhouse showed this phenotype. The implications of silencing for gene technology will be discussed.

540 (received after deadline)

INDUCTION OF A NOVEL COMPENSATORY ß-1,3-GLUCANASE IN ANTISENSE TRANSFORMED TOBACCO INFECTED WITH NECROTIZING VIRUSES

Beffa, R., Neuhaus, J.-M. and Meins, F., Jr., Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel

Vacuolar class I ß-1,3-glucanses (EC 3.2.1.39) have antifungal activity and are believed to be important in defending plants against infection by pathogens. We used antisense transformation to test this hypothesis and identify other possible functions of this enzyme. Here, we report that *N. sylvestris* and tobacco plants infected with viruses, Tobacco Necrosis Virus and Tobacco Mosaic Virus, respectively, can compensate physiologically at the level of enzyme activity for deficiencies generated by antisense transformation. Virus induction of class I ß-1,3-glucanase antigen was markedly inhibited in leaves of antisense transformants of both plant species obtained with different expression vectors. In both experimental systems a serologically distinct ß-1,3-glucanase activity was induced in antisense transformants by virus infection. This serotype did not accumulate in comparable healthy or infected tissues of empty vector transformants nor was it induced in antisense transformants by stress hormone ethylene. The compensatory ß-1,3-glucanase activity is an intracellular enzyme distinct from the isoforms of tobacco ß-1,3-glucanase described previously. We conclude that plants infected by viral pathogens can specifically compensate for a deficiency in the class I isoform by producing a functionally redundant protein. This argues strongly for an important function of ß-1,3-glucanases in pathogenesis.

Cell Proliferation and Differentiation

120

Effects of ionizing radiation on proteins controlling cell cycle progression.

Hain, J., Burkart, W.* and Jaussi R., Institut für Medizinische Radiobiologie, Paul Scherrer Institut & Universität Zürich, CH-5232 Villigen-PSI; *Institut für Strahlenhygiene / BfS, Ingolstädter Landstrasse 1, D-8042 Neuherberg/München, Deutschland

After irradiation with 7 Gy X-rays, V79 Chinese hamster lung fibroblasts undergo cell cycle arrest in the S and G_2 phase. These arrests are released on completion of DNA repair. A premature release occurs after treatment of the irradiated cells with caffeine. This release is accompanied by increased activity of the $p34^{cdc2}$ ser/thr kinase. We are now investigating which of the proteins regulating the $p34^{cdc2}$ activity are affected under these conditions. Protein levels of cdc25, cyclin A, cyclin B and their complex formation with $p34^{cdc2}$ are being determined after irradiation of the cells and after caffeine release of the cell cycle arrests.

121

CONTROL OF ENTRY INTO MITOSIS AND CELL CYCLE ARRESTS INVOLVE PROTEIN KINASES AND CAFFEINE SENSITIVE COMPLEXES.

Emery, G., Jaussi, R. and Crompton, N. Institut für Medizinische Radiobiologie, Paul Scherrer Institut & Universität Zürich, CH-5232 Villigen

Using flow cytometric methods we have demonstrated that the cell cycle arrests induced by either radiation or staurosporine in the G2 phase are very similar. Both arrests are equally sensitive caffeine. Here we present data of studies where the two inhibitors are applied consecutively, and then arrest caused by the first inhibitor is selectively released. The results demonstrate that although the cells appear to block at the same position in the cell cycle, the radiation block precedes, or is "recessive", to the staurosporine block. We also present data to show that in intact cells, staurosporine and caffeine display a form of reciprocal competitive inhibition in their abilities to induce and release arrest.

122

The function of a small GTP-binding protein in the regulation of cell cycle in higher plants.

Ferenc Nagy, Thomas Merkle and Thomas Haizel Friedrich Miescher Inst. P.O. Box 2543, Basel.

The life of a dividing cell is an orderly progression of events associated with growths, DNA replication and mitosis. Stringent controls prevent late events from occurring before completion of earlier events. Incompletely replicated DNA activates a regulatory mechanism, in fungi and mammalian cells, to ensure that mitosis strictly follows S phase. Unreplicated DNA is probably detected by a complex of RCC1, a DNA binding protein, and Ran, a Ras-related small GTP-binding protein in those organisms. We cloned the tobacco homologue of the Ran gene. The Nt-Ran1 protein shows high (>80 %) homology to fungal and mammalian Ran proteins. The Nt-Ran1 gene expressed predominantly in actively dividing tissues (root tip, apex). Moreover, overexpression of the Nt-Ran1 cDNA results in the complementation of the relevant fungal mutation. Therefore we propose, that the same control system also operates in the cell cycle in plants.

123

CELL CYCLE ARREST IN S PHASE OF IRRADIATED CELLS

Crompton, N., Saydan, N. and Jaussi, R. Institut für Medizinische Radiobiologie, Paul Scherrer Institut & Universität Zürich, CH-5232 Villigen

We are investigating radiation induced cell cycle arrests. The S phase cells in populations of exponentially growing V79 hamster fibroblasts were pulse labelled with BUdR. Then, the cells were exposed to x-rays (dose = 6 Gy) and the average velocity of the labelled cells through the S phase was monitored by flow cytometry. The cells do not all arrest immediately but over a period of 2 hours. The cells were arrested for an average of 0.6 hours. This arrest is independent of the delay to cells entering S phase, which can be observed in the unlabeled cell fraction. Caffeine abrogates the arrest induced by the radiation, and there is even an indication that it causes progression through S phase to be faster than in the control.

124

EVALUATION OF DNA INDEX, % S-PHASE, ESTROGEN AND PROGESTERON RECEPTORS AND METHYLTRANSFERASE IN HUMAN BREAST TISSUES WITH CARCINOMA.

Leoni, L.M, Camponovo, A., Pelloni, G., Ghisletta, M., Losa, G.A. , Laboratorio di Patologia Cellulare, Istituto Cantonale di Patologia, CH-6604 Locarno

Breast tissue biopsies of patients with ductal invasive carcinoma (82 cases, age range 36 to 89 years old) were homogenized and nuclei were isolated by means of two different procedures (lysis or gradient centrifugation after nitrogen pulverization). DNA index and proliferative activity (% S-phase) were estimated by four mathematical algorithms after flow cytometry analysis. The remaining tumor powder was homogenized, centrifuged at 100000 g and the high speed cytosol fraction assayed for estrogen (ER) and progesteron (PR) receptors. The activity of the methyltransferase-I (PMT-I), an enzyme catalyzing the methylation of the membrane phosphatidylethanolamine to phosphatidylcholine was determined in the microsomal pellet. 70 % of samples showed aneuploid DNA index and % S-phase ranging between 5-40 % without any correlation with both ER, PR and PMT-I activity. %S-phase from nuclei prepared with the two procedures gave similar values when evaluated with three algorithms (RFIT, SOBR and POLY), whilst the SFIT model yielded significantly higher values.

125

AGE INFLUENCES THE REPLICATIVE ACTIVITY AND THE DIFFERENTIATION FEATURES OF CULTURED RAT AORTIC SMOOTH MUSCLE CELL POPULATIONS AND CLONES.

Bochaton-Piallat M-L, Gabbiani F, Ropraz P and Gabbiani G. Department of Pathology, University of Geneva, CMU, CH-1211 Geneva 4.

Arterial smooth muscle cells (SMC) implicated in the formation of atheromatous plaque (Gabbiani et al., J Clin Invest 73:148, 1984; Kocher et al., Circ Res 56:829, 1985) or cultured from adult rats (Skalli et al., J Submicrosc Cytol 18:481, 1986) show decreased levels of specific cytoskeletal proteins such as α-smooth muscle (SM) actin, desmin and SM-myosin, assuming phenotypic features of fetal SMC. We have studied phenotypic features of populations and cloned arterial SMC cultured from rats of different ages. Newborn SMC cultured in the presence of fetal calf serum maintained differentiated features up to the 5th passage whereas SMC cultured from adult and old SMC did not. Old SMC showed the most important replicative activity. Newborn and adult SMC, cloned in primary culture, continued to express α-SM actin, SM myosin and in some cases desmin at the 5th passage. Compared to their parental populations, these clones express differentiated features in culture. In contrast, clones of old SMCs were poorly differentiated and expressed α-SM actin only in 80% of the cases. Thus, the capacity of SMC to differentiate in culture decreases when animals become old. Clonal populations may be useful to study the mecanisms of SMC differentiation and of atheromatous plaque formation. (Supported by the Swiss National Science Foundation. Grant Nr 31.30796.91).

126

THE 55-kDa REGULATORY SUBUNIT OF *DROSOPHILA* PROTEIN PHOSPHATASE 2A IS REQUIRED FOR ANAPHASE

Mayer-Jaekel, R.E., Ohkura, H.*, Glover, D.M.*, and Hemmings, B.A. Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel and *CRC Cell Cycle Group, The University, Dundee DD1 4HN, Scotland.

Protein phosphatase 2A (PP2A) encompasses a family of holoenzymes with a core structure consisting of a 36-kDa catalytic subunit and a 65-kDa regulatory subunit which can be associated with a third subunit of either 54, 55 or 72 kDa. We analyzed the spatial and temporal expression of several PP2A subunits during *Drosophila* development. The transcripts encoding the two core subunits are expressed ubiquitously, but the 55-kDa subunit (PR55) transcripts are mainly found in early embryos and at lower levels in the nervous system and in the gonads. PR55 expression in these highly proliferative tissues occurs in parallel with elevated levels of the two core subunit transcripts. A mutant strain, termed *aar*^{1} for abnormal anaphase resolution, was found to contain a P-element insertion within the PR55 gene. Larval neuroblasts show an increased mitotic index, overcondensed chromosomes and defects in anaphase where either chromatin is bridging the two poles or single lagging chromosomes fail to migrate to the poles. The mutant phenotype can be rescued by the wild type PR55 gene, suggesting that the PR55 containing PP2A holoenzyme plays a specific role in cell cycle regulation.

127

CHARACTERIZATION OF A PUTATIVE HUMAN HOMOLOGUE TO THE MITOTIC PROTEIN KINASE "POLO"

R.M. Golsteyn, Schultz, S.J. and E.A. Nigg
ISREC, Epalinges, 1066

We have used the polymerase chain reaction (PCR) to isolate and characterize cDNA fragments encoding human protein kinases. This method has been succesful in that several novel protein kinases have been isolated. One of these isolates has been cloned and has 52% sequence identity to the protein kinase "Polo", characterised in the fruit fly Drosophila. The precise function of this protein is not known, but analysis of Polo mutants reveals that the gene product is essential for chromosome condensation and microtubule spindle organisation during mitosis. Experiments to examine the subcellular localization and biochemical activity of the putative human homologue of Polo are in progress.

128

NUCLEAR LOCALIZATION OF CYCLIN A REQUIRES COMPLEX FORMATION WITH CDK CATALYTIC SUBUNITS

G. Maridor, P. Gallant, S. Dayan, R. Golsteyn, and E.A. Nigg

Swiss Institute for Experimental Cancer Research (ISREC), Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland.

The cell cycle is controlled by cyclin-dependent protein kinases. While B-type cyclins associate with p34^{cdc2} to trigger entry into mitosis, progression through S phase requires cyclin A, presumably in association with p33^{cdk2}. A- and B-type cyclins display strikingly different subcellular localizations, suggesting that they may target different cdk catalytic subunits to appropriate substrates. Here, we have used N- and C-terminal deletion mutants of cyclin A to determine the structural requirements for nuclear localization, as well as for complex formation with cdk catalytic subunits. We show that deletion of residues within the centrally located "cyclin-box", or deletion of as few as 15 residues from the C-terminus of cyclin A abolishes both nuclear localization and kinase association, whereas deletion of more than 100 residues from the N-terminus is without effect on either parameter. We conclude that nuclear transport of cyclin A is not mediated by a classical intramolecular nuclear localization signal, but instead depends on the formation of multiprotein complexes involving cdk catalytic subunits.

129

IDENTIFICATION AND CHARACTERIZATION OF HUMAN PROTEIN KINASES RELATED TO THE NIMA CELL CYCLE REGULATOR

S.J. Schultz, C. Sütterlin, and E.A. Nigg

Swiss Institute for Experimental Cancer Research (ISREC), Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland

We have used a PCR-based strategy to identify human protein kinases involved in cell proliferation. Degenerate oligonucleotide primers derived from conserved amino acid motifs shared between serine/threonine - specific protein kinases were used to amplify sequences from a leukemic human cell line, HL60. Partial sequences of over 40 protein kinases were identified and two of these, HuPK21 and HuPK36, showed homology to *nimA* of Aspergillus nidulans. The *nimA* protein kinase regulates entry into mitosis and is activated independently of the mitotic regulator p34^{cdc2}. A full length cDNA for HuPK21 and a partial cDNA for HuPK36 have been obtained. The amino-terminal catalytic domains of HuPK21 and HuPK36 show 48 % and approximately 40 % identity to that of *nimA*, respectively. Studies to characterize the substrate specificities, subcellular localizations, and cell cycle regulation of these protein kinases are in progress.

130

EFFECTS OF CALRETININ ANTISENSE OLIGONUCLEOTIDES ON WiDr CELLS.

J.-Ch. Gander, B. Schwaller, V. Gotzos, M.R. Celio, Institut d' Histologie et d' Embryologie générale, Université, Pérolles, CH-1700 Fribourg.

Calretinin (CR), a member of the EF-hand family of calcium-binding proteins, is present in WiDr cells (a cell line from a human adenocarcinoma) and strong immunohistochemical staining is observed at particular moments of the cell cycle: in G_1 and in mitotic cells. We postulated that this protein could intervene in some particular phenomena controlling the cell cycle. We therefore studied the effect of down-regulating CR expression by antisense oligo nucleotides (AS, oligo thioderivatives, 3μM), taking as controls parallel cultures containing non-sense oligonucleotides (NS) or non-treated cultures. In AS-treated cultures, cells grow as compact clusters that are neither present in the NS-treated cultures nor in the controls; calretinin immuno-reactivity is strongly decreased and the CR level, as determined by a Western Blot is lowered. G_1 cells accumulate and the mitotic index approaches zero along with the duration of the culture, as established by cytophotometric mesurements.
These results demonstrate that calretinin antisense oligonucleotides down-regulate CR synthesis and this manipulation strongly affects the proliferative cycle of WiDr cells.

131

Identification of a vertebrate *cdc2* mutant which is unable to complete the G1/S transition.

Nicole Schmitz, E.A.Nigg and Viesturs Simanis.. ISREC, Chemin Boveresses 155, 1066 Epalinges.

S. pombe strains have been constructed which should permit the genetic and molecular analysis of the events which occur in late G1 when cells become committed to the mitotic cell division cycle and the initiation of DNA synthesis. A number of mutants of the chicken *cdc2* gene were constructed during the course of analysis of phosphorylation sites some of which lead to the interesting phenotype of cold sensitivity when expressed in a *cdc2* null background, when the only source of $p34^{cdc2}$ is the chicken homologue of the gene, expressed from a multicopy plasmid. In order to create a genetically tractable strain, the wild-type chicken *cdc2* gene and one mutant into the *S. pombe* genome to create a strain in which cell cycle progression is dependent upon the chicken *cdc2* gene. Analysis of this strain indicates that the cells block predominantly before replication of DNA. In order to determine whether the cells were arrested before or after the execution of the start control their ability to conjugate at the arrest point was assessed. Consistent with a block before the traverse of start and commitment to S-phase, cells were able to conjugate with high efficiency. Further support for the view that this mutant is defective only for the G1-S transition is provided by the observation that it is able to complement a *cdc2A21* mutation in *trans*. This mutant is defective only for G2 function and not traverse of start. The double mutant is viable at the restrictive temperature of either of the parents and is no longer cold sensitive. Further work will concentrate on cloning genes involved in the traverse of G1 into S phase in fission yeast.

132

Dual role of the *S. pombe cdc16* gene: requirement for regulation of septum formation and maintenance of $p34^{cdc2}$ kinase activity in mitosis.

Christian Fankhauser, John Marks, Alexandre Reymond and Viesturs Simanis.
Unité de recherches sur le cycle cellulaire, ISREC, 1066 Epalinges.

In the fission yeast *S. pombe* a number of mutants have been identified in which S-phase and mitosis continue in the absence of septum formation and cytokinesis resulting in the formation of highly elongated multinucleate cells (*cdc7, cdc11,* and *cdc14*) or in which septum formation is deregulated leading to the formation of multiple septa without cytokinesis (*cdc16*). Genetic studies have suggested that the products of these four genes may interact in regulating the formation of the septum. In order to study the mechanisms responsible for regulation of septum formation and its coordination with mitosis, we have isolated these genes. This paper describes the cloning and analysis of the *cdc16* gene. The sequence of the predicted gene product ($p34^{cdc16}$) shows homology to the *BUB2* gene of *S. cerevisiae*. When *cdc16-116* cells were shifted to the non permissive temperature mitotic $p34^{cdc2}$ kinase activity decayed rapidly and cells failed to respond normally to the absence of the mitotic spindle, initiating septum formation even when mitosis had not been completed. Deletion of the gene demonstrated that it is essential for cell proliferation: spores lacking a functional *cdc16* gene germinated, completed mitosis and then formed multiple septa without undergoing cell cleavage. The *cdc16* gene product may be part of a feedback mechanism prevent premature exit from mitosis by maintaining high levels of $p34^{cdc2}$ activity until the initiation of anaphase thereby coordinating mitosis with septation, cytokinesis and entry into the next cycle.

133

DNA-specific florescent staining of nuclei of dividing cells investigated by confocal laser scanning microscopy (CLSM) without the use of an UV-laser

Markus Dürrenberger and Rosmarie Sütterlin

Interdepartmental Electron Microscopy and M.E.-Müller-Institute, Biocenter, University of Basel, Klingelbergstrasse 70, CH-4056 Basel

Most CLSM provide only two or three wavelength maxima for excitation of fluorescently labelled probes. The commonly used DNA-specific fluorochrome DAPI is excited at UV-wavelength thus requiring a CLSM equipped with an expensive UV-laser. For some time now, Molecular Probes, Inc. has offered DNA- and RNA-specific fluorescent probes that can be excited by an Argon-ion or Argon-Krypton laser.
Here we have investigated nuclei of dividing fibroblast cell lines in an CLSM equipped with an Argon-ion laser after fluorescent double labelling the spindle apparatus (tubulin) and the chromosomes (DNA). For this purpose, the spindle apparatus was labelled with anti-tubulin antibodies coupled to TRITC (excitation 540 nm, emission 570nm) and the chromosomes with TOTO-1 (excitation 509 nm, emission 533 nm). By this labelling protocol we got excellent separation of the two fluorescent signals one coming from the spindle apparatus and the other from the chromosomes of cells in anaphase. To explore the 3-D organization of these two mitotic structures in greater detail, serial optical sections (0.3 mm apart) were recorded from which (i) projections were calculated and (ii) 3-D data stacks computed and volume-rendered.

134

MITOSIS-SPECIFIC PHOSPHORYLATION REGULATES THE ONCOGENIC POTENTIAL OF POLYOMAVIRUS MIDDLE-T

Pérez, L. and Ballmer-Hofer, K., Friedrich Miescher-Institute, P. O. box 2543, CH 4002-Basel, Switzerland

Transformation of cells by polyomavirus is mediated by middle-T antigen, a protein able to form complexes with a cellular phosphatidylinositol-3-kinase, phosphatase 2A, and several members of the src family of tyrosine kinases. Middle-T has been shown to interfere with the regulation of $pp60^{c\text{-}src}$ during the cell cycle. $pp60^{c\text{-}src}$ and middle-T are targets of a cell cycle regulated serine/threonine-specific kinase, $p34^{cdc2}$. In middle-T we identified two threonine residues in positions 160 and 291, respectively, that are targets of a cdc2-like kinase. Both aminoacids were mutated to alanine residues and the mutant proteins expressed in 3T3 cells. While the 291A mutant behaved as wild type, 160Amiddle-T and the double mutant, 160A·291Amiddle-T, were transformation-defective. Interestingly, the defective mutant proteins were still able to form all the complexes with cellular proteins known to date suggesting that additional characteristics of middle-T are required for cell transformation.

135

MYRISTYLATION AND AMINO-TERMINAL PHOSPHORYLATION ARE REQUIRED FOR ACTIVATION OF c-SRC DURING MITOSIS.

Meili, R., Kaech, S. and Ballmer-Hofer, K., Friedrich Miescher-Institute, box 2543, CH-4002 Basel, Switzerland

$pp60^{c\text{-}src}$, a cellular tyrosine kinase, becomes transiently activated during mitosis. Activation is accompanied by phosphorylation of three sites in the amino-terminal regulatory domain of the protein. $pp60^{c\text{-}src}$ is negatively regulated by phosphorylation at tyrosine 527 and it has been shown that this site is transiently dephosphorylated in mitotic cells. Here we report that a non-myristylated mutant of $pp60^{c\text{-}src}$ is not activated and only partially phosphorylated at the amino-terminus in mitotic cells. Additional mutants lacking one (TTAc-src), two (AASc-src) and three (AAAc-src) cdc2 phosphorylation sites had slightly higher kinase activity than wt $pp60^{c\text{-}src}$ in interphase cells yet were not activated during mitosis. However, all four mutant proteins were still transiently dephosphorylated at tyrosine 527 during mitosis suggesting that myristylation and amino-terminal phosphorylation may be necessary but are clearly not sufficient for mitosis-specific activation.

136

SERUM AND PROTEIN-FREE CULTURE OF CHINESE HAMSTER OVARY (CHO) CELLS

Messi, F., Department of Biotechnology, ETH Zurich, CH-8093 Zurich

Chinese Hamster Ovary (CHO) cells can be cultivated in both static and agitated culture without any supplementation of serum, proteins or complex additives such as hydrolysates and extracts. In mechanically agitated cultures, the cell growth kinetics are comparable to those observed with serum-dependent culture processes. These results are achieved by:

- Using a convenient culture medium which satisfies both physicochemical and nutritional requirements of the selected cells.
- Ensuring a homogeneous dispersion of cells and media.

Furthermore, serum-independent CHO cells grown in the chemically defined medium FMX-8 demonstrate to be a very promising culture system for expression and production of recombinant proteins in biotechnological processes. Amounts up to 25 mg ml^{-1} urokinase plasminogen activator were recovered in batch cultures of transfected serum-free growing CHO cells cultivated in spinner flasks.

137

Influence of thyroid hormones on hepatic protein kinase C-isoforms

S. Dotti-Sigrist, D. Fabbro* and A. Jakob, Biochemisches Institut, Universität, CH-4051 Basel, *Ciba AG, Pharmaceutical Dept., CH-4002 Basel, Switzerland.

It was previously shown that hypothyroid rats express higher levels of hepatic α-form of protein kinase C (PKC). In the present study we investigated PKC-subspecies in primary cultures of hepatocytes of normal and hypothyroid adult rats. Measurements of serum levels of free thyroxine (T_4) and total trijodothyronine (T_3) showed about 10% of normal levels in hypothyroid rats. Cells were cultured for 72 h and treated either with vehicle (0.1 mM NaOH) or T_3 (1 μM) for 48 h. Cytosolic proteins were separated by SDS-gelelectrophoresis. Immunoblotting was performed with monoclonal antibodies against α, $ß_1$, $ß_2$, γ, δ, ε, η and ζ PKC-isoforms and showed the presence of PKC-subspecies α, $ß_2$, δ, ε, and ζ. Hypothyroid rats had higher levels of immunodetectable α-PKC than normal rats and lower levels of the other isoforms. Treatment of cultured hypothyroid cells with T_3 reversed α-, $ß_2$- and δ-PKC levels towards normal.

138

DD PK2 ACT AS AN UNUSUAL CATALYTIC SUBUNIT OF CAMP DEPENDENT PROTEIN KINASE

Anjard, C., Etchebehere*, L., Pinaud, S., Veron*, M. and Reymond, C. D., Intitut d'Histologie de l'Université, CH-1005 Lausanne, * Institut Pasteur, 75724 Paris Cedex 15

cAMP dependent protein kinase (cAPK) plays an essential role during the development of *Dictyostelium discoideum* . We have described the *Dd PK2* gene which encodes a putative protein of 648 aa. The C-terminal half of Dd PK2 present 54% indentity with mammalian cAPKs. We previously showed that overexpression of *Dd PK2* in *Dictyostelium* results in higher PKA activity and leads to rapid development. We had conclude that Dd PK2 is a catalytic subunit of cAMP dependent protein kinase since it is physically associated with the regulatory subunit of Dd cAPK. The PKA activity is inhibitable by purified R subunit and is fully recorvered in presence of cAMP. Futhermore, Dd PK2 co-eluate with cAPK activity upon partial purification in DEAE column.

139

TRANSFORMING GROWTH FACTOR-β1 INDUCES α-SMOOTH MUSCLE ACTIN EXPRESSION IN GRANULATION TISSUE MYOFIBROBLASTS AND IN QUIESCENT AND GROWING CULTURED FIBROBLASTS

DESMOULIERE, A., GEINOZ, A., GABBIANI, F., AND G. GABBIANI
Department of Pathology, CMU, 1 rue Michel Servet, 1211 Genève 4

Granulation tissue fibroblasts (myofibroblasts, MF) develop several ultrastructural and biochemical features of smooth muscle (SM) cells, including the presence of microfilament bundles and the expression of α-SM actin, the actin isoform typical of arterial SM cells. MF have been proposed to play a role in wound contraction and in retractile phenomena observed during fibrotic diseases.

We have shown that the subcutaneous administration of transforming growth factor-β1 (TGFβ1) to rats resulted in the formation of a granulation tissue in which α-SM actin expressing MF were particularly abundant. Other cytokines and growth factors such as platelet-derived growth factor and tumor necrosis factor-α, despite their profibrotic activity, did not induce α-SM actin in MF. In situ hybridization with an α-SM actin probe showed a high level of α-SM actin mRNA expression in MF of TGFβ1-induced granulation tissue. Moreover, TGFβ1 induced α-SM actin protein and mRNA expression in growing and quiescent cultured fibroblasts. These results suggest that TGFβ1 plays an important role in MF differentiation during wound healing and fibrocontractive diseases.

140

CHANGES IN EXPRESSION AND FUNCTIONAL ACTIVITY OF G PROTEINS DURING TERMINAL DIFFERENTIATION OF MURINE ERYTHROLEUKEMIA CELLS.

Kesselring, F. and Porzig, H., Department of Pharmacology, University of Bern, CH-3010 Bern

Using differentiable (6C8) and nondifferentiable (G3) subclones of an erythroleukemia cell line (RED-1), we tested whether factors promoting differentiation or proliferation affected G protein levels and G protein-mediated cellular signalling. Erythroid differentiation initiated by human recombinant erythropoietin (rhEPO, 1U/ml) and dimethylsulfoxide (DMSO, 1%) in 6C8 was associated with a 80% decrease in antibody-reactive or ADP-ribosylated membrane levels of Gαi and Gβγ, while Gαs remained unchanged. No loss of Gαi or Gβγ was observed in G3. Upon initiation of differentiation in 6C8 cells, basal and forskolin-stimulated adenylate cyclase activity (AC) decreased by 35% and 50% respectively, but maximal isoprenaline-stimulated cAMP formation remained unchanged. rhEPO alone, promoting cell proliferation but no differentiation, caused a 3-fold rise in β-receptor density, but no change in Gαi and basal AC activity. DMSO alone inhibited cell growth, reduced basal and forskolin-stimulated cAMP formation, but had no effect on G protein levels. In G3, containing twice as much Gi than 6C8, thrombine caused a PTX-inhibitable 25% decrease in isoprenaline-stimulated cAMP accumulation. This effect was not observed in 6C8. Our results suggest that significant changes in G protein-mediated signalling occur during erythroid differentiation. The drop in Gαi may help to initiate differentiation in committed erythroid cells.

141

INFLUENCE OF BIOTIN ON THE GROWTH AND DIFFERENTIATION OF CULTURED EPITHELIAL CELLS.

Sarasin, A. D., Malanga M. and Althaus, F. R.
University of Zürich - Tierspital, Institute of Pharmacology and Toxicology, Winterthurerstrasse 260, CH- 8057 Zürich

Biotin is a water-soluble vitamin that belongs to the vitamin B complex. Biotin deficiency has been associated with skin diseases, such as squamous, seborrhoic dermatoses and skin depigmentation. We have studied the effects of pharmacological doses of biotin on the growth and differentiation of cultured epithelial cells, namely outer root sheath cells (ORSc) isolated from anagen hair follicles of both humans and farm animals (bovine, swine). A slight, but reproducible, stimulation of cell proliferation has been observed at concentrations of this vitamin in the range 10^{-8}M - 10^{-6}M. The effect of biotin on the differentation of isolated ORSc has been examined both on monolayer and organotypic cultures. The latter were made possible by the use of a special device called Combi-ring-dish (CRD). The microscopic analysis of specifically stained specimens showed that cultured ORSc can be stimulated in vitro to produce an orderly structured and differentiated tissue comparable to normal epidermis.

142

EFFECTS OF CALCITONIN GENE-RELATED PEPTIDE ON CULTURED VASCULAR SMOOTH MUSCLE CELLS

Schnüriger V., Connat J.L., Anatomie et Physiologie Comparées, 30 quai E. Ansermet, 1211 Genève 4

Calcitonin gene-related peptide (CGRP) is issued from alternative splicing of calcitonin mRNA and mainly produced in nervous tissues. This neuropeptide is present in perivascular innervation and can induce vasodilatation in vivo and in vitro. However, this peptide was recently shown to have trophic effects, e.g. it induces a dopaminergic phenotype in neurons of the olfactory bulb or increases cell-surface acetylcholine receptor numbers. This peptide also stimulates proliferation of a vas deferens smooth muscle cell line or of cultured endothelial cells. We decided to test the effects of CGRP on rat aorta vascular smooth muscle cells (VSMC) used at the 5th passage. When the density of 10^4 cells/cm^2 is used, we observed that 10^{-7}M CGRP added every two days since seeding on cells grown in 10% FCS medium decreases significantly cell proliferation. We studied the expression of a specific marker of smooth muscle differentiation, the a-smooth actin isoform. CGRP decreases the level of a-smooth actin in the cells 4 and 8 days after the beginning of treatment, as shown on immunoblots. Thus, although CGRP reduces cell proliferation, it does not seem to induce differentiation of the cultured VSMC. In vivo, CGRP innervation occurs very early during the differentiation of the vessel wall, when the cells are not yet fully differentiated. It could be possible that at this moment, it stabilizes the growth of the vessel wall.

143

Caldesmon and calponin expression in cultured smooth muscle cells from human airways.

W. Durand-Arczynska, N. Marmy and J. Durand
Institut de Physiologie, 5 rue du Musée, 1700 Fribourg

The aim of the study was to explore in cultured smooth muscle cells (SMC) from human bronchi the expression of caldesmon and calponin, two proteins able to play a role in the regulation of contraction. The cells were found to express α-smooth muscle actin (αSMA), caldesmon and calponin after several passages. This was verified by immunocytochemistry, using the avidin-biotin-peroxidase technique with monoclonal antibodies and also by immunoblotting. Between passages 3 and 7, more than 95% of cells were positive histologically for each protein and specific bands corresponding to αSMA, to the heavy isoform of caldesmon (120 KDa) and to calponin were characterized on immunoblots. The expression of the heavy isoform of caldesmon and of calponin has been reported by others[1] to disappear in cultured SMC from the first passage. In sharp contrast, the present results show for the first time that cultured SMC from human airways continue to express these proteins.

[1]Gimona et al. (1992) Eur. J. Biochem. 205, 1067-1075.

144

Calmodulin-like activity of the tumour specific oncomodulin

J.K. Blum, Q.Ye and M.W. Berchtold
Inst. of Veterinary Biochemistry, Uni. Zürich-Irchel, 8057 Zürich, Switzerland

Oncomodulin (OM) is a Ca^{2+}-binding protein structurally closely related to parvalbumin. Its expression is restricted to neoplastic tissues and the placental cytotrophoblasts. The function of OM as a calmodulin (CaM)-like enzyme modulator is controversial[1,2]. Two types of experiments demonstrate that OM can act in a similar fashion to CaM in cancerous cell lines. Firstly, antisense oligonucleotides specific to the OM ATG region inhibited growth of a chemically transformed rat fibroblast cell line (T14) in a similar way as shown for CaM specific antisense probes. Secondly, the presence of an OM binding protein of 62-64 kD was demonstrated in T14 cells and OM expressing tissues of the rat (Morris hepatoma and placenta) by overlay assays employing biotinylated OM and CaM as probes. This putative target protein could not be detected in cells or tissues that do not exhibit OM expression.

1. McManus J.P., FEBS Letts. (1981) 126, 245-249
2. Klee C.B and Heppel L.A. Biochem. Biophys. Res. Commun. (1984) 125, 420-424

145

INDUCTION OF A CALMODULIN-BINDING PROTEIN (p64) by T_3 DURING RAT BRAIN DEVELOPMENT

Krebs,J.[1] and Honegger,P.[2] [1]Institute of Biochemistry III, ETH Zentrum, CH-8092 Zürich; [2]Institute of Physiology, University of Lausanne, CH-1005 Lausanne.

In the rat brain, triiodothyronine (T_3) regulates the expression of a number of neuronal and glial membrane markers during rat brain development. Here we report the specific induction of a CaM-binding protein (p64) at a very early stage of differentiation of a fetal rat telencephalon cell culture system which can grow under chemically defined conditions. A number of CaM-binding proteins in the range of 40 - 160 kDa could be identified which bind ^{125}I-CaM in a Ca^{2+}-dependent manner but only one was specifically induced by T_3. It is a soluble protein of 64 kDa which is expressed only in those cell cultures which have been exposed to T_3. The protein is visible already at very low T_3 concentrations (3×10^{-10}M). It seems to be specifically expressed in neurons since it is also visible in the presence of Ara-C which suppresses the growth of glia cells. In addition, it can be induced by retinoic acid which is typical for proteins containing a T_3-receptor responsive element in their promoter region. It is also interesting to note that p64 can be induced by T_3 already after 6 hours at which time a differentiation of the cells is not yet morphologically visible. The protein has been partially purified using a CaM affinity chromatography and its properties will be characterized. In this respect it might be of interest that a CaM-kinase which seems to be specifically expressed in brain and in T-lymphocytes has a M_r of 64 kDa.

146

BINDING AND EFFECT OF GROWTH FACTORS (GH, IGF AND INSULIN) ON CULTURED ADULT RAT CARDIOMYOCYTES (ARC)

M.Y. Donath, H.M. Eppenberger*, M. Eppenberger-Eberhardt* and J. Zapf, Dept. of Medicine, University Hospital, 8091 Zürich; *Inst. of Cell Biology, ETH-Hönggerberg, 8093 Zürich

Insulin and type I IGF receptors were demonstrated and characterized in long-term cultures of ARC. Type I IGF receptor number/surface area increased 8-fold during 9 days, whereas insulin receptor number/surface area remained unchanged. Thus, specific upregulation of the type I IGF I receptor occurs during redifferentiation of ARC in culture. No specific binding of GH was found. IGF I, but not GH, added to the cultures, increased granular density and spreading of cells after 7 days. After 16 days, IGF I-treated cultures showed, as compared to controls, a dramatic change in the extent of cross-striation as detected with immunofluorescence staining for myomesin. At the same time, αsm-actin accumulation decreased. GH had no effect. Obviously, IGF I specifically enhances myofibril development in ARC. Furthermore, insulin and IGF I stimulated glycogen synthesis over the same concentration range despite a 100-fold difference in the affinity for each other's receptor. Most likely, each of the two hormones acts on glucose metabolism in ARC via its own receptor.

147

THE EFFECT OF TRANSFERRIN ON CHICK BRAIN AND NEURONAL RETINA CELL CULTURES IS NOT ONLY ASSOCIATED WITH IRON TRANSPORT

Bruinink A. and Birchler F., Institute of Toxicology ETH and Univ. Zürich, CH-8603 Schwerzenbach.

Transferrin (Tf) has strong growth-promoting-activity on a large variety of cell types in culture. In order to test in how far nerve cells and glia cells are selectively influenced by Tf isolated from certain species and of different degree of iron occupancy, and in how far iron can replace Tf in the culture medium, the effects of holo and apo human and chick ovo Tf, and bovine apo-Tf, on viability and differentiation in serum-free cell cultures of embryonic chick brain and neuronal retina were determined. The effects were dependent on the Tf used, the origin of the cells in the culture and the parameter measured. Peak stimulation of viability and differention was observed after the addition of chick ovo apo-Tf at concentrations close to 40 mg/l. The effects of ovo-Tf could not be mimicked by the addition of iron. Our data suggest that Tf may interfere with processes other than iron uptake.

148

THE DIFFERENTIATION OF OLFACTORY EPITHELIUM NEURONS IS REGULATED BY SOLUBLE FACTOR(S) RELEASED BY THE OLFACTORY BULB

Dutly F., Sandoz Research Institute Berne LTD
CH-3001 Bern

The neurogenesis in the olfactory epithelium may involve two types of neuronal precursors: an immediate neuronal precursor which generates two postmitotic daughter neurons; and a neuroepithelial stem cell which is presumed to be the progenitor of the immediate neuronal precursor. Neurogenesis is ongoing even in adulthood. Ablation of the olfactory bulb causes neuronal death in the olfactory epithelium. We cultured the olfactory epithelium neurons with olfactory bulb-, olfactory epithelium- and pituitary-extracts. Only the olfactory bulb extracts were able to increase the differentiation of the olfactory neurons suggesting that their development is regulated by an unknown soluble factor released by their CNS target. We tried to determine whether any of the already known growth factor (NGF, FGF, TGFα, LIF, ...) had an effects on olfactory epi-thelium neurogenesis in vitro. None of the tested factor was specific for the survival or the differentiation of olfactory neurons.

149

The Amino-Terminus of Nerve Growth Factor Is Involved in the Interaction with the Receptor Tyrosine Kinase p140^{trkA}

*Kahle, P., #Burton, L. E., #Schmelzer, C. H. and *Hertel, C.; *F. Hoffmann-La Roche Ltd., Pharma Division, Preclinical Research, CH-4002 Basel, and #Genentech, Inc., South San Francisco, CA 94080, U. S. A.

The amino-terminus of nerve growth factor (NGF) is susceptible to proteolytic cleavage. A comparison of the bioactivity of highly purified full-length recombinant human (1-118)rhNGF and N-terminal truncated (10-118)rhNGF revealed lower potency of (10-118)rhNGF with regard to early NGF responses in neuron-like PC12 cells. Approximately fifty times higher concentrations of (10-118)rhNGF than (1-118)rhNGF were required to elicit the same extent of tyrosine phosphorylation of the NGF receptor tyrosine kinase p140^{trkA}, phospholipase $C_{\gamma-1}$, and the extracellular signal-regulated kinase ERK1. A similar reduced potency for induction of the transcription factor c-Fos was observed with (10-118)rhNGF compared to (1-118)rhNGF. The lower potency of (10-118)rhNGF in triggering early responses correlated with its 50-fold lower affinity for PC12 cells, compared with full-length rhNGF (produced in CHO cells or *E. coli*). Whereas (10-118)rhNGF had a more than 300-fold lower affinity for the high-affinity receptor p140^{trkA} than (1-118)rhNGF, amino-terminal truncation of NGF changed its affinity for the low-affinity receptor p75^{NGFR} only slightly. These observations suggest that amino acids 1-9 of NGF are important for binding to the signal transducing receptor p140^{trkA}. Proteolytic cleavage of the NGF amino-terminus, therefore, reduces its potency in starting several second messenger pathways leading to neuronal differentiation of PC12 cells.

150

Protein Kinases Expressed in Aggregating Brain Cell Cultures during Myelination

Miescher,G.C., Taylor,V., Pfarr, S., Steck, A. & Honegger,P.*
Department of Research and Neurological Clinic, 4031 Basel, *Institute of Physiology, 1005 Lausanne.

Serum-free aggregating rat brain cell cultures provide sufficient cell surface and paracrine interactions between neurons and glial cells for compact myelination. We are interested in the part played in these signalling pathways by protein kinases and have used a PCR cDNA cloning approach to catalogue the protein kinase genes expressed by these cultures. Seven previously described receptor protein kinases were identified: IGF1-R, trk B, bFGF-R, c-*met*, Tyro2, Tyro1, Tyro4. We have now proceeded to characterize a novel gene which is highly expressed in the brain cultures during differentiation and myelination. Like Tyro 1 and 4 it has all the features characteristic of the eck gene family and the protein has been demonstrated to have protein tyrosine kinase activity. We have used an affinity purified antiserum to a recombinant fusion protein to identify in the cultures a prominent phosphoprotein of 110 k molecular weight. This is in agreement with the open reading frame of 873 amino acids and the two possibilities for N-glycosylation sites. Expression studies are now under way to assess whether this gene has a role in myelination.

151

CHARACTERIZATION OF TYROSINE KINASES EXPRESSED IN THE EARLY *XENOPUS* EMBRYO

André Brändli and Marc Kirschner, University of California at San Francisco, Dept. of Biochemistry & Biophysics, San Francisco, CA 94122, U.S.A.

Receptor-type tyrosine kinases are activated by growth factors and control processes such as cell proliferation, cell survival and differentiation. Tyrosine kinases are also important components of signal transduction processes occurring during the establishment of the vertebrate body plan. In *Xenopus*, expression of a dominant-negative mutant of the FGFR-1 causes truncation of posterior structures in the embryo (Amaya *et al.*, Cell **66**, 257-270). We have used RT-PCR to determine the repertoire of receptor-type tyrosine kinases expressed in the early *Xenopus* embryo. Three different tissues were examined: unfertilized eggs to determine the maternal contribution, gastrulae (st.10.25) for early zygotic expression, and dorsal blastopore lips representing nascent mesoderm. We have identified ten tyrosine kinase. Three of the clones encode presumably amphibian homologs of FGFR-4, PDGFR-α, and ECK. The remaining clones represent novel members of the EPH/ECK, PDGFR, TYK2/JAK1 and CSK subfamilies. We are currently characterizing the expression of these tyrosine kinases in the early embryo by RNase protection assays, Northern blots and *in situ* hybridizations.

152

AN ACTIVATED c-erbB-2 ONCOGENE IMPAIRS KIDNEY AND LUNG FUNCTION AND CAUSES EARLY DEATH OF TRANSGENIC MICE

E. Stöcklin, F. Botteri and B. Groner; Friedrich Miescher Institute, CH-4002 Basel

The pathogenicity of the c-erbB-2 oncogene was evaluated in transgenic mice by introducing a DNA sequence comprising the promoter-enhancer region of the MMTV LTR and an activated allele of the human c-erbB-2 growth factor receptor gene into the germ line of mice. Transgene expression was observed in kidney, lung, mammary gland, salivary gland Harderian gland and in epithelial cells of the male reproductive tract. All transgenic mice expressing the c-erbB-2 receptor died within four months. Histopathological analysis showed severe preneoplastic lesions, especially in kidney and lung. The renal tubules displayed focal dilatation and proliferation of tubular epithelial cells. Widespread hyperplasia of bronchial and bronchiolar epithelium was found in the lung, where pseudopapillary proliferation of epithelial cells narrowed the bronchial lumen. The mammary gland was the most dramatically affected organ. The glands of two parous mice were underdeveloped, lacking lobular-alveolar structures and were lactation deficient. A virgin mouse developed a focal adenocarcinoma infiltrating the mammary fat pad. The expression of the c-erbB-2 protein was always restricted to the proliferating epithelial cells. Transgenic males were sterile with epithelial hyperplasia in the epididymis, vas deferens and seminal vesicles. The transgene is not uniformly expressed in the tissues where the MMTV LTR is transcriptionally active. Scattered transgene expression coincides with epithelial hyperplasia. Preneoplastic lesions in kidney and lung most likely cause organ failure and the early death of the transgenic mice.

153

TRANSCRIPTION FACTOR LEVELS, MILK PROTEIN GENE EXPRESSION DURING MAMMARY GLAND INVOLUTION

B. Jehn, E. Costello, A. Marti, N. Keon, F. Martin and R. Jaggi
Universität Bern, AKEF, Tiefenaustr. 120, 3004 Bern

We measured AP-1 and Oct-1 transcription factor activities throughout mouse mammary gland development. Very low AP-1 levels were found at all times. Oct-1 activity was high in the virgin gland, during pregnancy and lactation. An alarming decrease of Oct-1 activity was seen at day 2 to 3 of involution and Oct-1 activity was virtually undetectable between day 3 to day 12 of involution. Both, Oct-1 mRNA measurements and *in vivo* labelling experiments showed that Oct-1 mRNA is present and Oct-1 protein is synthesised in the epithelial compartment of the mammary gland during involution. We found that the DNA-binding activity was reduced when Oct-1 prepared from lactating mammary gland or synthesised *in vitro* was phosphorylated by PKA. *In vivo* PKA enzyme activity in the mammary gland was found to be considerably high at all stages of involution. Experiments are in progress to evaluate whether proteases are also involved in the observed changes in Oct-1 DNA-binding activities. Data will be presented on the correlation between the expression of milk protein genes (ß-casein, WAP) and possible markers for apoptosis (sulfated glycoprotein, TGF-ß, Clone 54, c-jun and junD) in the involuting mouse mammary gland and the observed loss of Oct-1 DNA-binding activity.

Cell-Cell and Cell-Matrix Interactions

154

INDUCTION OF CHONDROCYTE HYPERTROPHY AND EXTRACELLULAR MATRIX CALCIFICATION BY THYROXINE (T_4) IN SERUM-FREE CULTURE.

Alini M., and Poole A.R. Joint Diseases Laboratory, Shriners Hospital, Montreal, Quebec, Canada, H3G 1A6

In order to investigate the influence of biological factors on cell maturation and cartilage mineralization in the process of endochondral ossification it is necessary to isolate and separate the growth plate chondrocytes from their different zones. Using Percoll gradient centrifugation, we have successfully developed methods to separate five different viable subpopulations (A,B,C,D, and E) of chondrocytes from fetal bovine growth plates representing different in vivo maturational stages. They can each synthesize type X collagen, an extracellular matrix and, can subsequently calcify this matrix in a time dependent manner different for each subpopulation and depending upon their maturational stage at isolation. In this study we have compared the effect of T_4 in serum-free medium, with the effect of 10% FCS on in vitro maturation of the different chondrocyte subpopulations leading to expression of the hypertrophic phenotype. Thyroxine alone induced the synthesis of type X collagen and matrix calcification ($^{45}Ca^{+2}$ incorporation) effectively as well as 10% FCS. Moreover, the hypertrophic phenotype was expressed more rapidly with T_4 when compared to FCS. These preliminary results indicate that T_4 alone is sufficient to induce the maturation of mammalian growth plate chondrocytes leading to expression of the hypertrophic phenotype which culminates in the calcification of the matrix.

155

ATTACHMENT SITES OF ARC ON DIFFERENT SUBSTRATES

Sabine Koch-Schneidemann, Peter Gehr*, Hans M. Eppenberger
Institute of Cell Biology, ETH Hönggerberg, 8093 Zürich; * Department of Anatomy, University of Berne , 3000 Berne

Adult rat cardiomyocytes (ARC) were cultivated on five different substrates: gelatine, fibronectin, laminin-nidogen complex (laminin), E8 laminin-fragment, E1 laminin-fragment. Comparitive cell attachment assays have shown that ARC prefer adhesion to E8 laminin-fragment and laminin. It was shown by video time lapse (VTL) studies that, during the redifferentiation process of ARC in culture, the morphology of ARC grown on laminin, fibronectin and gelatine is undistinguishible, whereas the size of ARC grown on E8-fragment is larger, and when grown on E1-fragment definigly smaller than ARC on the whole laminin protein. Immunostaining for vinculin combined with reflection contrast microscopy were used to visualize the focal contacts of ARC on these substrates. Quantitative measurements, done with the help of a test line system, show that the number of adhesion plaques /μm^2 on gelatine, fibronectin and laminin are about the same. On the E8 fragment more attachment sites/μm^2 and on the E1 fragment less attachment sites/μm^2were counted than on whole laminin protein. This suggests that substrates influence the number of focal contacts. Correlating these results with the observations made in the VTL recording system, one can suggest that the number of adhesion sites/μm^2 increases in very flat and large cells.

156

Cell-substrate interactions regulate differentially cytochrome P-450 isoenzymes in cultured rat hepatocytes.

B. Saad, F. A. Scholl, and P. Maier Institute of Toxicology, ETH and University of Zürich, 8603 Schwerzenbach

The influence of cell-substrate interactions on the preservation and inducibility of microsomal cytochrome P-450 isoenzymes in cultured rat hepatocytes was investigated. Hepatocytes were cultured on collagen type I (COL), laminin (LM), fibronectin (FN) or liver crude membrane fractions/collagen type I (CMF/COL). The relative contents of P-450 isoenzymes were measured in ELISA using monoclonal antibodies. Hepatocytes cultured for up to nine days on CMF/COL, retained their relative cytochrome P-450 contents at 1.5-3 fold higher than cells cultured on COL, FN or LM. After exposure of hepatocytes cultured on CMF to PB from day 3-6, CYP3A proteins were enhanced more than two-fold and, depending on the exposure level, CYP2B1/B2 increased 1.3 - 6-fold. After exposure to 3-MC, a 3-fold increase of CYP1A proteins was found in CMF/COL- but also in LM-cultures. These results indicate that CMF/COL as substrate in rat hepatocyte cultures enables the cells to respond qualitatively similar to that observed in the liver.

157

Interaction of a transformed pancreatic B-cell line with extracellular matrix components

Karin Sadoul, Philippe A. Halban and Dominique Rouiller
Laboratoires de Recherche Louis Jeantet, CMU, CH-1211 Geneva 4

The four main cell types in the islet of Langerhans are A-, B-, D- and PP-cells, secreting glucagon, insulin, somatostatin and pancreatic polypeptide respectively. Islet architecture is highly ordered and both cell-cell and cell- extracellular matrix (ECM) interactions must be important for the organization and function of the islets of Langerhans.

To establish techniques for characterizing the latter interactions we have investigated adhesion and morphology of RIN-2A cells, a transformed B-cell line, plated on different substrates. Cells adhered best to poly-l-lysine (44%) and laminin (41%) compared to fibronectin (9%) and collagen (8%). Cells stayed rounded up on fibronectin, poly-l-lysine and collagen, whereas on laminin cells were spread out.

In order to identify cell surface receptors for molecules of the ECM, we used homology PCR amplification for integrin subunits. By comparison with the GenBank, our preliminary sequences could be identified as the previously unsequenced rat homologues of the integrin subunits $\beta 1$, $\alpha 3$ and α_V.

Applying these methods to primary B-and non-B-cells will allow the characterization of islet cell integrins. Comparison of primary and transformed B-cells may give further insight into cell surface changes possibly typical of insulinoma tissue.

158

EXPRESSION OF TENASCIN AND STROMELYSIN DURING NORMAL AND MALIGNANT GROWTH OF MAMMARY EPITHELIAL CELLS

V.Djonov, H.-J.Altermatt and A.-C.Andres
Abteilung für klinisch-experimentelle Forschung, Universität Bern, Tiefenaustr. 120, 3004 Bern

In the mammary gland, the structural organization changes drastically depending on the functional state and requires a repeated degradation and reconstruction of the extracellular Matrix (ECM). Massive changes in tissue architecture are also hallmarks of tumor development. We are analyzing histochemically the expression of the ECM protein Tenascin and of the metalloproteinase stromelysin 1 in various stages of murine mammary differentiation and carcinogenesis. Both proteins are of stromal origin and surround growing epithelium at puberty and postlactational involution. Similar expression and distribution was found in early premalignant lesions of mammary glands from Wap-ras transgenic animals. In contrast to the well defined distribution and the transient presence during normal growth, the expression of both, Tenascin and Stromelysin 1 increases and expands with tumor progression. Thus, the unscheduled activation of a normal cellular program may establish aggressive tumor growth already in the earliest stages of neoplastic development.

159

N-Cadherin mediated cell contact in adult rat cardiomyocytes.

Cecilia Hertig, Sabine Koch, Monika Eppenberger and Hans M. Eppenberger.
Institute of Cell Biology, ETH Hönggerberg, 8093 Zürich

Adult rat cardiomyocytes (ARC) in culture represent an ideal system to explore key questions of cell differentiation and cell contact. These cells do not undergoe cell division and grow by cellular hypertrophy. It is assumed that extending pseudopodia establish cell-cell contact, subsequently, the intimate contact structure , called intercalated discs, is regenerated and finally electric coupling is restored.

The N-cadherin isoform plays a key role in this adhesion process dependent of Ca^{2+} in ARC. Cadherins are also involved in morphogenesis and it is believed that the precise regulation of cadherin expression at the quantitative as well as qualitative level is crucial. A major step forward to reveal the adhesion process was to adapt microinjection into ARC of cDNA cloned in an appropriate expression vector. In the present study, the expressed chicken N-cadherin is followed by immunostaining with a specific antibody which discriminates between homologous isoforms of different species.

We report here that exogenous chicken N-cadherin is indeed expressed in ARC and specifically localize in the sites of contact. Cadherin overexpression does not disturb the precise localization and function of the endogenous homologue, moreover it is involved in contact and newly synthesized adherens junctions in ARC cocultivated with fetal rat cardiomyocytes. Finally, cDNA structure modification experiments are performed in order to elucidate as how cadherin localization and function are coordinated in heart muscle.

160

LARGE (350 kDa) AND SMALL (220 kDa) VARIANT OF TYPE XII COLLAGEN: DIFFERENTIAL EXPRESSION AND LIGAND BINDING

Koch, M., and Chiquet, M., Abt. Biophysikalische Chemie, Biozentrum der Universität, CH-4056 Basel

Type XII collagen is an extracellular matrix protein associated with collagen fibrils in vivo. The molecule has three very large, extended noncollagenous domains and a short collagen tail. Its non-collagenous domains are characterized by von Willebrand factor A (vWF A) domains and fibronectin type III (FN III) repeats. While the protein isolated from embryonic tendon has subunits of 220 kDa, we have recently purified from chick embryo skin fibroblasts a very large splicing variant with subunits of 350 kDa. These contain two additional vWF A and eight more FN III repeats, as well as chondroitin sulfate chain(s). We isolated a mAb which recognizes the additional domain and used it to separate the large variant from the small variant of type XII collagen. By comparison, we found that the small form binds more strongly to fibronectin in solid phase assays than the large one. Conversely, the large form binds to heparin-Sepharose in 0.15 M NaCl while the small one does not. Both variants affect collagen fibril formation in vitro, but in a distinct manner. In embryonic chick skin, small type XII collagen is localized to a defined layer everywhere in the dermis. In constrast, the large form is confined to the basis of feather buds. Our results indicate that the two forms of type XII collagen have distinct functions in morphogenesis.

161

Immunolocalization of a Novel Heterodimeric Proteoglycan: A Building Block of Some but not All Basement Membranes

J.C. Schittny[*], *B. Breuer*[+], *H. Kresse*[+], *and P.H. Burri*[*], [*]Institute of Anatomy, University of Berne, CH-3000 Berne 9, Switzerland, and [+]Institute of Physiological Chemistry and Pathobiochemistry, University of Münster, D-4400 Münster, Germany.

A novel 760 kDa dermatan sulfate proteoglycan has recently been isolated. It consists of two disulfide cross-linked core proteins (460 kDa and 300 kDa), each carrying one or very few dermatan sulfate chains of ~20 kDa. (Breuer et al. 1991, *J. Biol. Chem.* **266**: 13224-13232). Three different antisera (anti-300, -460, 760 kDa) were raised and used to study its light and electron microscopical tissue distribution. So far, immunostaining of the proteoglycan (immunofluorescence and immunogold) could be observed in some (e.g. corneal epithelium and Descemet's membranes), but not all basement membranes (e.g. capillaries, striated muscle, and liver); and also in corneal stroma, skin, smooth muscles, and in EHS-tumor. In Descemet's membrane the proteoglycan specific staining was predominately observed next to the cell-surface of the endothelium.

162

A NOVEL LAMININ VARIANT FROM CHICK HEART

Brandenberger, R., Brubacher, D., and Chiquet, M., Abt. Biophysikalische Chemie, Biozentrum, CH-4056 Basel

Laminins comprise a family of multidomain extracellular matrix glycoproteins. The classical laminin isolated from mouse EHS tumor is composed of three polypeptides designated A (400 kD), B1 and B2 (220 kD). Apart from classical laminin, other isoforms have been found where the A chain is replaced by a related M chain or the B1 chain by an S chain.We isolated laminins from chick heart and gizzard. By rotary shadowing, cross-shaped and T-shaped particles could be observed in both preparations. On SDS-PAGE under nonreducing conditions, a pattern of three laminin bands at 700, 800 and over 1000 kD could be detected in the heart preparation. In contrast to the gizzard preparation, the heart laminin, after reduction, contained a 350 kD polypeptide which is immunologically related to the M chain. We generated monoclonal antibodies against chick heart laminin. One mAb called 8/D-3 selectively labeled the myotendinous junctions of the dermamyotome in the 6-day-old chick embryo and similar structures in the legs of older embryos. This mAb is directed against a polypeptide present in heart but not in gizzard laminin. By affinity chromatography using this mAb, we separated from other heart laminin variants an 800 kD isoform with the likely subunit composition M/S/B2. Interestingly, this variant promoted neurite outgrowth in vitro, although the S chain has been postulated to contain a stop signal to the growing neurite.

163

Adhesion is essential for invasion of wounded soybean leaf tissue by the fungal pathogen *Phytophthora megasperma* f. sp. *glycinea*

Hua Ding and Hans R. Hohl
Institut für Pflanzenbiologie, Zollikerstr.107, 8008 Zürich

Phytophthora megasperma f. sp. *glycinea* (Pmg), race 1, was used to study the role of adhesion in colonization of soybean cv. Harosoy leaf disks through their cut edges. Invasion and colonization of the tissue was inhibited in the presence of Con A, IgG or D-mannose. Light microscopy revealed that only very few germ tubes attached to the cells and attempted penetration of the tissue. Of several sugars tested only glucose reversed the effect of ConA or IgG. Neither of the three substances, Con A, IgG or D-mannose, inhibited germination or germ tube growth of the pathogen substantially. The results suggest that inhibition of infection and colonization is due to the inhibition of adhesion between the fungal parasite and the host cell walls, and that adhesion is an essential prerequisite for these processes.

164

HUMAN COLON CARCINOMA CELLS EXHIBIT DISTINCT STAINING PATTERNS IN β 1,6 BRANCHING OF ASPARAGINE-LINKED OLIGOSACCHARIDES CORRELATED WITH METASTATIC POTENTIAL

Li W-P., Zuber C. and Roth J., Division of Cell and Molecular Pathology, Department of Pathology, University of Zürich, Switzerland.

Increased β1,6 branching of asparagine-linked oligosaccharides is directly associated with tumor cell metastasis (Dennis et al., Science 1987: 236, 582). The lectin *Phaseolus vulgaris* (PHA-L) can be used to detect β1,6 branches. We tested biotin- and digoxigenin-conjugated PHA-L for lectin blotting and histochemistry. Techniques using digoxigenin-conjugated PHA-L proved to be of higher specificity for lectin blotting and both more sensitive and specific for labeling of cell and tissue sections. In lectin blots of the parental HCT 116 line and the low invasive line HCT 116b a single reactive band of ≈162 kDa was detectable. In the highly invasive line HCT 116a the intensity of this band was greatly increased and a second major band of ≈170 kDa occurred. Morphometric analysis of cell surface staining by PHA-L in electron microscopy revealed a significantly higher density of labeling in the highly invasiv cell line. In sections of sporadic human colonic carcinoma, invasiv parts of the tumor showed higher PHA-L staining compared to the normal colonic epithelium. Collectively, these data indicate that differences in PHA-L staining seem to be correlated with differences in metastatic potential and may be an adjunct technique in the prognostic evaluation of tumors.
Supported by the Sassella Stifung Zürich, Switzerland.

165

S-100 PROTEINS ARE DIFFERENTIALLY EXPRESSED IN TUMOUR PROGRESSION

Pedrocchi, M., Schäfer, B.W. and Heizmann, C. W., Department of Pediatrics, University of Zürich, Steinwiesstr. 75, CH-8032 Zürich

Members of the S-100 calcium-binding protein family are good candidates as markers defining specific stages in mammary tumour progression. Their human genes are located in a cluster on chromosome 1q21, a region which is often involved in rearrangements observed in breast cancer. Additionally CAPL, a member of this protein family, has been associated with metastatic behaviour of *mouse* mammary tumour cell lines.
We found a correlation between the expression pattern of CAPL and the pathogenesis of *human* breast cancer cell lines. In particular MDA-MB-231, a cell line for which translocations in 1q had been found cytogenetically, showed a very high expression level. The expression of other S100 proteins of the cluster, however, did not vary dramatically, which suggests individual control mechanisms for the different members of the gene cluster. We will examine additional mammary tumour cell lines and will try to verify these results for clinically well studied primary tumours. We are testing those cells, in which we see an altered expression pattern, for genomic aberrations in the gene cluster region by the use of Southern blots and Pulse field electrophoresis.
Our preliminary study shows that S-100 proteins and CAPL in particular are possible markers for a high transformation level of human breast cancer cells.

166

CHARACTERIZATION OF A SEMI-SYNTHETIC MACROMOLECULE DESIGNED FOR TUMOR THERAPY

Jaussi, R. and Larsson, B., Institut für Medizinische Radiobiologie, Paul Scherrer Institut & Universität Zürich, CH-5232 Villigen

Solid primary tumors can be removed by surgery and radiotherapy. However, recurring tumors or metastases are often not eliminated by these treatments. We have designed chimeric molecules which are expected to fulfill therapy requirements imposed by small secondary tumors which may be spread throughout the body: A targeting part of the chimeric molecule which is called "DOG" (e.g. Epidermal Growth Factor) is crosslinked to a "SLEDGE" (e.g. a synthetic DNA) which carries a "LOAD" (e.g. a radioactive bisbenzimide derivative). An example of such a "DOG-SLEDGE-LOAD" vehicle is presented and its DNA "SLEDGE" is characterized.

537 (received after deadline)

COMPLETE PRIMARY STRUCTURE OF COLLAGEN XIV

Chantal Wälchli, Judith Trueb and Beat Trueb
Biochemie I, ETH Zentrum, 8092 Zurich

We have isolated and characterized several overlapping cDNA clones for chicken collagen XIV which span a total of 6.5 kbp. These clones contain an open reading frame of 5571 bp encoding the entire collagen XIV polypeptide. The predicted polypeptide has an estimated molecular mass of 205 kDa in its glycosylated form. It is composed of 1857 amino acids which are arranged in 16 individual subdomains, including a signal peptide of 28 residues. The large amino-terminal globular domain of collagen XIV (NC3) comprises 11 of these 16 subdomains. Two of them are related to the A modules of von Willebrand factor, eight show some relationship to the type III repeats of fibronectin and one is similar to the NC4 domain of collagen IX. The carboxy-terminal triple helical domain is composed of two collagenous segments (COL1 and COL2), which make up less than 14% of the entire molecular mass, and of two short noncollagenous domains (NC1 and NC2). A detailed analysis of our cDNA clones indicates that collagen XIV exists in two alternatively spliced forms which differ by 31 amino acids in their NC1 domain. The variant form of the polypeptide contains therefore 1888 residues with a total molecular mass of 208 kDa. Our results demonstrate that collagen XIV displays a complex multidomain structure resembling the one proposed for collagen XII.

Development

167

Pattern formation gene(s) in *C. elegans* ?

C. Wittmann, T. R. Bürglin*, G. Ruvkun*, M. Kaderli, H. Tobler and F. Müller,
Institute of Zoology, University of Fribourg, Pérolles, CH-1700 Fribourg
(e-mail: Wittmann@cfruni51.bitnet)
*Department of Molecular Biology, MGH Boston, MA

We are interested in the early development of *C. elegans*. The mainly invariant cell lineage led people to believe that maternal cytoplasmic components were the major strategy to bring about specification in the early embryo. However, zygotic RNA transcription begins as early as at the 8 to 16-cell stage and is appreciable one or two cell cycles later. Such a stepwise activation of transcription is observed and well studied in *D. melanogaster* where pattern formation genes, among them the HOM-C (homeobox-complex) genes, are expressed before general transcription begins. The early activation of transcription in *C. elegans* and the discovery of a large number of *C. elegans* homologs to genes important in other developmental systems, provide growing evidences for postmaternal contribution to cell fate determination.

We are working on *ceh-13*, a *C. elegans* homeobox containing gene with labial-like structure. It belongs to a cluster of four genes with sequence homologies to genes in the same relative order in the HOM-C in *Drosophila* and vertebrates. We are analyzing its pattern of expession by monitoring β-galactosidase activity in stable transgenic lines. Our preliminary results suggest that *ceh-13* plays an important postmaternal role in the *C. elegans* development. Furthermore, we are raising a polyclonal antibody to confirm the lacZ staining pattern and to possibly identify a maternal product.

169

Cell Communication across germ layers is involved in region specific expression of the POU-box gene *pdm-1* in the *Drosophila* endoderm

Affolter, M. & Gehring, W.J., Biozentrum der Universität Basel, Klingelbergstr. 70, CH-4056 Basel

During early *Drosophila* development, the midgut forms from two different endodermal primordia located near the embryonic poles, outside the expression domains of most segmentation genes. Despite this apparent unsegmented origin of the endoderm, the larval midgut epithelium shows striking functional and morphological differentiation along its anteroposterior axis. Recent experiments have provided evidence that region specific gene expression in the midgut epithelium is dependent on the expression, in the adhering visceral mesoderm, of homeotic selector genes that trigger expression of growth factor homologues. The midgut therefore represents an ideal system to study inductive events across germ layers in the *Drosophila* embryo.

We have recently found that the expression of the *Drosophila* POU-box gene *pdm-1* is repressed in two regions of the midgut, an anterior and a central region. Repression in the central domain is dependent on induction trough the visceral mesoderm. However, *pdm-1* repression uses a different genetic cascade then that previously reported to be involved in the induction of the homeotic gene *lab* in the midgut. Interestingly, the anterior repression domain of *pdm-1* is independent of the expression of known homeotic genes and characterized genes encoding secreted signalling molecules. To study in more detail the spatial regulation of *pdm-1* and *lab* expression through cell-cell communication, we have recently initiated a novel genetic screen in order to identify additional component involved in this process. These studies should help to elucidate the molecular basis underlying the transmission of positional information across germ layers, and might also provide a key to the understanding of induction mechanisms in vertebrate development.

168

ROLE OF THE *DROSOPHILA* SEGMENTATION GENE *GOOSEBERRY* IN SPECIFYING THE LARVAL DENTICLE PATTERN

Li, X. and Noll, M., Institute for Molecular Biology II, University of Zürich, CH-8057 Zürich

Cuticular pattern formation within each segment of the *Drosophila* embryo is specified by segment-polarity genes like *gooseberry* (*gsb*), *wingless* (*wg*), and *engrailed* (*en*). Expression of *gsb*, *wg*, and *en* is activated by pair-rule proteins but soon thereafter maintained by mutual regulatory interactions. Expression of the *gsb* gene, which encodes a paired-domain as well as a homeodomain, completely depends on *wg*, the homolog of the murine *Wnt-1* gene, and is reciprocally required for maintaining *wg*, but not *en*. We further present evidence that *gsb* specifies the ventral denticle pattern, the most conspicuous metameric feature of the larva, by suppressing denticle formation in *gsb*-expressing cells and their neighboring anterior cells.

170

REGULATION OF *l(3) gro*, A GENE INVOLVED IN NEUROGENESIS

W. Jost, B. Marte, A. Preiss, Abt. Zellbiologie, Biozentrum der Universität Basel, Klingelbergstr. 70, CH - 4056 Basel

The neurogenic locus E(spl) is defined as a genomic region containig at least 15 embryonically active transcrition units. Only one of them, the *lethal(3)groucho* gene is vital, whilst none of the others appear to be essential for fly survival. Seven of this genes are highly similar and share a basic helix-loop-helix motif of transcriptional regulators. These E(spl) bHLH genes are thought to be functionally redundant. Transformation and complementation analyses have shwon that both, the *l(3)gro* and the bHLH genes are indeed involved in *Drosophila* neurogenesis. For proper function the bHLH genes require a full maternal complement of *l(3)gro*. Maternal *l(3)gro* transcripts are distributed ubiquitously in early embryos, whereas zygotic *l(3)gro* expression becomes restricted to the peripheral and central nervous system and its primordia. However, the nuclear *l(3)gro* protein can be found ubiquitously throughout embryogenesis.
Analysing the transcriptional regulation of the *l(3)gro* gene in lacZ reporter-gene constructs we found zygotic regulatory elements located around the transcription start and additionally within parts of the first intron. Interestingly, the putative regulatory intron sequences contain target sites for bHLH proteins, rendering an involvement of E(spl) bHLH proteins in *l(3)gro* regulation possible.

171

A NOVEL APPROACH TO RESOLVE TEMPORAL AND SPATIAL EXPRESSION PATTERNS OF NEUROGENIC *ENHANCER OF SPLIT* (*E(SPL)*) GENES.

B. Werner, A. Preiss, Abt. Zellbiologie, Biozentrum der Universität Basel, Klingelbergstrasse 70, 4056 Basel

The segregation of neural from epidermal precursor cells requires a cell interaction mechanism that distinguishes both cell types and directs their respective differentiation. Involved in this postulated signal transduction pathway is the neurogenic *Enhancer of split* (*E(spl)*) gene complex. Its neurogenic function is composed of at least seven very similar genes encoding proteins with a basic helix-loop-helix (bHLH) motif sharing homology with mammalian transcription factors. They are candidates for repressors of the proneural genes thereby specifying epidermal fate in the expressing cells.
Ectodermal differentiation might well be dependent on the collective activity of the bHLH genes, despite their supposedly redundant function. Therefore, we have started a detailed study of the transcriptional activity of the individual *E(spl)* bHLH genes. The spatial and temporal expression pattern is highly dynamic and a direct comparison of the staining patterns is extremly difficult. Preliminary data suggest that the spatial activity of the various *E(spl)* bHLH genes is only partially overlapping. In order to increase resolution both spatially and temporally, the embryos were accurately staged and folded open by dissection. A newly developed double labeling technique might enable us to superimpose expression patterns of two *E(spl)* bHLH genes simultaneously and relate it to that of e.g. *engrailed* as positional marker.

172

MOLECULAR CHARACTERIZATION OF THE *L(3)DISCS OVERGROWN* LOCUS IN *DROSOPHILA MELANOGASTER*

Zilian, O., Bryant, P.*, and Noll, M., Institut für Molekularbiologie II der Universität Zürich, CH-8057 Zürich and *Developmental Biology Center, University of California, Irvine, CA 92717-2280, U.S.A.

In order to understand cell proliferation in imaginal discs of *Drosophila melanogaster*, we are studying the gene *lethal(3)discs overgrown(dco)*. Depending on the combination of *dco* alleles, disc anlagen degenerate or develop to hyperplastic imaginal discs. Genetic and molecular analysis allowed us to restrict the locus to a region of 55kb, which covers at least tree genes. Four EMS alleles of *dco* were analyzed on genomic Southern blots, but showed no obvious mutation. Recently, we got a fifth X-ray allele (from John Merriam, UCLA), whose phenotype is not analyzed yet; but it allowed us to exclude one candidate gene. A second candidate contains a homeobox. A corresponding cDNA was cloned from a total disc cDNA library. *In situ* hybridization with this gene on whole-mount embryos revealed transcripts in the developing nervous system, mesoderm and endoderm. The third transcript will be analyzed as well.

173

ANALYSIS OF THE ANTENNAL PHENOTYPE IN THE *DROSOPHILA* MUTANT *LOZENGE*

Reinhard F. Stocker, Nanaë Gendre*, and Philip Batterham§*
*Institute of Zoology, University of Fribourg, 1700 Fribourg
§Department of Genetics, University of Melbourne, Australia

We have studied the effects of 22 *lozenge* (*lz*) mutations on the antennal sensilla. The antenna of strong *lz* alleles lacks basiconic sensilla (BS), but shows a significantly increased density of coeloconic sensilla (CS). Intermediate alleles have few BS, they exhibit a highly increased density of trichoid sensilla (TS) but a normal CS density. BS on the maxillary palps are weakly affected even by strong *lz* alleles. Most of the strong and intermediate mutations are partially dominant for the antennal phenotype. None of 12 selected alleles complement each other indicating that they define a single cistron. Temperature shifts of the *lz^{ts1}* allele showed that gene activity is crucial around the larval-pupal transition period. Applying restrictive temperature before pupariation results in a 'novel' phenotype that is characterized by a dramatic decrease of the TS density, whereas restrictive temperature after pupariation leads to a 'normal' intermediate phenotype. Our data suggest that the *lz* gene controls at least five different functions in the antenna: its size, the overall number and density of sensilla, the proportions of the 3 types of sensilla, and the generation of BS. A model explaining the effect of *lz* on the sensillar pattern is proposed.

174

THE ROLE OF *EMPTY SPIRACLES* IN *DROSOPHILA* HEAD DEVELOPMENT

Walldorf, Uwe and Gehring, Walter J. Dept. of Cell Biology,
Biozentrum, University of Basel, Klingelbergstr. 70, CH-4056 Basel

The segmental organization of the *Drosophila* head is achieved by a flow of positional information from maternal gene products to the zygotic gap genes. Recent genetic analysis has identified three new gap genes involved in head development. One of these genes is the *empty spiracles (ems)* gene.

Mutations in *empty spiracles* cause severe defects in the head and the Filzkörper at the posterior end are missing. The *ems* gene has a DNA binding domain, the homeodomain, and two activating domains, a proline-rich domain and an acidic domain consistent with the role of the *ems* gene as a transcription factor. A 2.4 kb RNA is expressed in two phases of embryonic development. First expression is seen at the blastoderm stage in a single anterior band, correlating with its function as an anterior gap gene. The gene is expressed in the preantennal, antennal and intercalary segments and is required for the development of the antennal sense organ, the optic lobe and parts of the head skeleton. Later during embryogenesis *ems* is expressed in the posterior spiracles as well as lateral regions of each segment where the tracheal pits form and lateral neuroblasts originate. Using ß-gal fusions we could identify at least five different regulatory elements in the *ems* promotor region responsible for tissue specific expression of the gene. Since *ems* expression is dependent on the anterior and the terminal system, we used our ß-gal fusions to identify the target sites for this regulation. A 300 bp element responsible for the early expression which is dependent on the maternal gene *bicoid*, the key gene of the anterior system, was identified and studied in detail.

This analysis should allow us to elucidate the molecular mechanisms by which the morphogen *bicoid* regulates subordinate target genes like *ems* in the *Drosophila* embryo.

175

DNA DOMAIN BOUNDARIES IN THE BITHORAX-COMPLEX

Galloni, M.*, Von Almen, G.*, Sipos, L.+, Gausz, J.+, Gyurkovics, H.+,Schedl, P.°, and Karch, F.*
*Dept. de Zoologie et Biologie animale, 154 rte de Malagnou 1224 Geneve.
+Biological Research center, POBox521 H6701 Szeged Hungary.
°Dept of Biology, Princeton University, 8544 Pinceton NJ.

In our working hypothesis, the parasegment-specific functions of the bithorax complex are organized into independent DNA domains that are sequentially activated along the chromosome. Crucial to this model is the existence of boundary regions that insulate adjacent parasegment-specific regulatory regions. We have previously described two such boundary regions (*Mcp* and *Fab-7*) that insulate *iab-4* from *iab-5* and *iab-6* from *iab-7* respectively. We will present new deletions mutants that allow us to define the *Mcp* and *Fab-7* boundaries on regions of about 500 base pairs. Chromatin studies show that the DNA has a particular structure at these sites. Sequencing analysis reveals blocs of homology between *Mcp* and *Fab-7* We are presently cloning and sequencing homologous regions from *D. virilis*.

176

ANALYSIS OF EARLY FISH DEVELOPMENT - THE ROLE OF ACTIVIN-LIKE GENES

Wittbrodt, J., Rissi, M. and Rosa, F., Biocenter of the University of Basle, CH-4056 Basel

Cell-cell interactions through diffusible factors of the TGF-ß superfamily are thought to be involved in the specification and modification of cell fate in early vertebrate embryos. Using Medaka (*Oryzias latipes*) and Zebrafish (*Brachydanio rerio*) as experimental systems we are analyzing the role of homologous and newly discovered members of the TGF-ß family in fish. Screening of cDNA libraries from Zebrafish embryos with a degenerate oligonucleotide led to the discovery of two new members of the DVR subfamily. One of these is conserved throughout evolution and differentially expressed during embryonic development. Whole mount *in situ* analysis detected the message uniformly in 4-cell embryos. During late gastrulation the message disappears from the prospective dorsal region of the embryo. In later stages a specific signal is found in distinct parts of the brain. Using a PCR based approach we could isolate three activin clones in Medaka two of which display high homology to known activins in higher vertebrates. The three clones represent three distinct genes that are expressed in ovary and testis. The Zebrafish activin ßB homologue was isolated from a Zebrafish cDNA library using the corresponding Medaka probe. When expressed in COS cells the protein exhibits a strong mesoderm inducing potential in the animal cap assay. Variants with the ability to interfere with the wildtype activin were generated. The dominant suppression of the mesoderm inducing potential of the wild type activin ßB with these mutants could be demonstrated in the animal cap assay. These mutants will allow us to investigate the role of activins as well as of the newly discovered members of the TGFß family in transgenic fish *in vivo*.

177

AN EPISOMAL VECTOR FOR MOUSE EMBRYONIC STEM CELLS

Gassmann, M.*, Donoho G. and Berg P., Department of Biochemistry, Stanford University Medical School, Stanford, CA 94305, * Physiologisches Institut, Universität Zürich-Irchel, CH-8057 Zürich, Tel. 01/257 50 51.

We describe the construction and characterization of a polyoma-based vector that is maintained extrachromosomally in mouse embryonic stem (ES) cells. Vector pMGD20neo contains the polyoma (Py) virus origin of replication harboring a mutated enhancer (PyF101), a modified gene for Py large T antigen and a neomycin resistance gene. After electroporation, the vector is replicated in embryonic stem (ES) cells and is found as an extrachromosomal element in 15% of G418 resistant clones. The size and restriction patterns of the extrachromosomal DNA were identical to the transfected DNA. However, the vector or parts of it had integrated into the genome, suggesting that the extrachromosomal DNA might derive from one or more integrated copies. In one clone (1.19), the vector was maintained at 10-30 copies per cell for at least 22 passages (74 days) in the presence of G418. After supertransfection, clone 1.19 was able to replicate and maintain a second plasmid carrying the Py origin of replication but lacking a functional gene for Py large T antigen. Two vector-containing cell lines (1.19 and 1.24) have yielded several chimeric animals upon microinjection into host blastocysts and reimplantation into the uterus of pseudopregnant mice, indicating that the expression of Py large T antigen and the presence of extrachromosomal elements do not prevent the ES cells from populating an embryo. This system offers an useful tool to manipulate and analyze gene expression in ES cells without altering their pluripotential status.

178

The expression of calretinin in mesenchymal cells during development.

E. Kiraly, D.M. Vogt, A. Poncino, M.R. Celio, Institut für Histologie und allgemeine Embryologie, Universität Perolles, CH-1700 Fribourg

Calretinin (CR), an EF-hand Ca^{2+}-binding protein, is known to be a neuronal marker in adult mammals. During development we observed CR-like immunoreactivity in mesenchymal cells located in different parts of the growing embryo. The staining was strong around somites, vessels and in the serous membranes of both the chick and the rat. Furthermore in the rat it was present in the condensed mesenchymal tissue of the growing limb buds, nipple, vibrissae and the regions of body-bending. CR positive mesenchymal cells were mainly found in regions which are involved in rapid growing and remodelling processes. In these regions the cells express the cellular binding proteins for retinoic acid. Recently it was shown that CR occurs in the mitotic spindle in a cell line in culture (WiDr), indicating a function for this protein during the cell cycle. Furthermore, CR expression in these cells was shown to be dependent on factors in the serum which are removed by the stripping of the serum with activated charcoal (e.g. steroids, thyroids, retinoids). These observations led us to the conclusion that CR is specifically expressed in mesenchymal cells which are involved in the growth of the body along certain axes. It could be one of the intracellular proteins which control cell proliferation under the control of steroids.

179

CELL TYPE-SPECIFIC EXPRESSION OF THE MOUSE TYROSINASE GENE

Beermann,F. ¶, Ganß,R.# and Schütz,G.#
¶Swiss Institute for Experimental Cancer Research (ISREC), CH-1066 Epalinges and # Institute of Cell and Tumor Biology, German Cancer Research Center, D-6900 Heidelberg

Tyrosinase (EC 1.14.18.1) is regarded as the key enzyme in melanin synthesis, and is expressed in the retinal pigment epithelium, a cell layer derived from the optic cup, and in neural crest-derived melanocytes of skin, hair follicle, choroid and iris. The tyrosinase gene has been cloned and shown to map to the well-characterized *c*-locus (albino locus) of the mouse. Fusions of tyrosinase 5' sequences to a CAT reporter gene defined the regulatory region of the gene to 270bp 5' of the transcriptional start site.Further studies demonstrated that a functional tyrosinase minigene containing either 5.5kb or 270bp of 5' sequence was able to rescue the albino phenotype in transgenic mice. The transgene was expressed in a cell type-specific manner in skin and eye. During development of the mouse, the tyrosinase gene is expressed in the pigment epithelium at day 10.5 of gestation, and, in the hair follicle, from day 16.5 onwards. This developmentally regulated expression is largely reproduced in transgenic mice. Further studies will concentrate on the promoter region to identify *cis*-regulatory elements and the possible importance of further upstream sequences.

180

FINE STRUCTURAL IMMUNOCYTOCHEMICAL LOCALIZATION OF snRNP, hnRNP AND RIBOSOMAL PROTEINS IN MOUSE SPERMATOGENESIS

Biggiogera, M.°, von Schack, M.-L.°, Martin, T.E.², Gordon, J.³, Müller, M.* and Fakan, S.°. °Centre of Electron Microscopy, University of Lausanne, 1005 Lausanne; ²Department of Molecular Genetics and Cell Biology, University of Chicago; ³ABBOT Laboratories, North Chicago; *Laboratory for Electron Microscopy, ETH, Zürich.

Ultrastructural distribution of hnRNPs, snRNPs and of ribosomal proteins P1/P2 and L7 was studied during mouse spermatogenesis and spermiogenesis using specific antibodies and immunoelectron microscopy. hnRNPs and snRNPs were identified until the spermatid elongation phase when RNA synthesis is known to cease. Labeling for ribosomal proteins was no longer detectable during the cap phase. Nucleoplasmic RNPs first occurred in nuclear structural components comparable with those described in somatic cell nuclei. In later spermiogenic stages a new type of nuclear component,resembling perichromatin granules, was observed, often in clusters. Since these components were labeled with probes recognizing nucleoplasmic snRNPs, a non-nucleolar origin of these granules is suggested.

181

Intussusceptive microvascular growth in the chicken chorio-allantoic membrane may be implemented by capillary fusion

Patan, S., Haenni, B. and Burri, P.H.
Institute of Anatomy, University of Berne, Berne, Switzerland

Intussusceptive capillary growth is a new principle of microvascular growth originally described in the lungs of rats (Caduff et al., Anat. Rec. 216, 1986; Burri and Tarek, Anat. Rec. 228, 1990) and relevant for various organ systems (Patan et al., Arch. Histol. Cytol. 55, Suppl, 1992). Instead of sprouting the capillary network expands by insertion of slender transcapillary tissue pillars that give rise to full-size intercapillary meshes. In the lung, pillar birth was induced by disk-like interendothelial contacts between opposite capillary walls.
Thin pillars were also observed in the chicken chorio-allantoic membrane (CAM) microvasculature by in vivo and transmission electron microscopy (TEM; Patan et al., Anat. Embyol., in press). TEM-analysis of horizontal serial sections of the CAM aged 7 days suggests nowa mode of pillar formation related to capillary fusion: The intervening wall between two juxtapposed capillaries is successively thinned out on both sides of a centrally located core of a tissue pillar that is formed by collagen fibrils ensheated by extensions of endothelial-like cells. Following fusion of the endothelial leaflets the wall opens up on both sides of the pillars core which results in a free intraluminal tissue pillar. These findings suggest that there are many different modes of implementation of intussusceptive growth. The results also indicate that pillar formation is closely related to growth and remodelling of the vascular system.

182

Programmed Cell Death in Mammary and Prostate Glands: Defining the Apoptotic Window

W. Bielke, S. Saurer, G. Ke, R.R. Friis, AKEF, University Bern.

Programmed Cell Death is involved in many developmental and tissue organizational processes of cells which have fulfilled their biological roles. This is true for over 90% of the secretory epithelial cells in the mammary gland which die a few days after weaning. Similarly, most prostatic epithelial cells die 2-3 days after castration of the mature male. To define the early processes leading to apoptosis, we took advantage of a well-described subtraction protocol in combination with a novel, so called "coincidence" screening method. 1st strand cDNAs deriving from a) 2-3 days involuting mouse mammary glands and from b) 2-3 days post-castration prostates were hybridized in solution with mRNAs derived from the respective pre-apoptotic organs. Apoptosis-relevant cDNAs from novel transcribed genes remain single stranded and can be separated from the excess of cDNA/mRNA hybrids via hydroxylapatite chromatography. Single stranded fractions from "a" and "b" were individually used as probes for the coincidence screening of double lifts from a mouse involuting mammary gland library. By isolating only plaques which could be detected with both probes, we identified several genes which are strong candidates for participating in programmed cell death.

Protein Structure and Function, Enzymology

183

HETEROLOGOUS EXPRESSION OF FULL LENGTH SIALYL- AND GALACTOSYLTRANSFERASE IN SACCHAROMYCES CEREVISIAE

C. Krezdorn, R. Kleene, S. Ivanov, M. Watzele, G. Watzele and E.G. Berger; Institute of Physiology, University of Zurich Switzerland;
C.H. Hokke and J.P. Kamerling; Bijvoet Center, University Utrecht

The cDNA's of full length human ß1,4 galactosyltransferase (gal-T) from HeLa cells and α2,6 sialyltransferase (sialyl-T) from HepG2 cells has been cloned by PCR, ligated into a yeast/E.coli shuttle vector and placed under the control of a constitutive PHO5 promoter. Both enzymes were expressed in yeast strain and shown to be membrane-bound and enzymatically active. Immunoprecipitation of both enzymes from ^{35}S-metabolically labeled yeast cells revealed correct translocation, N- and probably O-glycosylation.
Gal-T was purified from detergent yeast extracts and shown to be kinetically similar to the human milk enzyme. Product analysis of both gal-T and sialyl-T activity by ^{1}H-NMR revealed gal ß1-4glcNAc and Neu5Aα2-6LacNAc, respectively.
Thus, we conclude that yeast is a suitable host for the heterologous expression of mammalian glycosyltransferases.
Supported by grant 2305.1 to EGB of the KWF.

184

HETEROLOGOUS EXPRESSION OF SPECTRALLY ACTIVE HUMAN CYTOCHROME P450 2D6

Kempf, A. and Meyer, U.A., Department of Pharmacology, Biocenter of the University of Basel, CH-4056 Basel

Cytochromes P450 are hemoproteins which catalyze the oxidation of a large variety of endogenous and exogenous chemicals. Numerous isozymes have been isolated and characterized by various means. The 3-dimensional structure is only known for bacterial (soluble) forms of P450, but not for the membrane-bound form of higher organisms. The human P450 2D6 is one of the best characterized isozymes and is involved in hepatic metabolism of over 30 drugs. Although it's functional and catalytic properties are well known, the 3-dimensional structure of the protein and the exact reaction-mechanisms remain unknown. -- We are presently developing expression systems with the aim to produce large amounts of P450's in order to study their structure and function. -- Transient expression in COS-1 cells is conveniant and well established for functional assays, but the amount of expressed protein is so low, that it usually cannot be detected spectrally. -- We therefore tested bacterial expression of CYP2D6. In an attempt to produce a soluble P450 which can easily be purified, we replaced the amino-terminal 30 amino acids, which presumably are involved in membrane anchoring of the protein, by a $[His]_6$tag. Spectral analysis revealed, that up to 3 mg of heme-containing P450 protein per l of bacterial culture could be produced, but only in the presence of ∂-ÁLA (a heme precursor). Preliminary data indicate, that the protein was not associated with the membrane fraction but could be purified from the soluble fraction using Ni^{2+}-chelate affinity chromatography and DEAE-anion exchange chromatography. The major problems encountered in this system were protein aggregation and a high proportion of apoprotein. -- We are presently testing insect cell culture (baculovirus), a eucaryotic system, that has the potential for producing large amounts of protein.

185

DIFFERENT REGULATION OF NAD(P)H:QUINONE REDUCTASE AND CARBONYL REDUCTASE EXPRESSION IN HEPG2 CELLS

Ruepp, B., and Wermuth, B., Universität Bern, Chemisches Zentrallabor, Inselspital, CH-3010 Bern

The genes of various detoxication enzymes, e.g. NAD(P)H:quinone reductase (NQR), contain transcription regulatory elements in their 5'-untranslated regions. These DNA-sequences exhibit enhancer activity in response to endogenous and foreign compounds such as polycyclic aromatic hydrocarbons and phenolic antioxidants. In addition to NQR, human hepatoblastoma (HepG2) cells contain a second quinone reductase: carbonyl reductase (CR). To investigate whether xenobiotics modulate the transcription of the CR gene similarly to the NQR gene, HepG2 cells were exposed to 3-methylcholanthrene (3-MC), ß-naphthoflavone (ß-Nf) and t-butylhydroxyanisole (BHA). The transcription rates were estimated indirectly from the amount of the expressed proteins on Western blots and from the menadione reducing activity of the cell extracts using rutin and dicoumarol as specific inhibitors of CR and NQR, respectively. After 3-MC and ß-Nf treatment NQR protein and enzyme activity increased time- and dose-dependent up to 2-fold whereas CR was hardly affected. The results indicate that the expression of CR is regulated by mechanisms different from NQR and other detoxication enzymes.

186

TISSUE SPECIFIC EXPRESSION OF THE FIBRIL-ASSOCIATED COLLAGENS XII AND XIV.

Chantal Wälchli and Beat Trueb, Biochemie I, ETH Zentrum, 8092 Zürich.

Interstitial collagen fibrils form the supporting scaffold of most tissues and organs. A new group of collagens, unable to build fibrillar structures by themselves but intimately associated with collagen fibrils, has recently been described and termed FACITs (fibril-associated collagens with interrupted triple-helices). This group includes, beside type IX collagen, the two closely related collagen types XII and XIV. These collagens seem to occur only in tissues containing type I collagen fibrils to which they may bind through their conserved COL1 domains.
Using three specific cDNA probes we have begun to investigate by in situ hybridization the tissue distribution of type XII and XIV collagen relative to that of type I collagen. In tissue sections of 17-day-old chick embryos we observed expression of collagen XII and XIV in the perichondrium and periosteum, whereas the bone matrix was negative. Like collagen I, collagen XIV is present in the connective tissue structures associated with skeletal and cardiac muscle and in the walls of small blood vessels; these tissues, however, seem to be devoid of collagen XII. Because of its more restricted tissue distribution, we conclude that collagen XII may serve a more specialized function than collagen XIV which shows the same distribution pattern as type I collagen.

187

BIOGENESIS OF LACTASE IN MATURING RABBITS

Wüthrich, M., Mantei, N. & Sterchi, E., Institut für Biochemie und Molekularbiologie, Med. Kinderklinik, Universität Bern und Laboratorium für Biochemie, ETH Zürich.

In most mammals expression of lactase activity is highest during the suckling period and falls off to a low residual level after weaning. The regulatory mechanisms of surface expression of lactase are unknown. Earlier studies have suggested that post-translational events may be involved (Sterchi et al. J. Clin. Invest. 86:1329, 1990). We have studied synthesis and processing of lactase in rabbits of different age using an organ-culture system. Enzymatic activity and lactase-specific mRNA were also assayed. Enzymatic activity was highest during the suckling period and declined sharply after weaning. Concommitantly with this decline intracellular maturation of lactase was retarded, leading to the presence of an increased amount of precursor lactase. In addition, the ratio of lactase-specific mRNA/specific activity increased 4-fold after weaning. This suggests that relatively large amounts of RNA were still synthesized in post-weaned rabbits with low lactase activity. These data are further evidence that post-translational events may have a regulatory role in lactase expression.

188

EXPRESSION OF THE $L_C^{8.9}$ LINKER POLYPEPTIDE FROM THE PHYCOBILISOME OF *MASTIGOCLADUS LAMINOSUS* IN *E. COLI*. RECONSTITUTION OF THE $(\alpha\beta)_3^{APC}L_C^{8.9}$ COMPLEX

Michael Betz, Walter Sidler and Herbert Zuber.
Institut für Molekularbiologie und Biophysik, ETH-Hönggerberg, CH-8093 Zürich, Schweiz.

In *M. lam.* the *apcC* gen encodes for the $L_C^{8.9}$. A 282 bp fragment was obtained by directed PCR-amplification from the *apcABC* operon. Primers were synthesized to introduce an NcoI site at the ATG-start codon, thus inserting the fragment into the expression vector pET3d. The construct was grown in the expression strain BL21(DE3)pLysE and induced by the addition of IPTG. Inclusion bodies were produced after induction, which were shown to consist of the $L_C^{8.9}$ by N-terminal sequencing over 31 steps and by SDS-PAGE. Reconstitution of the $(\alpha\beta)_3^{APC}L_C^{8.9}$ complex was performed both with $L_C^{8.9}$, α^{APC}, β^{APC} isolated from *M. lam.* as with overexpressed $L_C^{8.9}$. The two complexes have been shown to be identical with the native complex by means of sequence, SDS-PAGE, UV/VIS-, fluorescence- and CD-spectra. NMR-experiments did not show significant structural elements for the $L_C^{8.9}$.

189

OVEREXPRESSION OF $L_C^{29.5}$ LINKER POLYPEPTIDE FROM *M. LAMINOSUS* AND RECONSTITUTION OF THE ROD-CORE COMPLEX $(\alpha\beta)_6^{PC}\cdot L_{RC}^{29.5}(\alpha\beta)_3^{AP}\cdot L_C^{8.9}$

Sidler, W.,Rüegsegger, U. and Zuber, H., Institut für Molekularbiologie und Biophysik, ETH-Hönggerberg, CH-8093 Zürich, Schweiz.

The *cpcG2* gene encoding for one of the four rod-core linker polypeptides from the phycobilisome of the cyanobacterium *M. laminosus* PCC 7603 was overexpressed in *E. coli* BL21(DE3) cells, using the T7 RNA-polymerase system. $L_{CR}^{29.5}$ was successfully reconstituted with $(\alpha\beta)_3^{PC}$ to the $(\alpha\beta)_3^{PC}\cdot L_{RC}^{29.5}$ and with $(\alpha\beta)_3^{AP}\cdot L_C^{8.9}$ to the the rod-core complex $(\alpha\beta)_6^{PC}\cdot L_{RC}^{29.5}\cdot(\alpha\beta)_3^{AP}\cdot L_C^{8.9}$. A redshift of 16.4 nm in the absorption maximum, characteristic for a correctly reconstituted $(\alpha\beta)_6^{PC}\cdot L_{RC}^{29.5}$ complex was observed. The reconstitution of $(\alpha\beta)_6^{PC}\cdot L_{RC}^{29.5}\cdot(\alpha\beta)_3^{AP}\cdot L_C^{8.9}$ resulted in a blueshift of the emission maximum at 659 nm and was only 3 nm minor than $(\alpha\beta)_3^{AP}\cdot L_C^{8.9}$.

190

EVOLUTION OF BETALAIN SYNTHESIS:EVIDENCE FOR A HORIZONTAL GENE TRANSFER

L. Bindschedler, J. Fivaz, U. Hinz & J.-P. Zryd, Uni Lausanne, Lab. de phytogénétique cellulaire, 1015 Dorigny

Betalains are yellow and violet pigments found only in plants of the order *Caryophyllales* (e.g. *Beta vulgaris* & *Portulaca grandiflora*) and in a few fungal species, such as *Amanita muscaria*. Did betalain biosynthesis evolve twice, independently, or is there a common origin?
The betalain biosynthetic pathway is short and under the control of three *loci* in higher plants. Tyrosine is hydroxylated to DOPA, which is then cleaved by Dopa-4,5-dioxygenase to give the common betalain chromophore, betalamic acid.
We analysed DOPA-dioxygenase genes and their expression in plants and fungi and present evidence for a horizontal gene exchange.

191

AN EXAMPLE OF ANTI-PEPTIDE ANTISERA AS PROBES FOR PROTEIN FOLDING

S. Bornhauser, I. Jelesarov, A. R. De Pascalis and H. R. Bosshard
Biochemisches Institut der Universität Zürich, Winterthurerstr. 190, 8057 Zürich

Ferredoxin-$NADP^+$-oxidoreductase (FNR), a chloroplast enzyme, was used as a model to study the cross-reactivity of anti-peptide polyclonal antibodies with the parent native protein. Here we report results concerning the highly mobile and exposed N-terminal segment of the protein, which is invisible in the crystal structure of FNR. Three partially overlapping peptides covering 8, 10 and 20 N-terminal residues of FNR (S8, S10, S20, respectively) were synthesized. Rabbit antisera against these peptides were tested by solid-phase and solution-phase immunoassays. Of the three antisera only the serum against S20 reacted with native FNR to a significant degree. This same serum did not react with FNR lacking 8 N-terminal residues, and the reaction with S8 and S10 was very weak. The data suggest the following: 1. Peptide S20 may possess a folded structure and, therefore, give rise to conformation specific antibodies. 2. The folded structure of S20 may resemble the structure of the X-ray invisible N-terminal segment of FNR. Currently, molecular dynamics calculations are in progress to support these conclusions.

192

MAPPING EPITOPES WITH A CONFORMATIONALLY CONSTRAINED PHAGE COMBINATORIAL LIBRARY

Coulin, F., Allet, B., Losberger, C. and Wells, T., Glaxo Institute for Molecular Biology S.A., CH - 1228 Plan-Les-Ouates/Geneva.

We have constructed a library of peptides to find sequences which specifically bind to antibodies and other receptors. A peptide cassette consisting of ten random amino acids flanked by two cysteines was expressed at the amino terminus of the adsorption protein (pIII) of the M13 bacteriophage. The two flanking cysteines were added such that under oxidising condition the peptide expressed would have a cyclic conformation. It is therefore possible to model its three dimensional structure. In addition, since the conformation is restricted it is possible to determine the three dimensional structure of the epitope or receptor binding sequence using multidimensional NMR techniques. Results using an antibody against the reverse transcriptase enzyme of the virus HIV will be discussed.

193

STUDIES ON THE REFOLDING OF YEAST PHOSPHOMANNOSE ISOMERASE

Drijard, L., Payton, M., Wells, T. and Proudfoot, A., Glaxo Institute for Molecular Biology S.A., CH - 1228 Plan-Les-Ouates/Geneva.

Phosphomannose isomerase (PMI) catalyses the interconversion of mannose-6-phosphate and fructose-6-phosphate, which is the first step in the synthesis of glycoproteins. The enzyme therefore plays a key role in fungal cell wall biosynthesis. The enzyme is a zinc dependent metalloenzyme, but it is also inhibited by the addition of excess zinc. Renaturation of the enzyme by removal of the denaturant, either by infinite dilution, dialysis or stepwise dilution, does not result in any regain of enzyme activity. Re-activation has been found to depend on the inclusion of two factors, the reducing agent, dithiothreitol, and the zinc metal ion. Inclusion of the substrate has no effect. The effects of temperature, pH and ionic strength have been investigated. The basal level of *in vitro* refolding is increased by the inclusion of the bacterial chaperones. No effect is observed from the inclusion of a non-specific protein such as BSA. The effects of the GroEL/ES complex and DnaK are reported.

194

THREE DIMENSIONAL STRUCTURE OF HUMAN INTERLEUKIN-5

Proudfoot, A.[1], Graber, P.[1], Bernard, A.[1], Milburn, M.[2], Hassell, A.[2], Jordan, S.[2] and Wells, T.[1]
[1]Glaxo Institute for Molecular Biology S.A., CH - 1228 Geneva.
[2]Glaxo Research Institute, Research Triangle Park, NC 27709, USA

Interleukin-5 (IL-5) is a lineage-specific cytokine for eosinophilpoesis and plays an important role in diseases that involve elevated levels of eosinophils, such as asthma. Human IL-5 is a disulphide linked heterodimer of 115 amino acids. Crystals of recombinant human IL-5 have been produced which diffract X-rays to a resolution of 2.0 Å. Using a methionine auxotroph as the expression system, we have produced protein which is heavy atom labelled with two selenomethionine atoms per subunit. The location of the these two heavy atoms helped to define the chain tracing. Combined with other information from heavy atom derivatives produced by metal soaks, we have used this information to determine the structure of IL-5 to a resolution of 2.4 angstroms.

195

HOMOLOGY MODELLING OF THE THRRE DIMENSIONAL STRUCTURE OF Gq ALPHA SUBUNIT BASED ON P21 RAS

Lusti-Narasimhan, M.[1], Brown, F.K.[2], Allet, B.[1], Antonsson, B.[1], Bernard, A. and Wells, T.
[1]Glaxo Institute for Molecular Biology S.A., CH - 1228 Geneva.
[2]Glaxo Research Institute, Research Triangle Park, NC 27709, USA.

To understand the relationship between structure and function of G-proteins. Molecular modelling techniques were used to construct a model by homology and computational techniques to understand the specificity of the active site for GDP, GTP and other analogues. We have constructed a partial model of the alpha subunit of Gq from mouse using homology modelling with the 1.35 Å H-Ras 21 co-ordinates (Pai et al., 1990). No attempt was made to model those regions present in G-proteins but absent in ras. AMBER was used to parametize both GTP and Mg^{2+} and dock them into Gq. A few steps of energy minimisation was then carried out to yield a low energy structure. Gq from mouse brain has been cloned (Strathmann and Simon, 1990) and well expressed in *E. coli* (in which the protein is present in the inclusion bodies) and in insect cells with baculovirus (in which it is in the soluble phase). Gq was extracted from insect cells and purified on DEAE, hydroxyapatite, MonoQ and antibody affinity columns. The active subunit was measured using a ^{3}H-GTP binding assay.

196

THREADS IN THE ZIPPER: TRIPLE HELIX FORMATION OF MUTANT COLLAGEN I FROM PATIENTS WITH OSTEOGENESIS IMPERFECTA IS DELAYED

Steinmann, B., Raghunath, M. and Bruckner, P. Dept. of Paediatrics, University of Zürich, Steinwiesstr. 75, CH-8032 Zürich, Switzerland

It has always been postulated that, for sterical reasons, substitutions of a glycine of the repeating Gly-X-Y triplets of the collagen α-chains would interfere with collagen triple helix formation. We therefore measured the kinetics of triple helix formation of procollagen I in fibroblast cultures from controls and 7 patients with osteogenesis imperfecta (OI), a heritable connective tissue disorder caused by structurally abnormal collagen I. After a 4-min pulse-labelling with ^{35}S-methionine, the appearance of proteolytically stable and thus helical collagen molecules was followed for variable chase times; in controls, 50% of the molecules were fullyhelical after 14 min. In 6 of the 7 OI cell strains, all having a single Gly→Cys substitution at positions 94, 223, 526, 691, 748, and 988 of the helical domain of the α1(I)-chain, respectively, triple helix formation of abnormal collagen containing two mutant α1(I)-chains was determined by the appearance of α1(I)-dimers, which appeared later than the normal chains. Their delay ranged from 5 to 60 min and correlated inversely with the thermal stability of abnormal collagen containing α1(I)-dimers. Folding time and melting temperature of procollagen I in the seventh cell strain with a Gly→Cys substitution at position 1017, located outside of the helical domain in the C-terminal telopeptide, were normal. Here, we directly demonstrated the hitherto postulated delay in the zipper-like folding of abnormal collagen caused by structural defects affecting the helical part of the molecule, which is responsible for the overmodification of the α-chains N-terminal to the site of the mutation.

197

Folding of coiled-coil leucine zippers: Fluorescence energy transfer measurements in model peptides

Ch. Berger, H. Wendt, L. Leder and H.R. Bosshard
Biochemisches Institut der Universität Zürich, Winterthurerstr. 190, 8057 Zürich

The stability of coiled-coil α-helix dimers dependens on hydrophobic interactions between leucine side-chains in the inner core of the the dimer structure. We used synthetic peptides, based on a 29-residue sequence [1] to study the influence of sequence changes on coiled-coil stability. Exchange of two leucines by alanine produced a less stable coiled-coil. The structure collapsed to random coil when two residues of the solvent-exposed surface were replaced by proline. Stability was tested by CD spectroscopy in the far UV region. Fluorescent donor (coumarin) and acceptor (fluorescein) groups were linked to the N-terminus to follow association and dissociation of the coiled-coil by fluorescence energy transfer. The fluorescent peptides can be used to study the influence of sequence variations and solvent parameters on the dynamics of coiled-coil formation.

[1] O'Neil, K.T. and DeGrado, W.T. (1990) Science 250, 646-651.

198

CHARACTERIZATION OF THREE MOLECULAR FORMS OF HUMAN SERUM RETINOL-BINDING PROTEIN.

Jaconi[1], S., Rose[2], K., Saurat[1], J.-H., Siegenthaler[1], G. [1]Clinique de Dermatologie, Hôpital Cantonal Universitaire and [2]Département de Biochimie Médicale, Centre Médical Universitaire, 1211 Genève 14, Switzerland.

Retinol-binding protein (RBP) is the specific blood carrier for the transport of vitamin A (retinol) from the liver stores to the target tissues. Serum of patients with chronic renal failure (CRF) shows increased level of RBP compared to normal human serum (NHS). The amino acid sequence of the RBP described until now differs from gene's sequence by one amino acid residue.

We purified RBP species from both NHS and CRF sera and we compared their physical properties (retinoid binding, PAGE-immunoblotting and masses). The level of apo-RBP in CRF as analyzed by PAGE-immunoblotting was strongly increased and its retinol affinity was altered in comparison with NHS. SDS-PAGE-immunoblotting showed two major forms of RBP in patients with CRF whereas only one was detected in NHS. Mass spectrometry of RBP showed the presence of three molecular forms which expression varies significantly between NHS and CRF. One of these forms is in accordance with the amino acids sequence deduced from the gene, suggesting that the two others forms are post-translationally processed.

Increased levels of RBP concomitantly to altered expression of RBP species in CRF might interfer with retinol metabolism and could explain a defective delivery of vitamin A to the target cells.

199

Two-dimensional crystallisation of the light-harvesting core complex of *Rhodopseudomonas marina*

Rainer Meckenstock[a], Katarina Krusche[b], Rene Brunisholz[a] and Herbert Zuber[a]. [a]Institut für Molekularbiologie und Biophysik, ETH Hönggerberg, 8093 Zürich and [b]Institut für Zellbiologie, ETH Hönggerberg, 8093 Zürich.

The photosynthetic apparatus of *Rp. marina* consists of the light-harvesting core complex (B880) where the light energy is trapped and the reaction center (RC) where the energy conversion to chemical energy takes place.

We purified the B880-complex by ammonium sulfate precipitation and subsequent ion exchange chromatography. The resulting RC free protein complex was crystallised in two dimensions by microdialysis. The appearing crystals exhibited a hexagonal lattice with a lattice constant of 102 ± 3 Å. The optical difraction pattern showed spots up to 17 Å resolution. Image processing of these highly ordered areas revealed a sixfold symmetry of the ring like structure of the B880-complex.

200

ISOLATION AND CHARACTERIZATION OF THE ANTENNA COMPLEXES FROM THE PURPLE NON-SULFUR BACTERIUM *Rhodocyclus tenuis*

Qinghui Hu, Rene A. Brunisholz and Herbert Zuber
Institut für Molekularbiologie und Biophysik, ETH-Hönggerberg CH-8093 Zürich, Switzerland

The photosynthetic apparatus of the purple non-sulfur bacterium *Rhodocyclus tenuis* DSM 109 was found to be composed of two types of light-harvesting pigment-protein complexes, i) a core (B890) complex that surrounds and interconnects the membrane-bound reaction center, ii) a peripheral (B800-868) complex that is associated with the core complex. Experiments were performed to optimize conditions for the solubilization of both types of complexes by applying a combination of several different detergents like octyl-glucoside(OG), LDAO and SDS to the photosynthetic membrane. The two types of pigment-protein complexes were separated by subsequent sucrose gradient centrifugation, and, were analyzed by means of absorption and NIR circular dichroism spectroscopy. Separation and assignment of the polypeptide components of the peripheral (B800-868) and the core (B890-RC) complexes were performed by SDS gel electrophoresis, reverse phase HPLC and protein chemical analyses. The determination of the amino acid sequences of the apoproteins is in progress.

201

ANALYSIS OF THE POLYPEPTIDE COMPOSITION AND CIRCULAR DICHROISTIC PROPERTIES OF THE CHLOROSOME FROM CHLOROFLEXUS AURANTIACUS

Lehmann, R.P., Brunisholz, R.A. and Zuber, H.
Institut für Molekularbiologie und Biophysik, ETH-Hönggerberg
CH-8093 Zürich, Switzerland

The green photosynthetic bacterium Chloroflexus aurantiacus has a strong near infra-red absorption maximum at 740 nm. This absorption band is caused by the antenna system, the chlorosome, that comprises several thousands of bacteriochlorophyll c molecules and four polypeptides with the M_r 18, 11, 5.8, 5.7 kDa. We purified the 18 and 11 kDa polypeptides on reverse phase chromatography and started with the sequence determination. The role of the different polypeptides for the organisation of the photosynthetic pigments in the entire chlorosome is not understood. With the use of limited proteolysis on isolated chlorosomes we obtained changes of the circular dichroism characteristics. The alterations of the polypeptide pattern where analysed on SDS-PAGE and reverse phase HPLC.

202

PURIFICATION, CHARACTERIZATION AND CRYSTALLIZATION OF THE OUTER MEMBRANE PROTEIN MALTOPORIN FROM *Klebsiella pneumoniae*

Keller,T.A., Lustig,A. and Rosenbusch,J.P.
Biozentrum der Universität, Abt.Mikrobiologie, CH-4056 Basel

The outer membrane of *Klebsiella pneumoniae* contains a maltose-inducible protein homologous to maltoporin (LamB) from *Escherichia coli* K12. Its gene (*lamB*) encodes the precursor, a 429 amino acid polypeptide, which was placed under the IPTG-inducible *tac* promoter control and expressed from the plasmid pCW2 (Mol.Gen.Genet.(1992) **233**, 372-378) in the *E. coli* K12 strain pop6510 (dex$^-$). The protein was extracted from the outer membrane fraction using the non-ionic detergent octyl-polyoxyethylene (oPOE) and purified by column chromatography. The pure protein fully retained its maltodextrin-binding properties as tested by affinity-chromatography on a Starch-Sepharose column. The N-terminal 10 amino acid residues of the mature protein were sequenced (P.Jenö, Abt. Biochemie) and found to be identical with the *E. coli* protein. The sedimentation coefficient ($s_{20,w}$) was determined by analytical ultracentrifugation to be 6.3 ±0.1 S, sedimentation equilibrium centrifugation gave a mass of 136 kDa. These results are in agreement with the proposed trimeric structure (mature monomer with 404 amino acid residues, calculated M_r 45300). A preliminary screening of crystallization conditions has already yielded crystals of bipyramidal morphology.

203

CRYSTAL STRUCTURE OF TRUE REACTION INTERMEDIATES: ASPARTATE AND GLUTAMATE FORM STABLE KETIMINES IN ORTHORHOMBIC CRYSTALS OF ASPARTATE AMINOTRANSFERASE

Malashkevich, V.N., Toney, M.D. and Jansonius, J.N.
Biozentrum Univ. Basel, Abt. Strukturbiologie, CH-4056 Basel

The crystal structures of the complexes of chicken mitochondrial aspartate aminotransferase with the natural substrates L-aspartate and L-glutamate have been solved and refined at 2.3 Å resolution. Sharp electron densities in the active site correspond to the ketimine intermediates of the transamination reaction. Crystal absorption spectra indicate the presence of about 80% of the ketimine intermediate (330 nm band) in both cases. The second highest populated intermediate in the complex with L-aspartate is the external aldimine (430 nm band) while it is the pyridoxamine phosphate (PMP) form in the complex with L-glutamate. Addition of oxaloacetate to the crystals soaked in L-aspartate does not significantly decrease the 330 nm band, suggesting that the PMP-form of the enzyme is present in negligible amounts. On the contrary, the changes in the solution spectrum of the same complex upon addition of oxaloacetate indicate a high fraction of free PMP form. Accumulation of the ketimine intermediate in the crystal demonstrates that in the closed form of the enzyme, which is stabilized in the crystal form studied here, it is the thermodynamically most stable of the enzyme-substrate complexes. Apparently, the closed form disfavours hydrolysis of the ketimine intermediate.

204

Monovalent cation binding sites in the crystal structure of dialkylglycine decarboxylase: a structural explanation for the effect of K^+ and Na^+ on enzyme activity

Erhard Hohenester, Michael D. Toney, Sandra W. Cowan
& Johan N. Jansonius
Biozentrum der Universität Basel, Abt. Strukturbiologie, CH-4056 Basel

Dialkylglycine decarboxylase (DGD) is a bacterial PLP-dependent enzyme that catalyses the oxidative decarboxylation of α-dialkylamino acids such as 2-methylalanine or isovaline. DGD activity depends on the presence of K^+ ions, whereas Na^+ ions show an inhibitory effect. Recently, we have solved the crystal structure of DGD by multiple isomorphous replacement and solvent flattening. The structure is now refined (R = 0.178, good stereochemistry) to a resolution of 2.1 Å. In spite of low sequence homology the overall architecture of DGD is very similar to that of aspartate aminotransferase (AAT). We have identified two putative metal binding sites in the crystal structure of DGD that are not present in AAT. Site 1 is located in close vicinity to the active site and can be occupied by either a K^+ or a Na^+ ion depending on the buffer composition. Replacement of K^+ by Na^+ affects the conformations of the active site residues S80 and Y301* suggesting a structural explanation for the effects of these ions on enzyme activity. Site 2 is found on the periphery of the molecule and is located in a tight turn at the C-terminus of an α-helix. Although we cannot completely rule out the possibility that this site is a highly unusual Ca^{++} site, we believe that it is occupied by a Na^+ ion in the crystal structure of DGD thus representing a novel type of metal binding site in a protein.

205

Protein-Protein and Protein-Drug Interactions in a decameric Cyclophilin Cyclosporin Complex.

*Gaston M. Pflugel, Michel Sanner, *Tilman Schirmer, Joerg Kallen
*Johan N. Jansonius and Malcolm D. Walkinshaw
*Biocenter, University Basel and Sandoz Pharma AG, Basel

Human cyclophilin (Cyp) an ubiquitous intracellular protein of 165 amino acids is the likely target for the cyclic undecapeptide immunosuppressant drug cyclosporin A (CsA). Cyclophilins also have a peptidyl-prolyl isomerase (PPIase) activity and a chaperone activity by which they enhance the rate of protein folding. The crystal structure of a decameric cyclophilin cyclosporin complex has been recently solved by molecular replacement[1]. This structure provides the first detailed picture of the protein-drug interaction between **Cyp** and **CsA**. **Cyp** has an eight stranded anti-parallel β-barrel structure. The PPIase active site is located in a cleft on the surface of the barrel. **CsA** binds in this cleft like a coin in a slot. Two monomeric **Cyp-CsA** complexes form an intimate twofold-related dimer. A local fivefold axis builts up a decamer from such a dimer. The decamer has a doughnut-like structure in which the **CsA** molecules, largely excluded from contact with solvent, form a double inner ring, sandwiched above and below by **Cyp** pentamers. A detailed analysis of the monomeric **Cyp-CsA** receptor/drug interaction will be presented along with the other **Cyp-Cyp** protein/protein and **Cyp-CsA** protein/drug interactions found in the supramolecular "decameric **Cyp-CsA** sandwich".

1. *Gaston M. Pflügl, et al. (1992), THE X-RAY STRUCTURE DETERMINATION OF A DECAMERIC CYCLOPHILIN CYCLOSPORIN CRYSTAL COMPLEX
Nature, accepted for publication.

206

OVERPRODUCTION, LARGE-SCALE PURIFICATION AND CHARACTERIZATION OF THE CATALYTIC DOMAIN OF HUMAN POLY(ADP-RIBOSE) POLYMERASE

Höfferer L.*, Simonin F.§, Panzeter P.*, De Murcia G.§, Muller S.§, and Althaus F.R.*
§ IMBC of CNRS, Institute of Biochemistry, 15 rue René Descartes, 67084 Strasbourg, France
* University of Zürich - Tierspital, Institute of Pharmacology and Toxicology, Winterthurerstrasse 260, 8057 Zürich

The cDNA encoding the 40 kDa carboxy-terminal domain of human poly(ADP-ribose) polymerase was inserted in an expression vector, the recombinant protein was overproduced in E. coli, and purified to homogeneity. The 40 kDa catalytic domain has the same affinity (Km) for NAD^+ as the full-length enzyme, expresses abortive NAD^+ glycohydrolase activity, catalyzes the initiation, elongation, and branching of ADP-ribose polymers, but exhibits no DNA dependency. Its specific activity is approximately 500 fold lower than that of the whole enzyme, fully activated by DNA strand breaks. Surprisingly, the catalytic domain exhibits the processive mode of polymer attachment typical of full length poly(ADP-ribose)polymerase and is able to modify histones H1 and H2B. Finally, the polymer sizes formed by the 40 kDa fragment are influenced by histone H1.

207

FLUORESCENCE SPECTROSCOPIC EVIDENCE FOR DIFFERENCES IN SUBSTRATE BINDING CAPACITY BETWEEN OCTAMERIC AND DIMERIC MITOCHONDRIAL CREATINE KINASE

M. Gross, R. Furter, H.M. Eppenberger, T. Wallimann, Swiss Federal Institute of Technology, Institute for Cell Biology, CH-8093 Zurich, Switzerland

Mitochondrial creatine kinase (Mi-CK) forms octamers consisting of four stable homodimers and is supposed to participate in the formation of contact sites between the mitochondrial membranes. Previously, some evidence for limited active site accessibility in the octamer and slight differences in enzyme activity between octameric and dimeric Mi-CK have been reported by others. In the present study a 25% decrease in tryptophan fluorescence during the time course of octamer dissociation by the transition state-analogue complex ($Cr+Mg^{2+}$+nitrate+ADP) is demonstrated, and is shown to be due to enhanced quenching of an active site trp residue by the nucleotide substrate, suggesting a higher substrate binding capacity for the Mi-CK dimer in comparison to the octamer. Upon enzymatic removal of ADP, reoctamerization can also be followed on-line by monitoring a biphasic fluorescence increase. The individual dissociation and reassociation rate constants represent useful parameters for mechanistic studies on the octamer-dimer transition and for the systematic comparison of Mi-CK mutants. Dissociation rates of mutated proteins are presented and correlated with enzyme kinetical parameters. Both slower and faster decaying mutants have been investigated.

208

Mitochondrial creatine kinase activity is affected by amino acid changes at the reactive cysteine$_{278}$

Rolf Furter, Elizabeth M. Furter-Graves and Theo Wallimann; Institute for Cell Biology, Swiss Federal Institute of Technology ; ETH-Hönggerberg, 8093 Zürich.

Mitochondrial creatine kinase transfers a phosphate from ATP to creatine, yielding phosphocreatine. Previous investigations have shown that cysteine 278 can be chemically modified with sulfhydryl-specific reagents or creatine analogues, leading to an inactive enzyme.

We have taken a molecular approach to further elucidate the role of cys_{278} in the chicken cardiac mitochondrial creatine kinase (Mi_b-CK). Using site directed mutagenesis and heterologous expression of the protein in *E.coli*, the cys_{278} was replaced by a glycine and a serine. Under standard conditions C278G and C278S were reduced 40 to 250 fold in their enzymatic activities. The activity of both mutant proteins could be activated 5 to 75 fold by Cl^- anions. In contrast, the wild type protein is slightly inhibited by Cl^- anions. In addition, the pH optima of the mutant proteins are about 1 unit lower. Under optimal reaction conditions the mutant proteins show a V_{max} that is only about twofold lower than for the wildtype, but the K_m for creatine and phosphocreatine is about 10 to 20 fold increased. The mutant proteins are 5 to 10-fold more sensitive to inhibition by free ADP and ATP than the wild type protein. Our data suggest that cys_{278} plays an important role in maintaining the correct shape of the active site in Mi_b-CK for substrate binding, but the residue is not important for catalysis.

209

F102W rat parvalbumin as a fluorescent probe for studies on metal binding affinities and cation dependent conformational changes.

T.L. Pauls[1], I. Durussel[2], T. Petrova[2], J.A. Cox[2], I.D. Clark[3], A.G. Szabo[3] and M.W. Berchtold[1]. [1]University of Zürich-Irchel, 8057 Zürich, Switzerland; [2]Univerisité de Genève, 1211 Genève, Switzerland; [3]National Research Council, Ottawa, Canada K1A OR6.

Rat Parvalbumin (PV), an EF-hand type Ca^{2+}-binding protein, was expressed in E. coli and mutated by replacing a Phe at position 102 with a unique Trp in order to introduce a fluorescent label into the protein. Mass spectroscopy and NMR data indicated that the recombinant proteins rPV_{WT} and rPV_{F102W} have the expected molecular weight and retain their native structure, respectively. Both proteins contain two non-cooperative Ca^{2+}/Mg^{2+}-binding sites with single intrinsic affinity constants K_{Ca} of 2.4 10^7 M^{-1} and K_{Mg} of 2.9 10^4 M^{-1} for rPV_{WT} and K_{Ca} of 2.7 10^7 M^{-1} and K_{Mg} of 4.4 10^4 M^{-1} for rPV_{F102W}. Based on the highly similar metal binding properties of rPV_{WT} and rPV_{F102W} the latter protein was used to study cation dependent conformational changes. Trp-fluorescence emission and UV-difference spectra of PV_{F102W} indicate that the Trp-residue at position 102 is confined to a hydrophobic core. Upon Ca^{2+}- or Mg^{2+}-binding the structural organization of the region around the Trp is re-inforced and an electron-withdrawal effect takes place. Spectral studies indicate that in the Mg^{2+}-conformation the Trp-environment is less hydrophobic than in the Ca^{2+}-conformation. The conformational change upon binding Ca^{2+} increases linearly from 0 to 2 cations bound, indicating that the binding of both Ca^{2+}-ions contribute equally to the structural organization in this protein.

210

Aspartate Aminotransferase Y70H: Catalytic Activity and Substrate Specificity

Peng PAN, Rolf JAUSSI, Heinz GEHRING, §Sergio GIANNATTASIO and Philipp CHRISTEN. Biochemisches Institut der Universität Zürich, CH-8057 Zürich, Switzerland

Y70 of chicken mitochondrial aspartate aminotransferase (AspAT) was replaced with a histidine residue by oligonucleotide-directed mutagenesis. AspAT Y70H retained 13% of the activity of the wild type enzyme toward dicarboxylic substrates, whereas the activities toward aromatic amino acids were only about 0.6% of that of the wild type enzyme, corresponding to a 22-fold increase in the ratio of the activities toward these two types of substrates. The k_{cat}/K'_m values for aspartate and 2-oxoglutarate were decreased 40- and 70-fold, respectively. The spectrophotometrically determined pK_a value of the internal aldimine formed between pyridoxal 5'-P and K258 in the mutant enzyme was similar to that in the wild type enzyme. The dissociation rate constant of pyridoxamine 5'-P in AspAT Y70H proved to be increased only 3 times in contrast to the 80-fold increase in *E. coli* AspAT Y70F [Toney, M. D. & Kirsch, J. F.(1987) *JBC 262*, 12403]. These results as well as CD spectra of the coenzyme moiety indicate that the replacement of Y70 with an unprotonated histidine residue leaves the active site topochemistry essentially unchanged. However, upon protonation of H70 (pK_a' = 6.3) the catalytic activity of the mutant enzyme is reversibly abolished.

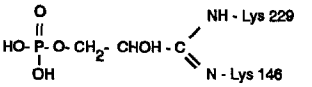
§*From the Centro di Studio sui Mitocondri e Metabolismo Energetico, C.N.R.,Bari, Italy*

211

STRUCTURE OF THE CROSSLINK FORMED AT THE ACTIVE SITE OF PARACATALYTICALLY INACTIVATED FRUCTOSE-1,6-BISPHOSPHATE ALDOLASE

Gupta, S., Hollenstein, R.‡†, Kochhar, S., and Christen, P., Biochemisches Institut and ‡Organisch-chemisches Institut der Universität Zürich, CH-8057 Zürich.

Oxidation of carbanionic enzyme-substrate intermediates by extrinsic oxidants may result in irreversible inactivation of certain enzymes (*EJB 63*, 223, 1976). Fru-1,6-P_2 aldolase was paracatalytically modified with [U-^{14}C] fru-1,6-P_2 plus $K_3Fe(CN)_6$ and digested with pronase. The isolated radioactive peptide contained 1 mol of a phosphorylated C_3 moiety per mol. Amino acid sequencing of a chymotryptic fragment had previously shown that the polypeptide chain was covalently crosslinked at active-site K229 and K146 (*PNAS 76*, 2527,1979). The structure of the crosslink was now investigated by MS, ^{1}H- and ^{13}C - NMR. Apparently, an amidine group connects the ε-amino groups of the two lysine residues:



†Deceased on 23 July, 1992

212

INTRODUCTION OF A HISTIDINE RESIDUE AT POSITION 17, 37 OR 140 OF ASPARTATE AMINOTRANSFERASE. EFFECTS ON REACTION AND SUBSTRATE SPECIFICITY

*Vacca, R.A., Christen, P. and Sandmeier, E., Biochemisches Institut der Universität Zürich, CH-8057 Zürich

Aspartate aminotransferase (AspAT) catalyzes the slow racemization of amino acids which results from protonation of the coenzyme-substrate adduct at C_α from the *re* side. Ionizable groups being absent on the *re* side, this protonation is effected by a water molecule, the diffusion of which into the interior of the enzyme is rate-limiting [*Eur. J. Biochem. 203*, 563, 1992]. In an attempt to enhance the rate of racemization, W140, I17, on the *re* side, and V37, in the plane of the coenzyme pyridine ring, were separately replaced by histidine residues. AspAT W140H racemizes alanine one order of magnitude faster (k_{cat} 3 x 10^{-2} min^{-1}) than the wild type enzyme. AspAT I17H slowly decarboxylates aspartate to alanine (k_{cat} 2 x 10^{-3} min^{-1}). AspAT W140H, I17H and V37H retain 20, 20, and 60 %, respectively, of the wild type activity toward dicarboxylic substrates, whereas in all three mutants activity toward aromatic amino acids is decreased by two orders of magnitude. Pyridoxamine 5'-phosphate dissociates from AspAT W140H 52 times faster than from the wild type enzyme.

**From the Centro Studi sui Mitocondri e Metabolismo Energetico, C.N.R., Bari, Italy*

213

CHANGING THE REACTION SPECIFICITY OF A B_6 ENZYME. NEWLY GENERATED BETA-DECARBOXYLASE ACTIVITY IN ASPARTATE AMINOTRANSFERASE R386A/Y225R

Graber, R., Sandmeier, E., Berger, P., Christen, P., Biochemisches Institut der Universität Zürich, CH-8057 Zürich.

The reaction specificity of pyridoxal 5'-phosphate-dependent enzymes is thought to be determined in part by stereoelectronic control. Orientation of a bond at Cα orthogonal to the coenzyme-substrate π system facilites its cleavage. Indeed, in aspartate aminotransferase (AspAT) the Cα–H bond of the substrate moiety is positioned in this way by interaction of its α-carboxylate group with R386, one of the four residues invariant in all aminotransferases. In order to change the orientation of the enzyme-bound substrate relative to the coenzyme ring, R386 was replaced by alanine and a new arginine residue was introduced at position 225 by oligonucleotide-directed mutagenesis. With this altered substrate binding site, AspAT R386A/Y225R decarboxylates L-aspartate to L-alanine 100 times faster (k_{cat} = 1.4 min^{-1}) than the wild type enzyme, whereas the transaminase activity towards dicarboxylic amino acids is decreased by 3 orders of magnitude (k_{cat} = 11 min^{-1}). Two reaction paths are followed: decarboxylation of L-aspartate to L-alanine and transamination-decarboxylation of L-aspartate to pyruvate.

214

MODIFICATION OF THE REACTIVITY OF THIOREDOXIN *f* BY SITE-DIRECTED MUTAGENESIS

DelVal, G., Maurer, F., Bonny, Ch., Spielmann, A., Stutz, E. and Schürmann, P., Laboratoire de Biochimie végétale, Université, CH-2000 Neuchâtel

Reduced thioredoxin *f* is an essential and specific activator protein of chloroplast fructose-1,6-bisphosphatase. Compared to the other plant thioredoxins it contains in the C-terminal part a unique third Cys residue which is surface exposed and easily modified by chemical modification (e.g.N-ethylmaleimide) and which may, in part, be responsible for the high specificity. The chemical modification results in reduced efficiency of activation. We have now replaced this Cys-73 by three different amino acids, Gly, Ser or Ala and studied the effects of these mutations on the properties of thioredoxin *f*. Whereas the Cys-73-Gly mutation produces a very unstable protein that may be partially uncorrectly folded, the other mutations, Cys-73-Ser and Cys-73-Ala, yield apparently correctly folded, stable proteins. However, there activation efficiencies are considerably reduced when compared to the wild type protein suggesting that Cys-73 is an important residue for the protein-protein interaction.

215

Fluorescence Studies with the III^{man} Subunit of the Mannose Transporter of *E. coli*.

Z. Marković-Housley, B. Stolz, K. Flükiger and B.Erni, Institute of Biochemistry, University of Berne, Freiestr. 3, 3012 Berne.

The functional form of the hydrophilic subunit III^{man} is a dimer. Each monomer of III^{man} consists of two structurally and functionally distinct domains, P13 and P20, which are both transiently phosphorylated. There are four tryptophan residues per monomer in positions 12, 33, 69 (P13 domain) and 182 (P20 domain). Intrinsic fluorescence studies were performed with III^{man}, the P13 and P20 domains, as well as with the following tryptophan to phenylalanine mutants: W12F, W69F and W12,69F. Results from fluorescence emission studies and fluorescence quenching by acrylamide indicate that Trp12 is a surface residue, Trp182 and Trp69 are buried, and Trp33 is intermediate in location. Analyses of fluorescence intensity measurement suggest that energy transfer occurs between Trp69 and Trp12. Trp12 is near His10 which is transiently phosphorylated. Trp12 probably plays an important role in the phosphoryl transfer reaction since its substitution by Phe results in a drastic decrease of enzyme activity.

216

Cysteine Mutants of the IIIMan Subunit of the Mannose Transporter of *E. coli*

B. Stolz, M. Huber and B. Erni, Biochemisches Institut d. Univ. Bern, Freiestr. 3, 3012 Bern.

IIIMan consists of two domains, P13 and P20, which are transiently phosphorylated on His10 and His175, respectively. IIIMan functions as a dimer and does not contain any cysteines. Cysteines were introduced at different positions to provide heavy-metal binding sites for crystallization. His86 was replaced by Asn. The mutant proteins were purified. The H10C, H175C, and H86N mutants are inactive. S72 has 5% of the wild-type activity. K48C and S110C have normal activity. DTNB modification of cysteines shows easy accessability of all cysteines with the exception of C10. Heterodimers between H10C and H175C, S72C and H175C, H10C and H86N, and between S72C and H86N are partly active while the other combinations of mutants are inactive. These results suggest that residues from different domains form functional units, and that the structural domains P13 and P20 do not coincide with the functional units.

217

ACETYLCHOLINESTERASE FROM MONKEY BRAIN: GLYCOSYLATION AND HYDROPHOBIC ANCHORING

Jian Liao[1], Bent Nørgaard-Pedersen[2], and Urs Brodbeck[1]
1) *Institute of Biochemistry and Molecular Biology, University of Bern, CH-3012 Bern, Switzerland,* and 2) *Department of Clinical Biochemistry, Statens Seruminstitut, DK-2300 Copenhagen S, Denmark*

Monkey brain acetylcholinesterase (AChE, EC 3.1.1.7) consists of about 15% hydrophilic, salt-soluble enzyme (SS-AChE), and of 83% amphiphilic, detergent-soluble form (DS-AChE). Sucrose density gradient centrifugation showed that SS-AChE was composed of about 85% tetramer (10.3 S) and 15% monomer (3.3 S). In amphiphilic DS-AChE, 85% tetramer (9.7 S), 10% dimer (5.7 S), and 5% monomer (3.2 S) were seen. The enzyme is N-glycosylated, no O-linked carbohydrate could be detected. Use of two monoclonal antibodies, one directed against the catalytic subunit, the other against the hydrophobic anchor, gave new insights on the subunit assembly of brain AChE. It is shown that in tetrameric AChE, not all of the subunits are disulfide bonded, and that two populations of tetramers exist, one carrying one, the other two hydrophobic membrane anchors.

218

MONOCLONAL ANTIBODIES AGAINST BRAIN ACETYLCHOLINESTERASES (ACHE) WHICH RECOGNIZE THE SUBUNITS BEARING THE HYDROPHOBIC ANCHOR

Jian Liao[1], Vibeke Mortensen[2], Bent Nørgaard-Pedersen[3], Claus Koch[2], and Urs Brodbeck[1], 1) *Institute of Biochemistry and Molecular Biology, University of Bern, CH-3012 Bern, Switzerland,* and *2) Department of Immunology,* and 3) *Department of Clinical Biochemistry, Statens Seruminstitut, DK-2300 Copenhagen S, Denmark*

Three monoclonal antibodies, mAb 132-4 (IgG_1), 132-5 (IgG_1), and 132-6 (IgG_3), specific for amphiphilic AChE from mammalian brain, were shown to mainly react with native tetrameric forms of AChE but not with dimeric and monomeric forms indicating that assembly of the tetrameric form of AChE by the hydrophobic anchors is essential for the epitope. Western blots after SDS-PAGE in non-reducing conditions showed that the mAbs reacted with tetramers, trimers, and heavy dimers carrying the hydrophobic anchors but not with light dimers or monomers carrying no anchor. Since in reducing conditions the mAbs did not recognize the hydrophobic anchor, it is concluded that disulfide bonding contributes to the epitope. The mAbs crossreacted with brain AChE from several sources indicating that the structure of the hydrophobic anchor and the assembly of mammalian brain AChE are similar.

219

DISTRIBUTION OF GLYCOSYL-PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE D mRNA IN BOVINE TISSUE SECTIONS

Stadelmann, B., Zurbriggen, A. and Brodbeck, U.
Institute of Biochemistry and Molecular Biology and Institute of Animal Neurology, University of Bern, CH-3012 Bern

It has been reported that mammalian serum, and to a lower extent mammalian liver, brain, pancreas, udder, and milk, contain glycosyl-phosphatidylinositol-specific phospholipase D (GPI-PLD) activity. However, the sites of synthesis have not been determined. In order to study in which cell(s) of the organism synthesis of GPI-PLD takes place, we undertook a systematic screening of 12 different bovine tissues. *In situ* hybridization experiments with a specific anti-sense RNA probe, derived from a bovine liver cDNA, revealed that GPI-PLD mRNA is present in mast cells of adrenal gland, lung, and liver. On the other hand, our probe detected no GPI-PLD mRNA in bovine pancreas, brain, and udder, although enzyme activity has been reported in these tissues. Northern blot analysis of total bovine liver RNA demonstrated two distinct GPI-PLD mRNAs of approximately 3.3 kb and 4 kb length suggesting that two forms of the enzyme may exist.

220

APOLIPOPROTEIN A-I IS AN ACTIVATOR OF GPI- SPECIFIC PHOSPHOLIPASE D (GPI-PLD)

Höner, M.[1], Bolli, R.[2], and Brodbeck, U.[1]
1) Institut für Biochemie und Molekularbiologie, Universität Bern, and
2) Zentrallaboratorium Blutspendedienst SRK, Bern, Switzerland

In bovine serum, GPI-PLD occurs in unusually high amounts of about 40 µg/ml. The purified enzyme is an amphiphilic protein the majority of which appears to be associated with the high density lipoprotein (HDL) fraction. The amphiphilic properties of GPI-PLD and its interaction with apolipoprotein A I (apo A), the major constituent of HDL, was investigated by sucrose density gradient centrifugation. In presence of 0.1 % TX-100 in the gradient, the enzyme sedimented with an apparent sedimentation constant of 6.0 S and formed aggregates up to 14.5 S in absence of detergent. In presence of apo A, the enzyme became desaggregated and formed a complex with apolipoprotein which sedimented at 7.6 S. In the aggregated state, the enzyme is virtually inactive; activation was seen by increasing amounts of apo A. Half maximal stimulation was obtained at a 10 fold molar excess of apo A over GPI-PLD. Desaggregation and stimulation of GPI-PLD was also obtained with TX-100 with optimal activity at 0.018%, the CMC of the detergent. From our data we conclude that in serum, GPI-PLD is in association with apo A which could provide a vehicle for the uptake of GPI-PLD into cells.

221

BIOSYNTHESIS OF GLYCOSYL-PHOSPHATIDYLINOSITOL PROTEIN ANCHORS IN MOUSE LYMPHOMA CELL LINES

Alessandro Puoti and Andreas Conzelmann*
From the Institute of Biochemistry, University of Lausanne and the *Institute of Biochemistry, University of Fribourg (CH)

We have structurally characterized two very polar glycolipids (CP_a and CP_b) which can be observed after metabolic labeling of mouse lymphoma cell lines S1A and EL-4 with either tritiated *myo*-inositol, mannose or ethanolamine. The glycosyl-phosphatidylinositol (GPI) protein anchor of S1A cells has also been characterized and it appears that the structure of its carbohydrate moiety is identical to the one of the CP_b lipid, namely: (*X*-)Manα1,2Manα1,6(*Y*-)Manα-GlcN-Inositol, *X* and *Y* being hydrofluoric acid-sensitive substituents (most likely *phosphoethanolamine*).
Mutant lymphoma $S1A^{-b}$, $EL\text{-}4^{-f}$ and BW^{-e} cell lines do not synthesize CP lipids, but accumulate abnormal inositol-containing lipids which are not present in the corresponding wild type cell lines. These lipids have been characterized and their structure suggests they are potential precursors of GPI anchor biosynthesis. As mutant lymphoma $S1A^{-b}$, $EL\text{-}4^{-f}$ and BW^{-e} accumulate GPI-related glycolipids having 3, 2 or 0 mannose residues respectively, we propose that these mutants are deficient in GPI anchor biosynthesis and that the accumulation of these incomplete GPI structures is due to the lack of the specific processing enzymes.

222

Betalain Biosynthesis: The Role of Tyrosine Hydroxylase

L. Müller, U. Hinz & J.-P. Zryd, UniL, Lab. de phytogénétique cellulaire, 1015 Dorigny

The betalains are a class of natural pigments found only in plants of the order *Caryophyllales* and in some fungi. In nature, the majority of pigment producing plants accumulate anthocyanins.The presence of both anthocyanins and betalains is mutually exclusive.

The first step in the betalain pathway, the conversion of tyrosine to DOPA, is catalyzed by the enzyme tyrosine hydroxylase. Subsequently, DOPA is converted, through the action of DOPA-4,5-dioxygenase, to betalamic acid, the betalain chromophore. Betalamic acid may condense with any amino acid or amine, giving rise to betaxanthins, or with cyclo-DOPA, to form betacyanins.

In the present study, tyrosine hydroxylase was isolated from the fly agaric mushroom (*Amanita muscaria*), purified and characterized. Purification was also attempted using beet root (*Beta vulgaris* L.) cell line cultures as the tissue source, but the enzyme proved to be very unstable. Some notable differences and some common features of the two enzymes will be discussed.

223

EVIDENCE FOR THE EXISTENCE OF TWO Δ^4-3-KETOSTEROID 5α-OXIDOREDUCTASES (5a-reductases) IN HUMAN PROSTATE.

Faller B, Farley D, Nick HP, CIBA Pharmaceutical Research, CH-4002 BASEL

The prostate is an accessory sex gland exclusively found in mammals. The motivation for the extensive study of this organ stems from the many pathological complications affecting this gland, the most important being benign prostatic hyperplasia (BPH). All forms of prostatic growth, be it normal or neoplastic exhibit androgen dependency. It is now clear that dihydro-testosterone (DHT) and not testosterone (T) is the important androgen in the prostate. Inhibition of 5AR, the enzyme which converts T to DHT, seems a promising approach for a pharmaceutical therapy of BPH. This task has become more challenging by the recent report of two cDNAs encoding different, homologous 5AR isozymes. Here we present kinetic evidence for the presence of two catalytically active isozymes (5AR1 and 5AR2) in human prostatic microsomes. The Km values for T were determined to be 19 µmol/l and 20 nmol/l for 5AR 1 and 5AR 2, respectively. These values are close to those determined for 5AR 1 and 5AR 2 expressed in yeast (25 µmol/l and 23 nmol/l). Under saturating T concentrations in human prostatic microsomes 55% of the specific activity (mole/min/mg of protein contained in microsomes) is related to 5AR 1 and 45% to 5AR 2.

224

TUMOR NECROSIS FACTOR (TNF) AND INTERLEUKIN 1 (IL1) INCREASE THE PRODUCTION OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR (U-PA) AND OF PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1(PAI-1) BY HUMAN COLON CARCINOMA (HCC) CELLS.

Trân-Thang, C., and Sordat, B., .Swiss Institute for Experimental Cancer Research (ISREC), CH-1066 Epalinges

Nine HCC cell lines (Co112, HT29, SW480, SW620, ISRECo1, CaCo2, CoSut, COLO205, SW1116) were treated with 20 ng/ml TNFα or 5 ng/ml IL1β for 24 h in serum-free culture conditions. PAs and PAIs were analyzed in supernatants by zymography using SDS-PAGE with copolymerized plasminogen-rich fibrinogen and by enzyme immunoassays for tissue-type PA (t-PA), u-PA, PAI-1 and PAI-2. . 8/9 and 9/9 cell lines increased their production of u-PA respectively from 4 to 50 fold after TNF treatment and from 2 to 100 fold after IL1 treatment. PAI-1 production was also augmented after TNF treatment from 2 to 500 fold in 5/9 cell lines and from 3 to 90 fold in 5/9 cell lines after IL1 treatment. There was no .correlation between increased production of u-PA and that of PAI-1. The cytokine stimulatory effect was shown to be time- and concentration -dependent. Northern analysis of polyA RNA extracts after cytokine treatment confirmed the stimulatory effect of TNF and IL1 on .of u-PA and PAI-1 expression. 2/9 and 1/9 cell lines also produced more t-PA after respectively TNF or IL1 treatment. No PAI-2 was detected in supernatants of the 9 cell lines examined. Using a glutaraldehyde immobilized ^{125}I-laminin degradation in vitro assay, we show that the increased u-PA production correlates with higher proteolytic degradation induced by the cytokine -treated cells.

Supported by the Swiss Cancer League and the Swiss Science Foundation

225

INFLUENCE OF PHORBOL 12-MYRISTATE 13-ACETATE ON THE SECRETIONS OF PROCATHEPSIN B AND CYSTATIN C BY HUMAN COLON CARCINOMA SW480 CELLS:

Keppler, D.; Corticchiato, O.; Abrahamson, M.; Chan, S.J.; and Sordat, B.
ISREC, CH-1066 Epalinges [S]/Lausanne.

We have shown recently that human colon carcinoma cell lines secreted both procathepsin B and cystatin C into culture media. Expression by colonic cells of the proteinase and the inhibitor also occurred at the mRNA level. We have now investigated wether the concomitant expression of these two antagonists by SW480 cells could be modulated by phorbol esters (PMA). In SW480 cells, secreted procathepsin B represents 17 to 19% of newly synthesized procathepsin B; the major part is targeted to lysosomes. Without PMA treatment, cycloheximide (CHX), an inhibitor of protein synthesis, drastically decreased secretion of procathepsin B, but decreased only by 16 to 17% the intracellular pool of cathepsin B. PMA induced a 5.5 fold increase in total cathepsin B synthesis upon 24-h of treatment. Both secreted procathepsin B as well as lysosomal cathepsin B increased after PMA treatment. Both increases were completely blocked by CHX. PMA had no effect on the 24-h accumulation of cystatin C in media, but CHX strongly inhibited its *de novo* synthesis. These results were confirmed on the mRNA levels for cathepsin B. The transcript of cystatin C however, decreased 1.3 fold upon 8-h of PMA stimulation, and at 24-h increased 1.4 fold the initial level, which explains the lack of effect of PMA observed above on the net cystatin C protein levels at 24-h. The expression levels of cathepsin B being increased in many human cancers, it is of importance to understand the dynamic of the balance between the proteinase and its endogeneous inhibitors. The present work shows that the tumor-promoter PMA induces, at 8-h, a switch in the expressions of cathepsin B and cystatin C.

Supported by the Swiss Science Foundation.

226

Cloning of a *Fasciola hepatica* cysteine-protease gene family

Volker Heussler and Dirk Dobbelaere.
Institute of Parasitology, University of Berne, Switzerland

Proteases play an important role in the life cycle of the liver fluke *Fasciola hepatica*, in particular during the invasion of the intermediate and final hosts. In addition, it is known that adult flukes secrete enzymes that can break down immunoglobulins.

Degenerate primers designed specifically for cysteine- and serine-proteases were used in the polymerase chain reaction using as starting material either DNA or RNA isolated from adult *F. hepatica*. Clones containing cDNA fragments of eight different cysteine-proteases (FCP1 - FCP8) and one clone encoding part of a serine-protease cDNA (FSP1) were generated. The homology at the nucleotide level among the different cysteine-protease clones varied from 50 to 80 %. A comparison of the predicted amino acid sequences with proteases of other organisms revealed homology to proteases of the cathepsin-L and cathepsin-B type.

Northern blot analysis of RNA isolated from adult *F. hepatica* revealed abundant protease transcripts of different length. FCP1 and FCP2 transcripts were particularly abundant. One protease cDNA was cloned in an expression vector and antibodies against the fusion protgein were produced in rabbits. Western blot analysis confirmed the abundance of proteases in adult *F. hepatica*.

227

DEVELOPMENTALLY REGULATED PROTEASE IN ALLOMYCES ARBUSCULA.

Mukti OJHA, *Laboratoire de Microbiologie générale, Sciences III, q. Ernest-Ansermet 30, 1211 Genève 4*

Allomyces, an aquatic fungus, has two predominant phases in the life cycle. The growth phase, which starts with the spore germination and mycelial growth occurs in balanced medium. Morphogenetic phase is induced by nutrient deprivation and involves the cessation of hyphal elongation and the transformation of the hyphal apex into sporangium in which the flagellated zoospores differentiate. Superimposed on this two developmental pathways is the activation and inactivation of two main intracellular neutral proteases. A cysteine-calpain-like protease characterizes the vegetative growth phase and a serine protease which characterizes the morphogentic pathway. Biochemical properties of the calpain-like protease has been adequately characterized (1, 2).

We present our results on the correlation between appearance and disappearance of the enzyme activity and the calpain-like antigen by immunoblotting. For example the antigen diasappears completely after 1 hour of induction of differentiation which correlates with the appearance of serine protease. A partial characterization of the serine protease is presented.

References: 1. Ojha & Wallace, J.Bact. 170, 1254-1260 (1988). 2. Ojha,M. In Calcium as an Intracellular Messenger in Eucaryotic Microbes (ed. D.H.O'Day, Acad.Press pp192-211, 1990)

228

Alanine aminopeptidase activities in poppy leaves

Benesova, M.[*], Fischer, A.[+] and Feller, U.[+] [*]Institute of Biochemistry and Microbiology, Faculty of Pharmacy, Komensky University, Bratislava, CSFR and [+]Institute of Plant Physiology, University of Bern, Altenbergrain 21, CH-3013 Bern

Aminopeptidases contribute to the complete degradation of polypeptides to free amino acids. Several forms with different affinities to N-terminal amino acids were identified previously in various plants. Alanine aminopeptidase extracted from poppy leaves (Papaver somniferum L.) was investigated with p-nitroanilides (pNA) of L-ala, of D-ala and of the dipeptide L-ala-L-ala. The activity against D-ala-pNA was below 15% of that against L-ala-pNA. The initial liberation of p-nitroaniline from L-ala-pNA was linear with time, while a delay was detected with L-ala-L-ala-pNA. This suggests that the liberation of the two amino acids from the latter substrate was caused by two independent events. The hydrolysis of L-ala-pNA was slightly inhibited (about 20%) by the addition of 8 mM L-ala (free amino acid) or of 8 mM L-ala-L-ala (dipeptide), whereas D-ala or dipeptides containing D-ala were not or much less effective. The inhibitory effect of L-ala-L-ala may be caused by the dipeptide itself or by free L-ala liberated from its cleavage.

229

CHARACTERIZATION OF THE UNFOLDING TRANSITIONS OF RECOMBINANT HUMAN TRANSFORMING GROWTH FACTOR *BETA*-2 BY CIRCULAR DICHROISM.

Runser, S. and Cerletti, N., Biotechnology Department. Pharmaceutical Division, CIBA Ltd., CH-4002 Basel.

Transforming Growth Factor Beta-2(TGF-ß2)is a disulfide-linked homodimer of *Mr* 25 kDa released from many cell types. Among numerous pleiotropic effects, it also influences the rate of proliferation of different cells, controls processes of osteogenesis, epithelial cell differentiation and immune cell function. In order to characterize the stability of rh-TGF-ß2, we have followed the unfolding transitions induced either by heat, urea or guanidinium-HCl (Gu-HCl) by using **Near, or Far-UV Circular Dichroism Spectroscopy**. Under **acidic** and **neutral** conditions, the **secondary** and **tertiary structures** are lost within a narrow range of chaotrop concentration (mid-point of transitions around 3.5M Gu-HCl and 5M urea). The protein exhibits a good heat-stability as the native tertiary (Near-UV CD) and backbone structures (Far-UV CD) are lost only at temperatures, respectively, higher than 70 °C and 80°C. After removal of the chaotrop(s) by dialysis or lowering the temperature, respectively, TGF-ß2 exhibits the original CD-spectrum as well as the initial bioactivity (growth inhibition of Mv-1-Lu Cells), demonstrating the **reversibility** of the unfolding transitions and the **remarkable stability** of this protein.

230

Infiltration of monocytes in glioblastoma tumors: possible involvement of MCP-1.

Desbaillets, I., Diserens, A-C.,Hamou M-F., de Tribolet, N. and Van Meir, E. CHUV, CH-1011 Lausanne

MCP-1 is a human monocyte chemotactic protein produced by several cell types such as keratinocytes, mesangial cells, fibroblasts, PMA stimulated PBMC and human glioma cell-line U-105MG. Presence of MCP-1 mRNA is demonstrated in permanent human glioma cell lines with or without stimulation by inflammatory cytokines. MCP-1 secretion in cell lines supernatants is analyzed by immunoprecipitation using a specific monoclonal antibody (E11). These *in vitro* data show that glioblastoma cells are able to release MCP-1 in the extracellular environment. To prove that this could also occur *in vivo* we first examined the presence of MCP-1 mRNA in biopsies of human low and high grade astrocytomas. Secondly, by immunohistochemistry, we are currently analyzing the presence of MCP-1 protein on frozen tissue sections using a polyclonal antibody. Finally, the *in vivo* secretion of MCP-1 will be tested in CSF and tumor cyst fluids of patients.

These results suggests that MCP-1 may play an important role *in vivo* in the recruitment of monocytes in tumor tissues.

231

PURIFICATION OF CITRATE SYNTHASE ISOFORMS FROM SOYBEAN COTYLEDONS

Escher C.-L., Caldelari D., Widmer F., Institut de Biologie et de Physiologie Végétales de l'Université, CH-1015 Lausanne

Citrate synthase (CS; EC 4.1.3.7) is found in two major pathways, *viz.* the Krebs and glyoxylate cycles. The latter is localized in the glyoxysomes, which appear at the beginning of oilseed germination and are converted into peroxisomes when fat reserves are depleted. The CS primary structure from various organisms has been determined, but at present no plant glyoxysomal CS sequence is known. Antibodies raised against mitochondrial and glyoxysomal CS are now necessary to screen a cDNA library.
Previous studies demonstrated that CS isoforms cannot be separated from crude extract, the mitochondrial form(s) being much more abundant than the glyoxysomal form. Consequently, a more appropriate purification method is based on density centrifugation (organelle isolation on sucrose gradients), membrane sedimentation and phase partition (CS is weekly adsorbed on membranes), as well as standard purification procedures. CS isoforms from the cotyledons of 5-day old soybean seedlings (*Glycine max*. L.) were thus obtained from SDS-PAGE, and used to raise antibodies.

232

PURIFICATION OF CALPAIN FROM HUMAN ERYTHROCYTES

John Anagli, Maurizio Molinari and Ernesto Carafoli
Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), 8092 Zurich, Switzerland

Most purification schemes of calpain (CANP) involve a number of chromatographic steps. The final preparations invariably contain impurities, including degradation fragments. To obtain a pure CANP preparation suitable for biochemical and structural studies, a new peptide-affinity column was developed, using a 27 amino acid peptide corresponding to the product of exon 1b of the endogenous inhibitor calpastatin. Crude preparations of CANP, isolated by a modification of the procedure described by Melloni et al.(1982) were incubated with a highly specific, irreversible, active site-directed synthetic inhibitor [(ZLLYCHN$_2$), Anagli et al., (1991)] which blocks the enzyme in the inactive 80 kDa form. The crude enzyme preparation was then loaded on the peptide affinity column in the presence of calcium. Pure CANP was eluted with an EDTA-containing buffer. The procedure allowed the isolation of CANP with a high grade of purity, i.e., a 80 kDa band wich accounted for more than 95% of the protein eluted from the column with the EDTA buffer.

233

IDENTIFICATION OF ISOCITRATE LYASE ISOFORMS IN SOYBEAN COTYLEDONS

Walther-Drapel, V., Widmer, F., Richter, H., Institut de Biologie et de Physiologie Végétales de l'Université, CH-1015 Lausanne.

Isocitrate lyase (ICL; EC 4.1.3.1) is a specific enzyme of the glyoxylate cycle, which plays a key role in the initiation of gluconeogenesis in germinating oilseeds. To investigate soybean (*Glycine max* L.) ICL we analyzed a crude extract and the glyoxysomal matrix from young (5-day) cotyledons using 2-D SDS-PAGE and immunoblotting techniques. Polyclonal antibodies against soybean ICL were raised in a rabbit injected with partially purified ICL recovered from SDS-PAGE. We identified two ICL isoforms, in agreement with the recent analysis of cDNA clones (*cf* poster by N. Guex *et al.*.) In addition, protein analysis (2-D) confirmed that the two isoforms were of glyoxysomal origin. Further investigations addressing the possible phosphorylation of ICL, as well as cotyledonary mRNAs analysis, are necessary to identify the gene products observed on 2-D SDS-PAGE and relate them to the cDNA clones.

234

GLYOXYSOMAL ACONITASE FROM SOYBEAN.

Cots, J., Richter, H., and Widmer, F., Institut de Biologie et de Physiologie Végétales de l'Université, CH-1015 Lausanne.

Aconitase (EC 4.2.1.3; an enzyme of the Krebs and glyoxylate cycles) was isolated from the cotyledons of 5-day old soybean seedlings (*Glycine max*. L.) using sucrose gradients. Significant activity was detected in the supernatant and mitochondria fractions, but not in the glyoxysomes fractions. Starch gel electrophoresis techniques, which separate five aconitase isoforms in soybean (Cardy & Beversdorf, Seed Sci. & Technol. (1984), **12**, 943-954) were used to analyze various extracts. While all five isoforms were present in the crude extract and the mitochondria fractions, the supernatant fractions contained one single form only. It is not uncommon to identify specific peroxisomal/glyoxysomal enzymes in the supernatant fractions (*e.g.* glycolate oxidase, isocitrate lyase and malate synthase), because of the fragility of these organelles. The aconitase activity detected in the same fractions might thus correspond to the glyoxysomal form of the enzyme. This is supported by differential inhibition phenomena (using glyoxylate and HgO) which characterize these fractions and the mitochondrial pool. Isolation of glyoxysomal aconitase might therefore be achieved independently of the possible premature disruption of glyoxysomes.

235

ISOLATION AND CHARACTERIZATION OF CYCLIC AMP-SPECIFIC PHOSPHODIESTERASE (PDE-IV) FROM HUMAN MONONUCLEAR LEUKOCYTES (MNL) USING AFFINITY CHROMATOGRAPHY

Truong, V. H., Rehmann, R., Müller, T., Sandoz Research Institute Berne LTD., CH-3007 Berne

Cyclic AMP-specific phosphodiesterase (PDE-IV) has been purified from human mononuclear leukocytes in four-step purification procedure.
The MNL suspension was sonicated, centrifuged (120'000xg), and applied to Q Sepharose (anion exchange). Active fractions eluted at 0.23-0.32 M NaCl. The affinity column for the next purification step was prepared from 4-[3-(carboxypropyloxy)-4-methoxyphenyl]-2-pyrrolidinone (J. Demnitz, Sandoz Basel) immobilised to EAH-Sepharose 4B (S. Fougier at. al, BBRC, 138, 205). After washing with 0.5 M NaCl PDE-IV was eluted with 0.5 M NaCl and 1 mM cAMP. Cyclic AMP was separated from PDE-IV on Mono Q (anion exchange). Activity eluted at 0.32 M NaCl. The overall recovery was 17% (30 µg protein, purification 1868x) from 1.1 - 10^{10} cells .
PDE-IV was concentrated and separated on gel filtration (Superose 12). The single peak of enzyme activity was associated with a single protein peak, which eluted at a M$_r$ of 100-140 kDa. Under non-denaturing conditions, Gel electrophoresis revealed one major band corresponding to ~120 kDa. Under denaturing conditions this band disappeared and several bands with lower apparent M$_r$ appeared, probably representing proteolytic fragments of PDE-IV.

236

Plasma glycoproteins detection using lectins on Western blots after mini two-dimensional electrophoresis.

O. Golaz, C. Walzer, D. Hochstrasser*, J.P. Von Wartburg, H. Turler$^+$, L. Balant, Clinical Research Unit, Psychiatric University Institutions of Geneva, Dpts of Internal Medicine* and Mol. Biology$^+$, CH.

Glycoproteins altered in the glycan moiety may be markers for diseases, e.g. in cancer, inflammations and alcoholism. In this study we combined the high protein resolution capacity of two-dimensional gel electrophoresis with the high sensitivity of glycoprotein detection by specific lectins. Human plasma proteins were separated by mini two- dimensional gel electrophoresis (7x9 cm), transferred to polyvinylidene difluoride membranes and incubated with biotinylated lectins. We focused on lectins reacting with sialic acid residues (Wheat germ and Sambucus nigra), β-D-galactose (Datura stramonium and Ricinus communis), α-fucose (Aleuria aurantia) and N-acetyl-glucosamine (succinylated wheat germ). Known plasma glycoproteins such as α1-antichymotrypsin, α1-antitrypsin, HS-glycoprotein, α1-acid glycoprotein, β-haptoglobin and transferrin were easily detected in ng amounts. This technique is suitable for studying changes in glycoproteins of body fluid and tissue extracts with high resolution, sensitivity and specificity.

237

BIOCHEMICAL CHARACTERIZATION OF A PALMITATED VACCINIA VIRUS ENVELOPE PROTEIN INVOLVED IN VIRION ENVELOPMENT AND RELEASE.

Schmutz, C., Kunz, B., Child, S.J., and Wittek, R. Institut de Biologie Animale, Université de Lausanne, CH-1015 Lausanne

The most abundant protein of the vaccinia virus envelope is the palmitated P37K protein. This protein is required for viral envelopment and subsequent release from infected cells. Little is known about the nature of the association of P37K with the envelope, whether P37K interacts with other proteins of viral or cellular origin, or the functional role played by the attached palmitate moiety. Therefore, a variety of experiments were performed in order to better characterize the protein and its interactions. Triton X-114 partitioning experiments and sodium carbonate treatment indicated that P37K has a hydrophobic nature and is tightly associated with the membrane. This might be due to the attached palmitic acid since computer analysis indicates that the protein is fairly hydrophilic and does not contain a well defined transmembrane domain. In addition, limited proteolysis of intact virions demonstrated that the P37K is localized to the inner surface of the viral membrane. Experiments are currently in progress in an attempt to identify other viral or cellular proteins which interact with P37K, in addition to studies involving proteolytic digestion of purified P37K to determine which portion(s) of the protein are subject to palmitation.

238

IMPROVING THE SENSITIVITY OF THE SEQUENCE PROFILE METHOD

Lüthy, R., and Bucher, P., Swiss Inst. for Exp. Cancer Research, Epalinges

The sequence profile method (Gribskov et al., PNAS 84:4355-4358) is a powerful tool to detect distant relationships between amino acid sequences. A profile is a table of position-specific scores and gap penalties, providing a generalized description of a protein motif, which can be used for sequence alignments and database searches instead of an individual sequence. A sequence profile is derived from a multiple sequence alignment. We have found several possibilities to improve the sensitivity of sequence profiles: 1. In the originally described method all sequences in the alignment are given the same weight. Usage of individual weights for each sequence avoids bias towards closely related sequences. These weights are automatically assigned based on the similarity of the sequences. 2. For the construction of the profile an amino acid mutation table is used. We have found that in some cases the original Dayhoff matrix works better, than the modified matrix used in the current implementation. 3. Gap penalty multipliers are parameters used to align profiles with sequence. Current implementations recommend constant values for all profiles. We developed a heuristic procedure to obtain profile specific gap penalty multipliers.
Profiles derived by the new method are more sensitive and selective in a number of cases where previous methods have failed to completely separate true members from false positives.

239

Two-Dimensional Gel Electrophoresis and Immunoblot Analysis of *Adoxophyes orana* Granulosis Virus

Li, X. and Benz, G., Entomologisches Institut, ETH-Zentrum, 8092 Zürich

High resolution 2-dimensional (2-D) gel electrophoresis was used to analyse an insect virus, *Adoxophyes orana* granulosis virus (AoGV). By SDS-PAGE 15-20 protein bands are resolved in the enveloped nucleocapsids (ENC), while by 2-D gel electrophoresis more than 40 proteins can be separated. Among them 8 protein spots appear repeatedly. They are $V35_{a, b, c, d}$; V34; $V33_{a, b}$; and V31. The advantage of 2-D electrophoresis is that those proteins with the same molecular weight but with different isoelectric points (IEP), can be well separated ($V35_{a, b, c, d}$; $V33_{a, b}$). They are possibly subunits of a compound protein in the ENC structure. These viral proteins separated by 2-D gel electrophoresis can be demonstrated again without doubt by immunoblot using three anti-virus antisera. Most viral proteins of the ENC have their IEPs ranging from pH 4.5-6.6, but V34 has its IEP at pH 8.5. The granulin has its IEP at pH 5.5. Two different commercial standards were used as monitor for the pH gradients, so that our 2-D electrophoresis profiles can be compared with those from other laboratories. A primary index of the 8 structural proteins of AoGV was established.

240

Does Ethylacetate influence the extraction of the Anilinothiazolinon (ATZ-) Amino Acids during automatic Edman-degradation ?

A. Ducret and H. Zuber, Federal Institute of Technology, Institute for Molecular Biology and Biophysic, CH-8093 Zürich

During automatic Edman-degradation, the N-terminus of a polypeptide is first coupled with phenylisothiocyanat (PITC) then specifically cleaved and extracted from the support. After conversion to its phenylthiohydantoin (PTH-) derivate, the amino acid is characterized by HPLC. Ethylacetate (S2-reagent), whose function is to wash excess PITC from the carrier, is known to represent a critical parameter. Small impurities may react with the coupling product or interfere with the PTH-amino acid detection. When small amounts of polypeptide were degraded we observed a up to two-fold detection enhancement by reducing the S2-delivery. Since this effect seems to depend on the support-type the presence of impurities (perhaps water) might influence in a not well understood way the extraction yield of the ATZ-amino acid.

241

High pressure freezing increases the vitrification depth.

N. Sartori, K. Richter, J. Dubochet, Laboratoire d'analyse ultrastructurale, Université de Lausanne, CH-1015 Dorigny

Freezing is a well known process to observe biological materiel in their aqueous environment. The vitrification depth which can be obtained with standard freezing techniques does not exceed 20 µm.
Using crystals of catalase suspended in 15 % sucrose, we show that by high pressure freezing, the depth of vitrification can reach at least 700 µm.

542 (received after deadline)

DEVELOPMENT OF NEW MICROTITRE ASSAYS FOR PREGNANCY-ASSOCIATED PLASMA PROTEINS A AND -B (PAPP-A, PAPP-B) FOR THE PRENATAL DIAGNOSIS OF DOWN SYNDROME AND OTHER TRISOMIES.

N.A.Bersinger, Dept. of Obstetrics and Gynaecology, University of Berne, Switzerland.

The risk of a pregnancy to be affected by Down syndrome (DS, Trisomy 21) and other chromosomal abnormalities increases sharply with maternal age. Conclusive prenatal diagnosis of such a pathology is only possible by karyotyping cells from the fetoplacental unit obtained by amniocentesis or chorionic villus sampling (CVS). These invasive procedures themselves carry some risk of abortion for a normal pregnancy; it is therefore necessary to detect as many abnormalities with as few amniocenteses/CVS as possible. When selection is based on maternal age only (usually 35 yr), less than half of all Down syndrome are detected since the vast majority of pregnancies occur at a maternal age of less than 35 years. Determination of alpha-fetoprotein (AFP) in maternal serum was found to be useful in DS screening. Tests for HCG and unconjugated oestriol have been added ("Triple Screen", "AFP-plus"), but 20-35% of DS still escape detection. PAPP-A has been found to be a good prognosticator in the 1st trimester. We have developed a time-resolved fluoroimmunometric assay for PAPP-A and an ELISA for PAPP-B to test in DS.

Receptors and Signal Transduction

242

EFFECTS OF DIFFERENT PROTEIN KINASE C (PKC) ACTIVATORS ON CELL PROLIFERATION AND PKC ISOENZYME EXPRESSION IN MESANGIAL CELLS

Andrea Huwiler[1,2], Doriano Fabbro[1] and Josef Pfeilschifter[2], 1: Ciba-Geigy Ltd., Basel; 2: Dept. Pharmacology, Biocenter, University of Basel, CH-4056 Basel

The isoforms of PKC present in glomerular mesangial cells were identified by immunoblot analysis. Mesangial cells were found to express 4 PKC isoenzymes, PKC-α, -β, -δ and ζ. No PKC-γ and -η isoforms were detected. On exposure to five structurally different activators of PKC (PMA, bryostatin 1, thymeleatoxin, dihydroteleocidin B, debromoaplysiatoxin, 100 nM each), a translocation and marked down-regulation of PKC-α and -δ is observed in response to all compounds investigated. In contrast, PKC-ε is only down-regulated in response to bryostatin 1 and thymeleatoxin. Moreover, PKC-ζ is resistant to down-regulation by all PKC activators examined. These data suggest, that it is possible to selectively activate and down-regulate PKC-isoenzymes and such compounds could be used to study the physiological functions of a particular PKC isoenzyme.

243

STUDIES ON THE SERINE/THREONINE PROTEIN KINASE *RAC* IN MAMMALIAN CELLS AND THE BACULOVIRUS EXPRESSION SYSTEM.

Ingley, E., Andjelkovic, M., & Hemmings, B.A.
Friedrich Miescher-Institut, PO Box 2543, CH-4002 Basel.

We recently reported the cloning of a new subfamily of serine/threonine protein kinases termed *RAC* , of which two closely related isofroms *RACα* and *RACβ* have been identified. *RAC* appears to represent the phylogenetic mid-point between protein kinase C and A. The N-terminal domain of *RAC* has limited homology with SH2 domains. More recently, *RAC* has been identified as the cellular homologue of *v-akt*, an oncogene encoding a 105 kDa phosphoprotein containing a truncated *gag* N-terminal of a wildtype *RACα*. In order to gain insights into its function and regulation we have over expressed *RAC* in COS cells and in the baculovirus system. To determine the functions of the various domains of *RAC* , N- and C-terminal deletions of *RAC* were expressed in COS cells, looking at their effects on subcellular localization and activity. *RAC* purified from the baculovirus system has been examined biochemically, including determining the sites on which it is phosphorylated in this system and its substrate site specificity. Specific properties of the various *RAC* domains, namely the SH2-like, kinase, and C-terminal regions, have also been investigated when expressed as GST fusions in bacteria.

244

THE SH2 DOMAIN OF THE PROTEIN TYROSINE KINASE p56^{lck}: AN ELEMENT THAT CONTROLS KINASE ACTIVITY AND INTERACTIONS WITH SIGNALING MOLECULES

Panholzer, B., Flint, N.A., Bannwarth, W., Amrein, K.E. and Burn, P., Department of Biology, Pharmaceutical Research-New Technologies, F. Hoffmann-La Roche Ltd., 4002 Basel, Switzerland

A number of proteins contain non-catalytical domains called SH2 and SH3. They are supposed to be important in mediating protein-protein interactions. We have used recombinant proteins containing the SH2 domain of the *src*-kinase p56^{lck} to study the binding of this region to tyrosine phosphorylated polypeptides. First, we demonstrated that recombinant *lck*-SH2 bound specifically to a synthetic phosphopeptide representing the tyrosine phosphorylated C-terminus of p56^{lck}. Second, we showed that a specific subset of tyrosine phosphorylated proteins present in transformed NIH3T3 cells bound to recombinant *lck*-SH2. Two of the bound proteins were identified as PI 3-kinase and p120-GAP. In summary our results suggest that the *lck*-SH2 domain has a dual function: it can bind the phosphorylated C-terminus of p56^{lck} an thereby restrict kinase activity or it can mediate intermolecular interactions with cellular signaling proteins.

245

REGULATORS AND TARGETS OF *src*-LIKE PROTEIN TYROSINE KINASES IN T CELL SIGNAL TRANSDUCTION

Flint, N.A., Gassmann, M., Panholzer, B., Jascur, T., Amrein, K.E., and Burn, P., Pharmaceutical Research-New Technologies, F. Hoffmann-La Roche Ltd, CH-4002 Basel, Switzerland

The T lymphocyte cell surface receptors CD4 and CD8 and the T cell receptor (TCR)-CD3 complex are crucially involved in T cell differentiation and activation. One of the earliest events that follows the triggering of T lymphocytes via these receptors is the tyrosine phosphorylation of a distinct set of cellular proteins leading ultimately to alterations in gene expression and the onset of replication. In our studies we addressed four major points. First, the involvement of *src*-like, nonreceptor protein tyrosine kinases in these early receptor-mediated signalling events. Second, the possible regulation of these protein tyrosine kinases by the CD45 phosphotyrosine phosphatase and the p50^{csk} protein tyrosine kinase. Third, SH2 and SH3 domain-mediated intramolecular and intermolecular interactions of these protein tyrosine kinases. Fourth, a potential link between receptor-mediated signalling pathways involving p21^{ras} and protein tyrosine kinases.

246

IDENTIFICATION OF A FUNCTIONAL MOLECULAR COMPLEX BETWEEN CD2, ζ-CHAIN AND p59^{fyn} PROTEIN TYROSINE KINASE IN HUMAN T LYMPHOCYTES

Gassmann, M., Amrein, K. E., zur Hausen, J. D., and Burn, P., Department of Biology, Pharmaceutical Research-New Technologies, F. Hoffmann-La Roche Ltd, 4002 Basel, Switzerland

The 50 kDa CD2 transmembrane receptor molecule mediates adhesion as well as signal transduction function in T lymphocytes and natural killer (NK) cells. One of the earliest events following CD2 engagement is phosphorylation on tyrosine residues of a distinct set of cellular proteins including the ζ-chain of the T cell receptor (TCR)/CD3 complex. Here, we investigated potential associations of the CD2 molecule with the *src*-family protein tyrosine kinases p56^{lck} and p59^{fyn} and the ζ-chain. Results from co-immunoprecipitation and co-capping experiments provided evidence for a specific association of CD2 with ζ-chain and p59^{fyn} *in vitro* and *in vivo*. They suggest that CD2-mediated signal transduction involves a multimolecular complex of at least three components: CD2, ζ-chain and p59^{fyn}.

247

EXPRESSION OF PROTEIN TYROSINE KINASES IN THE MAMMARY GLAND DURING THE ESTRUS CYCLE.

M.Flück, G.Zürcher, A.-C.Andres and A.Ziemiecki.
Abteilung für klinisch-experimentelle Forschung, Universität Bern, Tiefenaustrasse 120, 3004-Bern.

Mammary carcinoma is the most frequent malignancy and a major cause of death in women. In the Western world, approximately 10% of women become afflicted and the incidence rate is still increasing. Protein tyrosine kinases (PTKs) play a fundamental role in normal cell growth and differentiation and there is compelling evidence for their involvement in neoplastic disease. Using a PCR-aided gene family cloning strategy we have determined the constellation of PTKs expressed at various stages of mammary gland development, particularly during the estrus cycle. Our interest in the estrus cycle reflects the idea that perturbations in PTK expression contributing to uncontrolled epithelial cell growth may accumulate during the repetitive cycles. To identify possible candidate PTKs we want to know which PTKs are expressed during the normal estrus cycle and how their expression is controlled. We have isolated several known and unknown PTK clones expressed during the estrus cycle some of which show differential expression in the phases of the cycle.

248

Characterisation of three novel members of the eph/eck/elk subfamily of receptor tyrosin kinases

Blaschke,R.J., Andres,A-C., Zürcher,G., Reid,H.H. and Ziemiecki, A.
Abt. für klin.-exp. Forschung, Universität Bern, Tiefenaustrasse 120, 3004 Bern

The activation of receptor protein tyrosine kinases (PTKs) by external signals represent the initial step in a complex cascade that eventually result in differentiation phenomena observed in different states of normal and neoplastic development. We have isolated partial cDNAs for three novel putativ growth factor receptors (myk I to myk III) expressed in a differentiation dependent manner in the mouse mammary gland. Sequence analysis confirmed their affiliation to the eph/eck/elk subfamily of growth factor receptors for yet unknown ligands. Comparison of the carboxyterminal region of the predicted proteins revealed a conserved tyrosine residue that might be a phosphorylation target and therefore play a role in the regulation of interactions between the members of this PTK-subfamily and their prospective substrates. One of the novel growth factor receptors has a MW of approx. 120 kD and was shown to have an in vitro kinase activity. Expression analysis revealed an elevated expression in ras-transgenic tumors for all three putative receptors. In contrast, more differentiated myc-transgenic tumors did not show this overexpression. In addition, one of the identified PTKs (myk III) is differentialy expressed in different stages of the hormonaly controlled oestrus cycle.

249

EXPRESSION OF EF11, A RECEPTOR PROTEIN TYROSINE KINASE, DURING MAMMARY DIFFERENTIATION AND CARCINOGENESIS

A.-C.Andres, R.Blaschke, M.Flück, G.Zürcher and A.Ziemiecki
Abteilung für klinisch-experimentelle Forschung, Universität Bern, Tiefenaustr. 120, 3004 Bern

To understand the role of PTKs specifically in mammary biology we employed a PCR based cloning strategy to identify PTKs important for growth and differentiation of mammary epithelial cells. By this method *EF11*, a member of the *eph/eck/elk* family of receptor PTKs for unknown ligands, was isolated from the mammary epithelial cell line 31E. RNA analyses revealed that *EF11* is expressed as a 4.7 kb transcript in a variety of organs. In mammary glands *EF11* expression is at least partially of epithelial origin and can only be detected in mature mammary glands after puberty. Expression is downregulated during pregnancy induced differentiation of the mammary epithelium. This indicates a hormonal control of *EF11* expression which will be verified using a cell culture model system. Most importantly, mammary tumors of transgenic mice bearing an activated human *Ha-ras* oncogene showed a highly increased expression, suggesting that *EF11* may play an important role in mammary carcinogenesis as well as in its differentiation.

250

FUNCTIONAL CONSEQUENCES OF CD45 TYROSINE PHOSPHATASE INHIBITION

S. Arni, G. Senaldi, M. Poincelet, E. Rungger-Brändle and D. Hoessli. Department of Pathology, Centre Médical Universitaire, Genève.

The trivalent arsenical phenylarsenoxyde (PAO) that interacts with vicinal sulfhydryls was used to inhibit the tyrosine phosphatase (PTPase) activity of CD45, the major transmembrane glycoprotein of murine T cells. In an attempt to understand the mechanisms of CD45 regulation, the effects of the inhibitor on the following CD45 parameters were investigated: 1) metabolic incorporation of phosphate, 2) oligomerisation and 3) lateral mobility in intact cell membranes.

Low concentrations of PAO (1-5 μM) slightly increase (5-10%) the amount of phosphate incorporated into serines, without affecting the oligomeric state or lateral mobility of CD45. On the contrary, PAO concentrations above 25 μM result in a 40% decrease in phosphate incorporation into serines and increases the phosphate content in higher order CD45 oligomers, which no longer undergo patching and capping after antibody-mediated cross-linking.

Our results show that CD45 exists in a monomer-oligomer equilibrium that may be controlled by serine phosphorylation. We also suggest that CD45 PTPase activity is different in different order oligomers and may reflect distinct regulatory mechanisms and interactive capacities of CD45 with membrane proteins.

251

CHARACTERIZATION OF A HUMAN CALMODULIN-LIKE PROTEIN

Rhyner, J.A., Durussel-Gerber, I., Cox, J.A. and Strehler, E.E.
Lab. for Biochemistry, Swiss Fed. Inst. Technol., ETHZ, 8092 Zurich and Dept. of Biochemistry, Univ. of Geneva, 1211 Geneva

The coding region of the intronless human calmodulin-like gene (Koller, M. and Strehler, E.E. (1988) FEBS Lett. 239, 121-128) was overexpressed in E. coli and the recombinant 16,8 kDa Calmodulin-like protein (CLP) could be purified by Ca^{2+}-dependent Phenyl-sepharose chromatography. CLP showed a Ca^{2+}-dependent electrophoretic mobility shift characteristic for other high affinity Ca^{2+} binding proteins. CLP binds four Ca^{2+} with a mean affinity constant of 10^5 M^{-1} in low ionic salt buffer, a value about tenfold lower than that for CaM under comparable conditions. CLP was found to activate 3',5'-cyclic nucleotide phophodiesterase to the same extend as normal Calmodulin, albeit with a severalfold higher K_{act}. In contrast, the erythrocyte plasma membrane Ca^{2+}-ATPase could only be stimulated to 62% of its maximal CaM-dependent activity. These data suggest that CLP represents a novel member of the EF-hand Ca^{2+} binding protein family and may be considered a human calmodulin isoform.

Supported by Swiss NSF grant no. 31-28772.90.

252

PHOSPHORYLATION OF TWO CALCIUM-BINDING PROTEINS:POSSIBLE ROLE IN THEIR TRANSLOCATION TO THE MEMBRANE OF ACTIVATED HUMAN NEUTROPHILS.

Guignard F., Vaglio M., Mauël J.*, Markert M. Laboratory of Clinical Chemistry, CHUV and Biochemistry Institute*, CH-1011 Lausanne.

Two calcium-binding proteins of 8 kDa (Calgranulin A or p8) and 14 kDa (Calgranulin B or p14) have been shown to translocate from the cytosol to the membrane of human neutrophils upon calcium-dependent stimuli (J.Biol.-Chem.**267**:27,19379-19382,1992). When phorbol myristate acetate (PMA), a calcium-independent stimulus was used, no translocation was observed. Calcium was found to bind to both proteins on nitrocellulose blots after SDS-PAGE electrophoresis of cytosol or membrane preparations of resting and stimulated neutrophils. Interestingly, activation of the cells using both calcium-dependent and independent (e.g.PMA) stimuli strongly increased the level of phosphorylation of p14 compared with unstimulated neutrophils. On the contrary, p8 was phosphorylated in PMA-activated cells only, correlating with the absence of translocation of the protein to the membrane. To determine if protein kinase C was responsible of this phosphorylation, staurosporine was incubated with PMA-stimulated cells. The phosphorylation of p8, but not of p14, was completely inhibited. These results suggest that p8 and p14 are independently regulated by phosphorylation and that the absence of translocation of p8 with PMA could be a consequence of its phosphorylation by PKC.

253

MOLECULAR CLONING OF cDNA ENCODING CALMODULIN FROM *Neurospora crassa*.

Capelli, N., van Tuinen, D., Ortega Perez, R., Arrighi, J-F. and Turian, G. *Laboratoire de Microbiologie générale, Université de Genève, Sciences III, CH-1211 GENEVE 4.*

A full-length cDNA encoding *Neurospora crassa* calmodulin was isolated from a lambda ZAP II cDNA expression library by immunoselection using highly specific polyclonal antibodies purified as described previously (1). The open reading frame (ORF) encodes a protein of 148 amino acid residues with a calculated Mr of 16854 daltons. In addition, alignment of the predicted aa sequence showed 84.5 % analogy with vertebrate calmodulin. A single substitution (Phe13/Tyr) differentiates calmodulin from *Neurospora crassa* and an other Ascomycete *Aspergillus nidulans*.

Using site-directed mutagenesis, the complete cDNA was ligated into a pTrc promoter-regulated bacterial expression vector to allow expression of *Neurospora crassa* calmodulin in *Escherichia coli*. The expressed protein was purified from bacterial lysates by phenyl-Sepharose chromatography and exhibited an electrophoretic shift, in the presence of Ca^{2+}, identical to that of the native protein.

Southern analysis of restriction digests of genomic DNA indicates that calmodulin is encoded by a single-copy gene.

(1) van Tuinen, D., Barja, F., Turian, G., and Ortega Perez, R. D. **1988.** FEMS Lett. 55, 77-82.

254

OVEREXPRESSION AND FUNCTIONAL ANALYSIS OF THE 65kDa REGULATORY SUBUNIT OF PROTEIN PHOSPHATASE 2A

Favre B., Turowski P., Pallas D. C.*, and Hemmings B. A.
Friedrich Miescher Institut, P.O. Box 2543, 4002 Basel, *Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachussetts 02115

Protein phosphatase 2A (PP2A) consists of multiple different heteromeric enzymes which have a similar core, composed of the catalytic subunit, a 36 kDa protein, associated with a regulatory protein of 65 kDa, called PR65. PR65 was overexpressed both in insect Sf9 cells and in bacteria. In both expression systems, the PR65 was soluble and purified to apparent homogeneity. Gel filtration and non-denaturing polyacrylamide gel electrophoresis analyses revealed that purified PR65 could associate with the catalytic subunit of PP2A. This association led to an inhibition of the catalytic subunit towards various phosphorylated peptide substrates but stimulated its activity towards para-nitrophenyl phosphate. Studies with substrates phosphorylated on different sites by different protein kinases, cyclic AMP-dependent protein kinase, protein kinase C and casein kinase II, indicated that the degree of inhibition apparently depended mainly on the substrate structure but not so much on the sequence surrounding the phosphorylation site. Finally, association of the PR65 with the catalytic subunit conferred polycation-sensitivity to the heterodimeric form of PP2A.

255

STUDY OF OF HSP28 PHOSPHORYLATION BY TNF-ALPHA.

Mehlen, P., Rémy C., Chareyron, P., Briolay, J., and Arrigo, A.-P. Molecular and Cellular Genetics, CNRS UMR-106, Claude Bernard University, Lyon-I, France.

At normal temperature, the monokine tumor necrosis factor-alpha (TNF) was found to rapidly increase the level of phosphorylation of the unique alpha-crystallin-related stress protein hsp28. The phosphorylation of this chaperonin represents one of the earliest cellular effects described for TNF and was found to be C-kinase independent. In cell made thermotolerant to heat shock, the TNF-mediated hsp28 phosphorylation as well as the cytotoxicity to this monokine was greatly reduced. A similar decrease in TNF-induced- hsp28 phosphorylation was observed in stable transformants expressing high levels of the detoxifiant enzyme glutathion peroxidase. These different levels of hsp28 phosphorylation correlated with modulations in the activation of the transcription factor NF-kB. Our results indicate that the TNF-mediated hsp28 phosphorylation is linked to the presence in the cell of oxidizing agents, such as free oxygen radicals, known as potential second messengers. Hsp28 phosphorylation may therefore be linked to TNF-alpha signal transduction and/or to the cellular resistance to these oxidizing agents.

256

COLLISION COUPLING THEORY REVISITED: A MECHANISTIC ROLE FOR RECEPTOR LATERAL MOBILITY IN SIGNAL TRANSDUCTION

Jans, D.A., Institut für Medizinische Physik und Biophysik, Westfälische Wilhelms-Universität, Münster, Germany

The fluorescence photobleaching recovery technique has been applied to measure directly the lateral mobility of plasma membrane-localized hormone receptors and examine the role of receptor lateral mobility in signal transduction. Receptors for insulin and EGF are known to be largely immobile at physiological temperatures. This probably relates to their signal transduction mechanism, which appears to require intermolecular autophosphorylation (receptor aggregration) for activation. In contrast, G-protein coupled receptors must interact with other membrane components to effect signal transduction, and it is noteworthy in this regard that the adenylate cyclase activating vasopressin (VP) V_2-receptor is highly laterally mobile at 37°C. We have been able to reversibly modulate the V_2-receptor mobile fraction (f) to largely varying extents, and to demonstrate thereby a direct effect on the maximal rate of in vivo cAMP production at 37°C in response to VP. A direct correlation between f and maximal cAMP production indicates that f may be a key parameter in hormone signal transduction in vivo, especially at sub-K_D (physiological) hormone concentrations, consitent with mobile receptors being required to effect G-protein activation.

257

Blocking T-type calcium channels with tetrandrine inhibits steroidogenesis in bovine adrenal glomerulosa cells

M.F. Rossier, C.P. Python, A.M. Capponi, W. Schlegel[1], C.Y. Kwan[2], and M.B. Vallotton; Divisions of Endocrinology and [1]Infectious Diseases, University Hospital, CH-1211 Geneva, Switzerland, and [2]Dpt of Biomedical Sciences, Mc Master University, Hamilton, Ontario, Canada

Tetrandrine, an alkaloid extracted from a Chinese medicinal herb traditionally used in hypertension treatment, inhibited aldosterone production induced in bovine adrenal glomerulosa cells by either K^+ ion, angiotensin II (AngII), or adrenocorticotropin in a concentration dependent manner ($IC_{50} \approx 10$ uM). The inhibition of the response to K^+ by tetrandrine had a pattern very similar to that of Ni^{2+}, which is known to preferentially block T-type Ca^{2+} channels. In addition, tetrandrine dose-dependently prevented Ca^{2+} influx induced by K^+ or AngII, without affecting the Ca^{2+} release phase stimulated by the hormone. The effect of tetrandrine on voltage-activated Ca^{2+} channels was investigated, under voltage clamp, using the whole cell configuration of the patch clamp technique. T-type Ba^{2+} currents were isolated by recording the slowly deactivating currents elicited during repolarization of the cell to -65 mV after various depolarizing pulses. These currents were blocked by micromolar concentrations of tetrandrine. Their sensitivity to voltage activation was not affected by the drug, however, tetrandrine significantly decreased their deactivation kinetics. Tetrandrine also affected L-type currents, as assessed after T channels inactivation for 100 ms, but at higher concentrations of the drug.

In conclusion, tetrandrine affects with a similar efficacy aldosterone production, calcium influx and T-type calcium channel activity. This finding strongly suggests a role for these channels in calcium signalling and control of steroidogenesis in adrenal glomerulosa cells.

258

UNIDIRECTIONAL INTERACTION BETWEEN IP_3- AND CAFFEINE-SENSITIVE CALCIUM STORES IN RAT PHEOCHROMOCYTOMA (PC12) CELLS

B. Reber, J. Stucki & H. Reuter; Department of Pharmacology, University of Bern, CH-3010 Bern

We studied the interaction between bradykinin- and caffeine-induced Ca^{2+} release from intracellular Ca^{2+} stores in a clone of the rat pheochromocytoma cell line (PC12) treated with nerve growth factor (NGF) for 4-6 days. The bradykinin-induced Ca^{2+} release is mediated by inositol 1,4,5 trisphosphate (IP_3), while the caffeine-sensitive store can be depleted by Ca^{2+}-induced Ca^{2+} release (CICR). The effect of Ca^{2+} release from these Ca^{2+} stores on cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) was measured by means of Fura-2 single cell microfluorimetry. Caffeine application caused no or only a small Ca^{2+} release in cells kept at rest in normal culture medium. The caffeine-sensitive pool could be filled by Ca^{2+} entry into the cells through either voltage-activated Ca^{2+} channels or ligand-gated cation channels. Bradykinin application produced substantial Ca^{2+} release in cells kept at rest in normal culture medium. The response was enhanced after K^+-depolarization of the cells. The bradykinin-induced release of Ca^{2+} also caused depletion of the caffeine-sensitive pool by CICR. However, Ca^{2+} released from the IP_3-sensitive store was not sequestered into the caffeine-sensitive Ca^{2+} store. The caffeine-induced rise in $[Ca^{2+}]_c$ was blocked by ryanodine in a use-dependent manner. In addition, a substantial use-dependent ryanodine block resulted from the bradykinin-induced rise of $[Ca^{2+}]_c$ and subsequent CICR. By contrast, the K^+-induced rise of $[Ca^{2+}]_c$ caused only a marginal use-dependent ryanodine inhibition of Ca^{2+} release. Our results suggest an enhancement of the IP_3-induced $[Ca^{2+}]_c$ rise in the cytoplasm by CICR from the caffeine-sensitive pool. A mathematical model adequately simulates our experimental data.

259

α1- and β-ADRENERGIC REGULATION OF INTRACELLULAR Ca^{2+} LEVELS IN SINGLE BROWN ADIPOCYTES

Gillon-Zyzek E., Seydoux, J., *Pralong, W.-F. and Girardier L., Département de Physiologie et *Division de Biochimie Clinique, Faculté de Médecine, Centre Médical Universitaire, CH - 1211 Genève 4

The effects of adrenergic stimulation were, for the first time, directly measured on single brown adipocytes by monitoring the intracellular calcium levels. Preadipocytes from mouse brown adipose tissue were cultured according to conditions known to elicit the expression of the uncoupling protein and the β3-adrenoreceptor. Cells cultured for seven to ten days on cover-slips were loaded with the fluorescent Ca^{+2}-indicator fura-2AM. Changes of $[Ca^{2+}]_i$ were continuously monitored at 340 and 380 nm excitation and expressed as the ratio between these two wavelengths. The results obtained show that norepinephrine (10^{-7} M) induced, in most cells, oscillatory increases in $[Ca^{2+}]_i$, the α1-agonist phenylephrine (10^{-6} M) induced a rapid $[Ca^{+2}]_i$ increase, whereas both β1-selective agonist tazolol (10^{-8} M) and β-selective agonist BRL 37344 (10^{-9} M) induced delayed and sustained increases in $[Ca^{2+}]_i$. Parallel experiments performed on single cells from rat cultured brown adipocytes show similar results for α- and β-agonists; in this model however, norepinephrine induced $[Ca^{2+}]_i$ increases without oscillatory patterns. These results will be discussed in relation to adrenergic-mediated metabolic and membrane potential changes in this target tissue which possesses all the known adrenoreceptor subtypes.

260

INHIBITION OF HORMONE-INDUCED CYTOSOLIC $[Ca^{2+}]$ OSCILLATIONS BY BIGUANIDES IN SINGLE RAT HEPATOCYTES

J.J. Ubl, S. Chen & J.W. Stucki; Pharmakologisches Institut, Universität Bern, Friedbühlstr. 49, 3010 Bern

Stimulation of hepatocytes by agonists acting via phosphoinositide breakdown leads to oscillations of the free cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) concentration. We investigated the effects of metformin and phenformin, two antidiabetic drugs, on phenylephrine-induced $[Ca^{2+}]_{cyt}$ oscillations. Metformin and phenformin lowered the frequency of the $[Ca^{2+}]_{cyt}$ oscillations in a concentration-dependent manner with IC_{50}-values of 0.3mM and 3μM, respectively. Parallel application of the biguanides and insulin lead to an synergistic inhibition of the oscillations. By contrast, agents which cause an increase of the cellular cAMP level (glucagon, forskolin, di-butyryl-cAMP) reversed this inhibition. Measurement of the Mn^{2+}-influx into the cell via the quench of the fura-2 fluorescence at 360nm revealed that 0.5mM metformin and 3μM phenformin inhibited the hormone-stimulated Ca^{2+}-influx across the plasma membrane. In addition, by assessing the mitochondrial membrane potential in hepatocytes in situ with Rhodamine 123 we found no or only a weak depression of this potential by the biguanides in a concentration range sufficient to inhibit the oscillations.

261

β-ADRENERGIC UP- AND DOWNREGULATION IN CULTURED CELLS WITH DEFINED MEMBRANE DEFECTS

R. Styger and U.E. Honegger, Pharmakologisches Institut, Universität Bern, CH-3010 Bern

Cultured skin fibroblasts from patients with cerebrohepatorenal (Zellweger's) syndrome are characterized by decreased levels of plasmalogens and increased amounts of very long chain fatty acids. Despite of similar plasmamembrane alterations β-adrenoceptor densities, isoproterenol-, fluoride- and forskolin-stimulated cAMP responses were different among the cell strains tested. To get further information on the influence of these plasmamembrane alterations on β-adrenergic transmission, we investigated the extent and time course of isoproterenol-induced desensitisation and the following resensitisation by measuring the cAMP response to isoproterenol-stimulation.

In addition we compared β-adrenergic transmission in control and Zellweger cells with that of the same cell strains after exposure to hexadecylglycerol a precursor-compound which is known to nearly normalize plasmalogen levels in deficient cells.

262

Glucagon-like peptide 1 receptor is coupled to adenylyl cyclase but not to the formation of inositol phosphates.

Christian Widmann and Bernard Thorens.
Institute of Pharmacology, Bugnon 27, 1005 Lausanne

Glucagon-like peptide 1 (GLP-1) is a 30 amino acids long hormone which is secreted postprandially by intestine L cells. This hormone potentiates glucose-induced insulin secretion by pancreatic beta cells, thus defining it as a gluco-incretin. GLP-1 interacts with beta cells via a specific membrane receptor (the GLP-1 receptor) that has recently been cloned. This receptor belongs to the seven transmembrane segment receptor superfamily, members of which are coupled to G proteins. Previous studies have demonstrated that ligand occupied GLP-1 receptor activates adenylyl cyclase. This is also the case for the peptidic receptors which are the most closely related in sequence to the GLP-1 receptor. Some of these related receptors (i.e. the calcitonin receptor) are however also coupled to phospholipase C and thus lead to formation of inositol phosphates when stimulated by ligand binding. The presence and role of transduction mechanisms other than the one mediated by the formation of cAMP have not been clearly established in the case of the GLP-1 receptor. We therefore tested whether GLP-1 can activate, via its receptor, the formation of inositol phosphates and a consequent rise in free cytoplasmic calcium concentration. We show here that GLP-1 receptor-transfected fibroblasts produce cAMP with an EC50 close to the dissociation constant of GLP-1 from its receptor (about 1 nM). Similar findings were obtained using several insulinoma cell lines. In contrast, in no situation inositol phosphate production was observed. These results will contribute to our understanding of the interactions between GLP-1 transduction mechanisms and the glucose sensing machinery of the pancreatic beta cell.

263

CHARACTERISATION OF INSULIN RECEPTORS FROM PATIENTS WITH EXTREME INSULIN RESISTANCE.

Moritz, W., Böni-Schnetzler, M., and Froesch, E.R. Universitäts-spital, Abteilung für Endokrinologie und Stoffwechsel, Rämistr. 100, 8091 Zürich

Insulin receptors from 4 patients with the syndrome of extreme, type A insulin resistance were analysed for the presence of defects using EBV-transformed lymphoblast cell lines. The following parameters were examined: (1) insulin receptor number/cell, (2) affinity of insulin and (3) insulin regulated tyrosine kinase activity. Results: All patient receptors displayed normal ligand affinities (Kd of 1.3-2.6 x 10^{-10} M). P2 and P3 expressed markedly reduced receptor numbers (15% of controls) and P6 had virtually no insulin receptors (3% of controls). P1 had normal receptor numbers but impaired autophosphorylation activity. In the presence of insulin the number of ^{32}P incorporated/receptor was 0.35 ± 0.08 for controls (n=8) and 0.53 ± 0.16 for P1 receptors (n=4) and in the presence of insulin 2.72 ± 0.77 for controls and 0.81 ± 0.26 for P1. Thus, insulin receptors of P1 carry a defects that specifically impairs the activation of the kinase by insulin. The molecular localisation of the mutation should give us insight into the receptor structures involved in the transmembrane activation of the receptor kinase by insulin.

264

CALCITONIN RECEPTOR GENE: STRUCTURE, CHARACTERIZATION AND CHROMOSOMAL LOCATION

Zolnierowicz, S., Cron, P., Solinas-Toldo, S*., Fries, R*., Lin, H.Y#., Lodish, H.F#., and Hemmings, B.A.
Friedrich Miescher-Institut, Basel, Switzerland, *Eidgenoessische Technische Hochschule, CH-8092 Zuerich, #The Whitehead Institute for Biomedical Research, Cambridge, MA

The thyroid hormone calcitonin (CT) executes its action by binding to plasma membrane calcitonin receptors (CTRs). In order to study the CTR expression we have isolated the CTR gene from a porcine kidney epithelial cell line (LLC-PK_1) genomic library. The gene spans approximately 80 kilobase pairs and is composed of 13 exons. The presence of introns in CTR gene is in contrast to other members of G protein-coupled receptors genes such as for α_2, β_1, β_2-adrenergic and M_1-muscarinic receptors that are intronless. Interestingly, the seven putative transmembrane domains are encoded by seven separate exons (exons 6 to 12). The 542 bp fragment of the CTR gene promoter containing putative binding sites for the transcription factor Sp1 and the enhancer-binding protein AP-2 was cloned in front of the luciferase gene. Comparable levels of the luciferase activity were measured after transfection into LLC-PK_1, HeLa, COS-1, MCF-7 and T47D cell lines. The CTR gene was mapped to chromosome 9q11-12 by in site hybridization using a tritiated cDNA probe and fluorescence in situ hybridization using a genomic probe.

265

Photoaffinity labeling of rat calcitonin gene-related peptide receptors and adenylate cyclase activation: Identification of receptor subtypes

Daniel Stangl, Roman Muff, Caroline Schmolck, and Jan A. Fischer, Forschungslabor für Calciumstoffwechsel, Klinik Balgrist, Forchstr. 340, 8008 Zürich

Different biological effects of calcitonin gene-related peptide (CGRP) analogues have suggested receptor subtypes. Here we have investigated molecular forms of rat CGRP receptors, ligand binding and activation of adenylate cyclase. A single class of ^{125}I-α-human(h)CGRP binding sites was identified in rat cerebellum, liver and spleen with dissociation constants (K_d) of 206 ± 70 pM, 128 ± 23 pM and 229 ± 64 pM (mean ± SEM), respectively. Competition experiments showed the same rank order of displacement of ^{125}I-α-hCGRP binding in all the tissues examined with rat α-CGRP ≈ α-hCGRP ≈ β-hCGRP > α-hCGRP(8-37) > [acetamidomethyl-$Cys^{2,7}$]α-hCGRP > human amylin > salmon calcitonin. Photoaffinity labeling of CGRP receptors using ^{125}I-[CY-(4-azidoanilino)Asp^3]α-hCGRP revealed specifically labeled 71 kilodalton (kDa) binding proteins in the cerebellum, brainstem and spinal cord, of 74 kDa and 68 kDa in the liver, and of 75-90 kDa in the spleen. Enzymatic N-deglycosylation converted the labeled binding proteins into a common 48 kDa form (44 kDa with the molecular mass of the photoligand subtracted). In the presence of 100 μM guanosine-5'-O-(3-thiotriphosphate), the K_d of ^{125}I-α-hCGRP binding remained unchanged in the cerebellum, but was increased 3-fold in the liver and spleen suggesting interaction with GTP-binding proteins. In accordance, adenylate cyclase was stimulated by CGRP in the liver and spleen, but not in the cerebellum and brainstem. Furthermore, the linear analogue [acetamidomethyl-$Cys^{2,7}$]α-hCGRP enhanced cAMP formation in the liver, but not in the spleen. In conclusion, rat CGRP receptors with tissue-specific N-glycosylation but indistinguishable protein molecular mass have been identified in the cerebellum, brainstem, spinal cord, liver and spleen. Activation of adenylate cyclase by CGRP in the liver and spleen, but not in the central nervous system, and by the linear analogue in the liver alone provide evidence for CGRP receptor subtypes.

266

Purification and partial characterization of a high molecular weight GPI-anchored platelet membrane glycoprotein

J. Polgár, J.M. Clemetson and K.J. Clemetson, Theodor Kocher Institut, University of Berne, CH-3012 Berne

A variety of cell surface proteins are anchored in the membrane by glycophosphatidylinositol (GPI). Three GPI-linked proteins, DAF (CD55), Ly-6 antigen (CD59) and C8-binding protein, have been shown to be present on platelets. However, we found that PI-specific phospholipase C (PI-PLC)-treatment of human platelets resulted in the appearance of at least two additional novel GPI-linked glycoproteins (GP), GP 500 and GP 180, in the supernatant. Their presence on the platelet plasma membrane surface was demonstrated by periodate/borohydrate surface labelling . A three step procedure, involving affinity- and ion exchange chromatography and preparative agarose gel electrophoresis, was developed for purification of GP 500 from the supernatant of PI-PLC treated platelets.From 180 units of blood 300 ug of pure GP 500 could be isolated which was used to immunise rabbits and to prepare CNBr peptides.

267

Cross-linking studies on the platelet GPIb/IX/V

A.L. Kyriacou and K.J. Clemetson, Theodor Kocher Insitute, University of Berne, CH-3012 Berne.

The platelet glycoprotein (GP)Ib (140kDa) composed of the disulphide linked Ibα (140kDa) and Ibß (27kDa) chains, forms a noncovalent complex with GPIX (22kDa). GPIbα can be divided into 2 regions: The N-terminal 45kDa domain, which contains the binding sites for von Willebrand Factor (vWF) and thrombin and the macroglycopeptide domain, which is highly O-glycosylated. The 45kDa domain contains 7 leucine rich motifs (Ibβ and IX have each one) and between Ala_{200} and Lys_{297} a hinge region, containing a double loop linked by 4 Cys residues and a region rich in charged amino acids. This region may participate in a shear stress induced conformation change exposing the binding site for vWF. Cross linking was performed with bis(sulphosuccinimidyl)suberate and 3'3-dithiol-bis-(sulphosuccinimidylpropionate). A 200kDa band was detected in cross-linked platelets using gel electrophoresis and Western blotting. After tryptic cleavage some fragments still containing Ibβ and the 45kDa domain of Ibα were detected indicating that these regions are in contact.

268

Identification of the thrombin binding site on platelet GPIbα.

A.L. Kyriacou and K.J. Clemetson, Theodor Kocher Institute, University of Berne, CH-3012 Berne

Glycoprotein (GP)Ib, the major sialoglycoprotein on the platelet surface, has an important role in primary haemostasis. The 45kDa domain of Ibα, which forms a compact globular domain contains: a von Willebrand factor binding domain which is responsible for the adhesion of platelets to subendothelium and a thrombin binding domain. Binding of thrombin to this site increases thrombin induced platelet activation and aggregation, but is not essential. In order to identify and characterize the binding site for thrombin on Ib, we used glycocalicin (GC, the extracellular domain of Ibα), bovine thrombin (50IU/mg) and the cross-linking reagent bis-(sulphosuccinimidyl)suberate (BS^3). The GC-thrombin complex was purified by affinity chromatography on polyclonal antibodies and gel filtration. After tryptic cleavage a 60kDa peptide was obtained that still contains a part of the 45kDa domain of GC and the b-chain of thrombin. The analysis of this peptide is in progress.

538 (received after deadline)

INTEGRATED OPTICS BIOSENSORS

Ramsden, J.J., Biozentrum der Universität, 4056 Basel

The principle of this new type of sensor is the change of effective refractive index of an optical waveguide, which can be measured and recorded, caused by some change in the waveguide's environment, such as increased concentration of analyte. Selectivity and sensitivity may be conferred upon the basic sensor optical chip by depositing a phospholipid membrane on its surface. Many drugs partition favourably into bilayer lipid membranes. In this contribution, it is shown how the partition coefficient (PC) of any molecule for which the structural formula is known can be determined. If the PC is known, then the coated chip can be used as a sensor for the drug, with a sensitivity greatly enhanced compared with the uncoated chip.

Channels, Carriers, Pumps and Membrane Structure

269

WATER CHANNELS OF STIMULATED, GLUTARALDEHYDE-FIXED TOAD SKINS ARE SENSITIVE TO Hg AND ANIONS

Grosso A. & de Sousa R.C. Depts Physiology & Medicine, University of Geneva, CMU, 1211 Geneva 4, Switzerland

We have recently made two striking observations in toad skins exposed to Hg : (a) an exocytic effect leading to the appearance of long-lasting apical aggregates of intramembrane particles ; (b) anion-induced changes in osmotic water permeability (P_f), such that P_f is *low* in Cl-Ringer (Cl-R) and *high* in SO_4-R. Since these P_f changes appear to be related to the presence of apical water channels, we investigated if similar anion effects were found in skins stimulated with a hydrosmotic agent before exposure to Hg. To ensure a stable, high P_f, skins were first stimulated with isoproterenol, fixed with glutaraldehyde and then exposed to Hg added to the outer medium. In a typical example, representative of 6 skins, P_f (μm/sec) was 51 after exposure to isoproterenol and glutaraldehyde. Despite tissue fixation, Hg caused P_f to fall down to 28. After removal of Hg, P_f rose to 52 in SO_4-R and fell to 40 in Cl-R. These P_f changes could be repeated several times and, in each instance, P_f values in SO_4-R were close to those found prior to Hg exposure. In fixed, non-stimulated skins, anions had no appreciable effect on P_f. The results strongly suggest that : (a) the anion-induced changes in P_f reported here require the presence of apical water channels ; (b) glutaraldehyde fixation very likely abolishes the exocytic effect of Hg.

270

PERMEANCE MODULATING FACTORS OF APICAL WATER CHANNELS IN TOAD SKIN (*Bufo marinus*)

de Sousa R.C. & Grosso A. Depts Physiology & Medicine, University of Geneva, CMU, 1211 Geneva 4, Switzerland

In Hg-treated toad skins, anions induce striking changes in osmotic water permeability (P_f) : the latter is low in Cl-Ringer (Cl-R), whilst it increases 4- to 5-fold in SO_4-R. Since cells loose Cl and shrink in SO_4-R, the question arose as to whether cell volume and/or intracellular Cl concentration modulate the permeance of the water channel. To further investigate this problem, we looked at anion-induced P_f changes in anisosmotic conditions of the inner medium. When *isotonic* SO_4-R was replaced by *hypertonic* Cl-R, P_f fell to its basal value, as previously found with isotonic Cl-R. Conversely, when *isotonic* Cl-R was replaced by *hypotonic* SO_4-R, P_f rose dramatically. Since serosal hypertonicity is known to cause, by itself, an exocytosis of water channels in amphibian epithelia, we focused our attention on hypotonic media. In a group of 6 skins, P_f (μm/sec) was as follows : (A) 18.5±1.71 in *isotonic* Cl-R; (B) 54.6±1.22 in *isotonic* SO_4-R; (C) 111.3±4.71 in *hypotonic* SO_4-R. Statistically, $P<0.001$ for A *vs* B and $P<0.001$ for B *vs* C. In 4 of these skins, P_f was 106±5.6 in *hypotonic* SO_4-R and 21.6±3.7 in *hypotonic* Cl-R ($P<0.001$). The results suggest that in Hg-treated skins, apical water channels are sensitive to : (a) intracellular Cl concentration ; (b) cell volume and/or intracellular osmotic stimuli.

271

INFLUENCE OF THE EXTRACELLULAR pH ON THE ACTIVE IONIC TRANSPORTS IN THE CHICK EMBRYO

Abriel, H., Ksontini, R., and Kucera, P.
Institute of Physiology, University of Lausanne, CH-1005 Lausanne.

The blastoderm of the gastrulating chick embryo transports sodium ions in dorso-ventral direction. In this study, the blastoderms were mounted in Ussing conditions, allowing the measurement of short-circuit current (Isc) and total electrical conductance (Gtot). In order to test the influence of the extracellular pH, the dorsal and ventral compartments (with HEPES pH 7.3 in control conditions) were superfused with pulses of HEPES pH 6.6 of variable durations. The control values were : Isc = 19.2 ± 7.6 $\mu A/cm^2$ and Gtot = 2.4 ± 0.6 mS/cm^2 (n=11). When the replacement was done on the dorsal side, the Isc decreased by 13 ± 4 % and the Gtot by about 0.2-0.4 mS/cm^2. However, the decrease of Isc was transient : Isc progressively returned towards control values with a mean slope of 5.6 $\mu A/cm^2$ /h. Upon the return to pH 7.3, a rebound increase of Isc by 18 ± 4 % was observed. For each embryo, this rebound was linearly related to the slope of the Isc return and the pulse duration. On the contrary, the Gtot decrease was stable and returned to the control value upon pH 7.3. No such effects were observed on the ventral side. EIPA (1 μmol/l) had no influence on these effects. These results suggest an active proton extrusion mechanism in the embryonic cells.

H.A. is supported by a "UNIL-450ème" grant

272

ELECTROPHYSIOLOGICAL EFFECTS OF Cd^{2+} AND MERCURIAL COMPOUNDS ON TWO RENAL EPITHELIAL CELL LINES

Mingard, F., Hausel, P., Horisberger, J.-D. and Diezi J., Institut de Pharmacologie et de Toxicologie de l'Université, 1005 Lausanne

The kidney is a target organ of heavy metal toxicity. Two renal epithelial cell lines, with proximal ($LLC-PK_1$) or distal (MDCK-I) properties grown on collagen coated filters were used to study the short-term Na^+ transport modifications induced by Cd^{2+}, p-chloromercuri-benzoate (PCMB) and p-chloromercuriphenylsulfonate (PCMBS). Short-circuit current (Isc) and transepithelial resistance (Rt) were measured using a modified Ussing chamber allowing perfusion of the apical and basolateral sides. Cd^{2+}, PCMB and PCMBS (10 μM) at the apical side of **$LLC-PK_1$** cells increased Isc (1-4 $\mu A/cm^2$) within 15 min without change of Rt. In contrast, basolateral exposition with PCMB or PCMBS decreased the Isc (2-3 $\mu A/cm^2$) within 15 min. In the same condition, Cd^{2+} induced a transient decrease followed by a slight increase of the Isc. PCMB and PCMBS applied to the apical or basolateral side of **MDCK-I** cells stimulated Isc. Cd^{2+} (10 μM) applied to the apical side induced a slight increase of the Isc (0.1 $\mu A/cm^2$), while a basolateral exposition induced a marked and transient increase (2.5 μA/cm2) of the Isc. This stimulation was inhibited (20%) in the presence of the calcium channel blocker nifedipin. These observations suggest that Cd uptake/effect is mediated by dihydropyridine sensitive Ca^{2+}-channels, or that the electro-physiological effects are Ca^{2+}-dependent.

273

TIGHT JUNCTIONAL PERMEABILITY IN RAT HEPATOCYTES

Rahner, C. and Landmann, L., Anatomisches Institut der Universität, 4056 Basel

We examined junctional organization and permeability of tight junctions (TJ) in two models of impaired bile flow (cholestasis): bile duct ligation (BDL) and ethinylestradiol (EE). Linear density (expressed as junctional length per liver) which reflects the length of the paracellular cleft at the canaliculi was estimated on EM micrographs using conventional stereological methods. Cholestasis induced an increase by 53% following EE (174.1 ± 58.7 vs 114.0 ± 39.1 km in controls, n.s., n=5) and by 63% after BDL (202.5 ± 46.8 vs 124.0 ± 18.0 km in controls, $p < 0.01$, n=5). The number of strands reflecting the sealing capacity of the junctions was counted in freeze-fracture replicas. Cholestasis induced a decrease by 19% after EE (3.81 ± 0.29 vs 4.69 ± 0.36 in controls, $p < 0.005$, n=5) and by 26% following BDL (3.42 ± 0.55 vs 4.62 ± 0.32 in controls, $p < 0.005$, n=5). Taken together these morphological data suggest an increase in junctional permeability (controls < EE < BDL). An early (6-8 min) peak in bile of peroxidase (HRP) injected into the femoral vein has been demonstrated to reflect paracellular permeability. BDL induced an increase of HRP secretion by 730% (705.05 ± 211.63 vs 84.95 ± 40.53 ng/min in controls, $p < 0.001$, n=6) while no significant alterations were observed after EE (-42%, 82.48 ± 67.83 vs 142.63 ± 83.92 ng/min in controls, n.s., n=6). Our data indicate that BDL - a severe form of cholestasis - induces morphological and physiological TJ alterations which are compatible with increased junctional permeability. The morphological alterations induced by EE - a mild form of cholestasis - may, however, be to slight to allow an increase in biliary permeability for a molecule the size of HRP.

274

CONDUCTANCE OF GAP JUNCTION CHANNELS IN NEWLY FORMED CELL PAIRS

Bukauskas, F.F., Weingart, R. Department of Physiology, University of Bern, CH-3012 Bern

Cells of an insect cell line (Aedes albopictus, clone C6/36) were grown at low density (0.2 - 1·10^6 cells/ml). Two cells in close vicinity were pushed together to allow formation of gap junction channels. Flow of intercellular current was measured using the dual voltage-clamp method. We were able to study the conductance of newly formed gap junction channels. The channels exhibited multiple conductance states. The conductance of a fully open channel was 365 pS. The subconductance increment was 1/7 to 1/6 of the maximal conductance. Conceivably, the lowest conductance level reflects a residual conductance. The voltage gradient across the gap junction, V_j, had no effect on the conductance of a fully open channel, but affected the dwell time spent at particular conductance states. Small V_j gradients were associated with fully open channels, large V_j gradients with low subconductance states. These results offer an explanation for the V_j-dependent conductance of gap junction membranes (bell-shaped, non-zero for large V_j).

(supported by SNSF # 31-25333.88).

275

MUTATIONAL ANALYSIS OF SAXITOXIN AND DIVALENT CATION BINDING SITE OF THE VOLTAGE-GATED SODIUM CHANNEL. I. Favre, E. Schneeberger and L. Schild. Institut de Pharmacologie et de Toxicologie de l'Université. CH-1005 Lausanne.

Voltage-gated Na^+ channels can be pharmacologically distinguished with respect to their blocking affinities for saxitoxin (STX) and divalent cations. To understand the molecular mechanisms underlying channel block by these ligands and to localize their specific binding sites on the channel we combined site-directed mutagenesis and electrophysiological studies of channel function. We identified a Cys between the transmembrane segments S5 and S6 of the first homologous domain of the heart Na^+ channel (SKM2) responsible for its resistance to STX and its high affinity for Zn^{2+}. The corresponding Cys for Tyr mutation on the STX-sensitive Na^+ channel (SKM1) lowered its affinity for STX by more than 100 times, and resulted in 300 fold increase in Zn^{2+} affinity, suggesting a common receptor for STX and divalent cations, and similar mechanisms of channel block. Based on additional mutations we will present a detailed structural and functional analysis of this receptor, presumably located at the external mouth of the channel pore, which modulates the channel conductance.

276

Na^+ and K^+ currents in freshly dissociated human muscle satellite cells

Widmer, H.*, Hamann, M.*, Aubry, J.P.#, Baroffio, A.*, Bijlenga, P.*, and Bader C.R *. Departement of Physiology *, CMU, and GLAXO Institute for Molecular Biology, Geneva #.

Physiological properties of human muscle satellite cells (SC) are difficult to study *in situ*. In addition, SC cannot be easily distinguished from other cells in the suspension obtained from dissociated muscle. Therefore, we used a new method based on cytometric flow to obtain a purified suspension of SC (Baroffio et al., *in press*). Whole cell recording was then performed before the cells divided (i.e. during the first 24 hours after sorting). The mean capacitance of these small cells was 5 pF (n=55). Depolarization beyond a threshold of -45 mV elicited a transient Na^+ current which was abolished by removing external Na^+ and was inhibited by high concentration of tetrodotoxin (K_D= 3 µM). This current was expressed in 62 % of the cells and maximal conductance was 250 pS/pF ± 340 (S.D., n=44). An outward K^+ current was induced by depolarization from -100 mV to levels above -20 mV and was abolished by 20 mM external TEA. This current was expressed in 60 % of the cells and conductance at +40 mV was 63 ± 80 pS/pF (S.D., n=21). Thus it is possible to study SC before they begin to divide and we observe that they already express Na^+ and K^+ currents. Comparison with proliferating SC showed that in clonal culture conditions, 99% of the cells expressed the K^+ current. Furthermore, the K^+ conductance normalized to the cell capacitance increased by 5 to 10 times. By contrast, neither the percentage of cells which express the Na^+ current nor the Na^+ conductance normalized to capacitance are significantly affected by cell proliferation.

277

Regulation of K^+-permeability in isolated rat hepatocytes

S. Dotti-Sigrist and A. Jakob, Biochemisches Institut, Universität Basel, CH-4051 Basel, Switzerland

Several hormones enhance K^+-efflux from liver cells, possibly by opening K^+-channels in response to cell volume changes. We investigated the mechanisms of regulation of K^+-permeability of isolated perifused hepatocytes by measuring ^{86}Rb-efflux from preloaded cells. Under basal conditions ^{86}Rb-efflux remained stable for 90 min. Addition of 10 mM alanine resulted in cell swelling of 12% and an increase in ^{86}Rb-efflux of 20-25%. Cell swelling induced by hypotonic buffer also resulted in increased efflux whereas cell shrinking caused a decrease. Rising the concentration of Ca^{2+} from 1.27 mM to 2.54 mM in the medium decreased the ^{86}Rb-efflux and removing Ca^{2+} by 5 mM EGTA enhanced it independently of cell volume. Ca^{2+} and EGTA effects could be abolished by 0.5 mM 4-aminopyridine (4-AP) which had no influence on alanine induced ^{86}Rb-efflux. Vasopressin (10 nM) mimicked the effect of Ca^{2+}. In rat hepatocytes more than one type of K^+-channels appear to be operative since extracellular Ca^{2+}, EGTA, 4-AP and possibly vasopressin affect ^{86}Rb-efflux independently of cell volume changes.

278

Voltage-Dependent Proton Current in Human Skeletal Muscle Myotubes

Krause, R. M., Bernheim, L., and Bader, C. R. Division de Neurophysiologie Clinique, Hôpital Cantonal Universitaire, CH-1211 Genève 4 and Département de Physiologie, Centre Médical Universitaire, CH-1211 Genève 4

During exercise, large quantities of protons can be released in the cytosol of skeletal muscle fibers due to lactic acid generation. Intracellular pH can fall even below 6.5 during intense exercises. So far, three ion transporters have been suggested to participate in proton extrusion in skeletal muscle: the Na^+/H^+- and Cl^-/HCO_3^- exchangers and the lactate transporter. Here, we describe the presence of a voltage-dependent proton current (I_H) in clonally cultured human myotubes using the whole-cell patch-clamp technique. We find that, with pH_{in} = 6.3 and pH_{out} = 8.0, I_H is activated at voltages more depolarized than -50 mV, the maximal conductance being observed at voltages more depolarized than +10 mV. I_H can also be activated at pH_{in} and pH_{out} = 7.3. The reversal potential of the current depends on internal and external pH, and shifts of the reversal potential can be predicted by the Nernst equation. No or minute contributions of Na^+, K^+, Ca^{2+}, and Cl^- as charge carriers are found, although activation kinetics of I_H are influenced by physiological concentrations of external Cl^-. Various divalent cations block I_H in a voltage-dependent manner, being more efficient at hyperpolarized than at depolarized voltages. Cd^{2+} is the most efficient blocker. Physiological concentrations of extracellular Ca^{2+} also block I_H with Km of 0.9 mM at -38 mV and 8.1 mM at -8 mV. Variance analysis of the current noise indicates that the elementary event is below resolution of available techniques. A model of I_H activation suggests that under extreme conditions, the conductance can reach 40% of the maximum value after 10 action potentials.

279

CLONING OF A RAT RENAL BRUSH BORDER NA/SO_4 COTRANSPORTER.

Daniel Markovich, Jutka Forgo, Gerti Stange, Jürg Biber and Heini Murer. Institute of Physiology, University of Zürich, CH-8057 Zürich, Switzerland.

Brush border membrane Na/SO_4 cotransport is an essential step in proximal tubular sulfate reabsorption in the mammalian kidney. In order to structurally identify this transport system, we have used a functional cloning strategy based on expression cloning in *Xenopus laevis* oocytes. Injection of rat kidney cortex mRNA into stage VI oocytes showed a 3-fold stimulation in Na-dependent sulfate transport (Na/SO_4 cotransport), as compared to water injected controls. Size fractionation of this mRNA through a sucrose density gradient, gave rise to an even higher stimulation (6-fold over water) of Na/SO_4 cotransport by an mRNA fraction of 1.8-2.4kb in size. A cDNA library was constructed using the positive mRNA fraction and was screened, by the injection of in-vitro transcribed cRNA into oocytes and measurement of Na/SO_4 cotransport. Using sib selection to subdivide pools of colonies, the library was screened until a single colony was identified inducing Na/SO_4 cotransport. This clone (named NaSi-1) encodes a protein which specifically stimulates Na-dependent sulfate transport in *X.laevis* oocytes. NaSi-1 cDNA shows hybridization with RNAs from rat kidney cortex, small intestine and colon, and also some cross-species hybridization with mouse and rabbit kidney cortex RNA. We believe to have cloned an epithelial specific transcript that most likely is a rat renal brush border Na/SO_4 cotransporter.

280

REGULATION OF RAT HEPATOCYTE Na^+/H^+ ANTIPORT (NHA) AT THE mRNA LEVEL IN BILIARY CIRRHOSIS (BC), BUT NOT AFTER PARTIAL HEPATECTOMY (PHX).

A. Dällenbach, C. Elsing, E. L. Renner, J. Reichen, B. Humi and U. Marti, Department of Clinical Pharmacology, University of Berne.

We have recently shown that the activity of the rat hepatocyte NHA is increased in BC, i.e. 4 weeks after bile duct ligation (BDL; Elsing, Hepatology 16:119A, 1992), and during liver regeneration, i.e. 2h following PHX (Dällenbach, J Hepatol 16 : S7, 1992). This appeared, at least in part, attributable to an increased H+ sensitivity of the antiport. A possible NHA regulation at the transcriptional level, however, remained unexplored. AIM: To determine whether hepatocellular NHA is regulated at the mRNA level after BDL and/or PHX. METHODS: Poly-A RNA was prepared from rat livers 2, 8 and 20h after 2/3 PHX (or sham operation, SH) and from rat hepatocytes isolated 4 weeks after BDL (or SH), respectively. Northern blots were hybridized with a specific probe for the rat NHA (RAI; Krapf, JCI 88: 783, 1991). Autoradiographs were quantitated densitometricly using glyceraldehyde-3-phosphate dehydrogenase (GAPD) as internal standard. RESULTS: RAI and GAPD hybridized as single 5.8 kb and 1.3 kb bands, respectively, with mRNA extracted from both, BDL (or SH) hepatocytes and PHX (or SH) livers. Normalisation of individual RAI to respective GAPD signals revealed steady state mRNA levels of hepatocellular NHA to be increased from 2.6±SEM 0.6 (arbitrary units; n=3) after SH to 5.3±0.5 (n=5) after BDL ($p<0.02$). However, 2, 8 and 20h after PHX, steady state NHA mRNA levels averaging 0.51±0.08 (n=3), 0.66±0.09 (n=4) and 0.7±0.1 (n=5), respectively, were not significantly different from those after SH (0.4±0.1 (n=2), 0.6±0.1 (n=3) and 0.8±0.1 (n=2), respectively). CONCLUSION: Rat hepatocyte NHA is regulated at the mRNA level in BC, i.e. 4 weeks after BDL, but not during liver regeneration after PHX. Cause(s) and mechanism(s) responsible for the increased steady state NHA mRNA levels in BC remain to be determined.

281

PROLINE TRANSPORT IN TRYPANOSOMA BRUCEI

L'Hostis, C., Geindre, M. and Deshusses, J.; Dept. de Biochimie, CH-1211 Geneva 4

The characteristics of proline transport in the procyclic form of Trypanosoma brucei were studied on the cells and membrane vesicles. In the cells, uptake displayed Michaelis-Menten kinetics (K_m=19 uM and V_m=17 nmol/min/10^8 cells). The transport system was found to be highly concentrative (more than 200 fold). In competition studies with analogues only L-alanine, L-cysteine and L-azetidine-2-carboxylate inhibited proline uptake. Experiments on the cells with ionophores showed that proline uptake is not energized via an ion co-transport. Iodoacetate (10 mM) decreased proline uptake by half. KCN (1mM) inhibited proline uptake to a lesser extent and the degree of inhibition was proportional to the intracellular ATP concentration. Proline transport experiments on T. brucei membrane vesicles showed an uptake by the vesicles but only in the presence of intravesicular ATP. These results suggest that this proline carrier system is highly specific, ATP-driven and independent of an ion co-transport.

282

The Glucose Transporter of *Escherichia coli*: Overexpression, Purification and Characterization of Functional Domains

Buhr, A. and Erni, B., Institute of Biochemistry, University of Berne, CH-3012 Berne, Switzerland

The glucose transporter of *Escherichia coli* catalyzes transport and phosphorylation of glucose. It consists of a hydrophilic subunit (IIA^{Glc}) and a transmembrane subunit ($IIBC^{Glc}$). $IIBC^{Glc}$ consists of two domains. The N-terminal hydrophobic IIC domain spans the membrane eight times and contains the sugar binding site, the C-terminal hydrophilic IIB domain includes the phosphorylation site (C421). The coding sequences for IIB^{Glc} (residues 391-476) and IIC^{Glc} (residues 1-386) were subcloned separately and expressed as proteins with a His_6-tag for rapid purification. $IIB^{Glc}His_6$ complements the C421S mutant of $IIBC^{Glc}$ with a defect in the phosphorylation site and it also complements $IIC^{Glc}His_6$ *in vivo* and *in vitro*. $IIB^{Glc}His_6$ has been purified by Ni-chelate affinity chromatography.

283

Na^+-CURRENT INDUCED Ca^{2+}-TRANSIENTS IN CARDIAC MYOCYTES REVEALED BY CONFOCAL MICROSCOPY

P.Lipp & E.Niggli, Department of Physiology, University of Bern, 3012 Bern

An important step in the cardiac signal transduction coupling electrical excitation to contractile activity (ec-coupling) is the activation of Ca^{2+}-release from the sarcoplasmic reticulum (SR). Evidence is growing that in addition to the "physiological" activator (Ca^{2+}-current) the Na^+-current may serve as a release trigger. Release most likely results from an activation of the Na^+/Ca^{2+}-exchange to transport Ca^{2+}-ions into the cell. But until now evidence supporting this hypothesis was indirect. We used a new approach to measure the intracellular Ca^{2+}-concentration ratiometrically with a mixture of the two fluorescent Ca^{2+}-indicators Fluo-3 and Fura-Red. Ca^{2+}-current and Na^+-current induced Ca^{2+}-transients were analyzed in the absence and presence of 20 µM ryanodine. The Ca^{2+}-transients obtained under the different conditions were clearly distinguishable in agreement with the concept of Ca^{2+}-induced Ca^{2+}-release. In addition we were able to demonstrate small Ca^{2+}-transients obtained under conditions where Ca^{2+}-release was blocked by ryanodine. In the presence of ryanodine kinetic differences between Na^+-current and Ca^{2+}-current induced Ca^{2+}-transients could be found with the fast Ca^{2+}-indicator Fluo-3. These results therefore directly demonstrate the existence of Na^+-current induced Ca^{2+}-transients that under conditions of a functional SR may serve as a trigger for Ca^{2+}-release. In addition these findings support the existence of a restricted space underneath the sarcolemma.

Supported by the SNF.

284

SUBSTRATE DEPENDENCE OF NA-CA EXCHANGE 'GATING' CURRENTS

E. Niggli & P. Lipp, Department of Physiology, University of Bern, 3012 Bern.

Flash photolysis of 'caged' Ca^{2+} was used to produce intracellular Ca^{2+} concentration jumps in single cardiac myocytes. The rapid increase of intracellular Ca^{2+} activates Na-Ca exchange current and a brief 'gating' current. The gating current (I_{conf}) has been associated with a conformational change of the Na-Ca exchanger molecules after binding of Ca^{2+}. The amount of charge moved with I_{conf} reflects the number of exchanger molecules with the Ca^{2+} binding sites facing the inside of the membrane. Combined application of a rapid extracellular solution switcher and photorelease of intracellular Ca^{2+} enabled us to analyze the effects of Na^+_o and Ca^{2+}_o on the steady-state distribution of exchanger states. Compared to substrate-free conditions (Li^+ or NMG solutions), a short (~2 s) exposure to 10 mM Na^+ reduced I_{conf} to 92.2% ± 2.4% (mean ±S.E, n = 11). Addition of 1 mM Ca^{2+} had no significant influence on I_{conf} (99% ± 3.9%; n = 9). These results do not reflect the inward shift of molecules predicted by a consecutive transport model for the Na-Ca exchanger. We conclude that I_{conf} may arise from a conformational change of the Na-Ca exchanger molecules after Ca^{2+} binding other than a membrane crossing transition. Alternatively, the findings could be explained by assuming a two-step simultaneous mechanism for the Na-Ca exchange. Increases of I_{conf} observed after prolonged exposure to substrate-free solutions may result from uncontrolled changes of $[Ca^{2+}]_i$. Supported by SNF

285

EXPRESSION OF THE CARDIAC Na^+/Ca^{2+}-EXCHANGER BY THE VACCINIA SYSTEM

T. Iwata, D.Guerini, and E. Carafoli, Laboratory of Biochemistry III, ETH Zürich

The Na^+/Ca^{2+}-Exchanger is critical in the regulation of the contraction/relaxation cycle of cardiac muscle. Efforts have so far been only marginally successful to characterize this protein biochemically. The recent cloning of the corresponding cDNA and the expression of the protein in frog oocytes has established some principles on the structure of this protein. To make it amenable to biochemical analysis an expression system producing large amounts of the protein is needed. To this purpose we have used the vaccinia virus system, a system that allows great flexibility in the choice of the expressing cell lines and which is faster than other viral systems. Two different cell lines (HeLa and COS) were tested so far: in both significant expression of the exchanger-activity (Na^+-dependent/Ca^{2+} uptake or release) could be detected. After ^{35}S-Met labeling of the cells a band of a molecular mass of 120 kDa was observed, accounting for 3-5% of the total newly syntesized proteins.

286

A MUTATION IN THE FIRST TRANSMEMBRANE DOMAINE OF THE Na-K-PUMP INDUCES A HIGH AFFINITY FOR K^+.

Horisberger, J.-D., Canessa, C. and Rossier, B. C. Institut de Pharmacologie, Lausanne.

A cystein to phenylalanin (C113F) mutation in the outer third of the first transmembrane domain of the α subunit of Na-K-ATPase has been shown to decrease the binding rate of ouabain (EMBO J. 11:1681, 1992). We have studied the K^+ activation kinetics of this mutant by expressing it in Xenopus oocytes and measuring Na-K-pump current under voltage clamp conditions. In the absence of external Na^+, the apparent affinity for K^+ was increased, $K_{\frac{1}{2}}$ at -50 mV 45 ± 6 µM in the C113F mutant vs 135 ± 24 µM in wild type. The maximal K^+ induced current (Imax) was about 50% smaller in the C113F mutant. We studied then the ouabain-sensitive charge translocation under conditions of Na^+/Na^+ exchange (E1.Na <-->E2 + Na). The rate constant for a -50 to +50 mV voltage step was markedly slower in the C113F mutant, 53 ± 3 s^{-1} than in wild type, 288 ± 12 s^{-1}, while the backward rate constant of the same step was not affected. Kinetic analysis demonstrates that the change of this single forward step rate constant can explain the slower ouabain binding rate, the lower Imax and the higher apparent affinity for external K^+.

287

BIMODAL ACTION OF ALDOSTERONE ON Na,K-ATPase ACTIVATION AND CELL-SURFACE EXPRESSION IN A6 KIDNEY CELLS.

Beron, J. and Verrey, F., Physiologisches Institut der Universität Zürich, CH-8057 Zürich.

Aldosterone induces in A6 cells an early increase in active Na pumps, detected by measuring the initial rate of ouabain binding (Pellanda et al., Am. J. Physiol. 262: C899-C906, 1992). It has been postulated that this effect, which precedes a transcriptionally mediated increase in the rate of Na,K-ATPase subunit synthesis, could involve the translocation of Na pumps from an intracellular location to the cell surface. To test this hypothesis we used A6 cells maintained on filter cultures in serum-free medium. As expected, aldosterone induced an increase of the initial rate of ouabain binding (2.5 fold in 3 hours). This effect was parallel to but independent of the increase in transepithelial Na reabsorption. Changes in the pool of cell-surface Na,K-ATPase were evaluated using labeling of cell-surface proteins with sulfosuccinimidobiotin or enzyme-mediated radioiodination. Three hours after aldosterone (10^{-6} M) addition there was no increase in cell-surface Na,K-ATPase, while after 20 hours and 5 days there was a 1.7 and 2.5-fold increase, respectively. This increase was parallel to that of the total Na,K-ATPase pool measured by Western blotting and to that of the total pump sites measured by ouabain binding. We conclude that the late response to aldosterone is characterized by an increase in total and cell-surface Na,K-ATPase, while there is no indication that aldosterone promotes the translocation of pumps to the cell-surface. The early effect might be mediated by the activation of previously silent cell-surface pumps.

288

HYDROPHOBIC AMINO ACIDS IN THE C-TERMINAL OF Na,K-ATPase β-SUBUNITS ARE IMPORTANT FOR ASSEMBLY WITH α-SUBUNITS

Beggah, A.T., Jaunin, P., Peitsch, M., and Geering, K., Institut de Pharmacologie et de Toxicologie de l'Université, rue du Bugnon 27, CH-1005 Lausanne, Switzerland

Na,K-ATPase is a plasma membrane transporter that is responsible for the maintenance of the ionic homeostasis of animal cells. In the active form, Na,K-ATPase is composed of a catalytic α-subunit (Sα) and a β-subunit (Sβ). Subunit assembly is needed for the structural and functional maturation of the enzyme as well as for its transport from the ER to the plasma membrane. In order to characterize more precisely the structural determinants in Sβ which are implicated in subunit assembly, we have introduced point mutations in the last 11 amino acids of the C-terminal. By following the cellular accumulation of Sα, the glycosylation processing of Sβ and the cell surface expression of functional Na,K-ATPase in Xenopus oocytes after injection of cRNAs, we could show that mutations in the hydrophobic domain of the C-terminus abolish subunit assembly. On the other hand, mutations in the hydrophilic charged domain have no significant effect on the assembly process. Our data indicate that the hydrophobic domain in the C-terminal of Sβ either directly participates in the interaction with Sα or contributes to the acquisition of an assembly competent configuration of Sβ.

289

HETEROGENOUS GLYCOSYLATION OF Na,K-ATPase EVIDENCED BY LECTIN-BLOTS

M. Benalial and B.M. Anner, Laboratoire de Thérapeutique Expérimentale, Division de Pharmacologie Clinique, CMU, 1211 Genève 4.

The carbohydrate structures of blotted glycoproteins can be analysed by probing them with lectins (referred to here as lectin-blot analysis, extension of the widely used Western blotting technique). Lectins detect glycosylation by non-covalent binding to specific sugar groupings. We compared the lectin binding patterns of Na, K- ATPase (NKA), an integral membrane protein of intriguing heterogeneity, isolated from kidney and brain of different rat strains (spontaneously hypertensive rats SHR, Wistar Kyoto normotensive controls WKY, Milan hypertensive and normotensive strains MHS, MNS.). NKA is composed of an α catalytic subunit (112 kD) and a heavy glycosylated β subunit (55 kD). Yet, little information is available on possible heterogenous sugar groupings in NKA isoforms. Lectin-blot analysis was performed with a detection limit of about 0.1 µg glycoprotein with 5 different lectins, chosen for their well described and rather narrow binding specificities; these are conjugated to the steroid hapten digoxigenin by chemical coupling of digoxigenin-carboxymethyl-N-hydroxysuccinimide ester (Haselbeck A. *et al.* Anal. Biochem. 191 (1990). The different NKA preparations, electroblotted to a nitrocellulose sheet after gel electrophoresis (SDS PAGE), were incubated with the digoxigenin labelled lectins for 1h at 20°C. The bound lectins were detected by an alkaline phosphatase labelled antidigoxigenin antibody. The staining patterns were significantly different among the tested rat brain and kidney NKA preparations suggesting that sugar groupings confer additional heterogeneity to NKA. *SNSF* 31-25 666.88.

290

THE C-TERMINAL PORTION OF THE PLASMA MEMBRANE Ca^{2+} PUMP BINDS THREE Ca^{2+} IONS WITH VERY HIGH AFFINITY

Francesco Hofmann, Peter James, Thomas Vorherr and Ernesto Carafoli
Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), 8092 Zürich, Switzerland

The C-terminal portion of the plasma membrane Ca^{2+}ATPase (PMCA) contains different regulatory domains. A recombinant C-terminal fragment of the hPMCA1b isoform (E1079 - P1180) was used to study the role of two acidic amino acid stretches located on either side of the calmodulin-binding domain. The molecular mass of the recombinant C-terminal fragment, as determined by electrospray ionization mass spectrometry, was 39 mass units higher than the calculated value (12055 Da). This difference was due to an "EGTA insensitive" Ca^{2+} ion, which was located by chymotryptic proteolysis in a fragment corresponding to the last 37 amino acids of the expressed protein. Fluorescence experiments on the dansylated recombinant C-terminal fragment titrated with increasing amounts of free Ca^{2+} revealed two additional Ca^{2+} binding sites with affinities corresponding to k_Ds of about 30 and 300 nM, respectively. "Stains all" spectra of different synthetic peptides, corresponding to subdomains of the expressed protein, indicated that the site with the k_D of 30 nM was probably located in the acidic sequence on the N-terminal side of the CaM-binding domain; the site with the 300 nM k_D was apparently located on the C-terminal side of the calmodulin binding domain or, alternatevely, formed by the appropriate three dimensional arrangement of sequentially distant residues of the domain.

291

CONSTRUCTION AND CHARACTERISATION OF A CHIMERIC ER Ca^{2+}-ATPase, CONTAINING A PUTATIVE PHOSPHOLIPID BINDING DOMAIN OF THE PM Ca^{2+}-ATPase

S. De Jaegere, F. Wuytack, D. Guerini and E. Carafoli, Laboratory for Phisiology, K.U.Leuven and Laboratory for Biochemistry III, ETH Zürich

Plasma membrane (PM) and endoplasmic reticulum (ER) Ca^{2+}-ATPases differ not only in their membrane location but also in regulation properties. Regulation by phospholipids is peculiar to the PM-pump and different domains of the pump have been proposed to be involved in it. One of these domains, located between putative transmembrane helices 2 and 3, has been identified as a hot spot for alternative splicing producing at least at the mRNA level, three different isoforms. A chimeric ER-pump containing this region was constructed and expressed in the Baculovirus system.The chimeric protein was active, when tested for the formation of the phosphorylated intermediate from ATP , but showed differences with the ER-pump in the backward phosphorylation reaction with Pi. Different methods of reconstitution have been tried to study the pump in defined mixtures of phospholipids.

292

CONSTRUCTION, EXPRESSION AND LOCALISATION OF CHIMERIC SR/PM- Ca^{2+}-ATPases

D. Foletti, D. Guerini and E. Carafoli, Laboratory for Biochemistry III, ETH Zürich

Ca^{2+} -ATPases are membrane proteins transporting calcium out of the cytosol against a large chemical gradient. They are located in the intracellular membrane systems, i.e., the ER/SR-pump and located on the plasmamembrane (PM-pump). Cloning work has revealed that they belong to a family of highly homologous proteins, but hasn't provided any indications on the targeting to the different membranes. To obtain information on the targeting-signals or-sequence, two different chimeric proteins have been constructed, one made out of 150, the second out of 331 aminoacids of the N-terminal of the SR, followed by the remainder of the C-terminal sequences of the PM pump. The constructs were expressed transiently in the COS-cells and characterized biochemically. The membrane localization of the chimeric proteins was performed by immunocytochemistry with antibodies specific for the human PM-ATPase. Preliminary results indicate differences in the immunocytochemical staining compared to the PM-pump transfected cells.

293

QUANTITATIVE TISSUE DISTRIBUTION OF HUMAN PLASMA MEMBRANE CALCIUM PUMP ISOFORMS

Stauffer, T.P., Hilfiker, H., Carafoli, E., Strehler, E.E.*, Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), CH-8092 Zurich, Switzerland, * Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905, U.S.A.

The human plasma membrane calcium ATPase (hPMCA) gene family consists of at least four members; isoform variability is further increased by alternative RNA splicing at a number of "hot spots". We have used quantitative polymerase chain reaction (PCR) methodology to assess the distribution of the human PMCA1, 2, 3 and 4 mRNAs in various tissues. The results show that hPMCA1 and 4 correspond to ubiquitous isoforms whereas the expression of significant amounts of hPMCA2 and 3 is restricted to cerebral cortex and skeletal muscle. In contrast to the relative non-specific pattern of overall hPMCA gene expression, the different alternative spliced sub-forms of each gene transcript show a highly tissue-specific pattern of expression with a predominance of complex splicing options performed in the human cerebral cortex. Novel alternative spliced human PMCA mRNAs were detected by RT-PCR for isoforms 2, 3 and 4. A new nomenclature for the precise description of PMCA isoforms has been developed that takes alternative splicing at all "hot spots" into account.

Supported by Swiss NF grants Nos. 31-27103.89 and 31-28772.90

294

CHARACTERIZATION OF A CALMODULIN-STIMULATED Ca^{2+} TRANSPORT ACTIVITY IN TONOPLAST VESICLES FROM MAIZE ROOTS

Gavin, O., Pilet, P.E. and Chanson, A., Institute of Plant Biology and Physiology, Biology Building, CH-1015 Lausanne

Maize (*Zea mays* L., cv LG 11) root membranes were fractionated by dextran density gradient centrifugation. Marker enzymes were used to study the distribution of the different membranes in the gradients and a filtration technique was developed to measure $^{45}Ca^{2+}$ transport in sealed vesicles. Most of the Ca^{2+} transport activity was associated with the ER. However, a small part of this activity was associated with the tonoplast (corresponding to the activity of the H^+/Ca^{2+} antiport) and to the plasma membrane. When the Ca^{2+} transport was measured in the presence of exogenous calmodulin (1 µM), the calmodulin-stimulated activity was associated with the tonoplast vesicles only. This calmodulin-stimulated Ca^{2+} transport was insensitive to monensin, a proton ionophore, ruling out a direct effect of calmodulin on the H^+/Ca^{2+} antiport. We propose that a calmodulin-stimulated Ca^{2+}-ATPase is associated with the tonoplast of young maize root cells. Data will be presented on the characterization of this calmodulin-stimulated Ca^{2+}-transport activity.

295

FUNCTION AND EXPRESSION OF MULTIPLE DRUG RESISTANCE-LIKE GENES IN ARABIDOPSIS THALIANA

Siedler M., Ringli, C., and Dudler R., Institut für Pflanzenbiologie, Universität Zürich, CH-8008 Zürich

The products of multiple drug resistance (MDR)-like genes are integral membrane proteins that function as energy-dependant transporters of a diverse array of substrates in many eukaryotes. Best known are the mammalian P-glycoprotein drug transporters which are responsible for the multiple drug resistance phenomenon of many tumor cells. While some P-glycoproteins have recently been shown to have dual functions as drug transporters and chloride channels, other related proteins are involved in signal sequence-independent transport of peptides across membranes and heavy metal detoxification. To explore the possible function of MDR-like gene products in processes such as herbicide detoxification and signal-molecule transport in plants, we decided to analyze such genes in *Arabidopsis thaliana*. The genome of this species appears to contain a small number of MDR-like genes, two of which we have cloned and analyzed (*atpgp1* and *atpgp2*). We have studied the spacial and temporal expression pattern of *atpgp1* in transgenic plants using promoter/reporter gene chimeric constructs. To explore the function of this gene we have constructed transgenic plants overexpressing either the sense strand or the anti-sense strand of *atpgp1*. The results of these experiments will be presented.

296

CHARACTERIZATION OF AMINOPHOSPOLIPID TRANSLOCATION IN RED CELL VESICLES

#Zs. Beleznay, *A. Zachowski, *P. F. Devaux and #P. Ott; #Inst. Biochem. & Mol. Biol., University of Bern, Bühlstrasse 28, CH-3012 Bern/Switzerland; *Inst. Biol. Physico-Chimique., 13 rue P. M. Curie, F-75005 Paris/France

ATP-dependent aminophospholipid translocation was determined by EPR in the membranes of vesicles released from red blood cells. From the initial rates of phospolipid translocation and the concomitant ATP consumption, a stoichiometry of approximately one aminophospholipid molecule translocated per ATP molecule hydrolyzed could be obtained. The translocation was inhibited by vanadate in a concentration dependent way by up to 95 % for the spin-labelled phosphatidylserine and 59 % for the phosphatidylethanolamine analogue. Suramin (400 µM) also inhibited translocation, but only by 45 % or 32 %, respectively. Since both agents are known to be inhibitors of P-type ATPases the results strongly suggest that the aminophospholipid translocase of the red cell membrane acts *via* formation of a phosphoenzyme intermediate.

297

SOLUBILIZATION AND RECONSTITUTION OF THE TWO TONOPLAST PROTON PUMPS FROM *Rubus hispidus* CELL CULTURES

Perotti, E. and Chanson, A., Institute of Plant Biology and Physiology, Biology Building, CH-1015 Lausanne

Two proton-translocating activities are present on the tonoplast of higher plant cells: an anion-sensitive ATPase and a cation-sensitive pyrophosphatase (PPase). Both pumps generate an electrochemical potential difference of protons, serving as the driving force for several transport processes (uniport, antiport and symport) across the vacuolar membrane. To enhance our understanding of the molecular properties and transport mechanism of these two pumps, it was necessary to reconstitute them into liposomes and to compare the H^+-pumping activity with that of native tonoplast vesicles. The ATP- and pyrophosphate-dependent proton pumps from tonoplast-enriched vesicles prepared from *Rubus hispidus* cell cultures were solubilized in the presence of Triton X-100 and reconstituted into liposomes of soybean phosphilipids, using Bio-Beads SM-2 to remove the detergent. The specific activity of the two pumps was greatly increased by the solubilization-reconstitution procedure. Identical characteristics were found for pyrophosphate-dependent proton transport in native and reconstituted vesicles. However, the ATP-dependent proton transport of the reconstituted vesicles was no longer inhibited by KNO_3.

298

THE BEHAVIOUR OF THE CHLOROPLAST ATPase ACTIVITY IN AN APOLAR MEDIA

Kernen, P.[1], Degli Agosti, R.[1], Greppin, H.[1], Darszon, A.[2,1] and Strasser, R.J.[1], [1]Université de Genève, Laboratoire. de Bioénergétique, Jussy-Lullier and [2]UNAM, Instituto de Biotechnología, Cuernavaca, México

Spinach thylakoids were transferred into a reverse micellar phase (detergent CTAB, 1-hexanol and n-octane (CHO)). The macrocomplexes transferred into CHO displayed ion-dependent and methanol-enhanced ATPase activities similar to the membrane-bound enzyme in aqueous buffer. Both latent and methanol-modulated enzyme activities were highly dependent on the amount of water present, e.g. reducing water content from 7.2% to 3.6% decreased the specific ATPase activity 3 fold. Decreasing concentrations of water in CHO increased the fraction of bound water as shown by ^{1}H-Nuclear Magnetic Resonance. However the observed water-dependent ATPase activities did not fully follow the measured activity of water in the low water system, indicating that other regulatory factors are involved.

299

PROBING THE MEMBRANE TOPOLOGY OF CYTOCHROME P450S FROM THE YEAST *CANDIDA TROPICALIS*

Sanglard, D., Sengstag*, C. and Seghezzi, W.,Inst. für Biotechnologie, ETH-Hönggerberg, CH-8093 Zürich und *Inst. für Toxikologie, CH-8603 Schwerzenbach

The membrane topology of two alkane-inducible cytochrome P450s from the yeast *Candida tropicalis*, alk1 and alk2, was tested by construction of fusion proteins with part of invertase and histidinol dehydrogenase (invHIS4C) and expression in a *Saccharomyces cerevisiae his4* mutant. Depending on the localization of invHIS4C on the endoplasmic reticulum (ER) cytoplasmic or luminal side, the enzyme converts histidinol to histidine and allows the *his4* yeast strain to grow on histidinol supplemented medium. The N-terminal segments of alk1 and alk2 were fused to invHIS4C at different locations. The combination of this *in vivo* assay with subcellular immunoprecipitations of the expressed fusion proteins allowed us to establish that both P450s contain only one transmembrane domain with their N-terminus located in the ER lumen. Deletions removing the transmembrane domain of alk1 resulted in a less efficient targeting to the ER membrane but did not prevent insertion in these membranes. Furthermore deletion of a negatively charged peptide preceding the alk1 transmembrane domain in an invHIS4C protein fused after this domain caused the N-terminal end to have a positive net charge and to be oriented in the cytoplasm, thus translocating the remaining of the fusion protein into the ER lumen.

300

FLUORESCENCE QUENCHING OF LIGHT-HARVESTING PIGMENT-PROTEIN COMPLEX IN LOW WATER MEDIA

Gruszecki, W.I.[1], Kernen, P.[2], Krupa, Z.[1], Darszon, A.[2] and Strasser, R.J.[2], [1]Maria Curie-Sklodowska University, Lublin, Poland and [2]University of Geneva, Bioenergetics Laboratory

Light-harvesting pigment-protein complex of photosystem II (LHCII) transferred into a ternary system (isooctane-phospholipids-water) showed 1) efficient chlorophyll b to a excitation energy transfer and 2) energetic uncoupling of accessory xanthophyll pigments from chlorophylls. The light-induced fluorescence decrease of LHCII was found to be monophasic in the law water system and biphasic in aqueous buffer. During a dark intervall of 30 minutes the fluorescence changes were 50% reversible in buffer but completely reversible in the solvent system, indicating that only the fast component of fluorescence quenching, independent of energy transfer to xanthophylls, is active in the latter system. Non-conventional low water systems may provide unique possibilities to study chlorophyll deexcitation processes in photosynthesis.

301

The highly conserved ORF 31 downstream of *psbB* in *Chlamydomonas reinhardtii*, appears to be required for maintaining Photosystem II complex activity under conditions of limited chloroplast protein synthesis.

Caroline Monod, Michel Goldschmidt-Clermont, Jean-David Rochaix. University of Geneva, Dept. of Molecular Biology, Sciences II, CH-1211 Geneva, Switzerland.

We have identified for the first time a potential stress protein required by *C. reinhardtii* under conditions of reduced chloroplast protein synthesis. The importance of this potential stress protein is underlined by its conservation through evolution from *C. reinhardtii* to higher plants, not only with respect to its amino acid sequence but also with respect to its location in the chloroplast genome: ORF 31 is always located downstream of the *psbB* gene, that encodes one of the larger polypeptides of the Photosystem II complex. Having begun to characterize the function of the ORF 31 product is especially satisfying, since approximately half of the subunits comprising the Photosystem II complex are low molecular weight proteins of which the functions are unknown. Several of these small proteins are conserved from cyanobacteria to higher plants and are therefore believed to play a basic role in the structure and/or the functional capability of the PS II complex. Determining the role of these low molecular weight proteins is necessary to complete our understanding of the function of all the coding sequences that comprise the chloroplast genome in algae and higher plants.

302

GENERATION OF A MASTOCYTOMA LINE DEFICIENT IN GLYCOSYL-PHOSPHATIDYLINOSITOL ANCHORING

N. Déglon, A. Puoti, C. Beghdadi-Rais, M. Rousseaux, C. Bron, A. Conzelmann and N. Fasel. Institut de Biochimie, Université de Lausanne, 1066 Epalinges

P815 mastocytoma cells, expressing the glycosyl-phosphatidylinositol-anchored (GPI-anchored) murine T cell marker Thy-1 were mutagenized by treatment with ethyl methanesulfonate. Thy-1 negative cells were selected by successive negative sorting of anti-Thy-1-stained cells. The synthesis of an intracellular Thy-1 protein was demonstrated by immunoprecipitation in one clone selected for analysis. Furthermore, the expression of another GPI-anchored polypeptide was shown to be affected, suggesting a defect in GPI anchoring. Analysis of the putative intermediates in GPI biosynthesis using metabolic labeling with [^{3}H]myo-inositol and TLC confirmed this interpretation. This new glycosyl-phosphatidylinositol anchoring mutant is therefore a potential host for DNA mediated complementation using cosmids or cDNA expression libraries.

303

COOH-TERMINUS AMINO ACID REQUIREMENTS FOR GPI-ANCHOR ADDITION TO CELL SURFACE PROTEINS

C. Beghdadi-Rais, M. Schreyer, C. Bron and N. Fasel, Institut de Biochimie, Université de Lausanne.

All surface proteins anchored in the plasma membrane via a glycosylated form of phosphatidylinositol (GPI) are originally synthesized with a COOH-terminal hydrophobic region which is cleaved soon after translation and replaced by the preformed anchor. The signal which directs glycolipid addition is located in the COOH-terminal region of the precursor protein and has been shown to require a well-defined cleavage/attachment site positionned 10-12 residues upstream of the hydrophobic C-terminal domain. The GPI-anchored Thy-1 glycoprotein was used as a model system to further characterize the amino acid requirements of the spacer region separating the anchor acceptor site and the C-terminal hydrophobic segment. The spacer domain was replaced by different amino acid sequences and the correctly processed, GPI-anchored, molecules were selected by panning. This approach and the use of random amino acid sequences to modify the spacer element will provide informations on the structural requirements of this important domain.

304

Potential biosynthetic intermediates of glycophosphatidylinositol anchor biosynthesis in Saccharomyces cerevisiae

György Sipos, Alessandro Puoti and Andreas Conzelmann, Institute of Biochemistry, University of Fribourg

The carbohydrate part of the glycophosphatidylinositol-anchors (GPI-anchors) of Saccharomyces cerevisiae has recently been found to have the same core structure as other eukaryotic GPI-anchors, namely **Protein-ethanolamine-PO$_4$-(Manα1,2-or3Manα1,2->) Manα1,2Manα1,6Manα1,4GlcNa1,6Myoinositol1-PO$_4$-lipid (side chain in parenthesis).** Although [^{3}H]myo-inositol becomes integrated into newly made GPI-anchored proteines within minutes after addition to growing yeast cells, completed [^{3}H]myo-inositol labeled precursor glycolipids ready to be transferred onto proteins have not been found. Yet such free GPIs have been described in trypanosomes and mammalian cells.When yeast cells were metabolically labeled with [^{3}H]myo-inositol in the presence of mannosamine (2-amino-2-deoxy-D-mannose) we observed novel lipids some of which were sensitive to nitrous acid and to mild base treatment and resistent to PI-PLC. Since this combination of treatments is quite diagnostic for later GPI precursors, these novel lipids might be mannosamine-containing GPI-intermediates the further elongation of which is arrested because subsequent transfer of mannose onto the C$_2$ position of mannosamine is no more possible or they might accumulate because some mannosyltransferase of the GPI-pathway cannot work with GDP-mannosamine.Further studies address the intracellular addition of the Manα1,2 or 3Manα1,2- side chain. For this we used metabolic labeling with [^{3}H]myo-inositol of secretion mutant sec18. Labeled proteins retained either in the ER or the Golgi were isolated and the carbohydrate part of the anchor was released by hydrofluoric acid and then analyzed by paperchromatography in parallel with the appropriate standards. It turns out that the side chain is already present on the proteins retained in the ER and must be added in the ER.

305

EVIDENCE FOR A TRIPLE-INTERACTION BETWEEN α-ACTININ, (META-) VINCULIN AND ACIDIC PHOSPHOLIPID BILAYERS

Niggli, V.[1] and Gimona, M.[2], [1]Dept. of Pathology, University of Bern, CH-3010 Bern and [2]Institute of Molecular Biology, Austrian Academy of Sciences, Salzburg, Austria

The cytoskeletal component vinculin has been demonstrated by hydrophobic photolabelling to insert into bilayers of acidic phospholipids. We now show that the higher molecular weight variant metavinculin as well as α-actinin share the same property. Interestingly, preincubation of α-actinin with vinculin or metavinculin prior to the addition of liposomes strongly inhibited labelling of α-actinin under conditions of non-limiting liposome surface, but enhanced labelling of vinculin. In contrast, vinculin and metavinculin did not mutually influence their labelling. Using gel filtration chromatography, we could demonstrate that α-actinin still bound to the vinculin/liposome complex under conditions similar to those used for hydrophobic photolabelling with non-limiting lipid surface. Our results suggest formation of a triple complex consisting of vinculin,α-actinin and phospholipids. In this complex, both proteins interact at the bilayer, resulting in an altered conformation of the two proteins and,as a consequence,in modified bilayer interactions.

541 (received after deadline)

SINGLE CHANNEL ANALYSIS OF THE ANION CHANNEL FORMING PROTEIN

Schürholz, Th., Fakultät für Chemie der Universität Bielefeld, D-4800 Bielefeld 1

The plant pathogenic bacterium Clavibacter michiganense ssp. nebraskense secretes an anion channel forming protein into the culture fluid (Th. Schürholz et al., 1991, J.Membrane Biol. 123, 1-8). The channel inserts spontaneously into planar lipid bilayers and is strongly rectifying. At 0.8 M KCl the *single channel conductance* $G(V_m)$ increases exponentially from 21 pS at 50 mV up to 53 pS at V_m = 200 mV, 20°C. The anion concentration $K_{0.5}$, where $G(V_m)$ has its half maximum value, increases from 0.12 M at 50 mV to 0.24 M at 175 mV for channels in a soybean phospholipid bilayer. The voltage dependence of the single channel conductance, which is different for charged and neutral lipid bilayers, can be described either by a two-state flicker (2SF) model and the Nernst-Planck continuum theory, or by a two barrier - one-site (2B1S) model with asymmetric barriers. No voltage dependent conformational change has to be assumed in the latter case. The increase in the number of open channels after a voltage jump has a time constant of τ = 0.2 s at 50 mV and τ = 1 s at 200 mV. The electric part of the gating process is characterized by the (reversible) molar electrical work $\Delta G^{\ominus}_{el} = pz_gFV_m \approx -1.3$ RT, which corresponds to the movement of one charge of the gating charge number $|z_g| = 1$ across the fraction $p = \Delta V_m/V_m = 0.15$ of the membrane voltage V_m = 200 mV. Unlike chloride, fluoride shows a maximum of the single channel conductance at about 150 mV in the presence of Pipes (≥5 mM, pH 6.8) with $K_{0.5} \approx 1$ M. It is shown that the decrease in conductance is due to a blocking of the channel by the buffer.

Membrane Trafficking

306

ULTRASTRUCTURAL CHANGES IN MONOCYTES/MACROPHAGES INDUCED BY PHAGOCYTOSIS OF *BORRELIA BURGDORFERI*

MG Rittig [1], T Häupl [2], A Krause [2], GR Burmester [2]. 1) Department of Anatomy I ,2) Department of Medicine III, University of Erlangen, Germany

Lyme borreliosis is an inflammatory multisystemic disease caused by the spirochete *Borrelia burgdorferi*. This organism displays some unusual features which may contribute to the host's inability to eliminate the pathogenic spirochete. An electron microscopal (EM) study was performed to reveal ultrastructural alterations in human peripheral blood monocytes and murine bone marrow-derived macrophages caused by the uptake of this organism. Scanning EM showed that the monocytes/macrophages engulfed only parts of the extremely long spirochetes, while the remaining parts were projecting out of the cells. When two phagocytes started to internalize the same spirochete, transmission EM revealed that these cells fused at their contact regions. In addition, the murine macrophages developed enlarged cisterns of rough endoplasmic reticulum (rER) which were arranged in parallel rows along the cell periphery. These rER configurations separated from the macrophages creating cell membrane-bound rER bodies. In conclusion, release of catabolic enzymes out of leaky phagolysosomes, formation of syncytial cells of the foreign body type, and rER alterations are observed with monocytes/macrophages following the phagocytosis of *B. burgdorferi*. These findings may help to explain the host's inability to overcome the spirochetal infection in chronic disease situations.

307

ANALYSIS OF LIPOSOME-LYMPHOCYTE INTERACTION BY FLUORESCENT TECHNOLOGIES

B. Volet and B.M. Anner, Laboratoire de Thérapeutique Expérimentale, Division de Pharmacologie Clinique, C.M.U., 1211 Genève 4.

Liposomes are used to transfer drugs as well as various macromolecules to cells; they are believed to enter cells by endocytosis but it is not known if this is true for lymphocytes. We determined the liposome uptake by lymphocytes isolated from healthy donors: fluorescent liposomes prepared in the presence of 6-carboxyfluorescein (CF) by the detergent dialysis method were incubated with lymphocytes at either 4°C or 37°C during times ranging from 5 to 30 minutes in RPMI-1640 medium. As a control, lymphocytes were incubated in presence of free CF at a concentration corresponding to the total CF released by detergent lysed liposomes. After incubation, the cells were washed in PBS, observed under a fluorescence microscope and analysed either in a spectrofluorometer or in a FACScan. Fluorescence microscopy showed that the lymphocytes incubated with CF-liposomes were all diffusely fluorescent; they were more fluorescent when incubated at 37°C than at 4°C. However the control cells incubated with free CF also displayed some fluorescence although it was less pronounced. The fluorometry and the FACScan results confirmed and quantified these observations: cells were 4-5 times more fluorescent when incubated with CF-liposomes as with free CF which is probably taken up by pinocytosis. Moreover, the lymphocytes became more fluorescent with increased incubation time in the presence of CF-liposomes. The temperature dependence strongly suggests that the liposomes enter the lymphocytes by an energy-requiring process. SNSF 31-25666.88

308

CLONING STRATEGIES FOR THE ENDOPLASMIC RETICULUM GLYCOPROTEIN GLUCOSIDASE II.

Brada D., Ziak M. and Roth J. Division of Cell and Molecular Pathology, Department of Pathology, University of Zürich, 8091 Zürich

Glucosidase II is a resident glycoprotein of the endoplasmic reticulum in hepatocytes. It catalyzes the removal of the two inner α1,3-linked glucose residues of the oligosaccharide precursor chains of N-glycosylated glycoproteins.
The aim of this study was to clone the cDNA encoding pig glucosidase II in order to elucidate its protein structure. Two different approaches were established. (i) a λgt11 cDNA library was screened with antibodies raised against the SDS-denatured glucosidase II. Five positive clones were purified to homogeneity and their fusion proteins further analyzed. One of them showed immunoreactivity with antibodies recognizing the native enzyme. The fusion protein of another clone and the purified glucosidase II were digested with different proteases. The fragments were separated by SDS PAGE followed by Western blot analyses. The results indicate, that several fragments are shared by the fusion protein and glucosidase II. (ii) proteolytic fragments of glucosidase II were microsequenced. Oligonucleotide primers were synthesized according to these protein sequences and used for PCR amplification of DNA fragments coding for glucosidase II. Pig liver mRNA was prepared as a template. The amplified DNA fragments will be used for screening the λgt11 library. Combination of these two approaches should yield a full length nucleotide sequence information.

309

FUNCTIONAL DISSECTION OF THE COMPONENTS IN THE ELONGATION ARREST DOMAIN OF THE SIGNAL RECOGNITION PARTICLE.

Bui, N. & Strub, K. Dépt de Biologie Cellulaire, Université de Genève, 1211 Genève 4.

SRP, a cytoplasmic ribonucleoprotein, plays an essential role in sorting proteins to the ER. In addition to its signal recognition and targeting activity, SRP also contains an elongation arrest function which effects a specific arrest or pause in the biosynthesis of ER-targeted proteins in vitro. Two proteins, SRP 9 and SRP 14, and the Alu sequences of SRP RNA are essential for this function. SRP 9 and SRP 14 specifically bind SRP RNA only as a heterodimer. Furthermore, the two proteins form a dimer in the absence of SRP RNA. The heterodimer is therefore most likely an intermediate in the assembly of the RNA-protein complex. The analysis of a series of N- and C- terminal deletion mutants of SRP 9 and SRP 14 has allowed us to define regions in the proteins that are essential for dimerisation and for SRP RNA binding. These observations suggest that both proteins are involved in RNA binding.

310

YEAST Wbp1p AND Swp1p FORM A COMPLEX ESSENTIAL FOR OLIGOSACCHARYL TRANSFERASE ACTIVITY

te Heesen, S., Knauer, R.[1], Lehle, L.[1] and Aebi, M., Institut für Molekularbiologie I, Universität Zürich, Hönggerberg, 8093 Zürich, Switzerland, and [1]Institut für Zellbiologie, Universität Regensburg, W-8400 Regensburg, Germany.

N-linked glycosylation is an essential protein modification occuring in all eukaryotic cells. The central step is the co-translational transfer of the core-oligosaccharide assembled on the lipid carrier dolichol to selected Asn-X-Ser/Thr residues of nascent polypeptide chains. This reaction occurs in the endoplasmic reticulum and is catalysed by the enzyme N-oligosaccharyl transferase. In yeast, Wbp1p is an essential component of this enzyme. The *SWP1* gene was isolated as an allele-specific suppressor of a *wbp1* mutation. *SWP1* encodes an essential 30 kD type I transmembrane protein. The depletion of the protein results in a reduced enzyme activity similar to Wbp1p. Chemical crosslinking experiments indicates that Swp1p and Wbp1p form a protein complex essential for oligosacchary transferase activity.

311

TARGETING OF ERGIC-53 TO THE ER-GOLGI INTERMEDIATE COMPARTMENT

Itin, C.,-Schindler, R., Kappeler, F. and Hauri, H.-P., Department of Pharmacology, Biocenter of the University, CH-4056 Basel, Switzerland

ERGIC-53 is a marker protein for the ER-Golgi intermediate compartment (ERGIC). Cloning of the cDNA of ERGIC-53 revealed a transmembrane protein with type I topology. Surprizingly the 12 amino acid long cytoplasmic tail carries a KKXX ER retention motif although ERGIC-53 localizes to a post-ER compartment. This raised the question whether the KKXX is a functional motif. Therefore we replaced the cytoplasmic tail of a surface membrane protein (human CD4) with that of ERGIC-53. The construct was efficiently retained when transiently expressed in Vero and COS cells showing an ER-like pattern by immunofluorescence microscopy clearly different from endogenous ERGIC-53. Replacement of the two lysines by serines in this construct lead to surface appearance that was indistinguishable from expressed human CD4. This indicates that the KKXX-motif in the cytoplasmic tail of ERGIC-53 acts as a retention signal without conferring ERGIC localization. In contrast, overexpression of ERGIC-53 in Vero and COS cells lead to surface appearance suggesting saturation of ERGIC-53 retention, whereas low levels of expression in Vero cells revealed correct intracellular localization to the ERGIC. To test if the transmembrane domain of ERGIC-53 affects retention we attached the transmembrane and the cytoplasmic domain of ERGIC-53 to the luminal portion of CD4. This construct was efficiently retained in the ER without missorting to the plasma membrane indicating that the transmembrane together with the cytoplasmic domain is not sufficient for correct targeting to the ERGIC. A mutational analysis of the retention motif in the intact ERGIC-53 is in progress.

312

MEMBRANE TRANSPORT IN THE GOLGI APPARATUS IS INHIBITED BY ANTIBODIES AGAINST ß-COP

J. Scheel, R. Pepperkok*, G. Griffith*, and T.E. Kreis

Département de Biologie Cellulaire, Sciences III, Université de Genève, CH-1211 Genève, and * EMBL, Meyerhofstr. 1, D-6900 Heidelberg,

We tested the role of coat proteins (COPs) of non-clathrin coated vesicles in biosynthetic membrane transport by microinjection of epitope-specific antibodies into fibroblasts. The transmembrane glycocprotein (G) of the temparature-sensitive mutant tsO45 of vesicular stomatitis virus (VSV) was used as a model system for transport through the secretory pathway. One of the antibodies raised against synthetic peptides of ß-COP inhibits transport of VSV-G to the cell surface and reduces secretion of endogenous proteins. VSV-G accumulates in the *cis*-Golgi network (CGN) in injected cells at a site proximal to acquisition of resistance to Endoglycosidase H. Since transport of VSV-G from the *trans*-Golgi network to the cell surface is not inhibited under these conditions, we conclude that ß-COP is involved in a transport step between the CGN and the stack of Golgi cisternae.

313

TAGGING OF SECRETORY AND MEMBRANE PROTEINS WITH A TYROSINE SULFATION SITE

Birgit Leitinger and Martin Spiess
Dept. Biochemistry, Biocenter, University of Basel, CH-4056 Basel

The sulfation of proteins on tyrosine residues is a posttranslational modification that occurs specifically in the trans-Golgi. Proteins containing tyrosine sulfation sites can be selectively labeled with [^{35}S]sulfate in the trans-Golgi. In order to study intracellular transport out of this organelle for proteins that are not naturally sulfated we fused a nonapeptide corresponding to the tyrosine sulfation site of rat cholecystokinin precursor to the carboxy terminus of a type II transmembrane protein (subunit H1 of the human asialoglycoprotein receptor) as well as to a secreted protein (human α-1-proteinase inhibitor, A1Pi). Upon transfection into COS-7 cells these fusion proteins were sulfated in vivo. By performing pulse-chase experiments with [^{35}S]sulfate and measuring the appearance of radiolabeled A1Pi fusion protein in the medium we were able to measure the rate of transport of this protein from the trans-Golgi to the cell surface. A number of natural mutants of A1Pi which are defective in secretion, tagged with the tyrosine sulfation peptide, are currently being analyzed.

314

ANALYSIS OF THE FUNCTION OF CLIP-170 BY TRANSIENT EXPRESSION IN HELA AND VERO CELLS.

P. Pierre, J. E. Rickard, J. Scheel and T. E. Kreis, Département de Biologie Cellulaire, Sciences III, Université de Genève CH-1211 Genève 4.

CLIP-170, a microtubule binding protein isolated from HeLa cells, is involved in endosome-microtubule interactions, and the microtubule binding site was localized to its N-terminal domain using mutational analysis and in vitro translated proteins (Pierre et al., *Cell* 70: 887, 1992). Transiently overexpressed CLIP-170 and various mutants containing its N-terminal domain bind to microtubules in HeLa and Vero cells. Overexpression of CLIP-170 and some of the mutants leads to the rapid formation of rings of "bundled" microtubules in Vero cells. In HeLa cells, overexpression of CLIP-170 mutants leads to the stabilization and aggregation of microtubules which are, however, less dramatically altered than in Vero cells. When wild-type CLIP-170 is expressed in Hela cells, patchy structures are observed at the cell periphery. These structures are not observed with CLIP-170 mutants lacking the C-terminal domain. These structures are now investigated for their potential (endosomal) membranous origin. Overexpression of defined CLIP-170 mutants is being used to determine the exact role of CLIP-170 during endocytosis and to establish the function of its C-terminal domain.

315

SYNTHESIS AND PROCESSING OF HUMAN INTESTINAL LACTASE-PHLORIZIN HYDROLASE IN MDCK CELLS

Grünberg, J., Luginbühl, U. and Sterchi, E., Institut für Biochemie und Molekularbiologie, Universität Bern.

Maturation of Lactase-Phlorizin Hydrolase (LPH) (EC 3.2.1.23-62) requires proteolytic processing of a precursor (pro-LPH) to the mature enzyme (m-LPH). Subcellular site and function of this processing are not known. We studied the synthesis, processing and sorting of human LPH permanently expressed in MDCK cells. LPH was synthesized and proteolytically processed normally by the transfected MDCK cells, the molecular species corresponding to those found in human intestinal epithelial cells. To investigate sorting of LPH, transfected MDCK cells cultured on transwell filters, labelled with [^{35}S]-methionine and LPH was detected by surface immunoprecipitation. Most of the LPH synthesized was inserted into the apical (microvillus) membrane and small amounts were found basolateral. 42% of the LPH immunoprecipitated from the apical membrane was in the mature, i.e. proteoytically processed form. On the basolateral membrane only 20% was in mature form. These data show that sorting of LPH occurs prior to and independantly of proteolytic processing.

316

Transport, function and sorting of Lactase-Phlorizin Hydrolase (LPH) in polarized MDCK cells. **R. Jacob and H.Y. Naim, Institute of Microbiology, University of Düsseldorf, D-4000 Düsseldorf 1, Germany.**

Lactase-phlorizin hydrolase (LPH), an intestinal brush border glycoprotein, is synthesized as a single chain precursor (pro-LPH)(Mr = 215 - 230 kDa) that undergoes intracellular cleavage prior to insertion in the apical membrane. To assess the role of intracellular cleavage on the transport, function and sorting of pro-LPH/LPH a stable MDCK cell line was generated that expresses LPH. Biosynthetic labeling experiments demonstrated that the transport kinetics and posttranslational processing pattern of LPH in this cell line are virtually similar to those in intestinal cells. Moreover, the enzymatic activity was found to be indistinguishable from that of brush border LPH. The sorting of pro-LPH and LPH was studied by biosynthetic labelings of cells grown on filters. Here, we could demonstrate that the cleaved LPH molecule was directly sorted to the apical membrane, while uncleaved pro-LPH was found on the apical as well as on the basolateral membrane. The data suggest a correlation between cleavage and direct apical transport of LPH in MDCK cells.

317

ISOLATION AND CHARACTERIZATION OF CHROMAFFIN CELL MEMBRANE PROTEINS POTENTIALLY INVOLVED IN EXOCYTOSIS

Hodel, A., Schaefer, T. and Burger M. M.,
Friedrich Miescher-Institut, CH-4002 Basel

Two membrane proteins potentially involved in the exocytotic process are currently purified and characterized.

i) Proteins of light membrane fractions of bovine adrenal medullary tissue are solubilized in the detergent HECAMEG. From the family of proteins binding to fixed chromaffin granules in an affinity chromatography step a 130kD MW protein was selected. This protein can be found in medullary but not in cortical tissue fractions. Tryptic digestion followed by peptide separation on HPLC and microsequencing of selected peptides revealed 4 novel amino acid sequences. Degenerate oligonucleotides were used to prime PCR amplification reactions on a cDNA template synthesized from adrenal medullary mRNA. The DNA sequence of the 900bp PCR product was determined. No significant homology was found in databanks. The DNA fragment hybridizes to 8kb mRNA from adrenal medulla in Northern analysis. It is currently used to screen an adrenal medulla cDNA library.

ii) Syntaxin A, a plasma membrane protein of 35kD MW from rat brain synaptosomes was postulated to play a role in neurotransmitter release in nerve terminals (Bennett *et al.*, Science 257 (1992)). Using the PCR technique cDNA from adrenal medulla coding for bovine syntaxin was amplified and subcloned into an expression vector. The protein lacking its C-terminal hydrophobic stretch is expressed in *E.Coli*. The soluble form of syntaxin is used a) to characterize its interaction with purified chromaffin granules *in vitro*, and b) to test its role in exocytosis upon introduction into permeabilized chromaffin cells.

318

NUCLEOSIDE PHOSPHATASES OF THE PIG ZYMOGEN GRANULE MEMBRANE CONTAIN CARBOHYDRATE RESIDUES, PARTLY NEAR THE ACTIVE SITES

Spaans, M.C., Ammann, R.W., Freiburghaus, A.U.
Dept. of Internal Medicine, Univ. Hospital, H LAB 8, CH-8091 Zürich, Switzerland

Secretion of digestive enzymes involves zymogen granules in the pancreatic acinar cells. This energy utilizing activity may involve nucleoside phosphatases and/or specific attachment of cytosolic molecules to carbohydrate-bearing components of the zymogen granule membrane (ZGM). To characterize the carbohydrate-groups of the few ZGM proteins we used digoxigenin labelled lectins and anti-digoxigenin antibodies labelled with alkaline phosphatase. ZGM proteins were separated by non-denaturing electrophoresis in a 5–15% gradient polyacrylamide gel containing 0.2% CHAPS and 0.1 M TRIS/HCl, pH 9.5. At least four distinct protein bands were detected, all glycoproteins as confirmed with PAS-staining. The proteins were electroblotted to PVDF membranes and incubated with digoxigenin labelled lectins GNA, SNA, MAA, PNA, and DSA. Then anti-digoxigenin antibody conjugated to alkaline phosphatase was added to probe the bound lectins. 4-Nitroblue tetrazolium chloride was used as substrate for the alkaline phosphatase producing a violet color. The presence of glycosyl residues near the active sites of the nucleoside phosphatases was investigated by preincubating the gel stripes with substrates AMP, ADP, and ATP and with lectins Con A, WGA, SBA and RCA120. The nucleoside phosphatase activities were detected by visualization of the resulting lead phosphate precipitates in the gel forming in the presence of Pb^{++}. The various lectins bind to different glycoproteins of the ZGM and inhibit nucleoside phosphatase activities towards different substrates. The function of these glycoproteins during exocytosis remains to be elucidated.

319

Post-granular constitutive release in transformed (INS) pancreatic B-cells.

M. Neerman-Arbez and P.Halban. Labos Jeantet, CMU, 1211 Genève 4.

A novel secretory pathway has been described in which small vesicles bud from maturing granules, carrying with them an aliquot of soluble granular constituents for post-granular constitutive release. Proinsulin conversion yields equimolar amounts of insulin and C-peptide in granules. Whereas insulin is crystalline in granules, C-peptide remains soluble, and should thus be found enriched relative to insulin in the novel pathway. To study this, transformed B-cells were labelled (20';[^{3}H]leu) then chased. Labelled insulin (INS*) and C-peptide (CP*) were quantified by HPLC. CP*/INS* in 1h chase medium was 2.0, in keeping with exaggerated CP* release via the novel pathway. CP*/INS* in cells was 0.61, indicating that 39% of CP* was lost from them. Since only 1.2% CP* had been released, 37.8% was left unaccounted for. There was no CP* degradation in the medium; it was thus intracellular. To determine whether such degradation of CP* occurred in granules, release was stimulated (>40-fold) to discharge granule stores. CP*/INS* in medium after 1h stimulation was 1.1, reflecting a normal equimolar ratio in granules. Selective CP* degradation thus arose elsewhere in the cell, most probably in lysosomes. The post-granular constitutive pathway thus exists in transformed B-cells, but it only accounts for a minute loss of CP when compared with that due to intracellular degradation. The same is believed to apply to primary B-cells.

320

Proinsulin Processing in the Constitutive Pathway.

F. Vollenweider, J-C. Irminger and P.A. Halban. Laboratoires de Recherche Louis Jeantet, CMU, 1, rue Michel Servet, 1211 Genève 4.

Proinsulin (PI) is normally converted to insulin in the regulated pathway of pancreatic beta cells. However, we have reported that some conversion of both human and rat II PI occurs in transfected FAO (hepatoma) cells, which release proteins only via the constitutive pathway. We have further studied PI conversion in this pathway as follows:

-1) FAO cells were stably transfected with cDNA for rat I PI, which, unlike human or rat II PI, presents putative consensus sequences for cleavage by furin (a candidate constitutive pathway conversion endoprotease) at both its conversion sites. Products released to the medium were analysed by HPLC/radioimmunoassay. Insulin was the major form, with some PI but no conversion intermediates. This contrast with FAO cells expressing human or rat II PI which release mainly intermediates and PI.

-2) COS cells were transiently transfected with human PI cDNA; there was no conversion. Insulin and proinsulin but no intermediates were released when cells were cotransfected with furin, thereby confirming that furin can promote PI conversion.

In conclusion, proinsulin can be converted in the constitutive pathway. The interplay between endoproteases (including furin) and proinsulin structural domains determines the conversion products generated.

321

Structural Requirements for Proinsulin Cleavage at the B-chain/C-peptide junction.

J. KAUFMANN, J.-C. IRMINGER and P. HALBAN. Laboratoires de Recherche Louis Jeantet, Centre Médical Universitaire, 1211 Genève 4.

We mutated the first amino acid of the C-peptide of human proinsulin from Glu to Pro to determine the effect of proline immediately C-terminal to one of the two cleavage sites implicated in conversion. C1Pro or native proinsulin cDNA were stably transfected in AtT20 (pituitary corticotroph) cells. Kinetics of conversion of labelled (10'; [^{3}H]Leu) C1Pro proinsulin showed an important cellular accumulation of des-64,65 split proinsulin with only trace amounts of insulin appearing after 120 minutes chase, whereas conversion of native proinsulin progressively generated insulin in cells with a modest accumulation of des-31,32 split proinsulin. HPLC analysis of cellular immunoreactive material in steady state conditions revealed the majority as des-64,65 split C1Pro proinsulin; for native proinsulin transfectants the major product was fully processed insulin. C1Pro proinsulin is thus not cleaved efficiently between B-chain and C-peptide. To determine if this was simply due to replacement of the polar (Glu) C1 residue or a unique effect of C1Pro, we deleted the first 4 residues of C-peptide (highly conserved acidic domain), bringing Leu to the new C1 position; conversion was only modestly affected. We conclude that Pro C-terminal to the B-chain/C-peptide cleavage site induces a conformational change and thereby inhibits conversion.

322

Transfected AtT20 cells constitutively release des-31,32 split proinsulin.

J. C. IRMINGER and P. HALBAN. Laboratoires de Recherches Louis Jeantet. Centre Médical Universitaire, 1211 Genève 4.

Transfected AtT20 cells convert proinsulin to fully processed insulin and stock it in secretory granules. To see whether these cells also secrete insulin-like material via the constitutive pathway, we analysed stably transfected clones by pulse-chase experiments: cells were labelled for 10 min with [^{3}H]Leu and then chased for up to 120 min. Radioactivity in cell extracts and medium was analysed by HPLC. During the chase, des-31.32 split proinsulin accounted for approx. 50% of the released material while in cell extracts des-31.32 split proinsulin never exceeded 15% of the total insulin-like material. To distinguish between regulated and constitutive secretion, cells were first chased for 30 min under basal conditions and then incubated a further 30 min under basal or stimulated (forskolin/IBMX) conditions. Radioactivity in basal medium was 34% insulin, 51% des-31.32 split proinsulin and 15% proinsulin. In stimulated medium there was 46% insulin, 34% des-31.32 split proinsulin and 20% proinsulin. Insulin and proinsulin release was stimulated 2.8 fold while that of des-31.32 split proinsulin only 1.4 fold. These results suggest that a significant portion of newly synthesised proinsulin in AtT20 cells is released through the constitutive pathway after partial conversion to des-31.32 split proinsulin presumably by furin or a related endoprotease active in the constitutive pathway.

Cytoskeleton and Cell Motility

323

COMPARISON OF THE CYTOSKELETON OF FIBROBLASTS CULTURED IN THREE DIMENSIONAL COLLAGEN LATTICES OR IN MONOLAYER CULTURES

Degen, E., Kuhn, B., Meyer, R., Baschong, W., and Schreier, T., Biochemical Research and Development, Solco Ltd. Basel

The cytoskeleton of fibroblasts cultured in three-dimensional collagen lattices or as monolayers was studied by indirect immunofluorescence. The growth of fibroblasts in three-dimensional collagen gels closely approximates the in vivo environment, where they are surrounded by extracellular matrix. Here the cells were spindle-shaped and showed intense F-actin bundles of different thickness as well as cortical F-actin. The tubulin network was not very elaborated. In contrast, dermal fibroblast cultured as monolayers exhibited F-actin-microfibrils of equal thickness arranged in parallel arrays and intense tubulin networks. The effect of some substances used in wound healing were tested on cytoskeleton organization. In fibroblasts, cultured in three dimensional collagen lattices, Ascorbic Acid and the hemodialysate Solcoseryl resulted in dense F-actin-bundles, whereas TGF-ß and Retinoic Acid lead actin to be organized in aggregates disposed along the plasma membrane. None of these effects were observed in monolayer cultures.
In additional experiments, we studied both the intracellular microfibrils and the extracellular fibronectin deposition during cell migration in monolayer cultures. Actin and tubulin were organized in thin diffuse microfibrils not arranged in linear arrays. In the absence of any migration stimulating substance, only low amounts of fibronectin were deposited along their migration pathways. However, in presence of TGF-ß and the hemodialysate Solcoseryl, large amounts of fibronectin microfibrils were deposited oriented along the direction of cell translocation.

324

CHANGES IN THE ORGANIZATION OF THE CYTOSKELETON IN NEUROBLASTOMA CELLS TREATED WITH THROMBIN

Niclou S., Suidan H.S., Dürenberger M.* and Monard D. Friedrich Miescher-Institut, 4002 Basel and *Biozentrum der Universität Basel, 4056 Basel

Recent work from this laboratory has established that thrombin causes rapid neurite retraction via activation of cell surface receptors and modulation of intracellular metabolism (Suidan et al. 1992; Neuron, 8:363). Therefore, we examined what were the cytoskeletal reorganizations that occurred concomitantly with the changes in the neuronal cytoarchitecture. Anti-tubulin antibodies and phalloidin staining were used to label microtubules and actin filaments, respectively and analysis was carried out by confocal microscopy. We observed that after thrombin treatment microtubules and actin filaments appear to colocalize in arrays projecting from what seems to be an MTOC. The protein kinase inhibitor staurosporine which inhibits thrombin-induced neurite retraction prevented this cytoskeletal reorganization. The phosphatase inhibitor phenylarsine oxide mimics the effect of thrombin in causing neurite retraction but this involves changes in cytoskeletal arrangement which were distinct from those caused by thrombin.

325

Correlation of spatial and temporal changes of the fibroblast cytoskeleton with wound repair - a confocal laser scanning microscopy (CLSM) investigation

Rosmarie Sütterlin[1] Markus Dürrenberger[2] and Werner Baschong[1]
[1]M.E.-Müller-Institut am Biozentrum, Uni Basel, Klingelbergstr. 70, CH-4056 Basel
[2]EM-Zentrallabor Uni Zürich, Gloriastr. 30, CH-8028 Zürich

Synthesis rates and structural organization of cytoskeletal elements may strongly depend on a cell's phenotype, as this is for instance the case for fibroblasts during wound repair. These changes of cell shape and function have been emulated *in vitro* by cultivating dermal fibroblasts within collagen gel matrices. Here we have investigated the spatial arrangement of microfilaments, intermediate filaments, microtubules and fibronectin with respect to (i) the fibroblasts' location within the matrix and (ii) the progression of *in vitro* wound contraction by high resolution CLSM of multi-fluorescently labelled specimens. Serial optical sectioning enabled us to simultaneously observe the cytoskeleton, cell-cell contacts and cell-matrix interactions at near-theoretical resolution (~0.3 µm in x, y and z) at various depths within the collagen matrix.
Whereas the cytoskeletal organisation of the fibroblasts within the 3-D matrices was comparable to that *in vivo*, it markedly differed from that of cells growing at the surface of the matrix or conventionally in monolayer cultures.

326

PROTECTION AGAINST CELL DEHYDRATATION BY PROPANEDIOL (PROH 2M), A STUDY BY CRYOIMMUNOCYTOCHEMISTRY.

Salamin Michel L., Laboratoire d'analyse ultrastructurale, Université de Lausanne, CH-1015 Dorigny and **Campion E.**, Institut de biologie physico-chimique, 75005 Paris.

Chemical fixation promotes protein aggregation artefacts in cells. Water is then no longer retained and can cristallise or escape the cell during freezing procedures.
Using mouse embryons, we shaw that 2M PrOH, a cryoprotectant, prevents these fixation artefacts and that after freezing cells have a vitreous appearance.
Furthermore by cryoimmuncytochemistry according to Tokuyasu (1986), using antibodies against total actin and DNase I, a protein which binds preferentially G actin, we show that 2M PrOH prevents this aggregation by depolimerizing the cortical F-actin. This induces the formation of a gel more resistant to dehydratation.

327

CYTOSKELETON ASSOCIATED ANTIGENS IN AFRICAN TRYPANOSOMES

M. Imboden, N. Müller, A. Hemphill and T. Seebeck
Institute of General Microbiology, University of Berne, Switzerland

For many years the VSG (variant surface glycoprotein) was the only antigen from African trypanosomes that has been intensively investigated in terms of its suitability in diagnosis. By using molecular biological, immunological and immunocytochemical methods we have recently identified two strong, non-variable antigens MARP1 (microtubule associated repetitive protein 1) and GM6, that could be characterized as highly repetitive proteins located on the cytoskeleton of the parasite. A current study in our lab indicates that MARP1 and GM6 represent only two members of a large family of proteins that are high molecular weight components from the cytoskeleton and that at least in part exhibit antigenic properties. Future investigations will show whether these antigens can be used as agents in immunodiagnosis of African trypanosomiasis in human and cattle.

328

Protein Nr. VI, a microtubule associated protein (MAP) of Trypanosoma b. brucei

E. Detmer, A. Hemphill, N. Müller, and T. Seebeck
Institut für allg. Mikrobiologie, CH-3012 Bern

Most of the antigens recognized by Trypanosoma b. brucei infected mammals are cytoskeletal proteins, a fact illustrating the importance of the parasite's cytoskeleton in the immune response against an infection.
Trypanosomal cytoskeletons were prepared by Triton-X-100 extraction. Rats were immunized with these cytoskeletal proteins to raise an immune-response, the serum was collected and used for western-blots to detect the recognized proteins. One of these proteins, termed Nr. VI, was further characterized with immunofluorescence and immunogold-electronmicroscopy and found to be a MAP (microtubule associated protein).

329

THE GENES CODING FOR THE TWO PFR-PROTEINS IN TRYPANOSOMA BRUCEI

J. Deflorin and Th. Seebeck
Institut für allg. Mikrobiologie, CH-Bern

Besides the microtubular axoneme, the flagellum of the hemoflagellate *Trypanosoma brucei* contains another cytoskeletal element, called the paraflagellar rod (PFR). This structure is a highly ordered, three-dimensional fibrous network of yet unknown function, which runs parallel and in close contact to the axoneme.
The major structural components of the PFR are two immunologically and biochemically related proteins of 69 and 73kD. Two different PFR gene loci, each containing two identical tandemly linked genes (A and B, C and D respectively), have been found. It was shown that the gene-A codes for the smaller, 69kD PFR protein, whereas in the case of the gene-B no related full length transcript could be identified yet. Further we know that the bigger, 73kD PFR protein is encoded by the PFR gene-C in the second gene locus. The gene-D is only partially analized yet. Surprisingly gene-C only shows partial (65%) similarity to the gene-A and B, although coding for a very similar protein.

330

CHARACTERIZATION OF CLIP-170, A MICROTUBULE LINKER PROTEIN

J.E. Rickard and T.E. Kreis, Département de Biologie Cellulaire, Sciences III, Université de Genève, CH-1211 Genève 4

CLIP-170 is a microtubule-binding protein implicated in endosome-microtubule interactions (Pierre et al., 1992, *Cell*, 70:887). It has also been localized to desmosomal plaques in polarized epithelial cells (Wacker et al., 1992, *J. Cell Biol.*, 117:813), suggesting that it may function to link diverse cytoplasmic organelles to microtubules. In fibroblast-like cells, CLIP-170 accumulates at the microtubule plus ends (Rickard & Kreis, 1990, *J. Cell Biol.*, 110:1623), the site of polymer turnover. The interaction of CLIP-170 with microtubules is regulated by phosphorylation in vitro, and the phosphorylation of CLIP-170 in vivo is influenced by perturbation of the microtubule polymer state (Rickard & Kreis, 1991, *J. Biol. Chem.*, 266:17597). These data suggest a relationship between the phosphorylation and microtubule-binding activity of CLIP-170 and microtubule dynamics, and are consistent with a higher affinity of CLIP-170 for the stabilizing 'GTP-cap' conformation of tubulin. A working model will be discussed, and results of experiments in progress to test this model will be presented. Localization of CLIP-170 to microtubule plus ends, combined with its ability to interact with other cytoplasmic structures, may provide a cellular mechanism for control of microtubule turnover as well as promoting the microtubule-organelle interactions essential for cytoplasmic organization.

331

PARTIAL PURIFICATION OF MICROTUBULE-ASSOCIATED PROTEINS FROM *Neurospora crassa*.

Ortega Perez, R., Arrighi, J-F., Irminger-Finger*, I ., van Tuinen, D., Micheli, A.J., Capelli N., Andrey-T*, I., Edelstein*, S. and Turian, G.
Laboratoire de Microbiologie générale and Laboratoire de Biochimie, Université de Genève, CH-1211 GENEVE 4.*

Heat-stable proteins of the crude extract of *N. crassa* were purified by affinity chromatography on a calmodulin-sepharose 4B column (Lee, Y. C., and Wolff, J. 1984. J.Biol.Chem.259: 1226-30) and further purified by chromatography on a MonoQ column (Pharmacia). Using monoclonal antibodies against MAP 2, MAP 1, and tau (Sigma) or polyclonal antibodies against the 205K MAP from *Drosophila* (Goldstein, L.S.B., Laymon, R.A., and McIntosh, J.R. 1986. J. Cell Biol. 102: 2076-2087) we were able to demonstrate for the first time the presence of these cytoskeletal proteins in filamentous fungi. These proteins could be phosphorylated with a calcium-calmodulin dependent protein kinase also isolated from *Neurospora* (van Tuinen, D., Ortega Perez, R. D., Marmé, D., and Turian, G. 1984. FEBS Lett. 176: 317-320). The isoelectric point of each of these MAPs was determined. MAP-calmodulin complexes were obtained on a native slab gel using ^{3}H-calmodulin and were analyzed by fluorography of the gel. The MAPs thus isolated from *N.* crassa were able under appropriate conditions to induce the assembly of porcine brain tubulin into microtubules as visualized by EM using negative staining with uranyl acetate.

332

MBP-115, a novel microtubule-binding protein predominantly expressed in cells of epithelial origin

D. Masson and T. E. Kreis. Département de Biologie Cellulaire; Sciences III; Université de Genève; CH-1211 Genève 4.

Changes in microtubule dynamics and nucleation play a major role in the reorganization of the cytoplasm and in the establishment of polarity in epithelial cells (Bacallao et al., 1989, J. Cell Biol. 109:2817). Specific microtubule-binding proteins (MBPs) are presumably involved in these changes. We have identified MBP-115, a protein of 115 kDa in HeLa cells. Sequencing of a full-length cDNA clone coding for MBP-115 indicates that it is a novel protein. MBP-115 is predominantly expressed in cell lines of epithelial origin (Caco-2; MDCK) and it is localized on a subset of microtubules. In Caco-2 cells, the MBP-115 expression level increases upon polarization. Vero fibroblasts, which appear not to contain MBP-115, were used to investigate the role of MBP-115 on microtubule organization by transient expression. In such transfected cells, microtubules appear to be randomly nucleated and have become more resistant to nocodazole treatment. We are currently studying the role of MBP-115 in the establishment of cell polarity.

333

MAP2c variants in aggregated neuronal and glial cell cultures

Riederer B.M.*, Matus A.¶,Honegger P.#

Instituts d'Anatomie* et de Physiologie#, Université de Lausanne,
Friedrich Miescher Institut ¶, Basel

MAP2c is an embryonic microtubule-associated protein. It is a splice variant of MAP2b, lacking a central region, and is therefore of smaller molecular weight (65-70kD) than MAP2b (280kD). It is present in axons and in glial cells (Garner and Matus, J. Cell Biol. 106: 779, 1988; Tucker et al., J. Comp. Neurol. 271: 44, 1988)). In this study we have investigated the occurrence of MAP2c in aggregated rat telencephalon cultures, consisting either of a mixed population of neuronal and glial cells, or enriched in either neurons or glial cells. These cultures were tested with several monoclonal antibodies against MAP2, and with neurofilament- and glial fibrillary acidic protein-specific antibodies as markers for cell types. Differences in the composition of MAP2 variants were determined by electrophoresis and Western blots. MAP2c was found in two variants, one several kD larger than the other. Both variants existed in a phosphorylated form in mixed cell aggregates. The smaller variant was predominant in neurons, and the larger one in glial cells. Supported by SNF grants 31-26624.89, 31-33447.92 and 31-28809.90.

334

CYTOKERATIN-POSITIVE CELLS ARE OBTAINED FROM ENDOTHELIUM OF THE AORTA AND THE VENA CAVA

K. Spanel-Borowski and A. Fenyves
Anatomisches Institut der Universität Basel, CH 4056 Basel

Coexpression of cytokeratin filaments and vimentin filaments are described for "mesenchymal" epithelial cells, e.g. lens, thymus, as well as for some epithelial-like cells of mesodermal origin, e.g. Sertoli-cells, mesothelial cells. Endothelial cells of large blood vessels are known to possess vimentin filaments alone correlative with the mesodermal origin of these cells. We are engaged in studies to prove morphological heterogeneity of cultured endothelial cells. For this reason, we obtained endothelial cells by scraping the intima of the aorta or the vena cava of the cow. Confluent colonies of separate morphology were obtained from both blood vessels 14 days after cell seeding: "Cobble stone" monolayer for phentoype 1 and polygonal monolayer for phenotype 2. As detected by immunofluorescence localization, phenotype 1 showed a ring of actin filaments, bundled vimentin filaments, a meshwork of cytokeratin filaments, and single cilia. Phenotype 2 displayed a network of actin filaments and vimentin filaments, yet no cytokeratin filaments and no cilia. Only phenotype 1 expressed cell-cell-adhesion molecules of the E-cadherin family on the cell surface. We conclude: Separate endothelial cells can be distinguished in culture at the level of phase contrast microscopy. The occurrence of cytokeratin-positive cells questions whether endothelial cells are of mesodermal origin only.

335

NEUROFILAMENT TRIPLET PROTEINS IN DEVELOPING CAT CEREBELLUM

Riederer B.M.,Porchet R., and Marugg R.A.*
Institut d'Anatomie, Rue du Bugnon 9,1005 Lausanne;
*Neurochirurgische Universitätsklinik, Rämistr. 100, 8091 Zürich

Neurofilament proteins (NF) are typical components of the neuronal cytoskeleton. During postnatal development of cat cerebellum the expression and distribution of the triplet neurofilament proteins was studied with several monoclonal and polyclonal antibodies. Afferent fibers in medullary layer contained NF-M and NF-L already at birth, while NF-H appeared one week later. With progressing maturation all NF subunits appeared in mossy and climbing fibers, in axons of basket cells, and in efferent projections of Purkinje cells. In parallel fibers NFs appeared last, two months after birth. Differences in the immunostaining of NF-H in axons depended on the epitopes being phosphorylated. A non-phosphorylated sequence of NF-H was located exclusively in Purkinje cells and in some interneurons. These results correspond well with data from other reports. However, one antibody indicated the presence of a phosphorylated NF-H exclusively in Purkinje cell dendrites. Expression and phosphorylation of the individual neurofilament proteins are thought to be essential for the adult-type stabilization of the axonal and dendritic cytoskeletons. Supported by SNF grants 31.26624-89 and 31.33447-92.

336

Surface imaging of F-actin filaments and visualization of actin binding proteins

Roger Wepf#, Andreas Bremer*, Ueli Aebi* and Heinz Gross#
#Institute for Cell Biology, ETH Hönggerberg; *Maurice E. Müller-Institute, University of Basel

The function and structural state of F-actin filaments is controlled and modulated by actin-binding proteins (ABP). Visualization and structural analysis of such protein-protein interactions is difficult due to the low signal-to-noise ratio in TEM micrographs. Moreover the low signal-to-noise ratio found in TEM micrographs may not be increased considerably by averaging procedures, since most of the ABP's do not expose the same periodicity as the underlying F-actin helix. With the introduction of the "in-lens" Field Emission SEM (FESEM) a powerful tool for macromolecular structure determination has become available. We have found that the signal-to-noise ratio in secondary electron images from a FESEM is much higher than in TEM micrographs. This advantage of FESEM makes imaging of single proteins within a complex possible e.g. the two domain structure of the G-actin molecule within the F-actin filament. FESEM allows us to analysis the structure of single additional proteins (ABP's) or protein-complexes, such as the troponin complex, on the F-actin filament.

337

Towards an atomic structure of F-actin: facts and fiction

Andreas Bremer[1], Roger Wepf[2], Christian Hemm[1], and Ueli Aebi[1,3]
[1]Maurice E. Müller-Institute, University of Basel; [2]Institute for Cell Biology, ETH Hönggerberg; [3]Dept. for Cell Biology and Anatomy, Johns Hopkins University School of Medicine, Baltimore, USA

Actin is a part of the macromolecular machinery that controls intracellular transport and the cell shape as well as motility of cells and tissues. F-actin filaments polymerized *in vitro* from G-actin monomers are composed of two strands of subunits. An atomic ***model*** of the actin filament has been proposed [Holmes et al., *Nature* 347 (1990) 44] and we have investigated whether high-resolution EM data can be used to test this model and help developing it into an atomic ***structure***. We have optimized our strategy for digital image processing of F-actin filaments by including a number of refinement steps. The resulting 3-D reconstructions allow for meaningful comparisons since they are remarkably similar to electron densitiy maps computed from the atomic model. The resolution of our 3-D reconstructions is high enough that we can trace the hydrophobic loop of the actin molecule. However, there are also subtle differences between the EM data and the atomic model: we have found reproducibly less mass in region of the DNase I-binding loop of the actin molecule. In fact, recent refinement of the atomic model [K.-C. Holmes, personal communication] has now yielded a conformation of the actin molecule that is better in line with our reconstructions. Recent modelling [K.-C. Holmes, personal communication] has now also confirmed our previous mapping of the phalloidin molecule in 3-D reconstructions of phalloidin-stabilized F-actin filaments [Bremer et al., *J. Cell Biol.* 115 (1991) 689]. We are currently investigating the molecular basis of the structural changes that accompany the hydrolysis of the bound nucleotide during polymerization to ultimately explain them on the atomic level.

338

Cytoskeletal actin regulates human neutrophil respiratory burst activity by altering plasma membrane rigidity

M.E. Ehrengruber and D.A. Deranleau

Theodor Kocher Institute, University of Bern, CH-3012 Bern, Switzerland

Real-time fluorescence anisotropy measurements on chemotactically-stimulated neutrophils using trimethylamino diphenylhexatriene (TMA-DPH) as a rotational motion probe revealed sinusoidal oscillations in plasma membrane rigidity. These correspond to similarly-induced oscillations in actin filament assembly and cross-linking, apparent pseudopod size, and membrane-associated respiratory burst activity (Wymann et al., *J. Biol Chem.* **264**, 15829, 1989; *ibid.* **265**, 616, 1990). Increases in membrane rigidity are correlated with increases in membrane-anchored actin filament levels and burst activity. A more rigid environment would allow longer access to substrate and/or cofactors, and inhibit dissociation of the multisubunit regulatory burst enzyme (NADPH oxidase), suggesting a mechanism for its oscillatory behavior. Support for this idea comes from the fact that inhibiting subunit dissociation by chemical cross-linking stabilizes the activity (Tamura et al., *J. Biol. Chem.* **267**, 7529, 1992).

339

ANTI-ACTIN DRUGS AND HEAT-SHOCK INDUCE NUCLEAR DENSE GRANULES IN GERMINATION-INHIBITED CONIDIA OF *NEUROSPORA CRASSA*

Barja, F., Chappuis, M.-L. and Turian, G., *Laboratoire de Microbiologie générale, Sciences III, Université de Genève, 1211 Genève 4.*

F-actin has been reported to occur within the nuclei of *Physarum polycephalum*, *Neurospora crassa* and of higher eucaryotic cells. However, evidence for a functional role of nuclear actin in these systems is lacking.

By electron microscopy, we have observed nuclear dense granules on thin sections of conidia of *N. crassa* prevented to germinate, not only after heat-shock (46°C), but also in those treated with the anti-actin drugs cytochalasin A (125 µM) or cytochalasin B (167 µM). Dense RNA granules migrate out of the nucleus and disperse into the cytoplasm during conidial recovery of germination ability, after their shift-down to 25°C (1). Our results confirm and extend a preliminary study (2) suggesting that actin microfilaments are someway involved in the export mechanism of presumed preribosomal RNA into the cytoplasm.

(1) Ton That, T.C. and Turian, G. (1984). *Protoplasma* 120: 165-171.
(2) Turian, G., Barja, F. and Caesar-Ton That, T.C. (1992). *Cell Biol. Intern. Rep.* In Press.

Metabolism and Organ Physiology

340

A NEW METHOD FOR ASSESSING DIETARY HABITS AND STROOP PERFORMANCE UNDER FIELD CONDITIONS

Jiri Kos, Markus Hasenfratz and Karl Bättig, Behavioral Biology Laboratory, Swiss Federal Institute of Technology, ETH-Zentrum, CH-8092 Zurich

The assessment of dietary or subjective behavioural data has traditionally been done by paper-and-pencil methods which have certain disadvantages (absence of a time protocol, bias of earlier answers, time consuming handling of the data). Our method uses a pocketcomputer for all data inputs. Also, a version of the Stroop task was implemented. The PSION II Organiser LZ 64 pocketcomputer (142 x 78 x 29mm, 250g) offers 32Kbyte of working memory and up to 256 KByte of storage capacity using 2 EPROM cartridges. Programs and data can be transferred to a PC/AT and vice versa. The software so far consists of 3 program modules: assessment of dietary intake, presenting of questions using analouge-like scales and a numerical version of the Stroop task. On the PC side our software offers quick viewing of the data immediately after finishing the experiment. Every data input is stored with time and date so that the subject's compliance can easily be checked. The method is now being used in an experiment with 80 smokers and nonsmokers. Differences regarding dietary habits and Stroop performance are being investigated under field conditions. So far the computer and software have been well accepted by the subjects and appear to deliver reliable results.

341

RELATIVE CONTENT OF PHOSPHATIDYLGLYCEROL MOLECULAR SPECIES IN THYLAKOIDS OF FOUR PLANTS SPECIES DURING GROWTH UNDER VARIOUS TEMPERATURES

Xu, Y.N. and Siegenthaler, P.A., Laboratoire de Physiologie végétale, Université de Neuchâtel, CH-2000 Neuchâtel

The aim of this study was to determine the relative content of PG molecular species of thylakoids from four plant species under different growth conditions, in particular at various temperatures. In thylakoids of squash, potato, lettuce and spinach, two groups of 5 PG molecular species were identified : (1) 16:0/16:0, 18:0/16:0, 18:1/16:0, 18:2/16:0, 18:3/16:0 and (2) 16:0/16:1(3t), 18:0/16:1(3t), 18:1/16:1(3t), 18:2/16:1(3t), 18:3/16:1(3t). Their relative proportion was different in each plant species. When squash and spinach plants were grown under three temperature regimes (day 16°C/night 10°C; 24°/18°C; 32°/26°C), the PG molecular species of the group 2 increased at the expense of those of the group 1. On the other hand, low temperatures favored the formation of 18:2/16:0 and 18:3/16:0 as well as 18:2/16:1(3t) and 18:3/16:1(3t) molecular species. These results will be discussed in terms of the influence of growth temperature on the degree of unsaturation in these different PG molecular species. (Supported by the SNSF 3100-33693.92).

342

LACK OF SAP-RELATED, A PROTEIN HOMOLOGOUS TO THE SPHINGOLIPID ACTIVATOR PRECURSOR, CAUSES SEVERELY REDUCED VIABILITY IN *DROSOPHILA MELANOGASTER*

Hochstrasser, M., Hilfiker, A., Frigerio, G., Deleay, N., and Noll, M., Institut für Molekularbiologie II der Universität Zürich, CH-8057 Zürich

The SAP-related protein of *Drosophila melanogaster* has eight conserved domains that are homologous to the sphingolipid activator proteins, a group of lipid-binding proteins involved in the degradation of glycolipids. We have obtained a P element insertion (A177.1M3, from H. Bellen, Houston) located within a few basepairs of the putative 5'-end of the SAP-related transcript, at chromosomal location 100B1,2. Flies carrying this insertion on both chromosomes are viable and produce SAP-related. After mobilization of this P element, we have isolated 14 recessive mutations, which could be divided into two complementation groups. Eleven mutations showed severely reduced viability and number of progeny, 3 mutations showed embryonal lethality. On Southern blots, chromosomal defects could be detected in all 14 mutants. Deletions were found in 11 mutants, 3 mutants carried apparent insertions that resulted from incomplete excisions of the P element. The 3 largest deletions that were not lethal all extended towards the centromere, removing *Sapr* completely. The 3 lethal mutations all had large deletions that extended at least 8 kb towards the telomere, probably causing a lethal mutation in a gene located distally of *Sapr*.

343

INDIVIDUAL VARIATIONS IN THE PROPERTIES OF THE HIGH DENSITY LIPOPROTEIN ASSOCIATED TRYPANOLYTIC FACTOR OF HUMAN SERUM

P. LORENZ AND B. BETSCHART, SWISS TROPICAL INSTITUTE, BASEL

Several authors (Rifkin, 1978 & Hajduk et al. 1989) reported the association of a human serum factor, toxic to Trypanosoma brucei brucei (TrypanoLytic Factor = TLF) with High Density Lipoproteins (HDL). Analysis of the distribution of TLF in density gradients and gel filtrations of 6 individual sera indicated variations in the properties which did not parallel the distribution of typical HDL as regarding the size-, density- and apolipoprotein (AI, AII, CIII, E, J) -profiles . TLF activity in all sera could be found in a size and density range of typical HDL (200-400kD, 1.063-1.21g/ml), but in some sera additionally in fractions containing LDL sized molecules (and apolipoprotein B) and/or in a density range exceeding that of HDL (> 1,24 g/ml). Sera can contain one or two differently sized populations and one or two density classes of TLF. There was no correlation between any of the observed distributions and the lytic activity of whole serum which also varies considerably. These findings challenge the view of a classical HDL moiety being the lytic factor.

344

IDENTIFICATION AND EXPRESSION OF A NOVEL HUMAN FATTY ACID-BINDING PROTEIN: THE EPIDERMAL TYPE

Siegenthaler, G., Hotz, R., Chatellard-Gruaz, D., Jaconi, S., Saurat, J.-H. Clinique de Dermatologie, Hôpital Cantonal Universitaire, CH-1211 Genève 14

Fatty acid-binding proteins (FABPs) are tissue specific cytosolic proteins involved in fatty acid metabolism. Five types of FABP have been characterized but an epithelial-FABP has not been identified so far. Since lipids play an important role in skin, we investigated the presence of FABP in this tissue. Using PAGE-Autoradioblotting, we characterized an epidermal (E)-FABP distinct from liver-, heart-, intestine- and adipose tissue-FABPs. FABP analysis could be performed directly on protein extracts without prior partial purification. E-FABP has a Mr of ~15 kDa and binds oleic acid with high affinity but does not bind all-*trans*-, 13-*cis*- and 9-*cis*-retinoic acid nor all-*trans*-retinol suggesting a high specificity for lipids. Expression levels of E-FABP were low in epidermis, higher in human cultured keratinocytes and strong in abnormal epidermal differentiation (psoriasis). These findings suggest that epidermal cells might have a distinct fatty acid metabolism compared to other tissues.

345

MICRO-AFFINITY CHROMATOGRAPHIC SEPARATION AND CHARACTERIZATION OF DIFFERENT SERUM LIPOPROTEIN FRACTIONS IN THE MONGOLIAN GERBIL (MERIONES UNGUICULATUS)

Tschantz, J-Cl., Chiesa, A. and Sunahara, G.I., Nestec SA Research Centre, Vers-chez-les-Blanc, CH-1000 Lausanne 26.

A rapid and routine micro-affinity chromatography method coupled with spectrophotometric analysis was evaluated to determine the concentration of total and free cholesterol, triglycerides and phospholipids in the α- and ß- lipoproteins taken from 50 µl of serum.This procedure overcomes many of the limitations presented by other methods such as ultracentrifugation, electrophoresis and precipitation. Using this affinity chromatography method, we report that in 35 male gerbils (50-75 g; 3-4 months old) fed a commercial diet, the α-lipoprotein content (in mmol/L) is: 2.85 ± 0.81 [SD] and 0.33 ± 0.14, total and free cholesterol, respectively; 1.99 ± 0.41 phospholipids and 0.64 ± 0.42 triglycerides; whereas the ß-lipoprotein contains: 0.92 ± 0.26 and 0.22 ± 0.06, total and free cholesterol, respectively; 0.28 ± 0.17 phospholipids; and 0.49 ± 0.31 triglycerides.

346

EFFECTS OF HMG CoA REDUCTASE INHIBITORS ON THE LIPOGENESIS OF HUMAN RECONSTRUCTED SKIN

J Bailly, L Cicurel. Zyma SA, Development department, Nyon.

The conversion of acetate to mevalonate during lipogenesis is controlled by 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG CoA). We have studied the effect of 2 HMG CoA inhibitors, compactin (C) and 25-hydroxy cholesterol (HC), on the lipogenesis of human reconstructed skin (acetate incorporation and lipid profile). A dose dependent inhibition of the lipogenesis was obtained with HC (IC_{50}=200 nM), whereas a biphasic effect was observed with C (stimulation between 10 and 1000 nM, inhibition at 10 000 nM). Lipid profile analysis indicated that HC increased the proportions of mono- and di-glycerides as well as of wax esters. No effect on lipid profile was observed with C. These results suggest that all HMG CoA inhibitors are not acting in the same way on the keratinocytes lipogenesis.

347

OVERNIGHT GLUCOSE METABOLISM IN OBESE NON INSULIN DEPENDENT DIABETIC PATIENTS

Tappy, L., Schneiter, Ph., Riou, J.P., Jéquier, E., Institut de Physiologie de l'Université, CH-1005 Lausanne et INSERM U 197, Lyon, France.

An increased fasting hepatic glucose production has been demonstrated in non insulin dependent diabetes mellitus (NIDDM). In order to determine the nature of this increase in hepatic glucose production, glucose kinetics were assessed in 7 obese NIDDM patients (34±6% fat mass) and 6 lean controls (18±4%) from 10 pm to 7 am using 6,6 ^{2}H-glucose and U-^{13}C glucose tracer infusions. Total hepatic glucose output was increased in NIDDM (256±59 mg/min vs 182±38 in leans, $p<0.05$). Cori cycle activity, calculated as the difference in glucose kinetics obtained with 6,6 ^{2}H glucose and U-^{13}C glucose, was increased by 110% in NIDDM, and accounted for the totality of the increase in hepatic glucose production. Cori cycle activity correlated positively with fasting plasma glucose ($r=0.685$, $p<0.001$). It is concluded that increased Cori cycle activity is responsible for the increase in hepatic glucose production in NIDDM.

348

Glycogen kinetics in rat skeletal muscle during glucose infusion.

Erik Haesler, Luc Tappy, Jean-Pierre Felber, Eric Jéquier.
Institute of Physiology, Faculty of Medicine, University of Lausanne, Lausanne, Switzerland.

Muscle glycogen synthesis is a major determinant of insulin sensitivity in man. Alterations of glycogen synthesis and/or mobilization could be of importance in the pathogenesis of insulin resistance. To assess whether simultaneous muscle glycogen synthesis and breakdown take place in conditions promoting net glycogen synthesis, three groups of anesthetized rats were compared: a) infused during 2 hours with ^{14}C-labeled glucose followed by 2 hours of ^{12}C-glucose (4-hours group), b) infused during 2 hours with ^{14}C-glucose (2-hours group), c) not infused (control group). Glycogen concentrations (µg/mg muscle protein) and glycogen specific activities (SA; Bq/µg glycogen) were measured in the soleus muscles (mean ± sd):

	control	2-hours	4-hours
glycogen conc.	13.64 ± 5.58	15.91 ±2.18	17.89 ±3.34
glycogen SA	-	0.31 ± 0.14	0.25 ± 0.14

The difference in muscle glycogen ^{14}C specific activity observed between both perfused groups was consistent with the dilution of prelabeled glycogen with newly accreted unlabeled glycogen. Therefore, it is concluded that no important muscle glycogen breakdown occurs during net glycogen synthesis.

349

MEASUREMENT OF A LIGHT-INDUCED CHANGE IN THE O_2 CONSUMPTION OF SINGLE ROD PHOTORECEPTORS

Poitry, S., *Cornwall, C., and Tsacopoulos, M., Experimental Ophthalmology Laboratory, 22 rue Alcide-Jentzer, 1211 Geneva 4; *Department of Physiology, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118, U.S.A.

Measurements of the rate of O_2 consumption (QO_2) of photoreceptors in the vertebrate retina have always been hampered by the highly heterogeneous structure of the tissue. This difficulty has been circumvented in various ways, but never to full satisfaction. We report here measurements of the time course of stimulus-induced changes in the QO_2 of single rod photoreceptors isolated from the salamander retina. The method consists in pulling a rod photoreceptor into a close-fitting suction pipette so that the cell lies 10-20 µm recessed; the local partial pressure of O_2 immediately adjacent to the cell is then measured continuously, using an O_2-sensitive microelectrode, while the transcellular current is monitored *via* the suction pipette. In these conditions, a train of 90 light flashes delivered at 2 s intervals elicits a biphasic change of QO_2: the QO_2 increases during the first minute of stimulation, then decreases below its resting value, and finally returns to baseline a few minutes after illumination is stopped. We are now investigating how this change of QO_2 correlates with the intracellular levels of ATP, GTP and creatine phosphate.

350

THE "OXYGEN PARADOX" IN THE EMBRYONIC CHICK HEART

Raddatz, E., Rochat, A-C., Kucera, P. and de Ribaupierre, Y.
Institut de Physiologie de l'Université, CH-1005 Lausanne.

Spontaneously beating hearts were carefully excised from 2- to 4-day-old chick embryos and mounted in an airtight chamber in which the partial pressure of oxygen (PO_2) at the tissue level could be strictly controlled. The hearts rapidly (seconds) and reproducibly reacted to changes in PO_2 within the physiological range. The alterations of cardiac activity in response to variations of PO_2 were investigated using a noninvasive computerized microphotometric technique. The data acquisition and processing systems allowed us to determine instantaneous heart rate, myocardium shortening, velocities of contraction and relaxation. Hypoxia or anoxia transiently increased the beating rate and resulted in an incomplete relaxation (contracture), a decline in amplitude of shortening and velocities of contraction and relaxation. Reoxygenation slowed down or even stopped the cardiac activity. This effect was dependent on the severity and duration of the oxygen deprivation in all developmental stages studied. Such a reoxygenation-induced dysfunction appears to be the embryonic analog of the "oxygen paradox" in whole adult hearts. Moreover, the presence of glucose protected the myocardial function during anoxia and upon reoxygenation.

351

COORDINATION BETWEEN BREATHING- AND RUNNING RHYTHMS DURING AEROBIC AND ANAEROBIC EXERCISE.

Bernasconi, P., Bernasconi, A., Bührer, A., Bürki, P., and Kohl, J.,
Physiologisches Institut der Universität, CH-8057 Zürich

The purpose of the present study was to analyse the effect of training and training type on the incidence of coordination between running- and breathing rhythms under aerobic and anaerobic conditions. Furthermore, the effect of coordination on exercise efficiency was analysed. Three groups of volunteers either sexes participated in the study: 10 highly trained triathletes, 6 highly trained sprinters and 10 untrained medical students. All subjects exercised on a treadmill at work loads of 50%, 80% and 110% of their anaerobic threshold. Each work load was applied twice, once with spontaneous and once with paced breathing. Respiratory variables, heart rate and leg movements were continuously recorded by an automatic respiratory analysis system. The degree of spontaneous coordination during 50% AT was the same in all 3 groups. However, at 110% AT students coordinated less than at lower work loads and less than trained subjects. At 50% AT sprinters were less successful in increasing coordination by paced breathing than the other groups. The effect of coordination on exercise efficiency differed between groups and between work loads. In conclusion: (a) Training has no effect on incidence of coordination at low level aerobic exercise. (b) Endurance training increases incidence of coordination especially at anaerobic work load. (c) Higher degree of coordination is mostly associated with lower oxygen uptake for a given work load, however, other factors as e.g. the level of sympathetic tone influence this relationship.
(Supported by the Swiss National Foundation, grant Nr. 32-28983.90 and by Hermann Klaus Foundation.)

352

EXPANDABLE MODULAR MEASUREMENT SYSTEM FOR RESPIRATION ANALYSIS AND HUMAN SUBJECT MONITORING IN A LOW PRESSURE CHAMBER

Bührer, A., Gähwiler, M., Schuster, K., Koller, E. A.,
Physiologisches Institut, Universität Zürich-Irchel, CH-8057 Zürich

For our low pressure chamber we developed a measurement system for the breath-by-breath analysis and the subject monitoring. The system can easily be adapted for the acquisition of up to 24 instrumentation channels (e.g. for motion sensors). For the accurate registration of the measurement parameters under changing ambient conditions (e.g. ambient pressure) it is necessary to take into consideration that scaling factors can alter. For the optimal compensation of such systematic measuring errors we have configured our measuring system as follows:
All peripheral devices (mass spectrometer, pneumotachograph, pressure sensor, pulse oxymeter, etc.) send their signals to a 24-channel multi processor system called 'Data Preprocessing Unit' (DPU). This device corrects systematic measurement errors (e.g. time delays introduced by the various instruments) and transfers temporally correlated blocks of data to two personal computers. These handle the data storage, the on-line data processing, the numeric and graphic presentation of the results, the subject monitoring and the control of the whole measurement system.

353

ECG CHANGES DUE TO ALTITUDE IN ACCLIMATIZED AND NON-ACCLIMATIZED SUBJECTS

Kobza, R., Bührer, A. and Koller, E.A., Department of Physiology, University of Zurich, CH-8057 Zurich

The ECG changes occurring during acute exposure to high altitude are mainly due to the stimulation of the sympatho-adrenal system (EJAP 53:35-42, 1984). These changes were compared in ten acclimatized mountaineers returning from a Himalaya-expedition and in ten non-acclimatized volunteers (control). Both groups underwent in supine position a standardized stepwise ascent to 6000m (P_B 345 mmHg) in a low pressure chamber; the duration of the entire altitude exposure, apart from the adaptation time before ascent, was 2 h. Results: At 6000m the acclimatized subjects show significantly lower increase in heart rate, no significant P-Q shortening, significantly lower Q-T shortening and S-T depression (V_4, V_6) than the non-acclimatized control subjects. Conclusions: The hypoxia-induced ECG changes in the acclimatized subjects are similar to those found in betareceptor-blocked subjects at altitude and point to the economizing effect of altitude acclimatization which we have described for the respiratory and cardiovascular system (EJAP 62: 67-72, 1991).

354

MAXIMAL RATE OF BLOOD LACTATE ACCUMULATION DURING HIGH ALTITUDE EXPOSURE IN HUMANS

Kayser, B., Grassi, B., Ferretti, G., Colombini, A., Marconi C. and Cerretelli, P. Department of Physiology, CMU, University of Geneva, Switzerland and ITBA of CNR, Milan, Italie.

The reduction of maximal lactic capacity with altitude exposure may depend on a decreased substrate flow through the glycolytic pathway. If this is the case, provided unchanged blood lactate (La_b) kinetics at the end of exercise, the maximal rate of La_b accumulation ($\Delta[La_b]/\Delta t$) should be decreased at altitude. 6 males (32±4 years, x±SD) performed cycloergometric exercises of increasing duration up to exhaustion (30-45 s) at 200% of VO_2max, at sea level (SL1), after 7 (ALT1) and 27 (ALT2) days at 5050 m (Ev-K2-CNR Pyramid), and 7 days after return to sea level (SL2), as well as at a work load corresponding to VO_2max at ALT2 plus VO_2max at SL1 (ALT3). La_b accumulation ($\Delta[La_b]$) was determined for each exercise bout. After exhaustion, the kinetics of La_b were followed during recovery. $\Delta[La_b]$ was linearly related to exercise duration. The slopes of the individual regression lines, equal to $\Delta[La_b]/\Delta t$, were lower at ALT1 (0.09±0.02 ($P<0.01$) and at ALT2 (0.17±0.05) ($P<0.05$) than at SL1 (0.25±0.05) and SL2 (0.23±0.06). There was no difference between ALT2 and ALT3 (0.17±0.05). The kinetics of appearance and disappearance of $[La_b]$ in the recovery were similar in all conditions. $\Delta[La_b]/\Delta$ is thus reduced by altitude exposure. Such reduction, which is less marked after 27 days of acclimatization, is compatible with the hypothesis of a reduced substrate flow through the glycolytic pathway at altitude.

355

FATIGUE IN CHRONIC HYPOBARIC HYPOXIA

Binzoni, T.°, Kayser, B.+, Narici, M.V.*, Grassi, B.+ and Cerretelli, P.+.
°Dept. of Radiology and Physiology, CMU, 1211 Genève 4, CH; +Dept of Physiology, CMU 1211 Genève 4, CH and *ITBA of CNR, Milan, I.

Exhaustive dynamic exercise with large muscle groups in conditions of chronic hypobaric hypoxia may be limited by central rather than peripheral (metabolic) fatigue. Six subjects (32±4 SD yr) performed forearm (at maximum aerobic power) and leg (at VO_2max) exercises, at sea level (SL) and after a one month stay at 5050 m (ALT). Before and after exercise lactate (la) and gases were measured in arterialised blood. Electromyographic activity (EMG) of the forearm flexors and of the vastus lateralis was recorded during exercise and integrated (IEMG), and mean (MPF) and centroid (CPF) frequencies of the power spectrum were calculated. In forearm exercise, at the same load exhaustion time (t_{ex}) t_{ex} was similar in SI and ALT (5.55±2.39 vs 5.63±2.74 min). IEMG (+193%), MPF (-21%) and CPF (-14%) changed similarly during exercise in SL and ALT. By contrast, leg exercise in ALT could be performed at a 21% lower load than at SL for similar t_{ex} (SL 6.51±2.84; ALT 5.09±1.87 min). Whereas in SL, exhaustion was accompanied by an increase in IEMG (+66%) and decreases in MPF (-9%) and CPF (-8%), in ALT no EMG changes were found. In ALT La increased less (SL 12.86±1.45 mM; ALT 6.85±1.40) and changes in blood gases were significantly smaller then in SL. It is concluded that in conditions of chronic hypoxia the central nervous system may play a role in limiting exhaustive exercise of large muscle groups before the appearance of peripheral fatigue.

356

Regulation processes during regeneration of the lung after pneumonectomy.
C. Vallan, P.H. Burri and R.R. Friis*
Institute of anatomy and Laboratory for Clinical Experimental Research, University of Bern, CH-3012

In rats resection of lung tissue is known to trigger a compensatory growth leading to a more or less complete restoration of the lung structure and function. As yet the molecular mechanisms leading to such regeneration have been poorly investigated. Conceptually, the process of lung regeneration after pneumonectomy is analogous to that of liver regeneration after partial hepatectomy, a system which is far better characterised. One could expect that many genes up-regulated in this system would also be up-regulated after pneumonectomy. One example for such a gene is the hepatocyte growth factor (HGF), also called "scatter factor". Its expression is not liver specific. HGF has already been shown to be induced in the lung, after injury of the organism at distant site. Moreover, genes involved in remodelling of tissue, such as metalloproteinases and their inhibitors are likely to be found up-regulated in the process of regeneration of the lung.
We have prepared a Lambda gt11 (Lambda Zap) cDNA library using mRNA isolated from the lungs of pneumonectomized rats 3 days after surgery. Genes potentially involved in lung regeneration, as those described above are being cloned from this library, and their expression at different time after pneumonectomy is being analysed using Northern blots.

357

A NEW DYNAMOMETER FOR COMPARING DIFFERENT MODES OF MUSCLE CONTRACTION

Spengler Ch., Gähwiler M.*, Schuster K.*, Bührer A., Maag A.*, and Boutellier U.
Exercise Physiology, Swiss Federal Institute of Technology, and *Department of Physiology, University of Zurich, CH-8057 Zurich

We wanted to compare respiratory and circulatory responses to electrically induced dynamic leg exercise (EE) with responses to voluntary exercise (VE) or passive movement. For this purpose, torque and/or angular velocity of the lower leg must be the same for these different modes. Unfortunately, force produced by electrical muscle stimulation is not controlled as well as with voluntary contractions. So, for the same force production with EE and VE, voluntary work must be a copy of electrically induced work. For this reason, EE data must not only be recorded by a dynamometer but also shown to the subjects when performing voluntary exercise. Since available dynamometers do not include this possibility, we developed a new one with the following special features: (1) means of the torque-velocity-profiles, i.e. recorded in EE trials, are calculated and shown to the subjects on a feedback system, (2) torque-velocity-profiles are reproduced as velocity-profiles by the dynamometer for passive leg movements and (3) torque-velocity-profiles are presented to the subjects as torque-time-profiles for static reproduction of the dynamic work done before. These new features now allow comparisons of respiratory and circulatory responses to dynamic work, static work, and passive movement.

358

ENDOGENOUS PROSTAGLANDIN PRODUCTION MEDIATES ANGIOTENSIN II INDUCED ATRIAL NATRIURETIC PEPTIDE RELEASE IN CARDIOMYOCYTES

D. J. Church, M. B. Vallotton and U. Lang. Division of Endocrinology, Geneva University Hospital, Geneva, Switzerland.

Mammalian cardiomyocytes synthesize and secrete atrial natriuretic peptide (ANP), a vasorelaxant hormone exhibiting natriuretic and diuretic properties. We studied the roles of protein kinase C (PKC) and endogenous prostaglandin production in angiotensin II (Ang II)-induced ANP release in cultured, spontaneously-beating rat cardiomyocytes. Stimulation of cells with 0.1 μM Ang II lead to increases in particulate-bound PKC activity, cellular prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2) production, and in ANP secretion. A role for PKC in Ang II- induced ANP release was apparent insofar as the PKC inhibitor CGP 41521 (1 μM) strongly suppressed Ang II-induced ANP release, as did PKC down-regulation. Immunoblotting experiments indicated that Ang II induced the activation of both Ca^{++}-sensitive PKC α and Ca^{++}-insensitive PKC δ in these cells. Furthermore, Ang II-induced prostaglandin production and ANP secretion were strongly correlated, suggesting a role for prostaglandins in the response. This was confirmed by finding that the respective phospholipase A_2 and cyclooxygenase inhibitors quinacrine (10 μM) and indomethacin (10 μM) fully abolished Ang II-induced ANP secretion, while exogenous PGI_2 (1 μM) and PGE_2 (0.1 μM) induced significant increases in ANP release in this tissue. Our results suggest that Ang II induces ANP secretion in cardiomyocytes via a PKC-dependent pathway involving a myocardial prostanoid receptor.

359

NEUROPEPTIDE Y POTENTIATES THE INTRACELLULAR CALCIUM RESPONSE TO ANGIOTENSIN II IN HEL CELLS

E.Bürki, C.Centeno, M.Burnier, B.Waeber, H.R. Brunner
Division of Hypertension, CHUV, 1011 Lausanne

Neuropeptide Y (NPY) is an important component of the sympathetic nervous system and acts as a potent effector and modulator of vascular tone. *In vivo* and in *in vitro* vascular preparations NPY not only has a vasoconstrictor effect per se but also potentiates the effects of other vasoconstrictors such as norepinephrine and angiotensin II (AngII). The human erythroleukemia (HEL) cell line has proven a valuable tool to study NPY-mediated intracellular effects. These cells express a well defined Y_1 subtype NPY receptor coupled to calcium signalling and to inhibition of adenylate cyclase. HEL cells express a whole series of functional membrane receptors, among them an α_2-adrenoreceptor. We demonstrate here that they also contain AngII receptors of the AT_1 subtype. The receptor density is extremely low (<500 binding sites/cell) but affinity for AngII is very high (Kd in the sub-nanomolar range). We also demonstrate that these binding sites are coupled to intracellular calcium signalling. We have further studied potential calcium signalling cross-talk between NPY receptors and α_2-adrenergic or AngII receptors. While there exists no detectable synergism between stimulation by NPY and norepinephrine, the calcium response to AngII is potentiated by over 50% when HEL cells are first stimulated with NPY. The exact mechanism of this effect is under investigation.

360

STUDY OF VASCULAR PERFUSION PATTERN IN VARIOUS ORGANS BY ELECTRON AND LIGHT MICROSCOPY.

Martin König, John M. Lucocq, Thomas Pfäffli, and Ewald R. Weibel
Institute of Anatomy, University of Berne, 3012 Berne, Switzerland

To estimate the fraction of capillaries that are perfused under physiological conditions and thus available for gas exchange or substrate delivery, a morphometric analysis combined with a perfusion marker is required. To this end we developed a new method to demonstrate in vivo capillary perfusion by light and electron microscopy. To mark the blood plasma, 8 nm colloidal gold particles were prepared, coated with rabbit serum albumin, concentrated by centrifugation and dialysed against Ringer solution. In anaesthetised rabbits 4-5 ml of the final concentrate was injected via a catheter into the right atrium. Blood samples showed a slow decrease of gold particle concentration in plasma with a dose dependent half-time. After variable post-infusion times the circulation was interrupted by a snare around the heart, and the organs were fixed by immersion in Karnovsky fixative. Electron microscopic analyses of capillaries in heart, skeletal muscle, lung, liver, spleen, intestine and brain revealed colloidal gold particles homogeneously dispersed in the blood plasma. Morphometric analysis of heart, skeletal muscle and lung showed that the entire capillary bed was marked by the tracer even within two minutes following infusion. Silver enhancement of light microscopic sections showed areas with different staining intensities suggesting heterogeneous perfusion. We conclude that this plasma tracer allows quantitation of labelled capillaries using electron microscopy and, after silver enhancement, qualitative studies using light microscopy. (Supported by SNSF grant 31-30946.91)

361

MOTILITY OF RAT ILEUM IN VITRO

P. Bercik*, D. Armstrong, C. Emde, P. Dutoit*, R. Fraser, AL. Blum and P. Kucera*. *Institute of Physiology, University of Lausanne; Division of Gastroenterology CHUV-PMU, Lausanne.

Intestinal motility depends on interactions between myogenic, endocrine and neural factors. The influence of these factors on the contractile activity was studied using a 5-7 cm segment of terminal ileum, perfused arterially with oxygenated fluorocarbon solution and luminally with normal saline. Oral and aboral luminal pressures, luminal output and video images of the segment were recorded simultaneously for 2 hours.

Microscopy showed intact mucosa and enteric neurons indicating good loop viability. 2 motility patterns were observed: 1) High amplitude low frequency contractions (HALFc), with frequency of 0.27/min and oral and aboral peak pressures of 17 hPa and 15hPa, respectively. They originated mostly in the first oral cm of the loop and propagated for 3.5 cm. They were associated with fluid ejection (0.2 ml/contraction). 2) Low amplitude high frequency contractions (LAHFc), with frequency of 27/min and amplitude of 0.3 hPa. They propagated in both directions. Luminal distension increased frequency and amplitude of HALFc but did not affect LAHFc. Tetrodotoxin abolished HALFc and increased amplitudes and tonus of the LAHF. Butanedione monoxime allowed to describe the active and passive mechanical properties of the loop.

We conclude that the ileal segment separated from the CNS shows coordinated activity (HALFc are controlled by the ENS, LAHFc are of myogenic origin) and allows to study the role of the ENS in intestinal motility. *(Supported by SNRF Grant No.32/26369.89)*

362

EXPRESSION OF DESMIN AND ACTIN ISOFORMS IN HUMAN MUSCLE SATELLITE CELLS.

Baroffio, A.*, Bochaton-Piallat, M.-L.@, Gabbiani, G.@, Aubry, J.P.+, Kaelin, A.# and Bader, C.R.*, départements de physiologie* et de pathologie@, CMU, GENEVA ; +Glaxo Institute for Molecular Biology, Plan-les-Ouates, GENEVA; #département de pédiatrie, HCUG, GENEVA.

The expression of muscle-specific cytoskeletal proteins, such as desmin, α-striated (αsr) and α-smooth muscle (αsm) actin isoforms, was studied in quiescent, proliferating and differentiating human muscle satellite cells (HMSC), using immunocytochemical procedures. Quiescent HMSC, studied directly after enzymatic dissociation from muscle fibers, expressed desmin, but neither αsr nor αsm actin. Proliferating mononucleated HMSC cultivated as clones, contained desmin and both actin isoforms, αsr and αsm. Inside each clone, at least two subpopulations of cells were found, one containing both desmin and αsr actin, and another containing only αsm actin. When clones of HMSC were cultivated in a differentiation medium, part of the cells fused to form myotubes and strongly expressed αsr actin and desmin, whereas other cells remained mononucleated and contained either desmin or αsm actin. These results demonstrate that 1) only desmin is expressed in quiescent HMSC, 2) two different muscle actin isoforms are synthetized in HMSC when they proliferate, and 3) single HMSC give rise to an heterogeneous progeny, as concerns the expression of muscle-specific cytoskeletal proteins.

363

FUNCTIONAL SIGNIFCANCE OF ISOPROTEINS IN MUSCLE CYTOARCHITECTURE

Pierre von Arx, Tierry Soldati, Marius Messerli and Jean-Claude Perriard
Institute for Cell Biology, ETH, 8093 Zürich

We present the sorting and the interference with the sarcomeric organization of 2 isoprotein families: the Myosin Light Chains (MLC) and the Actins. MLC form an isoprotein family with members belonging to muscle and to non-muscle tissues; the Actin protein family is composed of six isoforms, which can be subclassed into cytoplasmic, smooth muscle, and striated muscle isoforms.
The isoform distribution is studied by microinjection of cDNA constructs, containing an VSV-epitope, into the nucleus of regenerating Adult Rat Cardiomyocytes (ARC). Immunological staining of the epitope showed that muscle isoforms integrate to the sarcomeric organization, which is not the case for cytoplasmic isoforms.
The expression of cytoplasmic γ-actin has a striking effect on cell morphology, and affects the myofibrillar organization. We show evidence that the thick filament organization can be independently maintained while the thin filament organization disappears, and that the third filament structure (titin) remains sarcomerically organized.

364

Characterization of myomesin as an essential protein in myofibrils

Bantle, St.; Perriard, E.; Eppenberger, H M., and Perriard J.-C.,
Institute for Cell Biology, ETH, 8093 Zürich.

Myomesin is a 185 kD myofibrillar protein which is located in the middle of the sarcomeres, and is one out of the three known M-band proteins: M-protein, myomesin and the muscle specific isoform of creatine kinase. It has been speculated that myomesin might bridge the gap between the titin filament system and the thick-filament system at the M-band, and therefore, it seems to be essential for the fundamental architecture of myofibrils. The aim of this project is to identify cDNA clones of myomesin which will lead to the protein sequence and enable us to examine functional aspects.

A chicken heart muscle λ-gt 11 cDNA expression library was screened by DNA hybridization and by a monoclonal antibody against myomesin. The used probe was a fragment of a cDNA which was found in earlier screenings of a leg muscle library by polyclonal antibodies against M-band protein. This cDNA is thought to contain skeletal muscle specific myomesin. We have identified 40 positive clones with the cDNA probe. Five of them were also detectable in the immuno-screening with the monoclonal antibody B4. The resulting derived protein sequences are compared to other myofibrillar components. The expression of these sequences was shown to be specific for sarcomeric muscle.

365

Analysis of Human Muscle Biopsies with PCR and *In situ* Hybridizations

K. Jostarndt; A. Puntschart, H. Hoppeler, R. Billeter
Institute for Anatomy, Bühlstr.26, CH-3000 Berne 9, Switzerland and Reasearch Institute of the Swiss Sports School Magglingen, CH-2532 Magglingen, Switzerland.

Human muscle adapts to changes in its pattern of use with alterations in the concentrations of key enzymes and structural proteins. Data from animal models indicate that such modulations are accompanied by changes in the steady state concentrations of their respective RNA's. Our focus is the adaptation of human muscle to exercise training. Muscle tissue is obtained from needle biopsies (Bergström technique). RT-PCR allows quantitative assessment of specific RNA's in as little as one cryostat section. A requirement of quantitative PCR is the control of the efficiency of the process during the logarithmic phase which in our hand is followed by the incorporation of radioactive label in its product. Thus we are able to distinguish less than 2 fold differences in RNA concentration.
A further approach involves *in situ* hybridization in order to localize particular RNAs in the different fiber types. We use ^{33}P labelled RNA probes in these experiments. This isotope has an energy spectrum comparable to ^{35}S, but probes labeled with ^{33}P nucleotides are less prone to oxidative modifications than ^{35}S probes, which eliminates some of the background problems. *In situ* hybridization with ^{33}P lableled myosin light chain 1f mRNA on chicken muscle gave signals that were at least as distinct as the ones with ^{35}S labeled probes. In human muscles hybridizations with probes for carbonic anhydrase III- and S100α-mRNA showed preferential localization in type I fibers., which corresponds to the localization of the respective proteins.

366

TISSUE QUANTIFICATION USING FUZZY LOGIC ALGORITHM

S. Kasas, K. Stoffel+ and M. Wisard*
Institut d'Histologie, Pérolles, CH-1700 Fribourg
+Institut d'informatique, Pérolles, CH-1700 Fribourg
*Service d'Urologie, CHUV, CH-1011 Lausanne

An involvement of the smooth muscle of the corpus cavernosum in the pathogenesis of impotence is still hardly debated. Ultrastructural changes have been described but it is not known if they are directly correlated to a modification of the muscle/connective tissue ratio. To try to answer this question a computer based method has been developed allowing the quantification of the muscle tissue in the corpus cavernosum.

Tissue samples from the corpus cavernosum of patients suffering from impotence were fixed in formalin and processed according to the usual technique for observation by light microscopy. The light microscopic images of the Goldner stained sections were digitalized with a CCD camera. To obtain maximal contrast between the muscle and the surrounding structures the slides were illuminated with a monochromatic light source.
The quantification was performed according to the gray level and the texture differences using an IBM PC computer.

We present here an image processing technique wich allows more reliable measurements by removing the inhomogenities in illumination, by normalizing each pixel sensitivity and segmenting the image using a fuzzy logic algorithm.

We believe that a fuzzy logic approach can give more precise results in tissue quantification, since it gives the possibility of considering several parameters, such as the gray level and the texture.

The preliminary results of these quantifications do not show significant differences between impotent and control patients.

367

pH-dependent formation of ethylenediaminetetracetic acid (EDTA) nanostructures

Michele Müller & André Haeberli
Medizinische Klinik, Thromboselabor, Inselspital, CH-3010 Bern

In order to localize the complementary polymerization sites of fibrin, limited proteolytic digestion of fibrinogen with plasmin resulted in the production of subdomains and smaller peptides. Following the original plasminolytic digestion of fragment D1 in the presence of EDTA we found that EDTA alone was eluted from a Superose 12 column at the same elution volume as the carboxy terminal γ-chain peptides. Pure EDTA tetrasodium salt in water showed a pH-dependent change of optical density detected in the ultraviolet region. The most important change was found between pH 4 and 8, corresponding to the deprotonation of the third ionizable group having a pK of 6.2. This observation is interpreted as formation of nanoaggregates by a mechanism which resembles the formation of lipid micelles. The absorbance/size change was reversible and was depended on the addition/removal of only 1 proton equivalent. The existence of nanoaggregates was confirmed by size-exclusion chromatography and dialysis experiments, as well by cryo-electron microscopy.

368

Molecular approaches to study glomerulonephritis in Swiss Bernese Mountain Dog

P.Werner*, U.Hübscher*, S.Arnold° and C. Schelling †
*Institut für Veterinärbiochemie und °Klinik für Geburtshilfe, Jungtier und Euterkrankheiten, Universität Zürich and †Departement of Veterinary Pathobiology, Texas A&M,College Station, USA

Recently idiopathic glomerulonephritis has been diagnosed in Bernese mountain dog (BMD) more than in other breeds. According to the known inbreeding rate a breed predisposition to develop this kidney disease has been demonstrated.Other hereditary glomerulopathies have been recognized in some breeds, e.g. an X-linked recessive glomerulopathie in Samoyed is a model of hereditary nephritis in man, e.g. Alport Syndrom caused by a type IV collagen defect.We collected blood from 150 BMD's of the Swiss breeding population and generated one-dimensional fingerprints by using minisatellites (Jeffrey's probes 33.15/33.6). The therby detected polymorphism between dogs within this breed was very small to directly use fingerprint-pattern as genetic markers in linkage analysis. We therefore started searching restriction fragment length polymorphisms (RFLP) using various single locus probes of human type IV collagen. The results so far indicated that polymorphisms exist in the collagen genes of the BMD. Studies are in progress to further characterize the degree of polymorphism in this breed and any disease association with molecular probes.

369

EXTRACELLULAR VOLUME CHANGES AND CONCENTRATIONS OF PLASMA NATRIURETIC FACTORS : Validation of a simple, non-invasive, battery-operated impedancemeter to monitor volume changes.

H. Favre, C.C.J. Farinelli, R. Donath, and L. Girardier, Departments of Medecine and Physiology, Faculty of Medecine, University of Geneva, CH-1211 Geneva 4, Switzerland

To investigate the role played by two natriuretic factors, atrial natriuretic peptide (ANP) and ouabain-displacing factor (ODF), in the regulation of the extracellular volume (ECV) under physiological conditions, changes in ECV have been induced in 5 volonteers male subjects. They were overnight-fasted and fluid-deprived before being submitted in random order to the following experiments at a week-time interval: ECV expansion (infusion of 21 NaCl 0.9%), plasma expansion (infusion of 500 ml albumin solution) and ECV reduction (diuretic administration). Changes in ECV were monitored by electrical body impedance (Z) using a new-developed quadripolar, 1 kHz, battery-operated impedance meter. The correlation between changes in Z and measured changes in ECV from the fluid balance sheet gives a $r=0.98$, $p<0.001$. The bivariant regression between Z and the inulin dilution space (IDS) was significant ($r=0.91, p<0.001$). Plasma ANP (corrected for passive dilution) is linearly related to changes in Z/IDS from -15 to +15 %, without changing the natriuresis. No significant change independent from passive dilution was seen in plasma ODF. This indicates that ANP but not ODF is responsive to physiological changes in ECV size but that ANP is not natriuretic in hydropenic conditions.

370

A TRUNCATED RAT 11ß-HYDROXYSTEROID DEHYDROGENASE (11ß-HSD 1B) IS INACTIVE IN OOCYTES AND TBM 18-23 CELLS

Duperrex, H., Gaeggeler, H.-P. and Rossier, B.C. Institut de Pharmacologie et de Toxicologie de l'Université, 1005 Lausanne

11ß-HSD 1a is the enzyme that converts active glucocorticoids (cortisol, corticosterone) into inactive compounds (cortisone, 11-dehydrocorticosterone) and vice versa. A truncated rat 11ß-HSD 1b has been recently cloned. Its coding sequence is identical to that of the previously cloned rat liver 11ß-HSD but the first 26 amino acids are missing. It has been proposed that 11ß-HSD 1b could insure mineralocorticoid specificity by inactivating glucocorticoids and preventing their binding to the mineralocorticoid receptor. To test this hypothesis we have injected 11ß-HSD 1b cRNA into oocytes and stably transfected 11ß-HSD 1b cDNA into a mineralocorticoid responsive cell line (TBM 18-23). Oocytes injected with the truncated cRNA were unable to induce dehydrogenase activity whereas oocytes injected with 11ß-HSD 1a cRNA inactivated corticosterone. Although transfected TBM cells highly expressed the truncated transcript, no enzymatic activity was detected. In contrast, TBM cells transfected with 11ß-HSD 1a cDNA rapidly converted 11-dehydrocorticosterone into corticosterone. These data suggest that 11ß-HSD 1b is inactive and therefore not involved in mineralocorticoid receptor protection. The molecular cloning of toad 11ß-HSD isoforms from liver and bladder tissue is in progress. This should help to better understand which isoform plays the critical role in mineralocorticoid receptor protection.

371

INDOLEAMINE 2,3-DIOXYGENASE ACTIVITY IN EYES

Malina H.Z. and Martin X.D., The University of Zurich Department of Ophthalmology, 8091 Zurich

Kynurenamine and 3-hydroxykynurenamine, 2 metabolites of tryptophan, are present in the lenses of humans and animals. However, the first enzyme of the tryptophan degradation pathway, indoleamine 2,3-dioxygenase (IDO), has not been previously found in the lens. Here, we measured IDO activity in an extract of bovine lenses. The metabolites formed were detected and measured by HPLC coupled with electrochemical and fluorometric detection. IDO activity was 1.48 nmol/g of lenses/hour. During the reaction, kynurenamine and 3-hydroxyantranilic acid are formed, indicating the presence not only of IDO but also of other enzymes of the tryptophan degradation pathway. IDO works by scavenging oxygen radicals and it probably has great importance in the protection of the lens against oxidative damage, which is considered to be a cause of cataract formation. Supported by Swiss National Fund grant 32-9527.88.

372

KYNURENINASE ACTIVITY (KA) IN BOVINE CILIARY BODY

Martin X.D. and Malina H.Z. The University of Zurich Department of Ophthalmology, 8091 Zurich

In previous studies we found that the human ciliary body is able to synthetize indoleamines and that the enzyme, indoleamine 2,3-dioxygenase (IDO) which cleaves the pyrrole ring of the indoleamines such as tryptophan and melatonin to produce kynurenines or kynurenamines respectively, is present in aqueous humor, ciliary body, and retina. Kynureninase catalyzes the hydrolysis of 3-hydroxykynurenine (3-HK) (yielding 3-hydroxyantranilate). KA has previously been found in many tissues but not in the eye. In this study we measured KA in iris/ciliary body. The measurement of 3-hydroxyantranilate formed from 3-HK was made by HPLC with electrochemical detection. The KA was 6 nmol/mg protein/hour. 3-hydroxyantranilic acid is an intermediate in the pathway to nicotinamides nucleotides and its accumulation in the lens could be responsible for senile cataract. Supported by Swiss National Fund grant 32-9527.88

373

THE KEY FUNCTION OF HUMAN PLACENTA IN THE TRANSPORT AND METABOLISM OF GLUTAMIC ACID *IN VITRO*.

A. Malek, R. Sager, B. Ugele, H. Schneider. Dept. Obstet. and Gynecol., University of Berne, Switzerland and I. Frauenklinik, University of Munich, Germany.

At the end of gestation most amino acids have a higher concentration in fetal than in maternal blood. In conterast, the level of glutamic acid (Glu) is higher in the maternal than in the fetal circuit. There is no net transfer for Glu across the placenta in either direction. Placental transport and metabolism of Glu was studied using an in vitro perfusion method of human placenta with separate closed circuits for maternal and fetal side (n=9). Glu was added at 250 µmol/l together with 1-^{14}C-Glu (5-10 µCi) to both circuits. Cold and radioactive amino acids were analyzed by HPLC or by enzymatic reaction. In addition, placental tissues before and after perfusion were homogenized and the activity of glutamine synthetase was determined by the highly sensitive γ-glutamyltransferase assay according to Levintow (1). Both cold and labelled Glu showed an uptake from both sides with a maternal to fetal gradient. The final Glu concentration after 4 hours of perfusion in the maternal and fetal circuit were 217±77 and 120±53 µmol/l, respectively. Although no glutamine (Gln) was added to the perfusate, final concentrations of Gln in the maternal and fetal circuit were 85±22 and 131±21 µmol/l, respectively. The release of Gln in both circuits was significantly correlated with the respective uptake of Glu. HPLC analysis of the ^{14}C-label has clearly shown a conversion of Glu into Gln with a higher fraction released into the fetal (20-41%) than into the maternal (11-29%) circuit. The overall production of Gln was 1.2-1.5 µmol/h/g tissue. The tissue homogenates showed a specific transferase activity of 686-704 mU/g tissue (n=10, insignificant change between before and after perfusion), which in analogy with other tissues (1) corresponds to an enzymatic Gln synthesis capacity of about 1-3 µmol/h/g tissue. This range is in good agreement with the actual production of Gln during the perfusion experiments. The results suggest that placental Gln synthesis may be limited by the respective enzyme activity under perfusion conditions. The activity of glutamine synthetase is highly dependend on ATP, indicating that the human placenta during in vitro perfusion is maintaining its energy metabolism. (1) Levintow, L. (1954) J. Nat. Cancer Inst. 15, 347-353.
(Supported by Swiss National Foundation grant # 32-9003.86)

374

THE POTENTIAL USE OF UREA LABELLED WITH ^{15}N FOR ASSESSING TOTAL BODY WATER IN HUMANS.

Schutz, Y., Qualm, Y., Cavelaars, A., Schneiter, Ph., Institute of Physiology, Faculty of Medicine, University of Lausanne, Lausanne, Switzerland.

The aim was to develop a new non invasive method to estimate total body water (TBW) based on the utilization of a single dose of urea labelled with ^{15}N (40 mg, 99% enriched, non radioactive isotope) administered per os diluted in water. Fourteen young subjects (63 kg, 22% body fat) were studied. The rate of elimination of ^{15}N urea in urine was determined over a 8-hour period using short urinary timed samples. The extrapolation of the enrichment decay at zero time (t_0) was used to obtain the urea space assumed to be equivalent to TBW. The TBW estimated from ^{15}N urea was found to be significantly lower (31.2±9.1 L, $p<.01$) than that assessed independently by a combination of bioelectrical impedance and anthropometric measurements (36.2±7.6 L), as well as from ethanol dilution space (36.3±8.8 L). Various factors which may explain the systematic underestimation of TBW based on ^{15}N urea will be outlined.

536 (received after deadline)

NUTRITIONAL REGULATION AND LOCALIZATION OF FATTY ACID SYNTHESIS IN LIVER.

Kikinis, Z., Cochary, E.F., and Paulson, K.E..
Tufts University, Boston, MA 02111, USA.

Malic enzyme, the donor of much of the NADPH required for *de novo* fatty acid synthesis, is tightly controlled by nutritional conditions. We examined the effects of fasting followed by refeeding a high carbohydrate/no fat diet on expression and localization of malic enzyme (ME) and fatty acid synthase (FAS) in mouse liver. *In situ* hybridization showed that both lipogenic enzymes were expressed at low basal levels in all hepatocytes in livers of mice fed a control diet. The temporal response of dietary-induced expression of both genes was studied at 0, 6, 12, 24, 36, 48 and 72 hours, and 8 days. *In situ* hybridization showed that ME and FAS mRNA was induced initially in periportal cells within 6 hours. By 24 hours, induced expression in periportal hepatocytes was maximal. This was coincident with expression of ME and FAS mRNAs which appeared to be maximal between 24 and 36 hours as measured by slot blot analysis. Both mRNA levels and portal expression of ME and FAS then decline. Transcription rates were measured by nuclear run-on assay and demonstrated that maximum transcription rates precede maximum mRNA levels by peaking at 12 hours. Furthermore, run-on assays showed that the periportal induction by carbohydrates is mostly a transcriptional response. These results suggest that ME and FAS appear to be regulated at the level of transcription.

Neurobiology

375

TRANSFER OF SUBSTRATE FROM GLIAL CELLS TO PHOTORECEPTOR-NEURONS

Tsacopoulos, M., Perrottet, Ph. and Veuthey, A.-L., Departments of Oto-neuro-ophthalmology and Physiology, CH-1211 GENEVA 4

The honeybee retina is a nervous tissue with a crystal-like structure and an extreme degree of metabolic compartmentation. Quantitative evidence shows that glial cells *in situ* phosphorylate glucose to glucose-6P and supply with a metabolic substrate the photoreceptors. Alanine is likely to be this substrate since more than 50% of $^{14}C(U)$-glucose is transformed to $^{14}C(U)$-alanine by transamination of pyruvate by glutamate. It is released into the extracellular space and then transferred to the photoreceptors where it enters the Krebs cycle. Light stimulation of the photoreceptors increases their O_2 consumption by 400%. This in turn send a signal to the glial cells inducing a rise in the rate of phosphorylation of glucose and of glycogen turnover. In parallel the concentration of alanine rises and the concentration of glutamate and of proline, both decrease by about 50%. Proline is the prefered precursor of glutamate. We conclude that glial cells, which contain most of the hexokinase activity, oversatisfy the respiratory requirements of photoactivated photoreceptors for metabolic energy substrate.

376

ENERGY METABOLISM IN PRIMARY ASTROCYTE CULTURES : STUDIES ON GLYCOGEN LEVELS AND LACTATE RELEASE. Q. Sorg and P.J. Magistretti. Institut de Physiologie, Université de Lausanne.

VIP and noradrenaline (NA) have been previously shown to promote glycogenolysis in primary cultures of mouse cerebral cortical astrocytes. Recently, we have observed a second, temporally-delayed, action of VIP or NA : following glycogenolysis, an induction of glycogen resynthesis is observed, resulting, within 9 hours, in glycogen levels that are 6 to 10 times higher than those measured before the application of either neurotransmitter. VIP pulses as short as one minute are sufficient to double, 9 hours later, glycogen levels. The induction of glycogen resynthesis triggered by VIP or NA is abolished by inhibition of transcription and translation. The effect of VIP and NA is mimicked by dibutyryl cAMP, suggesting the involvement of a cAMP Responsive Element in the long term effect of both neurotransmitters.

Studies performed on the same cultures have shown that astrocytes produce and release great quantities of lactate : in 30 minutes, as much as 1 µmol of lactate per mg of protein is measured in the extracellular medium; the release of lactate is linear at least during 6 hours; iodoacetate, a blocker of the glycolytic pathway, almost completely abolishes lactate release, while sodium azide, a blocker of oxidative phosphorylation, induces a 3-fold increase over basal levels. Furthermore, NA but not VIP enhances lactate output from astrocytes.

377

ROLE OF INTRACELLULAR CALCIUM IN ELECTRICAL PROPERTIES OF MAMMALIAN SCHWANN CELLS.

P.Jirounek & P.C. Brunet*, Département de Pharmacologie, CMU, 1211 Geneve 4

The electrophysiological properties of Schwann cells (SC) were studied on rabbit vagus nerves after complete Wallerian degeneration (DN) of axons. We found that the resting membrane potential of the remaining SC depended on $[K^+]_o$ in a similar manner as did normal nerve (NN). However, in opposite to the NN, the DN depolarized with decreasing $[Ca^{2+}]_o$ and hyperpolarized with increasing $[Ca^{2+}]_o$. The role of Ca^{2+} in these effects was thus studied by the use of Fura2. The results showed that $[Ca^{2+}]_i$ increased with increasing $[Ca^{2+}]_o$ and decreased with decreasing $[Ca^{2+}]_o$. On the other hand, we observed that $[Ca^{2+}]_i$ increased with decreasing $[K^+]_o$ and decreased with increasing $[K^+]_o$. These surprising results indicate that voltage dependent Ca^{2+} channels were not involved in these changes of $[Ca^{2+}]_i$. Furthermore, they suggest that SC might possess an electrogenic K^+/Ca^{2+} exchanger. On the basis of these results we propose a mechanism for $[K^+]_o$ and $[Ca^{2+}]_o$ homeostasis which simultaneously subtract K^+ from the periaxonal space and supplies axons with Ca^{2+} during the axonal electrical activity. In return, at the end of axonal activity, SC deal K^+ to axons, exchanging it for Ca^{2+}.

*Present address: Division d'Investigations Chirurgicales et Anesthésiologiques

378

HETEROGENEITY OF CEREBRAL ENDOTHELIUM: AN IN VITRO STUDY OF ASTROGLIAL CONTRIBUTION

Juillerat-Jeanneret,L.; Janzer,R.C.; Division of Neuropathology, Institute of Pathology, University of Lausanne

The blood-brain barrier phenotype of brain endothelium is induced by astrocytes. There is increasing evidence that in a single organ, microvascular endothelial cells are heterogeneous. To adress this question in the cerebral endothelium, we measured the activities of specific markers in endothelial cells of different origins, including two brain-derived endothelial cell lines, grown on plastic or on extracellular matrix components secreted either by a glial or a fibroblast cell lines, with or without the addition of astrocyte-conditioned medium.
The results demonstrate that subpopulations of cerebral endothelial cells exist, that astrocyte-derived soluble factors and components of the extracellular matrix can modulate the expression and function of endothelial cells, and that astrocyte-derived soluble factors have a selective growth inhibitory effect on endothelial cells of non-cerebral origin.

379

ROLE OF SCHWANN CELLS IN THE REGULATION OF THE POTASSIUM MICROENVIRONMENT IN MAMMALIAN NONMYELINATED NERVE FIBRES

A.Robert, J.-L. Balfroid & P.Jirounek
Département de Pharmacologie, CMU, 1211 Genève 4

Mechanisms regulating K^+ changes in the axonal microenvironment were studied in the rabbit vagus nerve. This preparation is well suited for this type of investigation because it contains a great number of small nonmyelinated axons with high membrane surface/volume ratio. By combining the techniques of ion selective microelectrodes with the sucrose-gap method, we measured simultaneously the changes in the extracellular potassium concentration ($[K^+]_o$) and the variations of the membrane potential. We have observed that the kinetics of the changes of $[K^+]_o$ strongly depended on the presence or absence of the perineural sheath (PS). In its presence, the changes in $[K^+]_o$, induced by electrical activity or by application of ouabain, GABA, caffeine and Ba^{++}, were much larger than in preparations where the PS was absent. We showed that at resting state, the $[K^+]_o$ in the extracellular space is subtly controlled by the $(Na^+\text{-}K^+)$ATPase and Ba^{++}-sensitive K^+ channels.
During and after electrical activity the changes in membrane potential did not correspond only to the variations of $[K^+]_o$. We hypothesize that this deviation of the membrane potential from the Nerstian behavior comes from a contribution of the Schwann cell K^+ currents, involved in the $[K^+]_o$ regulation.

380

CHARACTERIZATION OF NEWLY ISOLATED OLIGODENDROCYTE-SPECIFIC cDNA CLONES

N.Schaeren-Wiemers and M.E. Schwab
Brain Research Institute, University of Zurich, CH-8029 Zurich

Oligodendrocytes are a major glial cell type in the central nervous system (CNS). Beside their main function of myelinating axons, they have been shown to express, on their surface, proteins that are important for CNS development and regeneration. We differentially screened a rat postnatal day 16 (P16) spinal cord cDNA library with single-stranded cDNA of P16 spinal cord (plus probe) and with sscDNA of X-irradiated, oligodendrocyte free spinal cord (minus probe). Liver mRNA was used to remove "housekeeping genes" from both sets of probes by subtractive hybridization. Northern analysis showed that most of the clones obtained are either restricted to or more highly expressed in the nervous system. In situ hybridization showed that many of them are selective for oligodendrocytes. Besides already known genes (e.g. MOG) several clones represent novel genes. Four of these are exclusively expressed by oligodendrocytes, three are found in addition in sciatic nerve, most propably in Schwann cells. Some of the mRNAs have a perinuclear distribution in the cell bodies, but some are present also in the cell processes (as previously described for MBP mRNA). For some genes we have the full lenght cDNA, for others we are currently isolating and sequencing them. Functional studies and the production of antibodies are under way.

381

ZINC FINGER GENES EXPRESSED IN OLIGODENDROCYTES

Pott, U., Holz, A. and Schwab, M.E., Brain Research Institute, University of Zurich, August Forel-Str. 1, CH-8029 Zurich / Switzerland

Oligodendrocytes play an important role in development and function of the central nervous system (CNS). They provide the basis for fast saltatory conduction by elaborating the myelin sheath around the axons. Moreover, they produce proteins which inhibit neurite outgrowth; these proteins might be used to establish and maintain the wiring in the CNS. In order to understand gene expression in oligodendrocytes in more detail, we cloned putative transcription factors from this particular glial cell type.

A cDNA library constructed from differentiated oligodendrcytes was screened for members of the Krüppel family of zinc finger proteins. Eleven different cDNAs were obtained. Complete sequencing of four clones revealed that they code for new members of the zinc finger gene family. All proteins deduced from the cDNAs contain multiple Zinc finger repeats. The corresponding mRNAs range in size from 2 to 5 kb. As expected, all four genes are expressed in spinal cord and brain, the expression of three clones being higher in the CNS than in all peripheral tissues tested (with the exception of testis). In optic nerves, in which the oligodendrocytes have been eliminated by X-irradiation, the expression of two of the zinc finger clones was strongly repressed, similar to the typical myelin gene MBP. So far, direct cellular localization by in situ hybridization could be obtained for one of the clones; the corresponding mRNA is present in oligodendrocytes in all CNS areas, as well as in neuronal subpopulations.

382

THE C-DNA OF COP, A NOVEL OLIGODENDROCYTE SPECIFIC PROTEIN

C. SCHAEFER, A. HOLZ, N. SCHAEREN-WIEMERS AND M.E. SCHWAB
Institut für Hirnforschung der Universität Zürich, August Forel Str.1
8029 Zürich -Schweiz-

Myelin contains a variety of unique proteins (e.g. MAG, MBP, PLP, CNPase). Only a few proteins seem to be selectively expressed by central nervous system (CNS) oligodendrocytes as compared to peripheral nervous system (PNS) schwann cells. Because of the importance of oligodendrocytes in myelination, development and regeneration of neurons, we were mainly interested in oligodendrocyte specific c-DNAs.
The isolated COP (central nervous system oligodendrocyte protein) c-DNA corresponds to a m-RNA of 4.9 kb. The available c-DNA (3.4 kb) including the poly A tail does not show homology to any known oligodendrocyte or CNS c-DNA. Further sequence information is provided by isolating the COP gene from the rat chromosome. In northern blots the expression is restricted to the postnatal rat CNS and is higher at P16-20 than in the adult spinal cord. COP expression was detected by in situ hybridisation in developing oligodendrocytes in the rat CNS and in cultured, galC positive, oligodendrocytes.

383

Increased expression of the growth associated protein GAP-43 in the demyelinated rat spinal cord.

Josef P. Kapfhammer and Martin E. Schwab, Brain Research Institute, University of Zurich, August-Forel-Str. 1, 8029 Zürich.

Previous work has shown that the regional expression of a marker protein for fiber growth and plasticity, GAP-43, is mostly inverse to the degree of myelination of CNS gray matter areas in normal adult rats. This suggests a possible role of neurite growth inhibitory proteins from oligodendrocytes in suppressing sprouting and synapse rearrangement in the normal mature CNS. In order to study the relation between myelination and GAP-43 expression more directly, we have examined GAP-43 expression in the lumbar spinal cord of rats where myelination was suppressed by neonatal X-irradiation. In these spinal cords the typical regional downregulation of GAP-43 expression (as seen with GAP-43 immunohistochemistry) fails to occur and GAP-43 expression remains high in most parts of the unmyelinated spinal cord. Non-irradiated, myelinated parts of the spinal cord of the same rats show the normal developmental suppression of GAP-43. These results support the hypothesis that myelin and neurite growth inhibitors suppress sprouting in the normal rat CNS.

384

Cell proliferation during remyelination in aggregating brain cell cultures.

V. Comte-Miserez, P. Honegger and J.-M. Matthieu.
Laboratoire de Neurochimie, Pédiatrie, CHUV, 1011 Lausanne.

Demyelination was induced in aggregating brain cell cultures using a monoclonal antibody against myelin/oligodendrocyte glycoprotein (MOG) in the presence of complement. Seven days after having removed anti-MOG and complement and restored normal medium conditions, the cultures fully remyelinated. De- and remyelination were assessed by measuring myelin basic protein (MBP). 2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNP) was used as an oligodendrocyte marker and glutamine synthetase (GS) as an astrocyte marker. During this period, cell proliferation, determined by ^{14}C-thymidine incorporation was similar in remyelinating and control cultures.
Platelet derived growth factor (PDGF AA) given to demyelinated cultures induced cell proliferation and inhibited remyelination. PDGF AA removal allowed cells to differentiate into oligodendrocytes and myelinate. During remyelination if PDGF AA was added to AraC, proliferating cells were killed, but a partial remyelination could be observed. This indicates that mature and non dividing oligodendrocytes are able to remyelinate. Under those experimental conditions, GS levels remained stable indicating that PDGF AA affected only oligodendrocytes proliferation.

385

VASOPRESSIN RECEPTORS ARE LOCALIZED ON GLIAL CELLS IN THE SUPERIOR CERVICAL SYMPATHETIC GANGLION.

Tribollet E., Arsenijevic Y., Marguerat A., Dreifuss J.J., Bertrand D. and K. Maury, Department of Physiology, CMU, CH-1211 Geneva 4.

Specific binding sites for vasopressin (AVP) are present in high numbers in the rat superior cervical ganglion (SCG). Binding of AVP to these sites induces a massive increase in the turnover of phosphatidylinositol, which indicates that they are functional V_1 type receptors. Results of a previous study suggested that the major effect of AVP was not exerted on the principal noradrenergic neurones but rather on other cells (Kiraly et al., *PNAS*, 83, 5335-5339, 1986). In order to assess the cellular localization of AVP receptors in the SCG, we have labelled these sites with an iodinated V_1 antagonist in primary cell cultures obtained from SCGs of 5-6 days old rats. Binding was performed on living cells, 12 hours after enzymatic dissociation. Large noradrenergic neurones were not labelled, nor were small dopaminergic neurones. Silver grains were found overlying non-neuronal cells which on the basis of morphological criteria may be satellite cells rather than Schwann cells. We are presently further characterizing the cell type(s) bearing AVP receptors by using antibodies directed against various glial cell markers in combination with autoradiography.

386

EXPRESSION OF INTERMEDIATE FILAMENT PROTEINS IN OPTIC NERVE GLIAL CELLS OF *XENOPUS* TADPOLES

Rungger-Brändle, E. and Alliod Ch., Microscopie électronique, Clinique d'Ophtalmologie, HCUG, CH-1211 Genève 14

As part of the central nervous system, the optic nerve (ON) offers excellent possibilities to study regenerative capacities of white matter. Lower vertebrates are capable of regenerating ON and fully regain visual function. Soon after mechanical crush of *Xenopus* tadpole ON, glial cells phagocytize cell debris, migrate, and rearrange into a loose core. The acquisition of these novel features is accompanied by an alteration in the intermediate filament (IF) complement. Vimentin synthesis is selectively increased, whereas in the normal ON cytokeratin expression predominates. In order to study whether this shift in IF expression is realized in all glial cells or brought about by certain cell types only, freshly dissociated cells from ON were immunofluorescently stained for IF proteins and glial markers.

387

ANGIOTENSIN-II RECEPTORS MEDIATE REGULATION OF GLIA-DERIVED NEXIN (GDN) SYNTHESIS IN SCHWANN CELLS

Bleuel A., de Gasparo M.*, Püttner I.+ and Monard D., Friedrich Miescher Institute, *Pharmaceutical Division and +Biotechnology Department of Ciba, CH-4002 Basel

The aspartyl protease, renin, down-regulates the expression of GDN in Schwann cell cultures in a time- and dose-dependent manner. Angiotensin II (Ang II), the final member of the cascade resulting from the activation of angiotensinogen by renin, has the same effect. Ang II receptors, as well as mRNA for renin and angiotensinogen, have recently been found in various tissues, including the brain. Down-regulation of GDN is detected at both the mRNA and protein levels. On the other hand, GDN synthesis can be increased 10-fold if the cells are treated with synthetic ligands that specifically interfere with the binding of Ang II to its receptors. Northern blot analysis clearly showed the presence of the type I or "glial-specific" Ang II receptors, but in extremely low amounts. Ang II receptors also regulate GDN synthesis in Schwannoma and glioblastoma cell lines, but not in fibroblasts or neuroblastoma cells. These results indicate that some neuropeptides can regulate extracellular proteolytic activity.

388

EXPRESSION OF GLIA-DERIVED NEXIN IN TRANSGENIC AND NON-TRANSGENIC MICE

F.M.Botteri[1], I.M.Mansuy[1], M.Meins[1], C.Mondadori[2], G.Sansig[3], D.Sauer[2], H.van der Putten[3], and D.Monard[1].[1]Friedrich Miescher Institute, [2]Pharmaceutical Division, [3]Biotechnology, P.O.Box 2543, CH-4002 BASEL

Glia-derived nexin (GDN), also known as protease nexin 1 (PN-1), is a serine protease inhibitor, that *in vitro* promotes neurite outgrowth. *In vivo*, it is upregulated following lesion of the rat sciatic nerve and following transient global ischemia in the gerbil hippocampus. Therefore, GDN could influence both degenerative events and/or axonal regeneration *in vivo*. Spatial and temporal expression of GDN was examined by *in situ* hybridisation and immunohistochemistry. In the mouse embryo, the major sites of GDN expression are cartilage, bone, lung and heart, whereas it is detected in a few restricted areas of the developing nervous system. Postnally, GDN expression gradually increases in the brain and it is widely expressed in the adult nervous system. In addition, we generated several strains of transgenic mice carrying a chimeric Thy-1-GDN gene. This transgene directs expression of high levels of rat GDN in the brain. Transgenic mice of three lines were extensively analysed at RNA and protein level and went through a whole series of behavior tests. Transgenic GDN was shown to be biologically active by its capability to form SDS-stable complexes with thrombin. Furthermore, the role of GDN was evaluated in CNS regeneration processes. The overexpression of GDN did not result in detectable developmental, morphological, or physiological abnormalities.

389

WIDESPREAD DISTRIBUTION OF GDN/PN-I IN THE BRAIN CONFIRMED BY AN IMPROVED IMMUNOHISTOCHEMICAL METHOD

Pavlik, A., Bleuel, A. and Monard, D., Friedrich Miescher Institut2, CH-4002 Basel

The glia-derived nexin (GDN) or protease-nexin I (PN I) is an inhibitor of serine proteases which was shown to be constitutively expressed in the olfactory system and re-expressed in neuroglia following ischemic and excitotoxic insults to the brain. So far GDN/PN-I was visualised in the tissues fixed by formaldehyde-type fixatives. Recently we observed that its immunocyto/ histochemical detection with a monoclonal antibody was markedly enhanced by the addition of picric acid to the fixative. Since *in situ* hybridization has shown widespread expression of GDN/PN-I mRNA in the developing rat and mouse brain, we re-investigated the localization of GDN/PN-I protein in the neonatal and adult rat brain fixed with Bouin. We report here that - in addition to the expected localization in the olfactory bulb - we detected GDN/PN I immunoreactivity in many distinct neuronal and non-neuronal populations of the central nervous system of the rat.

390

TERM1: A SECRETED NEURON-SPECIFIC GLYCOPROTEIN EXPRESSED ON GROWTH CONES AND DEVELOPING SYNAPSES OF INDIVIDUAL IDENTIFIED NEURONS.

Therianos, S., Meier, T. and Reichert, H., Department of Zoology, University of Basel, CH-4051 Basel

The chemoaffinity theory postulates the existence of cell-specific molecular signals that uniquely identify individual developing neurons. Such molecules are thought to promote both accurate axon outgrowth and the formation of correct synaptic connections. Here we report on a candidate molecular label of this type that is expressed exclusively by two pairs of sibling interneurons in the developing central nervous system of the grasshopper. Our experiments indicate that during axogenesis, this molecule becomes enriched at the growth cones of these cells; while during subsequent synaptogenesis, it becomes concentrated at the cells' developing terminal arbors. In both cases immunoelectron microscopical studies show that the molecule is secreted by the labeled structures and becomes incorporated into the surrounding extracellular matrix. This molecule, which we call TERM-1, is a glycoprotein with a molecular weight of approximately 48 kD. The highly restricted spatiotemporal expression pattern of TERM-1 implies that individual developing neurons can acquire and retain unique molecular labels which may be important for neuron-specific outgrowth and target recognition. (Supported by the Swiss NSF).

391

PRODUCTION AND SECRETION OF SEVERAL EXTRACELLULAR PROTEIN FRAGMENTS OF THE NEURAL CELL ADHESION MOLECULE L1 IN CHO CELLS

Holm, J., Appel, F. and Schachner, M., Department of Neurobiology, Swiss Federal Institute of Technology, Hönggerberg, CH-8093 Zürich

Cell-to-cell interactions have been developed to a high degree of precision in the nervous system. Various cell surface and extracellular matrix molecules have been identified to be involved in neuron-neuron and neuron-glia recognition. In search for their particular functions, their structure was determined with the aim of relating specific functional traits to distinct structural features. Several neural cell adhesion molecules were shown to belong to the immunoglobulin superfamily and to contain structural motifs characteristic of extracellular matrix molecules, the fibronectin type III homologous repeats. The neural cell adhesion molecule L1, and its homologs in other species, such as human L1CAM and rat NILE, is one of these molecules. To analyze structure-function relationships of the neural cell adhesion molecule L1, we have expressed different fragments of the extracellular part of this glycoprotein in CHO cells[1]. The cDNAs were inserted into the pEE.14 eukaryotic expression vector which carries the human cytomegalovirus promotor and a glutamine synthetase gene as selection marker. For correct secretion of internal L1 protein fragments a signal sequence and a stop codon must be added to the cDNA. Therefore we constructed a set of vectors using the signal sequence of L1 followed by a Bgl II linker and stop codons in all three reading frames. The results showed a secretion of the internal L1 protein fragments carrying a signal peptide.

1: Appel et al., submitted

392

THE ROLE OF EXTRACELLULAR MATRIX MOLECULES AND MICROGLIA IN REGENERATION OF THE LEECH CNS

Masuda-Nakagawa, L., Brodbeck, D., Muller, K.J. and Nicholls, J.G., Dept. of Pharmacology, Biocenter, University of Basel, CH-4056 Basel

The aim of our experiments is to investigate how protein molecules in extracellular matrix of leech nervous system induce regeneration of identified nerve cells. In culture the processes of leech neurons on a laminin-like molecule are slender, straight and unbranched; on the plant lectin Con A they are thick, curved and branched, on enriched tenascin fractions the pattern of outgrowth resembles that on Con A. In the animal itself the laminin-like molecule appears during regeneration at the site of injury close to growing fibres. Metabolic labeling of the entire CNS shows that laminin synthesis is increased in the regenerating CNS. When the connective glial cells is killed by intracellular injection of protease, there is new sprouting and laminin accumulates. It therefore seems unlikely that glial cells produce the laminin that is induced in regeneration. Small phagocytes of the brain, microglial cells, also accumulate at the lesion site. Microglial cells isolated from the CNS are immunoreactive to laminin. We are currently investigating whether antibodies against laminin can inhibit regeneration of axons. Our results suggest that microglial cells may make laminin and bring it to sites of injury.

Supported by the Swiss National Fund Grant No. 31-27 814.89

393

EFFECT OF DEPOLARIZATION ON THE CYTOSKELETON OF LEECH NEURONS

M.D. Neely and M. Gesemann, Biocenter Univ. Basel, 4056 Basel, Switzerland

Leech neurons in culture growing on leech extracellular matrix (ECM) respond with neurite retraction to electrical stimulation or to depolarization with high extracellular K^+. This response is substrate dependent and requires influx of calcium (Grumbacher-Reinert and Nicholls, 1992, J.Exp.Biol. 167:1-14; Neely, J. Neurosci., in press). Time lapse studies reveal that the first morphological changes of neurons exposed to high extracellular K^+ include loss of filopodia and rounding-up of growth cones. Phalloidin, an agent that stabilizes microfilaments, prevents the neurites from retracting after depolarization. By contrast, taxol, a chemical that stabilizes microtubules, has no effect. Disruption of microfilaments with cytochalasin induces changes similar to the ones observed after depolarization: loss of filopodia, rounding-up of growth cones and neurite retraction. Depolymerization of microtubules has no effect on the neurites. Immunocytochemical studies reveal a loss of microfilaments, but not microtubules in the growth cones of leech neurons on ECM after depolarization. Neurons growing on Concanavalin A do not retract their neurites and show no changes in the cytoskeleton after depolarization. We are now exploring the mechanism by which depolarization causes disruption of the microfilaments in growth cones on ECM.

This work was supported by a grant from the Swiss Nationalfond No. 3127814.89 to J.G.N.

394

GLUCOCORTICOID MODULATION OF NEUROTROPHIN EXPRESSION IN HIPPOCAMPAL CELLS.

Scully, J.L. and Otten, U., Dept. of Physiology, University of Basel, CH-4051 Basel

Glucocorticoid hormones (GCs) are secreted in response to stress. The hippocampus is a particular target for GCs, and prolonged exposure exacerbates hippocampal damage due to ageing or neurological insults in both rodents and primates. Conversely, there is evidence that adrenalectomy destroys hippocampal granule cells in vivo. GCs have been found to decrease the expression of nerve growth factor (NGF) in rodent fibroblasts in vitro and sciatic non-neuronal cells in vivo. Since NGF and other members of the neurotrophin (NT) family are strongly implicated in the development and maintenance of the hippocampus, it is possible that GC effects are mediated via NTs. We were interested to see whether GCs could regulate NT expression in immortalized hippocampal cell lines which show characteristic features of hippocampal neurons. Using reverse transcription and amplification of DNA by the polymerase chain reaction (PCR), we have found that the expression of NTs in hippocampal cells is differentially modulated by GCs, and that the neuronal response may be developmentally regulated.

395

HUMAN TAG-1/AXONIN-1: cDNA CLONING, STRUCTURAL FEATURES, AND EUCARYOTIC EXPRESSION

Hasler, T.H., Rader, C., Stoeckli, E.T., Zuellig, R.A., and Sonderegger, P., Biochemisches Institut der Universität Zürich, CH-8057 Zürich

Axonin-1 of the chick and TAG-1 of the rat are homologous members of the immunoglobulin superfamily expressed either as membrane-associated GPI-linked or as soluble forms, and they are believed to play an important role in the axon guidance mechanisms in developing nerve tissues. Here we report the cloning of the human homologue from a fetal brain cDNA library. At the amino acid level, human TAG-1/axonin-1 has a identity of 91% with rat TAG-1 and 75% with chicken axonin-1. The encoded protein was transiently expressed in monkey COS1 cells, and a stable mouse myeloma cell line was established expressing human TAG-1/axonin-1. The transfected COS1 and myeloma cells showed immunoreactivity on the cell surface with polyclonal anti-chicken axonin-1 antibodies. Considerable amounts of the expressed protein were also detected in the cell culture medium. Immunostaining of cryostat sections of embryonic retinas with polyclonal anti-axonin-1 antibodies showed similar expression patterns in chicken and human samples at corresponding developmental stages.

396

HOMOPHILIC INTERACTION OF THE NEURONAL RECOGNITION MOLECULE AXONIN-1

Rader, C., Ziegler, U., Stoeckli, E.T., Osterwalder, T., and Sonderegger, P., Biochemisches Institut der Universität Zürich, CH-8057 Zürich

The axonal surface glycoprotein axonin-1 is thought to be involved in axon guidance mechanisms in the developing nervous system. The neurite outgrowth-promoting activity of axonin-1 has recently been demonstrated to result from a heterophilic interaction with Ng-CAM. Here we present evidence for homophilic binding among axonin-1 molecules. Heterologous expression by myeloma cells generated functionally competent soluble and membrane-bound axonin-1. Cell lines exposing membrane-bound axonin-1 at their surface formed large multicellular aggregates. Pairwise coincubations of transfected and parental myeloma cells revealed homophilic axonin-1 interactions across the intermembrane space as the molecular mechanism promoting stable cell-cell contacts. Interactions of axonin-1 with both Ng-CAM and other axonin-1 molecules might contribute to the formation of macromolecular networks at contact sites of growth cones and axons comprising molecules of both membranes, and thus, represent a mechanism for regulating neurite outgrowth and pathfinding.

397

Genomic clonong of the axonally secreted cell adhesion molecule axonin-1. R.J. Giger, R.A. Zuellig, and P. Sonderegger. Institute of Biochemistry, University of Zurich, CH-8057 Zurich, Switzerland.

Axonin-1 is a 135-kd glycoprotein of the chick. It is secreted from the neurites of cultured neurons and occurs also as a membrane-bound form. Axonin-1 is presumably the species homologue of TAG-1 of the rat. The cDNA of axonin-1 has recently been cloned (Zuellig et al., Eur. J. Biochem. 204, 453-463, 1992). The axonin-1 gene extends over more than 30 kilobases (kb) and consists of 23 exons giving rise to two mRNAs of 4000 and 7790 nucleotides (excluding the poly(A) tail). The deduced amino acid sequence contains a signal peptide of 23 amino acids, six immunoglobulin (Ig)-like domains, four fibronectin-type-III (FNIII)-like domains and 35 amino acids at the C-terminus. The first exon is located several kb upstream of the rest of the axonin-1 gene and encodes the first part of the leader sequence. The signal peptide is encoded by exon 2 and exon 3. Each Ig and FNIII domain is encoded by two exons. The last exon codes for the C-terminal 35 amino acids which are probably responsible for GPI-anchorage. Ribonuclease protection studies indicated two major transcription initiation sites at -153 and -178 bp upstream of the ATG start site. The 5' leader sequences are G+C rich (67%) and encoded by exon 1 and 2. The interrupting intron is of undetermined size

398

ISOLATION AND PARTIAL CHARACTERISATION OF METALLOTHIONEIN-3 (GROWTH INHIBITORY FACTOR) FROM BOVINE BRAIN

Pountney, D.L., Vodičková, H,. Vašák, M., Biochemisches Institut der Universität Zürich, CH-8057 Zürich.

Recently, a low molecular weight copper and zinc containing protein has been characterised from human brain tissue which is capable of inhibiting neuronal growth and survival in vitro and which was found to be down-regulated in the brains of Alzheimer's disease victims. This protein was shown to have around 70% sequence homology with mammalian metallothioneins (MT-1, MT-2), but with 68 rather than 60-61 amino acids, leading to its classification as MT-3. In order to investigate the structural differences between this protein and other MTs, a readily available source is required. To this end, an analogous protein has been isolated from bovine neural cortex based on its high metal content and similar elution on anion exchange chromatography. After incubation of the crude brain homogenate with excess cadmium salt, the purified protein contains cadmium and copper in the ratio 5:1. The apo-protein is cysteine rich and devoid of aromatic residues with an analytical molecular weight of 6975 obtained by ion-spray mass spectrometry. This compares with a calculated molecular weight of 6968 for the human protein. Difference absorption and circular dichroism measurements suggest that cadmium coordination is similar to that observed in other cadmium-containing MTs. Luminescence properties of the protein are consistent with the presence of a Cu(I) chromophore.

399

NEURITE OUTGROWTH AND SYNAPSE FORMATION IN INJURED SPINAL CORD OF NEONATAL OPOSSUM *IN VITRO*

Varga, Z., Blackshaw, S., Muller, K.J. and Nicholls, J.G.; Dept. of Pharmacology, Biocenter, University of Basel, CH-4056 Basel

The central nervous system (CNS) of newborn opossums has been used to study mechanisms of repair. The entire CNS is dissected and maintained in culture. Physiological experiments have shown that through-conduction becomes re-established 3-5 days after a lesion to the spinal cord. The principal aim of our experiments was to analyze by fluorescence and electron microscopy how neurites traverse the lesion and extend beyond it to form synaptic connections with targets. Neurons in dorsal root ganglia were stained with the carbocyanine dye (DiI) and their growth followed by video microscopy in living preparations. After photoconversion of DiI it became possible to observe the DRG neurites in electron micrographs and to search directly for synaptic connections. A further problem concerns the stage at which fibers lose the ability to grow through lesions. It is known that adult opossum CNS cannot regenerate after a lesion. Experiments in progress show that numerous processes traverse lesions made to the spinal cord of 3-6 day opossums; by contrast less repair was found in 11-14 day old opossums although they still survived well *in vitro*. It seems of interest that neurites grow less readily across lesions at the very time that oligodendrocytes start to appear.

Supported by grants from the Swiss Nationalfonds (31-30047.90) and from the International Foundation for Paraplegia.

400

OPPOSITE EFFECTS OF AXONE DAMAGE ON HEAT SHOCK PROTEINS (hsp 70) AND UBIQUITIN CONTENTS IN MOTOR NEURONS OF NEUROPATHIC RATS

[1] Lantéri-Minet M., [2] Desmeules J.A. and [3] Menetrey D.

[1]Laboratoire de Neuro-oncologie et Neurosciences Cliniques, Service de Neurologie du Centre Hospitalo-Universitaire de Nice, 06002 Nice, France. [2] Division de Pharmacologie clinique, Hôpital Cantonal Universitaire, 1211 Genève 14 Suisse. [3] I.N.S.E.R.M. U 161 Laboratoire de Neurophysiopharmacologie du Système Nerveux, 2 rue d'Alésia, 75014 Paris, France.

Alteration in the motoneurone contents of heat shock protein (hsp 70) and ubiquitin were studied in rats which had been subject to loose ligation of one common sciatic nerve. This results in a peripheral neuropathy which peaks at 14 days following ligation and is characterized by transient degeneration of both myelineted and unmyelineted nerve fibers, abnormal motor behaviors, allodynia and hyperalgesia of the hindpaw. Hsp 70 and ubiquitin are involved in protein metabolism and their expression is regulated during cellular stress. The unlesioned side was used as control. Motoneurone stainings for hsp 70 and ubiquitin were differentially altered at the peak of the neuropathy. Axon damage resulted in a decrease in hsp 70 labeling while ubiquitin staining increased. At the same time motoneurones undergoing axon damage overestained for immediate early gene encoded protein c-JUN and for nerve groth factor (rNGF). In contrast, no clear alteration was seen in the intensity of labeling calcitonin gene related peptide (CGRP). This study demonsrates that peripheral neuropathy resulting from loose ligation of the sciatic nerve not only produces sensory alterations as previously but also leads to pronounced alterations in motoneurone functionning that could partly explain the abnormal motor behaviours.

401

NEURAL INDUCTION OF AChR ε-mRNA AT ECTOPIC ENDPLATES

Rotzler, S., Herczeg, A. and Brenner, H.R., Dept. of Physiology, University of Basel, CH-4051 Basel.

During neuromuscular synapse formation, muscle fibres express the 'adult' subtype of acetylcholine receptors (AChR) as a consequence of the neural induction of AChR ε-subunit at the synapse. To examine whether the induction of ε-subunit and of adult AChR induction was dependent on the stage of motor neuron and/or muscle development, their expression was examined at ectopic endplates induced in extrasynaptic segments of *adult* rat soleus muscle by surgical redirection of the fibular nerve. In-situ hybridization and electrophysiological identification of AChR subtypes showed local induction of ε-mRNA and of adult AChRs at ectopic endplate sites, respectively. Similar observations were made when the foreign nerve was cut at the earliest stages of synapse formation and the muscles were kept active by exogenous stimulation. However, stimulation after denervation caused a redistribution of myonuclei expressing ε-mRNA. As the neural signal maintaining ε-mRNA expression after denervation appears to be associated with the basal lamina (BL) of the muscle fibre, we have now cultured myotubes on BL from muscle and found accumulations of AChRs where myotubes contacted synaptic BL. Experiments are in progress to determine the capacity of isolated synaptic BL to induce ε-mRNA in cultured myotubes.

402

DIFFERENTIAL EXPRESSION OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR GENES IN THE AUTONOMIC NERVOUS SYSTEM OF THE RAT

Rust G, Burgunder JM*, Lauterburg TE* & Cachelin AB. Dept of Pharmacology, 3010 Bern and *Dept of Neurology, Inselspital, 3010 Bern

Nicotinic acetylcholine receptors (nAChRs) play a key role for synaptic transmission in the ganglia of the vertebrate autonomic nervous system. Still little is known about subunit composition, function and regulation of these neuronal nAChRs. We have used the *in situ*-hybridization technique to determine the expression pattern of neuronal nAChR subunit genes in cryostat sections of rat autonomic ganglia and adrenal medulla. Radiolabelled cRNA probes were prepared *in vitro* from cDNA clones encoding α2, α3, α4-1, β2 and β4 rat neuronal nAChR subunits. Preliminary results suggest that the main nAChR subtype found in both sympathetic and parasympathetic ganglia as well as in the adrenal medulla may consist of α3 and β4 subunits. Whereas α2 mRNA expression was somewhat weaker than α3 and β4, the expression of α4-1 and β2 mRNA was very low. These results reveal an interesting contrast to the brain in which α4 and β2 seem to be the most common nAChR subunits. We are currently investigating the expression pattern of several other nAChR subunit mRNAs in the rat autonomic nervous system.

Supported by Swiss NF grant 31.31018.91 to ABC.

403

PPAR expression in the rat brain.

Braissant, O., Lemberger, T., W. Wahli, Institut de Biologie Animale, CH-1015 Lausanne

The peroxisome proliferators activated receptors (PPARs) belong to the nuclear hormone receptor superfamilly. Recently, our laboratory and others have shown that one possible group of putative physiological inducers of PPARs might be several polyunsaturated fatty acids (PFAs), among which figures also arachidonic acid.
The central nervous system is exceptionally rich in PFAs, and arachidonic acid was already proposed to be involved both in inter- and intracellular signalling mechanisms.Therefore, we investigated the expression of PPAR in different regions of the rat brain at several developmental stages by using in situ hybridization and Northern blotting experiments. As probe served a PCR-cloned DNA comprising a part of the ligand-binding or E domain of the rat PPAR-alpha.
The multiplicity of mRNA molecules hybridizing with our PPAR probe raises the question whether they correspond to different splicing variants or to various isoforms of the receptor.

404

NUCLEAR RECEPTORS OF THYROID HORMONES IN DORSAL ROOT GANGLIA AND SCIATIC NERVE DURING DEVELOPMENT AND REGENERATION.

Barakat-Walter, I., and Duc, C., Institut d'histologie et d'embryologie, Faculté de médecine, rue du Bugnon 9, CH-1005 Lausanne

Since the action of thyroid hormones on responsive cells is mediated through nuclear T_3 receptors (NT_3R) we detected the expression of NT_3R in the cell population of dorsal root ganglia (DRG) and sciatic nerve in rat. The expression of NT_3R was visualized by immunocytochemistry with the specific 2B3 monoclonal antibody. In the DRG of rat embryo, NT_3R immunoreactivity was first discretely revealed in a few neurons at E14, then strongly expressed by all the neurons at E17 and in the newborn rat. In adult rat, the DRG neurons continued to possess a clear NT_3R-immunostaining which slightly faded with age. In the developing sciatic nerve, Schwann cells exhibited a transient NT_3R immunoreactivity restricted to a short period ranging from E17 up to 10 postnatal days. Afterwards, Schwann cells became rapidly free of any detectable NT_3R immunostaining. However, transection of adult sciatic nerve caused a rapid reexpression of NT_3R by Schwann cells during axonal degeneration forty-five days after transection, NT_3R expression disappeared again in regenerated nerve. These results suggest that thyroid hormones could play a role in sciatic nerve development and regeneration. (SNF N° 31-33671.92).

405

EXPRESSION OF CYTOCHROME P450 AROMATASE (CYP19) IN THE DEVELOPING RAT BRAIN

Markus E. Lauber and Walter Lichtensteiger, Pharmakologisches Institut der Universität Zürich, Gloriastr. 32, CH-8006 Zürich

A central step in the sexual brain differentiation is the intraneuronal conversion of testosterone to estrogen. This conversion is catalyzed by an enzyme complex comprised of cytochrome P450 aromatase (CYP19) and the NADPH-dependent cytochrome P450 reductase. By means of activity assays and immmunocytochemistry, the former enzyme has been localized in specific brain regions, including the preoptic area, the amygdala, the anterior and posterior hypothalamic areas and others. We have designed oligonucleotides specific for rat cytochrome P450 aromatase and we are in the process of examining the expression of this gene in the developing rat brain by means of *in situ* hybridization and Northern blot analysis. The two methods have already been validated in the ovary of pregnant rats, where high levels of aromatase-specific mRNA have been detected in the corpus luteum. We will present data on the relative expression and regional distribution of mRNA encoding cytochrome P450 aromatase in the rat brain during normal, prenatal and postnatal development.

406

EARLY ONTOGENY OF MELANOTROPIN RECEPTORS IN RAT BRAIN: LOCATION IN FETAL BASAL GANGLIA

W. Lichtensteiger, B. Hanimann, M. Schlumpf, W. Siegrist* and A. Eberle*, Institute of Pharmacology, University of Zürich, Zürich, and Research Department (ZLF)*, University Hospital, Basel.

α-Melanocyte stimulating hormone (α-MSH) and related peptides are supposed to influence brain development. In view of a more detailed analysis of such actions, we studied the ontogeny of [^{125}I]Nle^4,D-Phe^7-α-MSH binding sites in fetuses and offspring of time-pregnant Long Evans rats by quantitative in vitro autoradiography. Binding sites are detected in lower brainstem, midbrain and thalamus by gestational day (GD) 15/GD 16. In hypothalamus, paraventricular nucleus and preoptic area are labelled by GD 18, while other typical locations (arcuate and ventromedial nucleus) acquire significant numbers of receptors only after birth. A striking feature is the high density of receptors in developing striatum, which increases rapidly from GD 18 and peaks between late fetal and first postnatal days. Cerebral cortex (virtually devoid of binding sites in adulthood) exhibits periods of transient receptor expression, e.g., in piriform cortex by GD 20 and 22, and in parietal cortex at the end of the first postnatal week. The presence of relatively high regional receptor densities at early stages and their transient occurrence suggest a role in developmental processes.

407

Regional development of μ- and k-opioid receptors in prenatally *benzodiazepine-exposed rats*

S. Inderbitzin, M. Schlumpf, W. Lichtensteiger, Institute of Pharmacology, University of Zürich, CH-8006 Zürich

The possible influence of prenatal administration of diazepam (2,5 mg/kg/d, s.c., gestation day 14-20) on μ-opioid receptor development was studied in striatum of female and male rat brains at two different postnatal ages (PN 14, PN 28), using the selective μ-opioid receptor agonist (^{3}H)-DAGO, whereas the influence of prenatal diazepam on k-opioid receptor development was investigated in striatum, midbrain and nucleus accumbens/olfactory tubercle at PN 14, PN 28 and 8 weeks in both, male and females, using the selective k-opioid agonist (^{3}H)-U69,593.
Preliminary data from Scatchard analysis indicate a probable decrease in the total number of k-opioid binding sites in the nucleus accumbens/olfactory tubercle region of prenatally diazepam-treated PN 14 male rats.

408

COMPARATIVE ANALYSIS OF DOPAMINE D_1 RECEPTOR ONTOGENY BY IN VITRO AUTORADIOGRAPHY AND *IN SITU* HYBRIDIZATION

Maier A., Schlumpf M., Beer H.F., Schubiger P.A. and Lichtensteiger W., Inst. of Pharmacology, Univ. of Zurich, Zurich and Paul Scherrer Inst., Radiopharmacy Div., Villigen PSI

The ontogeny of expression of Dopamine D_1 receptor mRNA in the rat brain and binding to the receptor were assessed at several time points from gestational day (GD) 14 to postnatal day (PN) 60 by receptor autoradiography and *in situ* hybridization. Long Evans rat pups and fetuses of different ages were frozen and sectioned coronally and sagittally at 10 μm. D_1 receptor binding, determined with the selective antagonist ^{125}I-SCH 23982 was first noted on GD 18 in the developing striatum, basal ganglia and the olfactory tubercle, reaching adult levels at around PN 14. The expression of D_1 receptor mRNA was studied by using a mixture of 3 specific ^{35}S-labelled oligonucleotides. On GD 14 messages were noted in the developing striatum, olfactory tubercle and retina. This study demonstrates that specific binding to D_1 receptors is present in midfetal brain.

409

The expression of calretinin by Cajal-Retzius cells during rat development.

D.M. Vogt, M.R. Celio, Institut für Histologie und allgemeine Embryologie, Universität Perolles, CH-1700 Fribourg

The Cajal-Retzius neurons have been considered as unusual neurons on account of their morphology and their fate during corticogenesis. They are situated in the marginal layer of the cortex and are the first cortical neurons to be generated. The function of Cajal-Retzius cells and their destiny in adulthood is still controversial. Calretinin (CR), an EF-hand Ca^{2+}-binding protein, is a neuronal marker in adult mammals. During the development of rat brain we observed strong CR-like immunoreactivity in the cells of the marginal layer. These CR positive cells appear as early as E12. They seem to be more numerous in younger than in older specimens. They exhibit typical morphological features of the Cajal-Retzius cells like horizontal orientation with usually one nearly rectilinear dendrite, a spindle-shaped cell body, round nucleus and, ultrastructurally, many roundish mitochondria. Furthermore strongly labelled CR positive cells were found in the lower part of the cortical plate. Again the cells were strikingly rich in mitochondria. In conclusion CR is expressed in early corticogenesis and seems to be a marker for Cajal-Retzius cells. It can therefore serve as a tool to resolve the long-standing problem of the fate of this conspicuous cell type during development.

410

ONTOGENESIS OF CALBINDIN D-28K IN THE DORSAL ROOT GANGLIA AND SPINAL CORD OF NORMAL AND TRISOMY 13 FETAL MICE

Ninomiya,T., Barakat Walter,I., Vuillemin,M., Pexieder,T. and Droz,B., Institut d'Histologie et d'Embryologie, Rue du Bugnon 9, 1005 Lausanne.

Trisomy 13 (Ts13) of the mouse i.e. triplication of one of its chromosomes, can be generated in the progeny of mice. The ontogenesis of calbindin D-28K (CaBP) was studied in dorsal root ganglia (DRG) and spinal cord of normal and Ts13 fetal mice. Cryostat sections of lumbosacral region were prepared from normal and Ts13 fetal mice (E12, E14, E16 and E18) and they were immunostained with polyclonal antiserum against CaBP. In DRG, the CaBP-immunoreactivity was detected at E12 in only about 3% of neurons in both normal and Ts13 mice. In later stages the percentage of CaBP-positive neurons dramatically increased in normal DRG to reach 22% at E18. In contrast, the percentage of CaBP-positive neurons in Ts13 DRG displayed a slight increase and it reached only 10% at E18. On the other hand, CaBP-positive neurons in normal as well as Ts13 spinal cord first appeared in ventral horn at E12, in intermediate gray matter at E14 and in dorsal horn at E16. CaBP-positive fibers were distributed in anterior funiculus from E14 onward. In spinal cord, the expression of CaBP-immunoreactivity did not show any difference between normal and Ts13 mice. In conclusion, CaBP-immunoreactivity was significantly reduced in lumbosacral DRG of Ts13 as compared with normal mice. No difference was seen in the spinal cord. This result suggests that an imbalance involving chromosome 13 in mouse embryo produces a reduction of CaBP synthesis during neurogenesis of DRG.

411

CORRELATION OF CALBINDIN D-28K AND CALRETININ WITH RAPIDLY ADAPTING MECHANORECEPTORS IN CHICKENS

Duc, C. and Barakat-Walter, I., Institut d'Histologie et d'Embryologie, Faculté de Médecine, CH-1005 Lausanne

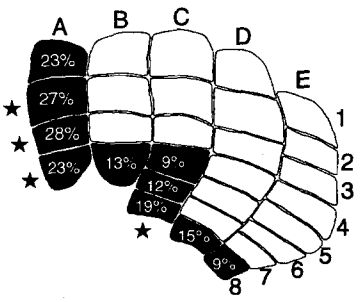
Calbindin D-28k (CaBP) and Calretinin (Calr) are two calcium-binding proteins which share a high homology. In the chicken dorsal root ganglia, CaBP is expressed by subpopulations of large A- and small B-neurons while Calr-immunoreactivity is restricted only to a subset of large A-neurons. The peripheral projections of these neurons to the tissues of hindlimbs were studied by detecting the CaBP-immunoreactive nerve fibers with a rabbit antiserum to CaBP (a gift from M. Celio, Fribourg) and Calr-immunoreactive axons with a rabbit antiserum to Calr (SWant, code no. 7686). In the skin, the CaBP- and Calr-positive thick myelinated axons were innervating specifically Herbst corpuscules while the CaBP-immunoreactive thin axons innervated Merkel corpuscules and the collar region of hair follicles. In muscles, both CaBP- and Calr-positive thick myelinated nerve fibers were forming selectively the equatorial innervation of muscle spindles. However, neither CaBP- nor Calr-positive axons could be observed in tendon organs. Most of the peripheral targets innervated by CaBP- or Calr-immunoreactive neurons are indeed known as rapidly adapting mechanoreceptors. These results suggest that these two calcium-binding proteins could be involved in rapid adaptation. (SNF 31-33671-92)

412

DIFFERENTIAL EFFECT OF NEONATAL EYE REMOVAL ON THE AREAL EXTENT OF THE BARRELFIELD IN MICE

G. Bronchti, N. Schönenberger, E. Welker, and H. Van der Loos, Institute of Anatomy, University of Lausanne, 1005 Lausanne, Switzerland.

We investigated the effect of neonatal eye removal on the tangential extent of the barrelfield in mice. Areas were measured in drawings made from tangentially cut Nissl-stained sections of somatosensory cortex. We compared areas of 29 barrels - shown in figure - corresponding to 29 mystacial vibrissae, between 60 days old mice enucleated at birth (n=13) and their intact littermates (n=13). Multivariate ANOVA showed that the barrelfield was larger in enucleated mice (by 6%; $p<0.0028$). This expansion was mainly due to the areal increase of barrels corresponding to the dorsalmost row of vibrissae (row A), and of a set of barrels corresponding to rostral vibrissae near the nose and mouth, whereas other barrels did not express any areal change in enucleated mice when compared to controls. The enlarged barrels are rendered black in the figure; number within barrels indicate the percentage of areal expansion in enucleated versus control mice; stars are placed beside barrels that showed significant areal increase ($p<0.0017$); other labels indicate nomenclature for barrels in rows (letters) and arc (numbers).



We now investigate functional aspects of this phenomena using electrophysiological as well as 2-deoxyglucose techniques. Support: Swiss NSF - 31-30932, and the Geigy Jubiläums Stiftung.

413

CHRONOTOPIC FIBER ORGANIZATION IN THE CHIASM OF THE CHICK EMBRYO

U. Drenhaus and G. Rager, Institut für Anatomie und Spezielle Embryologie der Universität, CH 1700 - Freiburg.

The growing retinal ganglion cell axon reveals three morphologically different parts: the growth cone, a distal and a proximal segment. Electron microscopic studies of the topographic distribution of these segments within the chiasm of embryos throughout the period of development allows conclusions with respect to the integration of optic axons as a function of their arrival time. In 4 to 19 days old embryos we found, firstly, that growth cones are predominantly located within the ventral sub-pial zone of the chiasm. Secondly, the maturity of axonal profiles shows a gradient along a ventro-dorsal axis with the oldest axons being located dorsally. With increasing age the gradient of maturity becomes steeper than during the early phase of development. We conclude that newly arriving axons are in general added at the ventral periphery of the developing chiasm which eventually results in a chronotopic organization of fibers.

The study was supported by the Swiss NF grant 31-30014.90

414

Cortical Changes Of Nerve Growth Factor Content After Middle Cerebral Artery Occlusion In Rat Brain

H. Paesold, M.T. Miss, G. Gerling, R. Wybrecht and H.P. Lorez
Pharma Division, Preclin. Research, F. Hoffmann-La Roche, Basel, Switzerland

The expression and role of nerve growth factor (NGF) in degenerative brain disease are poorly understood. Many in vitro and a few in vivo studies showed enhanced NGF synthesis by reactive astrocytes. Thus, reactive astrogliosis induced by neuronal necrosis might be expected to enhance NGF content of affected brain tissues. We measured NGF in rat cerebral neocortex from 2 days to 12 weeks after permanent unilateral middle cerebral artery occlusion (MCAO). NGF was measured by an ELISA using anti-NGF antibody clone 27/21 (Boehringer). MCAO performed in spontaneously hypertensive rats induced about 65% infarction of the ipsilateral cerebral neocortex. Reactive astrogliosis occurred around but not within the infarction most extensively from day 2 to day 14 after MCAO as shown by GFAP immunohistochemistry. Reactive microgliosis of the border zones was marked 2 days after MCAO as shown by OX-42 immunohistochemistry while the infarction was densely infiltrated with immunoreactive macrophages at day 5. The NGF content of infarcted cortex was unchanged 2 days after MCAO but reduced to $\leq$ 50% from 5 days to 12 weeks due to neuronal necrosis. In adjacent cortex comprising the gliotic border zone the NGF content was unchanged except for a modest increase by 30% at 2 weeks. Thus, in contrast to the working hypothesis, reactive gliosis did not lead to a marked and long-term increase in NGF content of the infarction border zone. The resulting pronounced net loss of NGF in the infarcted hemisphere could contribute to secondary post-infarction events, e.g. degeneration of cholinergic basal forebrain neurons.

415

Change Of Nerve Growth Factor Content In Rat Brain After Transient Global Ischemia

H. Paesold, M.T. Miss, R. Kettler, P. Schoch and H.P. Lorez
Pharma Division, Preclin. Research, F. Hoffmann-La Roche, Basel, Switzerland

Neuronal degeneration induces reactive gliosis. Based mainly on in vitro studies it is assumed that reactive astrocytes produce nerve growth factor (NGF). We used a rat model of transient forebrain ischemia to study temporal changes in the expression of brain NGF content induced by reactive gliosis. Adult rats underwent permanent bilateral common carotid artery ligation. Twenty-four hours later the animals were exposed to 7% O_2/93% N_2 for 17 minutes. This induced focal neuronal necrosis most markedly in cerebral neocortex and neostriatum and, to a lesser extent, in hippocampus. Two days to 12 weeks after hypoxia brain NGF was determined by a two-site ELISA using the anti-NGF antibody clone 27/21 (Boehringer). Northern blots were hybridized with a digoxigenin-labeled antisense NGF cRNA probe (Scott et al, Nature 302, 538, 1983) 2 weeks after hypoxia. Whereas the NGF content remained almost unchanged in hippocampus, a modest increase (by ≤ 50 pg /100 mg w.w.) occurred in cerebral cortex and neostriatum at 2 – 4 and 12 weeks, respectively. A very marked gliosis was present in cortex and striatum as shown by the increase in ^{3}H-PK 11195 binding to the mitochondrial benzodiazepine receptor, in monoamine oxidase B activity and in the number of OX-42– and GFAP–immunoreactive glial cells. The increase in NGF content at day 14 was not reflected by enhanced NGF gene transcription in either neostriatum or cortex. Considering the focal loss of neurons we conclude that reactive gliosis following forebrain ischemia leads to a moderately transient expression of NGF.

416

HIPPOCAMPAL SUPEROXIDE DISMUTASE (SOD) ACTIVITY DURING TRANSIENT CEREBRAL ISCHEMIA IN GERBILS.

Vallet, P .G., Huguenin, C., Charnay, Y. Institutions universitaires de Psychiatrie, Service Recherche, CH-1225 Geneva, Switzerland

It has been generally recognized that ischemia and subsequent reoxygenation produce reactive oxygen radical species, superoxide radicals in particular, in various tissues including brain. Although most of these studies have suggested that superoxide radicals are generated during reoxygenation period, some studies suggest that formation of radicals have occured during cerebral ischemia. Mongolian Gerbils were anaesthetized with fluothane 4% and clips were placed on the common carotid arteries for 6 min. Body temperature was maintened at 36-37°C. At the appropriate survival times, the animal were reanaesthetized and brains quikly removed. After isolation, hippocampi were frozen in isopentane. They were taken before ischemia, after ischemia and after 10, 30 and 60 min postischemia. SOD activity was measured in hippocampus by the modified method, originally developped by Kostyuk and Potapovich (1989). Results show that the SOD activity increases during ischemic period to reach a maximum at 30 min. At 60 min the activity is comparable to preischemic values.
These data are consistent with the hypothesis that cerebral ischemia is accompanied by hippocampal cell activation with an associated increase in SOD activity providing transient deleterious effects, which may be implicated in the delayed neuronal death process.

417

Neocortical Neurons Containing Calretinin are Resistant to Excitotoxicity via a Calcium-Dependent Mechanism

Lukas, W. and Jones, KA., Glaxo Inst. Mol. Biol., CH1228 Geneva

Calretinin (CR) is a member of a family of calcium-binding proteins that are localized in discrete populations of neurons in the brain. Its distribution in some areas of the hippocampus that receive dense glutamatergic inputs suggests that CR is aiding calcium homeostasis. To test for the possibility that CR can be neuroprotective, we induced neuronal cell death in 2 week-old cortical cultures from rat by treatment with the calcium ionophore A23187, or with brief applications of glutamate, NMDA or KA, and then stained with and antibody against CR. In control cultures 13% of all neurons were immunopositive while in all treated cultures the percentage of CR^+ neurons was significantly higher. This effect was dose dependent and most striking for cultures treated with the calcium ionophore where, after treatment for 3 hr at 3 µM, the percentage rose to 35%. We quantified the neuronal loss independently for both CR^+ and CR^- populations. For example, at a concentration of A23187 that induced a 45% loss of all neurons, there was a 50% loss of CR^- neurons and only a 15% loss of CR^+ neurons. This effect was not observed when calcium was removed from the medium. Selective sparing of CR^+ neurons continued when A23187 was added with glutamate receptor antagonists, and thus the selective cell killing by A23187 can not be explained by differences in intercellular glutamate receptor densities. These data suggest that CR can significantly attenuate short-term neurodegeneration induced by calcium overload.

418

SOME NEURONS DIE RAPIDLY AFTER LACK OF AFFERENT STIMULATION.

M.Catsicas, Y.Péquignot and P.G.H.Clarke, Institute of Anatomy, University of Lausanne, CH-1005 Lausanne.

We have investigated how neurons in the optic tecta of chick embryos depend for survival on their afferents from the retina. Activity-mediated effects were tested by blocking retinal action potentials (with tetrodotoxin, TTX); and other, "trophic", ones by blocking intraocular axoplasmic transport (with colchicine).

TTX (150 ng) injected into one eye at embryonic day 16 (E16) induced pyknosis (cell death) of neurons in the deep layer (SGC) of the contralateral tectum as early as 5 h after the injection; the number of pyknotic cells reached a peak at 13 h, and by 24 h the level of pyknosis had fallen to control levels. Neurons in the superficial layers (SGFS) of the tectum contralateral to the injection were not affected by the TTX .

Intraocular injection of colchicine (225 ng) at E16 caused pyknosis in both the superficial and the deep layers of the contralateral tectum. In the SGFS, pyknosis occurred as early as 9 h post-injection, reaching a plateau by 13 h and remaining essentially constant from then until the end of the period tested. In the SGC, significant pyknosis occurred 4 h later than in the superficial layers; the number of pyknotic cells did not seem to reach a plateau during the period tested.

This suggests that the survival of developing tectal neurons depends on an ongoing supply of substances released from the retino-tectal axon terminals, the release being activity-dependent in the case of the deep neurons but independent of activity in the case of the superficial ones.

419

UNBIASED CORRECTION OF CELL COUNTS IN HISTOLOGICAL SECTIONS: A NEW DEFENCE OF OLD STEREOLOGY.

Clarke, P.G.H., and Primi, M.-P., Institute of Anatomy, University of Lausanne, CH-1005 Lausanne.

It is standard to multiply raw counts of cells (or nuclei etc.) by a "correction factor" to compensate for sectioned cells being counted more than once. However, fashionable stereologists currently condemn the available correction factors, preferring newer methods such as the "Disector". We here derive a correction factor (C) that, unlike previous ones, is theoretically unbiased, even for cells of variable size and shape:

$$C = \frac{n_c}{n_r} = \frac{\sum_{i=1}^{m} T/(T - a_i)}{\sum_{i=1}^{m} (T + a_i)/(T - a_i)}$$

where n_C is the corrected count, n_r the raw count, T section thickness, m the sample size (10 to 20 cells), and a_i the length of a randomly selected whole cell or nucleus measured perpendicular to the section (by focusing up and down using an oil-immersion objective). This approach requires $T > a_i$ for all cells.

420

DISAPPEARANCE OF PERINEURONAL NETS AROUND PARVALBUMIN-NEURONS OF HIV-INFECTED BRAINS

Marco R. Celio, C. Dumas and Judith Miklóssy*, Institute of Histology, University, CH-1700 Fribourg and *Division of Neuropathology, Institute of Pathol., University, 1005 Lausanne

AIDS-patients often develop impairment of cognitive functions which is ascribed to a failure of nerve cells. Since the HIV-virus does not attack neurons, it is postulated that the normal activity of nerve cells is disturbed by infected glial cells. We have studied the fate of "perineuronal nets" (PN's) in the frontal and parietal cortex of six AIDS-patients, by lectin- and immunohistochemistry. PN's are a specialized extracellular matrix produced by glial cells around cortical parvalbumin-neurons. PN's cement the relationship between nerve and glial cells. In all six AIDS-cases we found a disappearance of PN's around parvalbumin-immunoreactive neurons of the cerebral cortex. We conclude that infection with the HIV-virus decreases the capacity of glial cells to produce the molecules of PN's. The absence of this extracellular matrix around parvalbumin-neurons may engender subtle physiological abnormalities in this population of inhibitory cortical interneurons, which could lead to mental deterioration.

421

PrP-deficient mice are resistant to scrapie

Büeler, H., Aguzzi, A.*, Autenried, P.#, Greiner, R.#, Sailer, A., Aguet, M., and Weissmann, C. Institut für Molekularbiologie I, Institut für Neuropathologie * und Biologisches Zentrallabor #, Universität Zürich.

Prusiner proposed that the transmissible agent (prion) causing spongiform encephalopathies such as scrapie in sheep or Creutzfeld-Jakob disease in man is identical with PrP^{Sc}, a modified form of the normal cellular protein PrP^{C}, and that prion propagation occurs by conversion of PrP^{C} or its precursor into PrP^{Sc}, elicited by PrP^{Sc}. Alternatively, the agent may consist of an as yet unidentified nucleic acid genome coated by host-derived PrP. We generated mice with deleted PrP genes ($PrP^{o/o}$) by homologous recombination in ES cells. Wild type (wt) and $PrP^{o/o}$ mice were infected intracerebrally with mouse-derived prions. While wt mice developed disease at 158 +/- 11 days and died shortly thereafter, all $PrP^{o/o}$ mice are healthy at > 280 days post infection. Moreover, brain extracts from infected wt mice transmitted the disease to wt mice, while brain extracts of infected $PrP^{o/o}$, to date, did not. This shows that PrP^{C} is essential for scrapie pathology and likely also for prion propagation. Introduction of the hamster PrP gene into $PrP^{o/o}$ mice renders them susceptible to hamster-derived but not to mouse-derived prions, demonstrating a specific relationship between incoming prion and resident PrP. These results are compatible with Prusiner's proposal, but do not, by themselves, exclude other possibilities.

422

CHARACTERISATION OF PrP BINDING PROTEINS

Bruno Oesch and Markus Moser
Brain Research Institute, University of Zurich, 8029 Zurich, Switzerland

The molecular mechanisms causing scrapie and other prion diseases are still unknown. Interactions of the PrP isoforms with cellular proteins of normal or infected animals have previously been analyzed using either ligand blots or binding of PrP to native proteins in unfixed sections. A 45 kDa ligand (Pli 45) was shown to be glial fibrillary acidic protein (GFAP) which is produced by astrocytes. We have purified Plis of higher molecular weight using ion exchange and reverse phase chromatography on an HPLC system. A 60 kDa Pli has been purified to homogeneity. Sequence analysis should allow us to clone a corresponding cDNA. Attempts to clone Plis by expression screening of a cDNA library in E. coli will also be reported. Further analysis of cellular proteins interacting with PrP may give us insight into the normal function of PrP as well as the cellular processes underlaying the degenerative changes observed in prion diseases.

423

EXPRESSION OF WILD-TYPE AND MUTANT SYRIAN HAMSTER PRION PROTEINS IN TRANSGENIC *DROSOPHILA MELANOGASTER.*

Raeber, A.J., Kornberg, T.B., and S.B. Prusiner
Departments of Neurology and Biochemistry and Biophysics, University of California, San Francisco, CA 94143, USA

The scrapie agent belongs to a novel class of transmissible pathogens termed prions that cause spongiform encephalopathies in humans and animals. An abnormal isoform of the host encoded prion protein (PrP) was found to be a major and essential component of the infectious scrapie prion.
In order to explore the fruit fly *Drosophila melanogaster* as a new model system to study neurodegenerative diseases caused by prions, transgenic flies were generated by introducing wild-type and mutant Syrian hamster PrP genes into the *Drosophila melanogaster* germ line by P element-mediated transformation. Induction of the transgenes placed under the control of the *Drosophila* heat shock protein 70 gene promoter resulted in the synthesis of full-length hamster PrP. The recombinant protein is shown to be posttranslationally modified similar to authentic hamster PrP, which includes two asparagine-linked oligosaccharides and a glycolipid anchor. We were unable to detect a phenotype associated with the expression of the PrP transgenes.

424

Immunoprobes in the antenna of *Drosophila*

K.F. Störtkuhl, N. Gendre, V. Keller and R.F. Stocker
Institute of Zoology, University of Fribourg, 1700 Fribourg, Switzerland

We have isolated and tested several monoclonal antibodies (mab) in order to identify antennal specific proteins.

Mab ca51/2 (kindly provided by A. Hofbauer, Institute of Genetics, Würzburg, Germany) recognizes sensory neurons in the third antennal segment (funiculus), which can be correlated with basiconic sensilla (BS). No staining in the funiculus is observed in the mutant *lozenge* lacking BS. In Western blots of homogenized antennae, the mab recognizes a protein with a molecular weight of 43,3kdal. In a similar preparation made from *lozenge* antennae, this protein band is missing. Therefore it seems that the 43,3kdal protein is associated with BS.

Mab na21/2 (provided by A. Hofbauer) stains sheath cells correlated with coeloconic sensilla (CS) and BS. The latter show an additional labeling in their shaft. However, in *lozenge*, mab lacks labeling of BS associated sheath cells. On Western blots of a homogenate of wild type antennae the mab recognizes two protein bands of 46,7kdal and 42,2kdal. The larger band is missing in preparations from *lozenge* antennae. The mab is likely to cross-react with two colocalized antigens, the larger protein being correlated with BS.

Mab I24B5 and VG2 generated in our laboratory stain structures in the third antennal segment. VG2 recognizes structures correlated with BS and CS, whereas the antibody I24B5 stains specifically CS.

425

A FAMILY OF PUTATIVE AVIAN GLUTAMATE RECEPTORS

F. Del Principe, A. Moser, S. Oertle, P. Streit and Ottiger H.P.
Institut für Hirnforschung, Universität Zürich, CH-8029 Zürich

Glutamate receptors form part of a major excitatory neurotransmitter system. We have cloned several potential non-NMDA glutamate receptor subunits (GluR's) in pigeon brain. The primary structures of three glutamate receptor subunits have been analyzed. Based on amino acid sequence similarities the obtained clones revealed that they encode for AMPA-sensitive glutamate receptor homologues to rodent GluR-B, GluR-C, and GluR-D. Furthermore, within the brain regions analyzed by *in-situ* hybridization histochemistry (i.e. telencephalon, cerebellar cortex, optic tectum and subtectal nuclei) all three subunits showed distinct and regionally specific expression patterns. Most of the differences were seen in cell types of the cerebellar cortex. Functional analysis of these proteins in transfection assays is currently being performed. Polyclonal antibodies to a C-terminal peptide of GluR-B/C confirm the presence of an immunoreactive band around 100 kDa in Western blots of pigeon brain extracts. In the cerebellar cortex of pigeon and rat these antibodies produced similar immunocytochemical staining patterns. These results indicate a high degree of evolutionary conservation for GluR subunits.

426

IMMUNOSTAINING FOR AMPA-SELECTIVE GLUTAMATE RECEPTOR SUBUNITS IN CEREBELLAR SLICE CULTURES

Bi, X.-N., Gähwiler, B.H., Wenthold, R.* and Streit, P., Institut für Hirnforschung, Universität Zürich, CH-8029 Zürich; *NIDCD, NIH, Bethesda, MD 20892

An immunocytochemical study has been carried out at the level of light microscopy to determine the localization of AMPA-selective glutamate receptor (GluR) subunits in cerebellar slice cultures. Cultures prepared with standard techniques from newborn rats were fixed after 2-3 weeks 'in vitro' and processed for immunocytochemistry using antibodies to the C-termini of GluR1, GluR2/3 and GluR4 (Wenthold et al., J. Biol. Chem. 1992, 267: 501-507) in combination with an avidin-biotin-peroxidase procedure. Antibodies to GluR1 stained astrocytes in the cultured slice and in the outgrowth zone as well as some Purkinje cells. Cell bodies and dendritic trees of Purkinje cells were strongly labeled by antibodies to GluR2/3, while the staining of granule cells was weaker. Immunoreactive Purkinje cell axons could occasionally be followed to their terminals around unstained neurons in deep cerebellar nuclei. Antibodies to GluR4 labeled certain small multipolar cells in the vicinity of Purkinje cells. Whether these cells represent Golgi epithelial cells or interneurons has to be determined. The differential labeling patterns obtained with these antibodies to GluR subunits show major similarities but also interesting differences to those described in cerebella 'in situ'.

427

INTERACTION BETWEEN IONOTROPIC AND METABOTROPIC GLUTAMATE RESPONSES IN THE HIPPOCAMPUS

Anita Lüthi, Beat H. Gähwiler and Urs Gerber, Brain Research Institute, University of Zürich, CH-8029 Zürich

Glutamate, the most prevalent excitatory neurotransmitter in the mammalian brain, activates both ionotropic and metabotropic receptors. We have investigated the interaction between these receptor types by studying the effects of NMDA receptor activation and arachidonic acid application on metabotropic responses. The single-electrode voltage-clamp method was used to record metabotropic responses in CA3 pyramidal cells of rat hippocampal slice cultures. Metabotropic responses were evoked by bath application of 1-amino-cyclopentyl-trans-1S,3R-dicarboxylate (ACPD), a selective agonist. ACPD application (10 µM, 30sec) resulted in an inward current associated with a decrease in potassium conductance. Following bath application of NMDA (10 µM, 30sec), the ACPD-induced inward current was potentiated by 40± 20%. In contrast, bath application of arachidonic acid (AA, 40 µM) resulted in a decrease of this current by 40±10%. The effects of AA were mimicked by stimulation of the endogenous phospholipase A_2 (PLA_2) through melittin from bee venom (0.5 µM) or by direct PLA_2 application (1 µg/ml). Mepacrine, a PLA_2 inhibitor, partially inhibited the melittin effects. Thus, AA reduces postsynaptic metabotropic responses, however, does not appear to be involved in the potentiation of the ACPD-mediated responses by NMDA receptor activation.

428

ENHANCEMENT OF AMPA RECEPTOR-MEDIATED SYNAPTIC TRANSMISSION IN HIPPOCAMPUS BY THE PHOSPHATASE INHIBITOR CALYCULIN A

Figurov, A. and Muller, D. Department of Pharmacology, CMU, 1211 Geneva 4.

Several recent reports indicate that mechanisms of synaptic transmission and plasticity in hippocampus involve activation of the enzyme calcium/calmodulin protein kinase II and enhancement of phosphorylation processes. Another way to enhance the activity of this kinase, as shown by phosphorylation assays, is to treat slices with antagonists of phosphatases such as calyculin A. Here we tested the effects of this compound on synaptic responses in hippocampal slices. At the concentration of 1 uM, calyculin A reproducibly increased the size of synaptic responses elicited by stimulation of Schaffer collaterals. This enhancement averaged 29% in 15 experiments. The effect was observed only with AMPA-dependent synaptic responses, but not NMDA-mediated potentials. Other properties of plasticity such as paired-pulse facilitation or long-term potentiation (LTP) were unaffected by calyculin A. LTP could still be induced in the presence of the drug and the enhancement produced by calyculin A was comparable on control and potentiated responses. These results suggest that the efficacy of AMPA-mediated synaptic transmission in hippocampus can be controlled by a phosphorylation mechanism which is probably not involved in LTP expression (Work supported by FNRS 31-29338.90 and 31.30980.91).

429

ALTERATIONS IN HIPPOCAMPAL GLUTAMATE RECEPTOR mRNA EXPRESSION FOLLOWING CHRONIC EPILEPSY *IN VITRO*

Andrea M. Moser, Michael Müller, Beat H. Gähwiler, Scott M. Thompson and Peter Streit, Brain Research Institute, University of Zürich, CH-8029 Zürich

We have used organotypic hippocampal slice cultures as a model system with which to investigate the long-term consequences of epileptic activity. Cultures were prepared with standard techniques from 5 day old rat pups, and allowed to mature for 14-18 days. They were then treated with 500 µM picrotoxin for 2-3 days in serum-free medium. The expression of glutamate receptor (GluR) mRNAs was examined after 48 hrs by *in situ* hybridization using ^{35}S-labelled subunit specific oligonucleotides as probes. The most striking change in mRNA expression was observed for two AMPA-sensitive GluR subunits (GluR-A & GluR-B). Compared to control cultures, mRNA levels for these subunits in picrotoxin-treated cultures decreased to less than half the levels observed in untreated sister cultures, as judged by the labelling intensity on autoradiographic films. Levels of GABA receptor subunit mRNAs were apparently unaffected. Nissl staining of these cultures following *in situ* hybridization showed virtually no nuclear or perikaryal damage to the neurons, indicating that cell death did not account for the decrease in mRNA expression. In contrast, with 72 hrs exposure to convulsants, large numbers of degenerating cells in all hippocampal regions were apparent in Nissl stained material. Such experiments will allow us to examine the mechanisms underlying excitotoxic injury and the control of receptor expression under normal and pathological conditions.

430

BICUCULLINE-INDUCED EPILEPTOGENESIS IN THE MOUSE CINGULATE CORTEX MAINTAINED IN VITRO. M. Kiraly and P.J. Magistretti. Institut de Physiologie, Université de Lausanne.

Intracellular recordings were obtained from biocytin-labelled neurons in cortical layers II-VI in a submerged slice (300 µM thick) preparation. Electrical stimulations of the white matter via bipolar electrodes elicited four types of synaptic potentials in all cells tested (n = 56) : the non-NMDA and the NMDA receptor-mediated early and late EPSPs, blocked by CNQX and AP5 respectively, and the $GABA_A$ and $GABA_B$ receptor-mediated early and late IPSPs, suppressed by bicuculline (BIC) and 2-hydroxysaclofen, respectively.

Following prolonged bath application (10 min.) of BIC 20 µM, cingulate cortex neurons fired in response to electrical stimuli, paroxysmal discharges of 1-2 sec. duration, and spontaneously emitted epileptiform bursts at a rate of about 1 per min. Both the stimulus evoked and the spontaneous events presented an early and a late component, consisting of an initial depolarizing shift followed by a long duration depolarization with superimposed action potentials. The late component was suppressed at hyperpolarized membrane potentials and was abolished by AP5 50 µM, whereas CNQX 20 µM suppressed both the early and the late components, as well as the spontaneaous activity.

These results suggest that in the absence of GABAergic inhibition synaptic potentials play a dominant role in epileptogenesis in that NMDA receptors would amplify the paroxysmal depolarization initiated by the activation of non-NMDA receptors.

431

DOPAMINE-GLUTAMATE INTERACTIONS IN RAT ENTHORINAL CORTICAL NEURONES IN VITRO

Pralong, E. and Jones, R.S.G., University Department of Pharmacology, Oxford OX1 3QT, UK

Recent hypotheses propose an imbalance between dopamine (DA) and glutamate transmission in schizophrenia. We have investigated DA-glutamate interactions using intracellular recording of rat enthorinal cortical (EC) cells *in vitro*. DA (0.1-1 mM) hyperpolarized most layer II cells (22/38) but depolarized most layer V cells (15/21). The hyperpolarization was mediated by D2 receptors whereas depolarizing responses were neither D1 nor D2 mediated. Synaptic responses of layer II cells were complex and exhibited fast and slow excitatory postsynaptic potentials (EPSPs) mediated by AMPA and NMDA receptors, and fast and slow inhibitory postsynaptic potentials (IPSPs) mediated by $GABA_A$ and $GABA_B$ receptors, respectively. DA (50-500µM) reduced reversibly (in a dose-dependant manner) the amplitude of the untreated synaptic potentials, e.g. 100 µM DA reduced the fEPSP by 29+/-4 % (mean +/_ sem, n = 23). Pharmacologically isolated components of the synaptic potentials were also studied. These results indicate that both D1 and D2 receptors modulate the NMDA and the AMPA mediated glutamate transmission although D1 receptors play a more prominent role. In addition, the fIPSP did not appear to be directly modulated by DA.

Supported by the Royal Society and the Swiss National Foundation.

432

INTRACELLULAR RESPONSES OF CERVICAL MOTONEURONS TO INTRACORTICAL STIMULATION OF THE RAT MOTOR CORTEX

Babalian, A., Liang, F. and Rouiller E.M. Institute of Physiology, University of Fribourg, Rue du Musée 5, CH-1700 Fribourg.

In order to reinvestigate the disputed issue of the latency distribution of EPSPs elicited in cervical motoneurons by intracortical stimulation (ICS) of the forelimb area of the rat motor cortex, intracellular recordings were obtained from motoneurons in the cervical cord, identified antidromically by peripheral nerve stimulation and/or by intracellular biocytin staining. With ICS of 0.6-1.5 mA, most motoneurons responded with monophasic EPSPs (65 out of 66), while only one exhibited an IPSP. The amplitude of the EPSPs progressively increased with intensity, whereas their latency did not significantly vary. The mean EPSPs latency was 8.28 ms (SD=1.61 ms), ranging from 4.7 ms to 12.55 ms. Based on the earliest potential recorded from corticospinal (CS) axons in the dorsal funiculus and morphometric measurements of the conduction distance, the conduction velocity in the CS tract was estimated to range from 9 to 20 m/s. These results suggest that cervical motoneurons receive indirect (presumably disynaptic) inputs from fast conducting CS axons or direct corticomotoneuronal contacts via slower conducting CS axons. The biocytin labeled motoneurons exhibited extraordinary long dendritic trees, extending laterally near the edge of the spinal cord and medially up to the midline of the spinal cord, and also one or several recurrent axon collaterals, with numerous boutons *en passant* and *terminaux* contacting most likely neighbouring cervical neurons.

433

REGULATION OF c-*fos* EXPRESSION BY VIP IN PRIMARY CULTURES OF CEREBRAL CORTICAL ASTROCYTES AND NEURONS. J.-L. Martin[1], S. de Rham[1], T. Bardoscia[1], N. Stella[1,2] and P.J. Magistretti[1]. Institut de Physiologie, Université de Lausanne, Lausanne[1], and INSERM U114, Collège de France, Paris[2].

The expression of c-*fos* was examined in primary cultures of astrocytes and neurons by Northern blot analysis. Cultures were prepared from mouse cerebral cortex; neonates (1-2 days) and 16-17 days embryos were used for astrocytes and neurons respectively. In astrocytes, VIP induced c-*fos* expression in a concentration-dependent manner, with an EC_{50} of 1 nM. The stimulation of c-*fos* expression was time-dependent, being detectable at 30 min and maximal between 3 to 4 hours. The levels of c-*fos* mRNA returned to control values within 9 hours. Similar effects were observed when VIP was applied as a 10-minute pulse. In the presence of fetal calf serum (5%), the induction of c-*fos* by VIP was markedly inhibited. The expression of other immediate-early genes such as c-*myc*, c-*jun*, c-*src*, c-Ha-*ras* and c-*mos* was not affected by VIP. The expression of c-*fos* was also examined in primary cultures of cortical neurons. In this cell type also, VIP 1 µM increased the amount of c-*fos* transcripts. In addition, glutamate as well as the glutamate agonists AMPA and kainate stimulated c-*fos* expression, whereas NMDA and *t*-ACPD were inactive.

434

NMDA Receptor Blockade Did Not Result in Neuroprotection in Ischemic Stroke of a Hypertensive Rat Strain

E. Tschudin, G. Gerling, F. Taverne, R. Wybrecht and H.P. Lorez
Pharma Division, Preclin. Research, F. Hoffmann-La Roche, Basel, Switzerland

In male spontaneously hypertensive rats (SHR/Kyo Nlbm; systolic tail blood pressure about 230 mm Hg) permanent middle cerebral artery occlusion (MCAO) consistently induced large cortical and occasionally small neostriatal infarctions. We tested the applicability of this model for the evaluation of neuroprotective drugs. MK-801 (Dizocilpine®), a non-competitive NMDA receptor antagonist, was administered pre- and/or post-occlusion. The cortical infarction size was measured 2 days after MCAO in serial, frontal paraffin sections stained with toluidine blue, using a computerized analysis system. MK-801 did not unequivocally reduce the infarction volume in SHR in contrast to male normotensive Fischer 344 rats, in which we observed a reduction in infarction size of about 50% after treatment with MK-801. Similar differences in neuroprotective efficacies between SHR and normotensive strains were frequently ascribed to a different size of the penumbra. In support, Fischer 344 rats showed an extended border zone in the caudal cortex exhibiting scattered neuronal necrosis, which was much smaller in our SHR. However, besides different vascularization, other mechanisms can not be excluded.

435

A NEW HIGHLY SENSITIVE ASSAY FOR MEASURING NEUROTOXICITY AND NEUROPROTECTION IN CULTURED NEURONS

Mattenberger, L., Gut, A.-M., Flores, N. and Kato, A.C., Division de Neurophysiologie Clinique et Département de Pharmacologie, C.M.U., CH-1211 Genève 4

Cultured neurons from the CNS provide a useful tool for the study of neurotoxic agents and for substances which can prevent neuronal cell death. We have used 14-day old dissociated cultures from the ventral part of the embryonic rat spinal cord (E14). Glutamate (500 µm, 15-30 min) was added to the cultures and 24 hrs later, a morphological degeneration of the neurons was observed. Two biochemical assays were used to quantitate the neurotoxicity : lactate dehydrogenase (LDH) was measured in the supernatant and choline acetyltransferase (ChAT) in the cells. The latter proved to be a more sensitive and rapid measure for neurodegeneration. MK801 (20 µM) and ketamine (100 µM) acted as neuroprotectors as demonstrated by a decrease in LDH and an increase in ChAT activity. Results using methylprednisolone, lazaroids and neurotrophic factors as neuroprotectors will be presented.

436

CHARACTERIZATION OF RAT PINEAL NITRIC OXIDE SYNTHASE (NOS)

N.C. Schaad[1,2], D.C. Klein[1], B.H. White[1] and P.E. Schulz[2], [1]Lab. Dev. Neurobiol., NICHD, NIH, Bethesda, MD 20892, USA [2]Div. of Clin. Psychopharmacol., IUPG Bel-Air, CH-1225 Chêne-Bourg

The rat pineal gland responds to norepinephrine (NE) treatment with a 100-fold increase in cGMP formation. The function of cGMP in the pineal gland, as well as the mechanism of its formation is not known precisely yet. Since nitric oxide (NO) is a potent stimulator of the soluble form of guanylate cyclase and sodium nitroprussiate elevates cGMP levels in pinealocytes, we have characterized NOS in the pineal gland with our modification of the method published by Bredt *et al.* (PNAS, **86**:9030-33). Pineal NOS activity was about 50% of the activity measured in the cerebellum. The activity is strongly dependent on calcium and is inhibited by CaM inhibitors. These results are similar to those reported for brain NOS, and suggest the presence of a constitutive, Ca^{++}/CaM -dependent form of NOS in the rat pineal gland. Developmental studies indicate that NOS is present before the cGMP response to NE treatment is first detected and circadian studies indicate that it does not change significantly on a 24-hour basis in the adult.

437

DELAYED INCREASE OF EXTRACELLULAR ARGININE (ARG), THE NITRIC OXIDE (NO) PRECURSOR, FOLLOWING ELECTRICAL WHITE MATTER STIMULATION IN RAT CEREBELLAR SLICES.

C. Hansel[1], A. Batchelor[2], M. Cuenod[1] J. Garthwaite[2], T. Knöpfel[2] and K.Q. Do[1]. [1]Brain Res. Inst., Univ. of Zürich, Switzerland ; [2]Dept. of Physiology, Univ. of Liverpool, U.K.

Amino acid levels were measured in perfusates from L-shaped rat cerebellar slices installed in a Krebs-filled three-compartment system following electrical white matter stimulation. The lateral compartments housed white matter and a cortical section containing parallel fibres respectively, whereas the central compartment housed cortical structures at the point of bending. This arrangement allows electrical stimulation while the perfusion medium passing the central chamber can be collected for amino acid analysis with HPLC. Following a 2-minutes stimulation period of either 2 Hz (n=6) or 5 Hz (n=5) Arg levels were significantly rised above levels already present in the Krebs-solution (6.62±1.75 pmol/min respectively 7.31±2.66 pmol/ min). Arg is the precursor of NO, a neuronal messenger in the brain, which is synthesized by NO synthase. Since Arg and the NO synthase are located in different cell types it can be suggested that Arg passes through the extracellular space in order to replenish the precursor pool for NO synthesis.

438

IMMUNOCYTOCHEMICAL DETECTION OF PGD SYNTHASE IN CHICK SPINAL CORD.

M.-F. Vesin and B. Droz. Institut d'Histologie & d'Embryologie, Université de Lausanne, CH-1005 Lausanne.

In chick spinal cord an active synthesis of prostaglandin D_2 (PGD_2) was first demonstrated by bioconversion of [^{14}C] arachidonate and further by PGD_2 enzyme immunoassay. In an attempt to determine the cell types which synthesize PGD_2 in spinal cord, antisera raised to two PGD synthase isozymes (the rat brain or spleen enzymes) were used for immunocytochemical staining. Under specified conditions of processing, the antibody directed to the brain enzyme gave raise to a strong specific immunostaining restricted to : 1) neurons of lamina II and III; 2) small elements apparently arranged around motoneuron perikaryons and dendrites. These elements in the electron microscope correspond to small Nissl bodies located just below the plasmalemma. In contrast, no immunoreaction was observed when spleen PGD synthase antibody was used.

These experiments point to neurons as source of PGD_2 via a rat brain-like PGD synthase. SNF N° 3.397.-0.86

439

MODULATION OF PGE2 BY UNSATURATED FATTY ACIDS IN CHICK SPINAL CORD

C. Billotte and M.-F. Vesin, Institut d'Histologie & d'Embryologie, Université de Lausanne, CH-1005 Lausanne

Prostaglandin (PG) E_2 is one of the major prostanoids formed in chick spinal cord homogenates. In the present study variations of PGE_2 synthesis are investigated. Results show that the basal level of PGE_2 is low. In contrast: 1) addition of arachidonate (AA) greatly enhanced PGE_2 formation in a dose and time dependent manner. Others unsaturated fatty acids do not markedly modify basal accumulation of PGE_2, but interestingly lower the AA response. 2) addition of *Crotalus* phospholipase A_2 (to induce endogenous AA release) promotes a smaller but noticable PGE_2 formation, as to compare to exogenous AA. This limited synthesis of PGE_2 may be due to concomittant release of other unsaturated fatty acids.
From these experiments it is concluded that PGE_2 synthesis is modulated by unsaturated fatty acids. Since these fatty acids are released in large amounts during injury, they could contribute to limit PG formation. SNF N° 3.397-0.86.

440

Pharmacological characterization of the NK-1 antagonist, (+)-CP-96,345, in the hemisected spinal cord preparation of the rat and gerbil

M. Lepre, H.-R. Olpe and F. Brugger
Research and Development Dept., CIBA, CH - 4002 BASEL, Switzerland

Three distinct types of neurokynin (NK) receptors have been identified in the peripheral and central nervous system. Receptor binding studies indicated that all three tachykinin receptors are present in the spinal cord. Recently, a new nonpeptidic NK-1 specific receptor antagonist, (+)-CP-96,345, has become available. (+)-CP-96,345 displays high affinity for NK-1 receptors as shown in binding assays and has been demonstrated to antagonize the excitatory effect of SP in the spinal cord in vivo and in vitro. However (+)-CP-96,345 shows different affinities depending on the species tested. As previously reported, (+)-CP-96,345 blocks the SP-induced depolarizations more effectively in the gerbil (pA2: 6.79) than in the rat spinal cord (pA2: 4.69). It was the goal of this study to further characterize (+)-CP-96,345 in the hemisected spinal cord preparation of the rat and gerbil in vitro.
SP-sulphone, a specific NK1 agonist, induced dose-dependent depolarizations of the ventral roots, which were potently blocked by (+)-CP-96,345 in the gerbil spinal cord. In the rat spinal cord (+)-CP-96,345 was much less potent. The amplitudes of the depolarizations were almost unaffected but a reduction of the duration was observed. [β-Ala8]NKA (4-10), a specific NK2 agonist, induced depolarizations similar to SP-sulfone. Interestingely (+)-CP-96,345 partially blocked the effect of the NK2 agonist in both species. It was difficult to study the interaction with the specific NK-3 receptor agonist, senktide, because this peptide has a desensitizing action even at low concentrations.

441

EFFECT OF DIVALENT CATIONS ON THE CURRENT GENERATED BY VASOPRESSIN IN A MOTONEURONE.

Alberi S., Dreifuss J.J. and Raggenbass M., Department of Physiology, CMU, CH-1211 Geneva 4.

Vasopressin increases the excitability of facial motoneurones in newborn rats by generating an inward current which is sodium-dependent, TTX-insensitive and voltage-dependent (Raggenbass et al., *J. Neurosci.* 11, 1609-1616; 1991). When facial neurones were voltage-clamped at their resting membrane potential, reducing the extracellular calcium concentration from 2 to 0.01 mM, while keeping the magnesium concentration at 1 mM, caused a 1.3- to 2.2-fold increase in the amplitude of the vasopressin current, the average relative increase being 1.7 ± 0.1 (mean ± SEM, n=13). This effect was fully reversible. It was specific, since lowering extracellular calcium did not affect the inward current elicited by activation of AMPA receptors also present on facial motoneurones (n=3). Lowering extracellular magnesium also increased the vasopressin response, although only by a factor of 1.3 ± 0.1 (n=6). These data suggest that in the physiological solution (2 mM Ca, 1 mM Mg) the response of facial motoneurones to vasopressin is partially blocked. Increasing the extracellular calcium from 2 to 5 mM did not further reduce the amplitude of this response (n=7). I/V curves suggest that the potentiation of vasopressin current in low calcium may be due to the disappearance of a region of negative slope conductance.

442

ACTION OF VASOPRESSIN ON HYPOGLOSSAL MOTONEURONES : UNUSUAL PROPERTIES OF EVOKED INWARD CURRENT.

Palouzier-Paulignan B., Dubois-Dauphin M. and Raggenbass M., Department of Physiology, CMU, CH-1211 Geneva 4.

The effect of vasopressin on hypoglossal neurones was studied in brainstem slices of newborn rats, using CsCl-filled micropipettes and the single-electrode voltage clamp technique. Vasopressin, applied at 0.1 to 1 μM, generated a non-inactivating inward current in almost all neurones tested. Antidromic invasion following electrical stimulation of the hypoglossal nerve and morphological characterization of biocytin-injected neurones indicate that part of the vasopressin sensitive cells were motoneurones. Hypoglossal neurones formed two classes. *A*) Cells in which the effect of vasopressin persisted in the presence of TTX and/or in a low calcium/high magnesium perfusion solution. Current-voltage relations suggest that the vasopressin current was voltage-dependent and reversed at potentials ranging from -20 to 0 mV. *B*) Neurones in which the action of vasopressin, although *postsynaptic*, could be suppressed or attenuated by TTX and/or by increasing the concentration of extracellular Ca or Mg. Our results suggest that in class *A* neurones vasopressin affects membrane channels located at or near the cell soma, whereas in class *B* neurones the primary effect would be mainly dendritic and transmitted toward the soma by a mechanism which is TTX-sensitive and modulated by divalent cations.

443

MAPPING OF [^{3}H]-VASOPRESSIN RECEPTORS IN THE BRAIN BY DETECTION OF β-PARTICLES DISTRIBUTION WITH A HIGH RESOLUTION RADIO-IMAGER.

Michel Dubois-Dauphin, Jean-Marc Theler, Jean Jacques Dreifuss, Departments of Physiology and Clinical Biochemistry, CMU 1211 Geneva 4.

The distribution of vasopressin binding sites in the brain of the jerboa was studied using a newly developped β-radio imager. Brain sections on microscope slides were incubated for 1 hour with [^{3}H]-vasopressin. The slides were then introduced in the radio imager. Each β-particle emitted from brain sections generated a light spot of 1 mm in diameter, which was red by a CCD camera. The number of events and the coordinate of the center of gravity were recorded. After 1-20 hours, an image representing the distribution of [^{3}H]-vasopressin bound in brain sections was generated. Specific [^{3}H]-vasopressin binding was detected in brain regions such as cortex, insula Calleja, pallidum, the amygdala and the hippocampus. The binding was quantified directly in cpm from the images obtained. The linearity of the method allowed a relevant measurement of non-specific binding and thus its substraction from images representing the total binding. This method provides a high resolution map of histological sections within a few hours, allows direct quantification of emitted β-particles and can detect all isotopes emitting β-particles.

444

REDUCED BINDING OF OXYTOCIN, BUT NOT OF VASOPRESSIN, IN THE BRAIN OF SENESCENT RATS.

Arsenijevic Y., Dreifuss J.J. and Tribollet E., Department of Physiology, Centre Médical Universitaire, CH-1211 Geneva 4.

A reduced arginine vasopressin (AVP) innervation is seen with aging in some parts of the rat brain and this can be restored by administering testosterone to senescent rats (Goudsmit et al., *Brain Res.* 473, 306-313, 1988). Previous studies in which we mapped AVP and oxytocin (OT) binding sites in the rat brain revealed marked changes during development and in relation to gonadal functions (Tribollet et al., *J. Neurosci.* 9, 1764-1773, 1989). In the present work, we labelled AVP and OT receptors throughout the brain of young (3 months old, n=4) and old (20 months old, n=4) male rats using two ^{125}I-labelled ligands, one specific for V_1 AVP receptors, the other for OT receptors. Analysis of autoradiograms by an image analyzer revealed a reduction of OT binding in various areas, but not of AVP binding. OT receptors were markedly decreased in the hypothalamic ventromedial nucleus, the central amygdaloid nucleus, the islands of Calleja, i.e. in regions where binding of OT is reduced by castration in rats of either sex (Tribollet et al., *Brain Res.* 511, 129-140, 1990). Binding was also reduced in the ventral hippocampus. Experiments are in progress to assess whether these changes can be reversed by administration of gonadal steroids.

445

Somatostatin Receptors in the Mammalian Brain

Klemens Kaupmann, Christian Bruns, Klaus Seuwen, Daniel Hoyer, Franz-Werner Kluxen, Graeme I. Bell* and Hermann Lübbert. Preclinical Research, Sandoz Pharma Ltd., CH-4002 Basel; *University of Chicago, Chicago, Illinois 60637, USA.

Somatostatin is an important regulator of endocrine and brain functions. In the central nervous system, somatostatin exerts its functions as a neurotransmitter and as a neuromodulator by binding to specific receptors on the plasma membrane. Based on their pharmacological characteristics several somatostatin receptor subtypes had been postulated. During the past year at least four different SRIF receptors have been cloned by us and by other groups. The deduced amino acid sequence of these receptors display sequence and structural homology to the family of G-protein coupled receptors. The distribution of the receptor mRNAs in mouse and rat tissues was studied by northern blot analysis and *in situ* hybridization. At least three of the genes are expressed in cortex and hippocampus, although the expression levels vary. In substantia nigra moderate expression of SSTR2 RNA and low expression of SSTR1 RNA was found. SSTR1 was also expressed in spinal cord. SSTR2 is the most widely expressed SRIF receptor with relatively high expression levels in cortex, hippocampus, striatum, spinal cord, substantia nigra, lung and spleen. The highest levels of SSTR3 RNA were found in the cerebellum and the olfactory bulb. SRIF receptors were stably expressed in HEK 293 human embryonic kidney cells. These cell lines are now used as tools to analyse receptor ligands and to characterize the receptor second messenger coupling.

446

MULTIPLE PHOSPHORYLATION SITES CONFER FUNCTIONAL MODULATION OF THE $\alpha1\beta2\gamma2S$ $GABA_A$ RECEPTOR CHANNEL BY PROTEINKINASE C

Kellenberger, S., Malherbe, P.* and Sigel, E., Pharmakologisches Institut der Universität, CH-3010 Bern and *Hoffmann-La Roche, CH-4002 Basel

Activation of protein kinase C (PKC) results in down-modulation of the $GABA_A$ receptor. In order to identify phosphorylation sites of PKC, the recombinant subunit combination $\alpha1\beta2\gamma2S$ was expressed in Xenopus oocytes. The resulting receptor channel could be modulated either by oleoylacetylglycerol (2 µM), or stereospecifically by 4ß-phorbol 12-myristate 13-acetate (ß-PMA; 10 nM). 14 Ser or Thr residues of consensus phosphorylation sites for PKC in the large intracellular domains of α1, ß2 and γ2S were altered individually by site-directed mutagenesis. Mutant subunits were co-expressed with wild type subunits to give an $\alpha1\beta2\gamma2S$ combination. The mutations S410A in ß2 and S327A in γ2S led to a 40-50 % reduction of the short-term effect of ß-PMA on the GABA current amplitude. Our results identify two single serine residues as phosphorylation sites of a PKC endogenous to Xenopus oocytes. Upon co-expression, the effect of the two mutant subunits was not additive. This indicates, that phosphorylation of both sites is required for a full, PKC mediated down-regulation of GABA currents.

447

Development of currents in neurons of the inferior colliculus

K. Maury and D. Bertrand
Dpt of Physiology, CMU, 1211 Geneva 4

Ionic currents where recorded from neurons isolated from the inferior colliculus of newborn or young rats. Newborn neurons display large voltage activated outward currents that, by analogy, can be classified as I_A and I_K currents. Recordings from more than 100 cells never showed significant inward currents that could trigger a regenerative electrical activity. In contrast, neurons dissociated from young rats (30 days) display large and fast inward currents of about 2 nA. Thus, we concluded that newborn neurons are immature in their electrical properties and have not yet expressed channels responsible for regenerative activity. Attempts to identify the activity of ligand-gated receptors in newborn neurons have revealed that, although immature, these cells are already expressing ligand-gated channels. This indicates that the development profile of voltage dependent and ligand-gated channels undergo different time courses. In view of our findings it appears that the inferior colliculus, a critical structure for processing of auditory information, pursue its maturation during the weeks following birth.

448

MINIATURE EXCITATORY SYNAPTIC CURRENTS CORRECTED FOR DENDRITIC CABLE PROPERTIES REVEAL QUANTAL SIZE AND VARIANCE. D.Ulrich & H.-R.Lüscher, Dept. of Physiology, University of Bern, 3012 Bern, Switzerland.

Non-NMDA receptor mediated miniature excitatory postsynaptic currents (mepscs) were recorded from motoneurons in organotypic cultures of embryonic rat spinal cord. Amplitude histograms of mepscs were unimodal and skewed towards larger events. The mean of the modes obtained from 10 experiments was -18 pA with a maximal amplitude range of -4 pA to -160 pA for individual mepscs. Current transients to a short voltage pulse were used to estimate the passive cable parameters of the motoneurons. The membrane time constant and electrotonic length were 20 ± 2 ms and 0.96 ± 0.13 (n=15), respectively. The mepscs were subsequently corrected for imperfect space and voltage clamp. The resulting amplitude histograms could be fitted by the sum of two gaussian curves using a maximum likelihood estimator, revealing a mean quantal size of -48 pA with a coefficient of variation of 0.28. The conductance underlying the elementary event was 750 ± 139 pS with probably not more than 54 channnels involved. Our data suggest that quantal size and its variance are masked by the cable properties of neurons, that quantal variance is close to its counterpart at the neuromuscular junction and that simultaneous release of elementary quanta occurs occasionally. (SNF 31-27553.89)

449

IS QUANTAL SIZE IN NEURON-NEURON CONNECTIONS STABLE?

Dityatev, A.E. and Clamann, H.P. Institute of Physiology, University of Bern

Quantal analysis of fluctuations of PSPs in neuromuscular junctions has shown that the coefficient of variation (CV_Q) of quantal size (Q) obtained by analysis of evoked and by spontaneous miniature potentials is about 30%. Estimates of CV_Q from neuron-neuron connection are 0 or 5%. Quantal size of miniature currents show a variation of 30% or more. To resolve this paradox we carried out an analysis of the accuracy of the deconvolution method used for estimation of CV_Q. Histograms of 500 hippocampal EPSPs were simulated with different values of ratio of Q to the standard deviation of noise σ (Q/σ = 1, 1.5, 2, 2.5, 3) and different values of CV_Q (0, 15, 30%). A bootstrap method was used for setting the weights of components used for simulation. Q and CV_Q were estimated as parameters of a unimodal quantal model because it shows a low bias of estimates. Significant underestimates of CV_Q (using maximum likelihood method) were obtained for values of $Q/\sigma < 2.5$. Correct estimates of CV_Q were obtained for CV_Q=15% when Q/σ was ≥ 2.5 and in part for CV_Q=30% when Q/σ was equal to 3. CV_Q could sometimes be estimated correctly by using dependence of the mean estimate of Q on CV_Q because the estimated Q was maximal for the correct value of CV_Q. Values of CV_Q in different preparations can be explained by underestimation of this parameter by the maximum likelihood estimator.

Supported by Grant No. 31-27973.89 from the Swiss National Fund.

450

MOLECULES INVOLVED IN THE PULSATILE RELEASE OF NEUROTRANSMITTERS

Falk-Vairant, J., Cavalli, A. and Dunant, Y., Département de pharmacologie, Centre Médical Universitaire, CH-1211 Genève 4

The quantal, or pulsatile, nature of transmitter release is an essential feature of rapidly transmitting synapses. We recently succeeded in reconstituting the process of ACh release in *Xenopus* oocytes injected with mRNA from cholinergic neurons. Antisens polynucleotides complementary to cDNA of the proteolipid mediatophore 15 kDa subunit were shown to parallely inhibit expression of the mediatophore and of transmitter release in mRNA primed oocytes, whereas other neuronal functions were not affected (with C. Leroy, E. Meunier, N. Morel, M. Israël, CNRS, Gif-sur-Yvette, France).

We are presently investigating whether the release of other neurotransmitter can be express in reconstituted systems as well. Also, a method is being developped to test whether transmitter release in such systems is pulsatile like in the natural synapses.

451

EXCITATION RELEASE COUPLING IN CHOLINERGIC NERVE TERMINALS

Y. DUNANT, A. PARDUCZ[1] and P. CORREGES*.

Département de Pharmacologie, Centre Médical Universitaire, CH - 1211 Genève 4
[1]Biological Research Center, Hungarian Academy of Sciences, H-6701 Szeged.

A brief stimulation was applied to the nerve-electroplaque synapse of *Torpedo* electric organ. During and after this period of activity, synaptic transmission and the metabolism of calcium were monitored : ^{45}Calcium accumulated in stimulated tissue as a function of the number of stimuli. Histochemical localization showed that the number of calcium deposits in synaptic vesicles transiently increased at the end of activity and declined later.
Rapid freezing of the tissue followed by freeze fracture revealed membrane openings (pits) in the presynaptic membrane. While the number of pits did not change during the tetanic stimulation, it increased soon after, reaching a maximum value at 1 min after the tetanus.
It is concluded that synaptic vesicles sequester calcium ions in synaptic terminals during activity and expel them afterwards by exocytosis.
An other approach consists in by-passing the normal calcium channels by using a Ca-ionophore. The miniature potential obtained in this way have a different amplitude and time course than those obtained under normal conditions. From this, information can be obtained upon the relationship between calcium entry and pulsatile transmitter release.

452

SIGNAL TRANSDUCTION MECHANISMS OF THE NEUROTROPHINS BDNF AND NT-3 IN CULTURED BRAIN NEURONS.

*Widmer, H.R., Knüsel, B., *Kaplan, D.R. and Hefti, F., University of Southern California, Los Angeles 90089-0191, CA; and *NCI, Frederick, MD.*

Neurotrophins stimulate *trk*-type protein kinase receptors, however, further intracellular signalling steps in brain neurons are poorly understood. The present study aimed at investigating the role of phospholipase-Cγ1 (PLC) and phosphatidylinositol (PI) hydrolysis in the signal transduction mechanisms for BDNF and NT-3. We found that both factors stimulate PI hydrolysis in primary cell cultures of fetal brain neurons in a dose and time dependent manner. The absence of extracellular calcium resulted in a decrease of the PI response. The effects on PI hydrolysis were preceded by a rapid stimulation of phosphorylation of PLC-γ1 mediated by activated *trk*-type kinase receptors. Stimulation of *trk*-phosphorylation, PLC-γ1 phosphorylation and PI hydrolysis occurred in cultures from all major brain areas. While pure neuronal cultures were responsive no effects were observed in non-neuronal cultures. K-252b which selectively blocks neurotrophin actions by inhibiting *trk*-type receptor proteins prevented the PI hydrolysis and phosphorylation of PLC-γ1 mediated by BDNF and NT-3. Chronic treatment of cultures with BDNF or NT-3 downregulates these responses. The findings provide evidence that *trk*-mediated selective phosphorylation of PLC-γ1 followed by PI hydrolysis is involved in the signal transduction of BDNF and NT-3 in the brain and that brain neurons functionally respond to these factors during early development.

453

LONG-TERM BRAIN CELL AGGREGATE CULTURES

Zurich, M.-G., Monnet-Tschudi, F., Matthieu, J.-M.* and Honegger, P. Institut de Physiologie, Université de Lausanne et *Service de Pédiatrie, CHUV, Lausanne.

Brain cell aggregate cultures offer a unique model to study brain development and plasticity. In order to adjust the culture conditions for long-term experiments (e.g., to study demyelination and remyelination in vitro), aggregate cultures were prepared either from 15-day or from 16-day rat fetuses, and grown for 50 days in chemically defined medium. The following parameters were measured at different time points to monitor maturation and maintenance: protein, DNA and myelin basic protein content; neuron- and glia-specific enzyme activities. It was found that in cultures prepared from 15-day fetuses, the presence of a defined mixture of albumin-bound lipids (Albumax, Gibco-BRL) enhanced and prolonged the maturation of neurons and glial cells, and stabilized the cells in long-term cultures. Cultures prepared from brains of 16-day fetuses expressed the highest levels of all parameters measured, and supplementation with Albumax was more effective for neuronal than for glial parameters. Cell density and developmental stage of the cells at culture initiation appeared to be the most critical parameters for optimal conditions in long-term cultures.

454

SUPRAVITAL STAINING OF NEURONES IN ORGANOTYPIC CULTURE WITH METHYLENE BLUE

L.Stoppini, P.A.Buchs, D.Muller
Dept of Pharmacology C.M.U. 1211 Geneva 4.

Ehrlich (1886) showed that methylene blue possesses an exceptionnaly high affinity for both perikarya and fine varicose nerve fibres. When methylene blue (0.0025%, final concentration) is added in the medium of organotypic cultures kept on porous and transparent Millicell membrane (see Stoppini et al. J. Neurosci. Methods 37 (1991) 173-182), fibers and then soma begin to stain after 15 min of incubation in a 5% CO2 incubator. At that point only a few cells are stained, giving a Golgi-like picture of the tissue. A longer incubation time results in a progressively greater number of cells that take up methylene blue. This rapid and simple method can be used as an interesting test for the viability of the tissue (dead cells do not stain), and allows the visualization of the fibre network and the cellular organization in a still living tissue. If desired, the tissue can then be fixed with ammonium heptamolybdate which precipitates intracellular methylene blue and processed for either light or electron microscopy. The preservation of the fixed tissue is excellent, thereby allowing to analyze at different levels the connectivity established between different groups of cells. This approach can be useful for the study of the morphological characteristics of identified neurones. (Work supported by FNRS 31-29338.90 and 3130980.91)

455

ULTRASTRUCTURAL LOCALIZATION OF CALCIUM IN HIPPOCAMPAL ORGANOTYPIC CULTURES

Buchs Pierre-Alain, Stoppini Luc and Muller Dominique, Departement of Pharmacology, CMU, 1211 Geneva 4.

To study the morphological correlate of synaptic transmission and plasticity in hippocampal organotypic cultures, we have developed a method that allows to identify calcium at the ultrastructural level, and thus to recognize activated from silent synapses, based on the accumulation of calcium triggered by electrical stimulation. For the visualization of calcium, we developed a new technique which consists in precipitating calcium after fixation in a selective and high electron-dense reaction product using the oxidative properties of osmium.

Calcium deposits are clearly recognized as fine particles present in various subcellular compartments such as synaptic vesicles and mitochondria. Accumulation of calcium was also observed in smooth reticulum-like structures localized in presynaptic terminals, dendrites and dendritic spines. Another interesting feature of this technique is the possibility to observe calcium in synaptic clefts and more specifically in active zones. After brief high frequency stimulation trains, changes in the distribution of calcium took place, specifically in postsynaptic spines. A significant increase in the number of calcium-containing spines was observed (about 15% of spines with calcium deposits versus 5% in control conditions), and the calcium deposits were generally accumulated in reticulum-like structures (Work supported by De Reuter Fondation and FNRS 31.29338.90 and 31.30980.91).

456

REGULATION OF SEROTONIN RECEPTOR EXPRESSION IN NEURONAL PRIMARY CULTURES

Karin Schmuck, Montserrat Foguet, Jukka A. Hartikka, and Hermann Lübbert
Preclinical Research, Sandoz Pharma Ltd., CH-4002 Basel

Serotonergic neurons located at the base of the mammalian brain innervate practically every region of the brain and the spinal cord. These neurons exhibit spontaneous electrical discharges in a rhythmical way. The basic activity of the target neurons is regulated by the rhythmical activation of multiple 5-HT receptors. The firing frequency of the serotonergic neurons is modulated by a feedback mechanism involving serotonin autoreceptors regulating intracellular cAMP levels, while the sensitivity of the target neurons is controlled by the differential expression of 5-HT receptors. We have investigated the effects of cAMP on the development and the functional properties of serotonergic neurons in culture, and the influence of serotonergic ligands on the expression of their receptors on the target neurons. To analyze gene expression in primary cultures, a quantitative RT-PCR approach using internal standards was developed. Cultures of rat brain serotonergic neurons were continuously treated with cAMP analogues. Increased cAMP levels had three effects: First, the neuronal morphology was changed towards that typical for mature serotonergic neurons. Second, the expression of tryptophan hydroxylase, the rate-limiting enzyme in serotonin production, was increased in dibutyryl-cAMP treated cultures. Third, the expression of the inhibitory autoreceptor (5-HT_{1A}) was down-regulated. These results suggest the existence of a mechanism by which the neurons react to synaptic input regulating intracellular cAMP levels. Neuronal cultures from rat cortex were treated with specific 5-HT receptor agonists and antagonists and the consequences for receptor expression were analyzed. Ligand binding *in vivo* has been shown to affect serotonin binding. The *in vitro* approach allows the analysis of the underlying molecular mechanisms.

457

PATTERN OF INNERVATION OF THE RAT CEREBRAL CORTEX BY SEROTONERGIC MESENCEPHALIC OR RHOMBENCEPHALIC RAPHE NEURONS IN ORGANOTYPIC SLICES.

SCHMID E. and HORNUNG J.-P., Institute of Anatomy, University of Lausanne, Bugnon 9, 1005 Lausanne.

The serotonergic neurons are grouped in a column of raphe nuclei extending from the mesencephalon to the pyramidal decussation, divided in an anterior group projecting rostrally and a posterior one caudally. The dorsal and median raphe nuclei located in the mesencephalon innervate the cerebral cortex. In organotypic co-cultures of brainstem and forebrain slices, serotonin (5-HT)-containing neurons survive and reinnervate the cerebral cortex. This system allow to test whether serotonergic neurons of the anterior or the posterior group grow in a specific cortical target. To this end, a section of the brainstem of the met-myelencephalic border or of the mesencephalon was co-cultured with a section of the fronto-parietal cortex from 2-day-old rats. By 2 days in culture (DIV2), numerous growth cones appeared at the tip of the 5-HT-immunoreactive axons. The 5-HT-containing axons colonized the cerebral cortex between DIV4 and DIV7, branched through all layers and started to form a plexus from DIV10 to DIV15. A second process of maturation took place between DIV20 and DIV30: a dense axonal network developed at the bottom of the slice and a second one in the overlying neuropil with regularly spaced varicosities. Although the cerebral cortex is not a repulsive substrate, this second phase of maturation was never observed in cultures containing rhombencephalic serotonergic neurons.

458

SEROTONERGIC INNERVATION OF INTERNEURONS CONTAINING CHOLECYSTOKININ IN THE CAT CEREBRAL CORTEX.

HORNUNG J.-P., Institute of Anatomy, University of Lausanne, Bugnon 9, 1005 Lausanne

The mammalian cerebral cortex is innervated by two parallel serotonergic pathways originating from the dorsal and median raphe nuclei. The median raphe projection predominates in the upper layers of the neocortex, where, in certain areas, it forms baskets which surround the soma and proximal dendrites of GABA-containing neurons. Among the heterogenous population of GABAergic cortical interneurons, those contacted by the serotonergic median raphe baskets belong to the subtype containing the calcium-binding protein calbindin, in rat, marmoset, and human. In the cat cortex, presence of calcium-binding proteins in the target neurons of the serotonergic baskets was investigated using double-labelling immunocytochemistry. No calcium-binding proteins (calbindin, parvalbumin, calretinin) were detected in those neurons. As a second marker in GABAergic neurons, several neuropeptides (neuropeptide Y, somatostatin, substance P, cholecystokinin (CCK)) were analyzed. Of those, only CCK-immunoreactivity was observed in the target neurons, using DAB-reacted sections, and fluorescent labelled preparations viewed with confocal microscopy (BioRad 6000, Cancer Research Institute, Lausanne). Variation in the neurochemical composition of the cortical neurons innervated by serotonergic baskets reveal species-specific modes of regulation of these circuits.

459

INVESTIGATION OF NEURAL INFORMATION PROCESSING IN THE AUDITORY THALAMUS BY COHERENCE ANALYSIS. A.E.P. Villa*, D.R. Brillinger and F. de Ribaupierre , *Institut de Physiologie, UNIL, CH 1005 Lausanne, Switzerland and Department of Statistics, UC Berkeley, Berkeley, CA 94720, USA.*

Simultaneous recordings of single unit spike trains were carried out in the auditory sector of the thalamic reticular nucleus (RE) and medial geniculate body (MGB) of nitrous oxide anesthetized cats. Coherence and partial coherence analyses of 171 pairs of units, one in RE and one in MGB, were used to investigate the degree of linear time invariant association between the cells as a function of frequency. For 79 pairs of units the comparison of coherences and partial coherences during spontaneous activity and during stimulation by white noise bursts, indicate that the functional connectivity is modified by stimulus delivery. Overall the stimulus tended to decrease association of firing at very low frequencies (below 5 Hz) and increased association at frequencies higher than 80 Hz. Average null levels were estimated in order to group pairs of units corresponding to the same thalamic subdivisions. Removal of linear effects by partial coherence indicates that the medial division of MGB and RE are preferentially associated at discharge rates at 15 spikes/sec. The techniques presented here provide a valuable improvement in detecting modulations of neural information processing induced by a stimulus without being necessarily time-locked to its time course.

460

INTRA- AND INTERHEMISPHERIC NEURONAL INTERACTIONS IN THE CAT AUDITORY CORTEX

de Ribaupierre, F., Simm, G., de Ribaupierre, Y., Vallélian J.-F. and Clarke, S. Inst. de Physiologie de l'université, CH 1005 Lausanne

Functional significance of interhemispheric connections for the auditory system was approached by recording 8 spike trains simultaneously from 7 microelectrodes placed in the primary (AI), anterior (AAF) and posterior (PAF) auditory cortices on both sides of a nitrous oxide and nembutal anesthetized cat. Neuronal interactions were studied by cross-correlation techniques for 256 single unit pairs during spontaneous and acoustically driven activity.

For 116 pairs with both members within the same hemisphere, interactions observed were typical for the presence of a shared common input (CI), in 17 % of the pairs. These CI occurred between pair members located within the same (AI-AI) or across different areas (AI-AAF), within the same cortical depth or between superficial and deep layers, and for quite a range of best frequency (BF) separations.

Pairs with members in opposite hemispheres presented CI for 9 % of them. These CI were also observed for all combination of areas, layers and BFs separations, with a preference for short BFs separations and different associations of binaural properties. It is quite significant that these interhemispheric neuronal interactions were only about half less frequent than those within one hemisphere. They could be due to the presence of local collaterals in the hemisphere of origin, of the callosal axons.

461

DISCHARGE PROPERTIES OF SINGLE NEURONS IN THE DORSAL NUCLEUS OF THE LATERAL LEMNISCUS IN THE RAT.

Bajo, V.M.[1,2], Villa, A.E.P.[1], Rouiller, E.M.[3], and de Ribaupierre, F.[1]. (1.Institut de Physiologie de l'Université, CH-1005 Lausanne. 2.Dpto. de Biología Celular y Patología, Universidad de Salamanca, España. 3.Institut de Physiologie de l'Université, CH-1700 Fribourg.

Single unit properties of the Dorsal Nucleus of the Lateral Lemniscus (DLL) were studied in six Wistar albino female rats. Spike trains of 71 units were recorded along 9 electrode penetrations in the DLL. Activity was recorded during spontaneous activity and binaural white noise bursts stimulation. In spontaneous activity, the distribution of firing rate showed three distinct unit populations with peaks at 1.5, 3.5 and 8.5 spikes/s. Peri-Stimulus Time Histograms (PSTH) were computed on several hundreds of responses to 200 ms noise bursts, delivered at 20 dB above the threshold. The mean latency to the first peak of the response was 21 ms and the signal-to-noise ratio was 125.8. The most common response pattern to noise bursts was an excitatory onset response followed in some cases by an inhibition. The majority of DLL neurons responded to binaural noise bursts stimulation (80%). Most units presented an excitatory response to the contralateral stimulation with an inhibitory response to the ipsilateral stimulation (EI, 18%) or without it (EO, 56%).

(Supported by PB 90-052-C02-01 and EX92 34938144, and SNSF 3130-025128 and 31-28572.90)

462

STRUCTURAL ORGANIZATION OF THE GENICULOSTRIATE PATHWAY IN THE TREE SHREW

Kretz, R., Nay, P., and Rager, G.,
Anatomisches Institut der Universität, CH-1700 Fribourg

In ten tree shrews (Tupaia belangeri) wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) was injected into electrophysiologically defined areas of the dorsal lateral geniculate nucleus. We investigated the geniculostriate pathway by using the highly selective TMB-AHM method (Olucha et al. 1985, J. Neurosci. Meth. 13,131) in order to detect histochemically the anterogradely transported tracer substance WGA-HRP. The results obtained so far not only confirm the general laminar organization of the geniculostriate projection in the tree shrew, but they also reveal a clear bandlike pattern (patches) in layers I and IIIb. This periodicity - distances between maxima of 250 to 450 µm - can be seen in coronal and in tangential sections and can easily be distinguished from the more or less regular staining pattern of layer IV and the most basal part of sublayer IIIc.

Supported by Swiss NSF grant No. 31-30014.90

463

ASSOCIATION AND INTRINSIC CONNECTIONS OF THE HUMAN EXTRASTRIATE VISUAL CORTEX REVEAL MODULAR ORGANIZATION

S. Clarke, Institut de Physiologie, Rue du Bugnon 7, 1005 Lausanne

Human extrastriate visual cortex contains several functionally specialized areas, some of which can be identified by anatomical criteria alone (Clarke, Eur. J. Neurosci. Suppl. 5 (1992) 157).

I have now studied intrahemispheric corticocortical connections of these visual areas. Connections were traced anterogradely with the Nauta-method for degenerating axons from a small lesion in upper area 19. They were distributed discontinuously; dense afferents were found around the lesion and in visual areas V3, V3A, as well as in upper V1 and V2 and lateral V4. Weak afferents were found in VP, V5, lower V2, central V1 and medial V4. Lower V1 was devoid of afferents. The tangential distribution of intrisic connections, studied in the cortex medial to the lesion, varied in density in a columnar fashion. Thus, there is evidence for modular organization of human extrastriate visual areas, namely i) selective connectivity between some human visual areas or parts of them; and ii) columnar organization of intrinsic connections.

464

CORTICOMOTONEURONAL CONNECTIONS IN THE RAT: ELECTRON MICROSCOPIC EVIDENCE FROM DOUBLE-LABELING

[1]Liang, F., [2]Blümcke, I., [2]Blümcke, B., [1]Moret, V., [3]Ryser, U. and [1]Rouiller, E.M., Instituts de Physiologie (1), Histologie (2) et Botanique (3), Université de Fribourg, CH-1700 Fribourg.

To confirm that close appositions of corticospinal (CS) axon terminals with spinal motoneuronal dendrites or somata, as seen in light microscopy, correspond to direct corticomotoneuronal (CM) connections in the rat, possible CM synapses were investigated with electron microscopy (EM). Injections of wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP) into motor cortex, and cholera toxin subunit B (CB) into forelimb muscles, labeled CS axons and spinal motoneurons, respectively. Cervical cord sections were processed for visualizing them and flat-embedded in Epon. Semi-thin and ultra-thin sections were then prepared. WGA-HRP labeled CS axon terminals were distributed in Rexed's laminae I-X and, to a lesser extent, in the lateral funiculus of the white matter. CB labeled motoneuronal dendrites extended profusely in the gray matter and in the lateral funiculus of the white matter, widely overlapping the termination zone of CS axons. Synapses containing round vesicles were identified at EM level between the WGA-HRP labeled CS axons and the CB labeled motoneuronal dendrites. In conclusion, direct CM connections, believed to be a prerogative of primates, are also present in the rat. There is also evidence that this connection is of functional importance.

465

A cervical primary afferent input to vestibular nuclei as demonstrated by retrograde and anterograde transport of wheat germ agglutinin-horseradish peroxidase in the cat.

S. Bankoul and **T. Goto**[1]; Inst. of Anatomy, University of Fribourg, CH-1700 Fribourg, [1]Laboratory of Neurophysiology, Rockefeller University, New York 10021-6399

Wheat germ agglutinin-horseradish peroxidase conjugate (WGA-HRP) was microiontophoretically injected into the caudal half of the medial (MVN) and descending (DVN) vestibular nuclei. In other experiments WGA-HRP was pressure injected into spinal ganglia (SG) C_2-C_4.
Injections into either the caudal part of the MVN, DVN or the border between the two nuclei revealed retrogradely labeled cells in ipsilateral spinal ganglia C_2-C_5. After injections into SG C_2-C_4 anterogradely labeled fibers were observed in the caudal part of the ipsilateral MVN and DVN. These results strongly suggest that the caudal part of the MVN and the DVN is the target region for rostral cervical primary afferents entering the vestibular nuclear complex. These findings corroborate similar results found in the rat (Neuhuber and Zenker, 1989, J. Comp. Neurol. 280:231-253; Bankoul and Neuhuber, 1990, Exp Brain Res 79:405-411)
This work was supported by grant NSO2619

466

RESPONSES OF DOPAMINE NEURONS TO REWARD AND CONDITIONED INCENTIVE STIMULI IN MONKEYS LEARNING A COGNITIVE TASK

Schultz W, Ljungberg T and Apicella P, Institut de Physiologie, Univ. de Fribourg, CH-1700 Fribourg

Lesion studies showed that midbrain dopamine neurons are involved in movements, motivation and cognition. In order to assess their role in learning and cognition, we investigated their electrophysiological activity while monkeys learned to perform a spatial delayed response task via two intermediate tasks. The behavioral apparatus contained two response levers, and the animal had to touch, after a delay of 2-3 s, the correct lever previously indicated by an instruction cue. While learning each task, 25% of 76 dopamine neurons responded to liquid reward, whereas only 9% of 163 neurons responded to this stimulus when task performance was established. Reward responses were significantly more numerous and pronounced during but not after learning in area A10, as compared to areas A8 and A9. Dopamine neurons responded also to the two conditioned stimuli that were important for task performance, the instruction cue as the first stimulus in each trial indicating the target of the upcoming arm movement (58% of 76 neurons during and 44% of 163 neurons after learning), and the trigger stimulus as conditioned incentive stimulus eliciting an ocular reaction and an arm reaching movement by predicting reward (38% during and 40% after learning). None of the dopamine neurons showed delay-related sustained activity typical for striatum and frontal cortex. These data demonstrate that dopamine neurons respond to the most significant attentional and motivating stimuli during learning and performance of a cognitive task. Their activity does not reflect the specific cognitive components of the tasks, such as preparation of movement, expectation of external stimuli, expectation of reward, or working memory. Rather, dopamine neurons appear to participate in attentional and motivational processes necessary for learning of behavioral tasks.

467

BRAIN AROMATIZATION OF TESTOSTERONE AND EMOTIONAL BEHAVIOUR IN ROMAN HIGH- AND LOW-AVOIDANCE (RHA/RLA) RATS.

Steimer, Th., Driscoll, P.* and Schulz, P., *Division de* Psychopharmacologie, I.U.P.G., CH-1225 GENEVE and * Institut für Verhaltenswissenschaft, ETH Zentrum, CH-8092 ZURICH

Oestrogens seem to play a modulatory role in the regulation of emotional behaviour. In the male, neuroactive oestradiol-17β (E2) is formed by aromatization of testosterone in various hypothalamic and limbic areas. Aromatase activity (AA) was measured using an in vitro radiometric assay in microdissected samples obtained from RHA and RLA rats, which differ in emotional reactity. Tissue concentration of dopamine (DA) and its metabolite DOPAC were also measured by HPLC/ED in frontoparietal cortical (FPC) samples to assess the activity of the mesocortical dopaminergic system, which is involved in emotional behaviour. Under baseline conditions, there was a significant difference ($p<0.005$) in AA between RHA and RLA rats at the level of the bed n. of the stria terminalis (BST), which contains oestrogen-sensitive neurons projecting to the ventral tegmental area (VTA). DA and DOPAC in the FPC did not differ. In a separate experiment, rats subjected to a mild stress (open field and elevated plus-maze for 10 min) showed a marked decrease in AA in all areas and the BST difference was abolished. However, both DA and DOPAC were markedly increased in the FPC of RLA as compared to RHA rats ($p<0.01$), indicating differential activation of the mesocortical dopaminergic system in RLA vs RHA rats. These preliminary results suggest that AA may play a role in the control of emotional behaviour in part through testosterone-derived E2 acting on oestrogen-sensitive neurons in the BST projecting to the VTA, which could regulate the sensitivity of the mesocortical dopaminergic system.
[This research is supported by grant 31-29996.90 from the FNSRS]

468

SOCIAL RANK DETERMINED IN A COMPETITIVE SITUATION AND ITS RELATION TO VOLUNTARY ALCOHOL CONSUMPTION IN RATS.

Kessler, M., Welzl, H., Bättig, K., Lab. Behavioral Biology, ETH Zürich, CH-8092 Zürich

The present experiment investigated a possible relationship between dominance and alcohol consumption in male Wistar rats. Rats were housed in groups of 3 animals per cage (triads) and competed daily for a limited number of food-pellets after being food deprived for 22 hrs. Based on each individual's competition-score (number of food pellets gained per session), rats were either labeled as being dominant (D), intermediate, or subordinate (S). Clear and stable rank orders developed in 10 out of 15 triads (1). After 32 days of adaptation to alcohol drinking (alcohol in increasing concentrations was substituted in place of water every other day), alcohol (9% v/v) consumption was measured in a free-choice, two bottle situation during 6 days in single-housed D- and S-rats. Dominant rats consumed significantly ($p \leq 0.05$) more alcohol (3.56 g/kg/day) than subordinate rats (2.14 g/kg/day). This finding is in agreement with neurochemical data indicating that dominant behavior is inversely related to the activity of brain 5-HT-systems. On the other hand, low 5-HT activity is related to increased chronic alcohol drinking. Our result suggests that social rank is a non-pharmacological factor which influences alcohol consumption.
(1) Gentsch et al. (1988). *Behav. Brain Research* 27:37.

Immune System and Allergy

469

AFRICAN TRYPANOSOMES: VSG AND NON-VARIABLE ANTIGENS CROSS-REACT WITH SELF-REACTIVE SPECIFICITIES FROM UNINFECTED HOSTS

Müller, N,. Imboden M., Hemphill A., Kunz, S., Detmer, E., Mansfield, J. # and T. Seebeck

Institute of General Microbiology, University of Berne, Switzerland
#Dept. of Animal Health & Biomedical Sciences, Madison,WI, U.S.A.

Based on the use of molecular biological, immunological and immuno-cytochemical methods we could identify several non-variable antigens from African trypanosomes that are located on the cytoskeleton. A recent study revealed that two of these antigens, proteins I/6 and MARP1, immunoreact with sera from various uninfected hosts (mouse, bovine, human) and that at least one, I/6, is immunologically related to a cytoskeletal component from mouse 3T6 fibroblasts. In the case of VSG (variant surface glycoprotein) analogous results were obtained. In sera from different host organisms native antibodies could be detected which immunoreact both, with VSG specific epitopes and with epitopes from different 3T6 proteins. In addition, evidence could be provided that, in mice, these antibodies are produced by a $CD5^+$B-cell compartment of the immune system. These data indicate that self-reactive specificities in uninfected hosts crossreact with strong trypanosomal antigens and thus create an immunological environment which is pre-primed to trypanosomal infections.

470

Diagnosis of trypanosomiasis using phage presentation repertoire libraries

P. Weber and T. Seebeck
Institut für allg. Mikrobiologie; CH-3012 BERN

It has been demonstrated by our group that mammals, infected by *African Trypanosomes*, raise an early antibody response to a small number of trypanosomal proteins. We have characterized the molecular structure of several of these early antigens since we intend to use them for diagnostic purposes. As a technique for producing these proteins we are currently establishing the expression and presentation of the antigens at the surface of M13 bacteriophage. One of our goals is to use such recombinant phages as polypeptide carriers for immunodiagnosis.

471

HALOTHANE HEPATITIS : THE E2 SUBUNIT OF THE PYRUVATE DEHYDROGENASE COMPLEX CONFERS MOLECULAR MIMICRY OF TRIFLUOROACETYL-PROTEIN ADDUCTS

Urs Christen, Paul Jenö*, and Josef Gut; Departments of Pharmacology and *Biochemistry, Biocenter of the University, CH-4056 Basel

A monoform anti-TFA antibody, directed against trifluoroacetyl-protein adducts (TFA-protein adducts) that are elicited in tissues of experimental animals and humans upon exposure to the anesthetic agent halothane, recognizes cross-reactive proteins of 64 kDa and 52 kDa in several tissues of rats and the liver of humans not previously exposed to the drug. These cross-reactive proteins were shown to confer molecular mimicry of TFA-protein adducts. Here, by the use of the anti-TFA antibody as an immunoaffinity matrix, the protein of 64 kDa was purified from rat heart. The amino acid sequence of six internal tryptic peptides exhibited 100% identity with the corresponding deduced amino acid sequence of the dihydrolipoamide acetyltransferase component (E2 subunit) of the rat liver pyruvate dehydrogenase (PDH) complex, as encoded by the clone pRMIT [Gershwin, M.E. et al, (1987) J. Immunol. 138, 3525-3531]. Similar to the hapten-derivative N-ε-trifluoroacetyl-L-lysine, free lipoic acid, the prosthetic group of the E2 subunit, inhibited on immunoblots the recognition by anti-TFA antibody of the E2 subunit, of the not yet identified protein of 52 kDa, and of TFA-protein adducts. Additionally, both lipoic acid and N-ε-trifluoroacetyl-L-lysine did abolish the precipitation of the native E2 subunit by anti-TFA antibody from solubilized rat heart mitochondria. These data suggest that lipoic acid is actively involved in the molecular mimicry of epitopes present on TFA-protein adducts by the E2 subunit of the PDH complex.

472

Direct inhibition of T-cell proliferation by anti-MHC class II antibodies.

Dirk Dobbelaere, Margarete Eichhorn* and Volker Heussler. Institute of Parasitology, Uni Bern, Switzerland and *KfK, IGT, Karlsruhe, FRG.

We have analysed the effect of antibodies directed against MHC class II antibodies on the proliferation of T-cells. We have shown that anti-class II antibodies exert a direct effect on T-cells causing an acute block in their proliferation. The inhibition does not involve apoptosis. It is also not due to interference with the state of activation of the T-cells, since the trancriptional activator NF-kappaB, which regulates interleukin 2 and interleukin 2 receptor gene expression, remains activated in arrested cells. Interleukin 2, interleukin 2 receptor and *c-myc* gene expression, which are characteristic for T-cell activation, are also unaffected. In adddition, the rapid arrest of DNA synthesis caused by anti-class II antibodies is entirely reversible. By analysing the cell-cycle phase distribution of inhibited cells, it could be shown that cells in all phases of the cell-cycle are inhibited. The signal transduction pathway that results in inhibition was shown to be independent of protein kinase C and extracellular Calcium Tyrosine kinase inhibitors, however, partly reduced the level of inhibition and, conversely, phosphatase inhibitors enhanced it. Antibodies directed against MHC class II molecules have been demonstrated in a number of autoimmune conditions and also in patients with AIDS. Anti-MHC class II-mediated inhibition of T-cells may therefore play an important role in a number of autoimmune diseases and contribute to the pathogenesis of HIV-infection.

473

HLA-DPB1 ALLELIC POLYMORPHISM IN CAUCASOIDS AND LINKAGE DISEQUILIBRIUM WITH HLA-A,-B,-DR ANTIGENS

Grundschober, C., Sanchez-Mazas, A., Jeannet M. and Tiercy J.-M. Transplantation Immunology Unit, Hôpital Cantonal Universitaire of Geneva, Department of Anthropology, University of Geneva.

The extensive polymorphism displayed by the HLA antigens of the major histocompatibility complex is of crucial importance to understand the regulation of immune responses in normal and pathological situations. Because of their high degree of diversity, HLA genes also represent useful markers for human population genetics. The development of the PCR technology and of the oligonucleotide typing methods now allows large scale analysis of the polymorphic HLA loci. By using a combination of 21 SSO probes, DPB1 allelic polymorphism was analysed in a sample of 135 healthy individuals from a local panel of blood donors. 17 of 22 possible alleles were detected with 4 alleles accounting for >72% of all DP alleles. Significant linkage disequilibrium was found for the following haplotypes: A1-B8-DRB1*0301-DPB1*0101, B44-DRB1*07-DPB1*1101, DRB1*1302-DPB1*0301, and DRB1*04-DPB1*0201. In our panel, heterozygosity levels are: H=0.87 for HLA-A, 0.92 for HLA-B, 0.90 for HLA-DRB1 and 0.82 for HLA-DPB1. These values are strikingly similar despite different frequency distributions.

474

RESISTANCE OF CYTOLYTIC T LYMPHOCYTES (CTL) TO PERFORIN-MEDIATED KILLING

Müller Ch. and Tschopp J.
Université de Lausanne, Institut de Biochimie 1066 Epalinges

The stimulus-secretion model of CTL-mediated lysis proposes the release of the lytic pore-forming protein perforin into the intercellular space between killer and target. CTL lines, in contrast to tumor target cells, are resistant to cell-mediated lysis and they also withstand lysis by the isolated perforin. These observations suggest a self-protection mechanism of CTLs against their own lytic protein.
We radioactively labeled proteins of cytotoxic granules and studied the binding of perforin to different cell lines. Studies performed at 4°C revealed that equal amounts of perforin bound to susceptible target cells as well as to resistant CTLs in a Ca^{2+}-dependent manner. Cell-bound perforin was treated with trypsin. Perforin on the CTL line B6.1 was more accessible to the protease than perforin bound to the tumor target cell K562. This indicates a difference in conformation or accessibility. We propose a mechanism whereby CTLs have an inhibitory molecule, which prevents membrane insertion and subsequent polymerisation of perforin.

475

Calreticulin (Ro/SS-A auroantigen) is a granule-associated molecule of cytolytic T lymphocytes and is released during target cell lysis

Dupuis, M., Schärer, E., Krause, K.-H., and Tschopp, J..
Institut de Biochimie, CH-1066 Epalinges

The delivery of lytic components harbored in granules of CTLs by exocytosis is an effective mechanism for target cell destruction. Purified granules contain perforin, granzymes and proteoglycans as major components. We report the purification of an additional major 60-kD granule-associated protein from human LAK cells and mouse cytolytic T cells. The NH_2-terminal amino acid sequence of the polypeptide was found to be identical to calreticulin. Calreticulin is a calcium storage protein. In CTLs, calreticulin colocalizes with the lytic perforin to the lysosome-like secretory granules, as confirmed by double-label immunofluorescence confocal microscopy. Moreover, when the release of granule-associated proteins was triggered by stimulation of the T cell receptor complex, calreticulin was released along with granzymes A and D. Since perforin is activated and becomes lytic in the presence of calcium, we propose that the role of calreticulin is to prevent organelle autolysis due to the protein's calcium chelator capacity.

476

ROLE OF SPECIFIC V_β^+ T CELLS IN MURINE AUTOIMMUNE GASTRITIS

Claeys,D.*, Karapetian,O.*, Schreyer,M.+, Saraga,E.*, Louis,J.&, Acha-Orbea,H.+, Jaisser,F.", Rossier,B." and Krähenbühl, J.P.*, *Swiss Inst. for Exp. Cancer Research, &Inst. of Biochemistry and +Ludwig Inst. for Cancer Research, CH-1066 Epalinges, *Inst. of Pathology and "Inst. of Pharmacology, University CH-1005 Lausanne

Autoimmune gastritis (AIG, chronic atrophic gastritis Type A) is accompanied by autoantibodies to H,K-ATPase of the gastric parietal cells. Neonatal thymectomy can induce gastritis in the fundic mucosa of BALB/c mice and constitutes a useful model for human AIG. Murine AIG appears to be T cell mediated. Two recent reports suggest a close link between BALB/c.AIG and $V_\beta6^+$ and $V_\beta11^+$ T cells. To establish whether T cells expressing specific V_β elements play a direct role in AIG we have 1) analyzed the frequency of potential autoreactive $V_\beta6^+$ and $V_\beta11^+$ T cells in 5 month-old control and AIG positive mice by FACS analysis of stomach lymph node lymphocytes 2) examined in congenic BALB.D_2. Mls-1^a the effect of clonal deletion of Mls-1^a reactive $V_\beta6^+$ T cells on the onset of AIG. To identify protonpump autoreactive T cells we have cloned and sequenced the α and β subunits of the BALB/c H,K-ATPase for generating the autoantigens.

477

Demonstration of ternary immunophilin calcineurin complexes with the immunosuppressants cyclosporine and FK506

G. Woerly, L.Weber, M. Zurini and B. Ryffel

Abstract

The specificity of CsA binding to the major intracellular receptor protein, cyclophilin A and B as well as the interaction with the phosphatase calcineurin was investigated. Binding of PL-CS, a photoaffinity probe of CsA, to recombinant human cyclophilin A and B is saturable and specific. Although PL-CS binds calcineurin in the absence of cyclophilin and calmodulin, this binding is non-specific, since it does not discriminate between active and inactive CS derivatives. In the presence of cyclophilin cyclosporin- calcineurin binding becomes specific.

Ternary complexes containing an equimolar ratio of cyclophilin A or B, PL-CS and calcineurin are resolved using chemical-crosslinking technique. The formation of these complexes is specific, calcium, but not calmodulin dependent and is inhibited only by cyclosporines, which bind cyclophilin. The drug-immunophilin complex binds only to the calcineurin A subunit; The proteolytic 40 kD product of calcineurin A retains the binding properties, suggesting that the N-terminal domains are not necessary for the complex formation. Similarly, a trimeric complex of FKBP, FK506 and calcineurin can be demonstrated. As expected these complexes are only competed by homologous derivatives. In vivo labelling of Jurkat T-cells revealed, that similar immunophilin-drug-calcineurin complexes occur in vivo.

Key: Photoaffinity labeling, chemical-crosslinking, calcineurin, immunophilin, Jurkat cells

478

CYCLOSPORIN DERIVATIVES INHIBIT INTERLEUKIN 1β INDUCTION OF NITRIC OXIDE SYNTHASE IN MESANGIAL CELLS

Heiko Mühl and Josef Pfeilschifter, Department of Pharmacology,Biocenter, University of Basel, CH-4056 Basel

Treatment of mesangial cells with recombinant human interleukin 1β (IL-1β) dose-dependently increased nitrite formation due to the induction of a mcarophage-type of nitric oxide synthase. Addition of the immunosuppressive drug cyclosporin A or its derivatives, cyclosporin G and cyclosporin H, dose-dependently inhibited IL-1β-induced nitritie generation. Half-maximal inhibition was observed at concentrations of 0.9 μM, 2.0 μM and 3.8 μM of cyclosporin A, cyclosporin G and cyclosporin H, respectively. Two other immunosuppressive drugs, FK506 and rapamycin (10 μM each) did not affect IL-1β-induced nitrite production, suggesting that inhibition of nitric oxide synthase activity is not related to the immunosuppressive activities of the investigated drugs. In summary, cyclosporins may protect the kidney from damage resulting from cytokine-stimulated mediator release and subsequent inflammatory reactions.

479

NITRIC OXIDE PRODUCTION BY HUMAN NEUTROPHILS

M. Markert, B. Carnal and J. Mauël*
Laboratory of Clinical Chemistry, CHUV and Biochemistry Institute*, CH-1011 Lausanne.

Production of low levels of nitric oxide (NO) by human neutrophils has been demonstrated recently. The possible relationship between this pathway and the superoxide (O_2^-)-generating system was evaluated. Incubation of neutrophils with L-arginine (L-arg) (1 mM,1h at 37°C) did not increase O_2^- production except with PMA as a stimulus (18.7+/-6.8 vs 14.3+/6.1nmol/5min.10^6cells,n=9,p<0.01). In contrast, no effect of L-arg was found on NO production measured as nitrite (NO_2^-) with the Griess reagent. Maximum NO_2^- production was obtained during adherence of neutrophils to plastic surfaces in comparison to resting cells in suspension (2.49+/-0.23 vs 0.74+/-0.19 nmol/h.10^6cells). NO2- could be further stimulated by agonists only in cells in suspension. Upon adhesion, O_2^- was also produced at a higher level compared to cells in suspension but could be further stimulated with PMA. L-canavanine inhibited NO_2^- and O_2^- production at different maximal concentrations of 10 and 5 mM respectively. These data suggest at the same time a relationship and a dissociation between NO_2^- and O_2^- release.

480

Cloning and expression in mammalian cells of porcine tumor necrosis factor alpha: Examination of biological properties

G. Bertoni, B. von Niederhäusern, P. Kuhnert, C. Hertig, H. Pfister, U. Pauli and E. Peterhans

Institute of Veterinary Virology, University of Bern

The use of swine models to study diseases like viral hemorrhagic fevers and endotoxic shock syndrome, where tumor necrosis factor alpha (TNF-α) could play a crucial role, have been hampered by the lack of the necessary immunological tools such as recombinant TNF-α and specific bioassays to measure this important immunoregulatory molecule. We have therefore cloned a full length complementary DNA (cDNA) of the porcine TNF-α gene and expressed it in porcine and murine cells . The cloned cDNA was introduced into the pBMGNeo expression vector (Karasuyama, H. and Melchers, F., 1988, Eur. J. Immunol., 18: 97-104) and transfected by electroporation in porcine (PK(15)) and murine (L929) cell lines. TNF-α bioactivity was detected in the supernatant of the transfected cells using a standard L929 bioassay or a newly developed PK(15) bioassay. This bioassay is 100 to 1000 times more sensitive than the L929 bioassay suggesting a partial species-specificity of the porcine TNF receptor(s).

481

THE EFFECT OF PRENATAL DIAZEPAM EXPOSURE ON RAT CYTOKINE PRODUCTION BY RAT SPLENOCYTES

A.A. Schreiber, K. Frei*, W. Lichtensteiger, and M. Schlumpf, Institute of Pharmacology, University of Zürich, Section of Clinical Immunology and Dept. of Neurosurgery, University Hospital, Zürich, Switzerland

We previously found that prenatal exposure of rats to diazepam resulted in a marked reduction of lymphocyte proliferation and of LPS-stimulated TNF-α release from rat splenocytes during postnatal development. Both of these defects recovered in adulthood. We further examined the mitogen-stimulated release of IL-6, a product of activated macrophages and lymphocytes. IL-6 was significantly lowered at 2, 4, and 8 weeks of age in splenocytes after prenatal diazepam exposure. In contrast to TNF-α, this decreased release was still observed in adulthood. In search for mechanisms underlying the disturbed cytokine release, we are currently investigating the liberation of IFN-γ and PGE_2.

482

DISTRIBUTION OF THE INTERFERON-γ RECEPTOR IN MICE

Car, B.D., Oznen, L., Garotta, G, Steckholzer, U. and Ryffel, B.
Institut für Toxikologie der ETH und der Universitat Zürich
Schorenstr. 16, 8603 Schwerzenbach, Switzerland.

Production of Interferon γ (IFN-γ) by activated T-lymphocytes and natural killer cells results in a pleotropic array or responses, including antiviral activity, stimulation of macrophages and NK-cells and the induction of MHC class II molecules in a variety of cell types. That these responses are transduced through a single cellular receptor prompted our investigation into the topographic distribution of this receptor in mice. Affinity purified rabbit-anti mouse antibodies directed against the murine IFN-γ receptor were utilized in morphologic and fluorocytometric investigations. Striking patterns of highly positive cells were observed in the liver, spleen and kidney. Hepatic staining was restricted to Kupffer cells which were markedly positive, while coexpressing the macrophage marker F4/80 and MHC class II molecules. This coexpression by macrophage-dendritic cells of the splenic red pulp was confirmed by immunofluorescence and FACS analyses. Renal positivity was restricted to glomeruli which contained cells expressing much IFN-γ receptor antigen in the mesangium. By FACS analysis activated T- and B-lymphocytes and well as NK-cells were determined to express the IFN-γ receptor. The close proximity of IFN-γ receptor bearing cells to cells capable of producing IFN-γ implicate these cells in IFN-γ-mediated pathologic events in the mouse. Hepatic graft-versus-host inflammatory responses and certain murine glomerulonephridities are proposed as disease processes involving inappropriate stimulation of the IFN-γ-receptor.

483

Targeted Disruption of the Murine IFN I Receptor Gene

U. Müller, L. Reis, S. Hemmi, and M. Aguet
Institute of Molecular Biology I, University of Zürich

Interferons were discovered by virtue of their antiviral activity but they exert additional activities including inhibition of cell growth, antitumor action, effects on cellular differentiation, and immunoregulatory functions. The α and β (type I) IFNs are thought to act through a common receptor; the gene encoding one of its chains was cloned, however at least one more chain is required for activity (Uzé et al.). To define the physiological role of IFN-α/β, we generated mice devoid of the known receptor chain by homologous recombination in ES cells. Homozygous IFN-α/β$R^{0/0}$ mice were viable, anatomically normal and fertile. The type I receptor was functionally inactive as shown by the lack of antiviral response of primary IFN-α/β$R^{0/0}$ embryonic fibroblasts (PMEFs) to natural murine type I IFN-α/β; the response to IFN-γ was unaltered. Northern blot analysis of PMEF-RNA with probes derived from several IFN-inducible genes showed that mutant cells did not respond towards natural murine type I IFNs, rIFN-β and human recombinant IFN-α2/α1 (to which normal mouse cells are responsive), indicating that the disrupted receptor chain is essential for the response to the IFNs tested. Hematological and immunological characterization of the targeted animals is underway.

484

Growth of *Theileria*-infected T-cells inhibited by anti-sense IL2R/Tac RNA

Margarete Eichhorn and Dirk Dobbelaere. KfK, IGT, Karlsruhe, FRG and Institute of Parasitology, Uni Bern, Switzerland

T-cells that are infected with the protozoan parasite *Theileria parva* acquire the ability to proliferate in an uncontrolled manner. High levels of activated NF-kappaB can be found in their nucleus and the IL2R and IL2 genes are constitutively expressed. Expression of these genes is critically dependent on the presence of the parasite in the host cell cytoplasm. To establish whether IL2R/Tac gene expression is essential for continuous proliferation, anti-sense IL2R/Tac RNA experiments were carried out. Theileria-infected cells were permanently transfected with plasmid constructs designed to express either sense or anti-sense IL2R/Tac RNA under control of a cadmium-inducible metallothionein promotor. The neomycin resistance gene was co-transfected to provide a selection marker. Several clones of cells transfected with either sense or anti-sense constructs were generated. It was found that clones transfected with anti-sense IL2R/Tac constructs grew more slowly than cells containing sense constructs, also in the absence of cadmium. This was due to the continuous basic activity of the promotor. Reduced amounts of IL2R/Tac mRNA were also found in cells transfected with anti-sense constructs and, in proliferation assays, significant inhibition of cells containing anti-sense constructs could be induced by the addition of cadmium to the culture medium. These data show that proliferation of Theileria-infected T-cells used in this study is at least in part dependent on the expression of a fully functional IL2R.

485

IN VITRO AND IN VIVO EXPRESSION OF THE INTERLEUKIN-2 RECEPTOR ON HUMAN MELANOMA CELLS

S. Carrel[1], S. Salvi[1], F. Hartmann[1], M. Schreyer[1], S. Blum[1], B. Azzarone[2], L. Zografos[3], D. Rimoldi[1]
[1]Ludwig Institute for Cancer Research, Epalinges, Switzerland
[2]Hôpital Paul-Brousse, INSERM U268, Villejuif, France
[3]Hôpital Ophtalmique Universitaire, Lausanne, Switzerland

Melanoma cells can secrete several cytokines and express various cell surface molecules, such as the intercellular cell adhesion molecule ICAM-1, class II histocompatibility antigens, and the CALLA antigen, typically found in cells of the immune system. We have investigated the possible expression of interleukin-2 (IL-2) receptors in melanomas using monoclonal antibodies specific for the p55/α chain (TAC antigen) and the p57/β subunit. Flow cytometric analysis of cultured melanoma cells showed the presence at low level of the TAC antigen and of the β chain on the cell surface of several lines. Similar results were obtained in vivo by immunohistochemistry on cryosections prepared from cutaneous and ocular melanoma explants. Positive staining of various intensity was readily observed for the α chain of the IL-2 receptor in a high percentage of tumor cells. The β chain could also be detected, although with a weak staining and in a limited number of specimens. Analysis of RNA from melanoma cell lines by Northern bolt showed the presence of typical 4.0 Kb transcripts of the p75 subunit, while mRNA for the p55 chain could be detected using combined reverse transcription/PCR analysis. Together these results suggest that melanoma cells may express high affinity receptor for IL-2.

486

Interleukin-1 dependent stimulation of interleukin-6 production in cultivated human dermal fibroblasts is linked to the presence of fetal calf serum or platelet-derived growth factor

S.P. Inaebnit, E.R. Waelti+, C. Luethy, O.H. Oetliker and U.N. Wiesmann, Dept. of Pediatrics and Pathology+, University of Berne, Switzerland.

Interleukin-1α (IL-1) is a potent inducer of various pro-inflammatory cytokines in vivo and in cultured cells. Factors such as platelet-derived growth factor (PDGF) or FGF are assumed to amplify induction by co-stimulation of cytokines and/or upregulating IL-1 receptor numbers on cells. Fetal calf serum (FCS) contains various polypeptide factors, including PDGF, and stimulates interleukin-6 (IL-6) production. Therefore cultured human dermal fibroblasts were incubated in MEM supplemented with 0 to 10% FCS for 24h. Without IL-1 stimulation the concentration of IL-6 in the supernatant rose in a dose dependent manner (0 to 7.5 ng IL-6/ml). Upon additional stimulation with IL-1 (15 U/ml) IL-6 concentrations were about 100-fold higher in the presence of increasing FCS concentrations (30 to 750 ng IL-6/ml).The role of PDGF-BB in the production of IL-6 after IL-1 stimulation was investigated by incubating fibroblasts in medium containing 0.5% FCS with IL-1 (15 U/ml), PDGF-BB (20 ng/ml), and both IL-1/PDGF-BB, respectively, for 24h: IL-1 moderately stimulated IL-6 production (140 to 290 ng/ml), whereas PDGF-BB alone had only little effect (1 to 15 ng IL-6/ml). However, IL-1 together with PDGF-BB enhanced IL-6 production to very high levels of 400 to 450 ng/ml. Our findings suggest that IL-6 production in fibroblasts stimulated by IL-1 is dependent on the presence of serum. PDGF is one major though possibly not the only factor in serum acting synergistically with IL-1 to produce high levels of IL-6.

487

Interleukin-6 receptor density is higher in cultivated human follicular outer root sheath cells than in interfollicular epidermal keratinocytes and decreases with confluency

E.R. Waelti+, A. Limat*, S.P. Inaebnit°, T. Hunziker*, U.N. Wiesmann°, Dept. of Pathology+, Dermatology* and Pediatrics°, University of Berne, Switzerland.

In skin, effects of interleukin-6 (IL-6) on cell motility, proliferation and differentiation of keratinocytes are discussed. We therefore studied IL-6 receptor densities on follicular outer root sheath cells (ORS) and interfollicular epidermal keratinocytes (IEK) in preconfluent and confluent cell cultures. Binding studies and Scatchard analysis were performed using recombinant ^{125}I-IL-6 added in low to saturating doses. The number of binding sites for IL-6 on ORS cells grown to preconfluency ranged from 1380 to 7350 per cell. ORS cells grown to confluency showed a much lower number of binding sites, i. e. 240 to 430 per cell. In contrast, the number of binding sites on IEK was 680 per cell when grown to preconfluency and 80 to 130 per cell in confluent cultures. The Scatchard plot disclosed one type of high affinity receptor with a K_d-value of 43 pM and 7300 receptors per cell on ORS cells grown to preconfluency. IEK grown to preconfluency exhibited a K_d-value of 51 pM and 580 receptors per cell.
Our results support the concept that IL-6 is involved in enhanced cell motility and proliferation of ORS cells, which occurs e.g. in re-epithelialization of superficial wounds.

488

PROTEOLYTIC ACTIVITY AND CYTOKINES LEVELS IN BRONCHOALVEOLAR LAVAGE FLUID

Juillerat-Jeanneret,L.; Leuenberger,Ph.; Division of Pneumology, CHUV, Lausanne

In some pathologies, proteolytic enzymes released by pulmonary cells may affect the biological functions of hormones. To adress this question, we measured the activities of various peptidases and the level of cytokines (IL6 and TNF-α) by bioassay and immunoassay in bronchoalveolar lavage fluid of patients with various pathologies. We found that neutral aminopeptidase (APN/CD13) activity was correlated to the number of neutrophils and dipeptidylaminopeptidase IV (DPPIV/CD26) activity to that of lymphocytes. TNF-α levels measured by immunoassay and bioassay were well correlated (n=68, r=0.87), but we demonstrated the presence of a substance interfering with the bioassay of IL6. Interfering capacity was more marked in samples with low IL6 biological and immunological levels. It is hypothetized that, depending of its N-terminal sequence, IL6 might be a substrate for DPPIV/CD26, but not for APN/CD13. However it is premature to link proteolytic activities and loss of IL6 biological activities.

489

Interleukin-1 and cell-to-cell contacts stimulate interleukin-6 in fibroblasts in fibroblast/keratinocytes co-cultures

W. Liu°, Y. Cao°, S.P. Inaebnit°, E.R. Waelti+, A. Limat*, T. Hunziker* and U.N. Wiesmann°, Dept. of Pediatrics°, Pathology+ and Dermatology*, University of Berne, Switzerland.

Interleukin-6 (IL-6) production of cultured dermal fibroblasts can be stimulated by adding interleukin-1 (IL-1) to the culture medium. Mixed co-cultures of fibroblasts with keratinocytes produce large amounts of IL-6 (Waelti et al., J. Invest. Dermatol. 1992: 98, 805-808). The production of IL-6 in fibroblasts is stimulated to a certain extent by keratinocyte conditioned medium. In a two-chamber co-culture system keratinocytes secreted IL-1 and fibroblasts subsequently produced considerably increased amounts of IL-6, but less than in co-cultures of the two cell type on either side of a porous membrane. Cell contacts through the pores greatly stimulated IL-6 production. The extent of IL-6 production was dependent on the pore size (3 μm pores > 0.45 μm pores). The IL-6 synthesis could partially be suppressed by 10 μM hydrocortisone. Our results support the evidence that in fibroblast/keratinocyte cultures the production of IL-6 is stimulated by IL-1 but also by factors depending on cell-to-cell contacts.

490

REGULATION OF IL-8, GROα AND ENA-78 IN LUNG TYPE-II EPITHELIAL CELLS, FIBROBLASTS AND MONOCYTES

Schnyder-Candrian, S. and Walz, A. Theodor Kocher Institut, University of Bern, CH-3000 Bern 9, Switzerland

Interleukin-8 (IL-8), GROα and ENA-78 are potent neutrophil-stimulating peptides inducing chemotaxis, respiratory burst and the release of granule enzymes. All three peptides are structurally related and belong to the CXC-family of the chemokines. We have studied the kinetic of induction of IL-8, GROα and ENA-78 by northern blot analysis, neutrophil-stimulating activity and ELISA measurements in human adult lung fibroblasts, lung type-II epithelial cell line A549 and blood monocytes. Upon stimulation with IL-1, IL-8 and GROα mRNA were rapidly induced in fibroblasts and epithelial cells. Maximal mRNA levels were reached at 2-4 hrs, but levels remained elevated up to 24 hrs. ENA-78 mRNA was strongly induced in epithelial cells, but no such mRNA was detected in adult lung fibroblasts. In lipopolysaccharide stimulated monocytes, however, ENA-78 mRNA was transiently induced at 18 to 24 hours, whereas IL-8 and GROα mRNA reached maximal levels at 4 to 8 hrs after induction. This difference in regulation has been confirmed with specific ELISA measurements, demonstrating that ENA-78 peptide is released from monocytes when IL-8 activity is downregulated. This suggests a different pathophysiological role for ENA-78.

491

CONTRIBUTION OF GROUP II PHOSPHOLIPASE A_2 (PLA_2) TO CYTOKINE-STIMULATED PROSTAGLANDIN E_2 (PGE_2) SYNTHESIS IN MESANGIAL CELLS AND OTHER GLOMERULAR CELLS.

Josef Pfeilschifter[1], Casper Schalkwijk[2], Vreny A. Briner[3] and Henk van den Bosch[2]
1: Department of Pharmacology, Biocenter, University of Basel; 2: University of Utrecht, The Netherlands; 3: Inselspital Bern

Interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα) stimulate PLA_2 and PGE_2 synthesis and secretion by mesangial cells. Co-incubation with a neutralizing monoclonal antibody to PLA_2 attenuates IL-1β and TNFα-induced PGE_2 production by 45% and 52%, respectively. Addition of purified PLA_2 to unstimulated mesangial cells causes a marked release of arachidonic acid and an increased synthesis of PGE_2. Moreover, addition of purified PLA_2 to glomerular epithelial and glomerular endothelial cells augmented both, arachidonic acid release and PGE_2 synthesis, with the endothelial cells being especially sensitive. These data suggest that expression and secretion of PLA_2 triggered by proinflammatory cytokines crucially participates in glomerular inflammatory processes.

492

ADRENALECTOMY (ADX) INCREASES PHOSPHOLIPASE A2 (PLA2) mRNA, PROTEIN AND ENZYME ACTIVITY.

B. Vishwanath, F.J. Frey, M.F. Dallman, B.M. Frey. Div. of Nephrology, Univ. of Berne, Switzerland; Dept. of Physiology, UCSF, San Francisco.

Clinical evidence suggests that acute withdrawal of chronic glucocorticoid therapy with suppression of endogenous cortisol production in RTX patients is associated with an increased susceptibility to rejection. Furthermore, experimental and clinical observations revealed an enhanced immunological and inflammatory response in glucocorticoid deficiency. A key enzyme of the inflammatory response is the PLA2, which releases arachidonic acid. In order to elucidate whether glucocorticoid deficiency causes PLA2 up-regulation, the expression of PLA2 mRNA and protein, as well as the enzyme activity, were quantified in rats with and without ADX. The mRNA of PLA2 was quantified by polymerase chain reaction using a constant amount of a modified PLA2 cDNA transcript as an internal standard. PLA2 message and activity varied from organ to organ with the lowest values in kidneys. The PLA2 mRNA was increased by 116 ± 24 % in various tissues of ADX rats. This up-regulation of PLA2 mRNA was not due to a nonspecific effect of ADX since the mRNA levels of other proteins (c-fos, c-myc, c-erbB, methallothionein-II) remained unchanged. The increase in PLA2 mRNA in ADX rats was reflected by a corresponding increase in tissue (kidney, lung, spleen, liver) PLA2 enzyme activity and protein content as assessed by quantitative Western blot analysis. Thus, ADX increases PLA2 message, protein and activity, an observation in accordance with the increased susceptibility to inflammatory reactions in glucocorticoid deficiency.

Microbiology and Virology

493

DIFFERENT ACYLATION OF SFV GLYCOPROTEINS IN VERTEBRATE AND INVERTEBRATE CELLS: NEW ASPECTS ON THIS MODIFICATION ?

Schärer, C.G. and Koblet, H., Institute of Medical Microbiology, University of Berne, Friedbühlstrasse 51, CH-3010 Berne, Switzerland

The modification of proteins by covalent attachment of fatty acids, such as palmitic acid, is a common but mandatory event in the maturation of enveloped viruses. We are studying the acylation of envelope proteins of Semliki Forest virus (SFV), an RNA virus containing the spike proteins E1, E2 and E3. E1 and E2 become acylated in vertebrate (Vero) and invertebrate cells (A. albopictus C6/36). We found considerable differences between these cells. In C6/36 cells, mainly E1 acted as primary acceptor of palmitic acid whereas p62, the precursor of E2 and E3, was weakly labeled. The palmitoylation of E2 (or p62) seems to be delayed as compared to E1 and might be coupled with the cleavage of p62. Further data suggest that the small amount of acylated p62 contains less bound palmitic acid residues within its cytoplasmic domain than its cleavage product E2. This domain is responsible for the spike-nucleocapsid interaction during virus budding. The role of acylation in this interaction will be discussed.

494

CELL FUSION BY THE ENVELOPE GLYCOPROTEINS OF PERSISTENT MEASLES VIRUSES WHICH CAUSED LETHAL BRAIN DISEASE

R. Cattaneo and J.K. Rose, *Dpt. of Pathology, Yale Medical School, USA and Institut für Molekularbiologie I, Universität Zürich, CH-8093 Zürich*

Measles virus (MV) rarely induces lethal diseases of the human central nervous system characterized by reduced expression of the viral envelope proteins, and by lack of viral budding. The MV envelope contains two integral membrane proteins, fusion (F) and hemagglutinin (H), and a membrane-associated matrix (M) protein. Previously, analysis of MV genes from autopsy material indicated that the M protein and the F protein intracellular domain are often drastically altered by mutations. Here we present evidence that truncation of the F protein intracellular domain does not impair fusion function and we suggest that this alteration interferes with viral budding. Unexpectedly, certain combinations of functional F and H proteins were unable to induce syncytia formation, an observation suggesting that specific F-H protein interactions are required for cell fusion. We also found that three of four H proteins of persistent MVs are defective in intracellular transport, oligosaccharide modification, dimerization, and fusion-helper function. Thus, MVs replicating in brains at the terminal stage of infection are typically defective in M protein and in the two integral membrane proteins. Whereas the M protein appears dispensable altogether, partial preservation of F and H protein function seems to be required, presumably to allow local cell fusion. Certain subtle alterations of the F and H proteins might be instrumental for disease development.

495

Membrane Fusion Activity of the HIV Envelope Protein

Sammy Frey', Mark Marsh* Toon Stegmann'
'Biocenter, Basel and *MRC, London

Human immunodeficiency virus (HIV), the causative agent of AIDS, is an enveloped virus. The virus has one integral membrane protein, a non-covalently linked complex of gp120 and gp41, which are derived from the same precursor, gp160, a product of the *env*. gene. While gp120 recognizes the viral receptor, CD4, gp41 is thought to be responsible for the ability of the viral membrane to fuse with the host cell membrane.

To study the fusion mechanism, large quantities of concentrated gp 120/ gp41 are needed. To avoid having to deal with live virus, part of the HIV genome containing the *env* gene and the viral regulatory element *REV* was cloned into a vector for glutamine synthetase selection and CHO cells were transfected with it. After subcloning, stable expression of the protein on the cell surface was obtained, as demonstrated by indirect immunofluorescence with antibodies specific for gp41. Here, it is shown, that fusion of these gp 120/ gp41 expressing cells with CD4 expressing cells, but not with CD4 negative cells, was obtained. We intend to functionally reconstitute the protein from these cells to study the fusion mechanism.

496

CHARACTERIZATION OF THE EARLY EVENTS IN SEMLIKI FOREST VIRUS (SFV) INFECTION OF VERO CELLS USING PLASMA MEMBRANE VESICLES (PMV) AND LIVING CELLS

Julen, R. and Koblet, H., Institute of Medical Microbiology, University of Berne, Friedbühlstrasse 51, CH-3010 Berne, Switzerland

In previous work we demonstrated the usefulness of chemically induced PMV for studying virus binding per se omitting endocytosis. Here we present data characterizing SF viral binding to the cell surface using this model and, for comparison, living cells. Thereby the need of divalent cations for virus attachment could clearly be demonstrated. Our results further suggest an involvement of sialic acids in SFV binding sites. Both, removal of sialic acid residues from PMV by neuraminidase treatment and free sialic acid present in the incubation mixture led to a reduction of PMV binding ability. These results could be confirmed by experiments using living Vero cells. Similar treatments strongly reduced the susceptibility of the cells to SFV infection. Studying fusion between viral and plasma membranes represents another application of PMV.

497

CAN INCOMING CAPSID PROTEINS CONTROL VIRAL DNA REPLICATION?

N.Previsani, M.C.Colomar, B.Hirt
ISREC-1066 EPALINGES

The T-lymphocyte-derived cell line EL4 is a host for the immunosuppressive strain of minute virus of mice MVMi. The prototype strain MVMp, in contrast, grows specifically in A9 fibroblasts. For lytic growth of MVM in lymphocytes, two regions of the i genome are required: iE mapping from nt 1084 to nt 2070 and iL from nt 3523 to nt 4339. For growth in fibroblasts, pL (the region from MVMp analogous to iL) is sufficient.

Here we focus on how iL and pL function in EL4 cells. Coinfection experiments using MVMi and a recombinant MVMi virus modified to contain pL in place of iL (MVMp3) showed that MVMi cannot help the replication of the hybrid p3 viral DNA in trans. Since pL lies in the coding region for the capsid proteins, this result led to a hypothesis that incoming capsid proteins remain associated with viral DNA and inhibit viral replication in cis. To test this hypothesis we are transfecting purified MVMp3 RF DNA into MVMi-infected EL4 cells.

If the incoming capsids of MVMp were responsible for the absence of MVMp3 DNA replication, the transfected p3 DNA should be able to replicate.

498

Identification and Preliminary Characterization of the Bovine Herpesvirus-1 Transactivator Protein bICP0.

C. Fraefel, M. Ackermann, M. Engels, E. Loepfe, B. Vogt, and M. Schwyzer, Institut für Virologie, vet. med. Fakultät der Universität Zürich, 8057 Zürich

Bovine herpesvirus-1 (BHV-1) contains four immediate-early (IE) genes involved in regulation of the productive cycle of replication. A spliced IE RNA (2.9 kb) and an unspliced early RNA (2.6 kb) are 3' coterminal and exhibit a common open reading frame with a coding potential of 676 amino acids. The putative protein contains a cysteine-rich zinc finger domain and is a strong transactivator in transient expression assays. Because of the structural and functional homology to ICP0 of herpes simplex virus, we named it bICP0. In order to identify the viral protein, we produced polyclonal antisera against synthetic peptides in rabbits. The peptides were synthesized according to the predicted amino acid sequence of bICP0. Each peptide represented a specific epitope of the predicted protein, including the C- and N-termini. Sera against the C-terminus and the N-terminus stained nuclei of BHV-1 strain Jura or K22 infected cells in an in situ immunoassay (black plaque). The bICP0 protein was expected to enter the cell nucleus because of its gene regulating activity. Western Blots revealed a viral protein of about 100 kD, which was synthesized both under IE conditions and at later times of the BHV-1 infection.

499

THERMOSENSITIVE MUTATIONS IN THE ROUS SARCOMA VIRUS *gag* GENE PROTEIN P10 AFFECTS VIRUS PARTICLE FORMATION AND INFECTIVITY

P. Dupraz and P.-F. Spahr, Dept of Molecular Biology, Sciences II, 30, quai Ernest-Ansermet, CH-1211 Geneva 4

The *gag* gene of Rous sarcoma virus (RSV) encodes a protein, p10, of unknown function, located between MA and CA in $Pr76^{gag}$. This protein has a high content of proline and glycine residues and is very hydrophobic. We have inserted Mlu-1 linkers (Thr-Arg) in its sequence at five different positions: 934, 973, 1003, 1048 and 1075. Upon transfection in CEFs cells at 36°C, these mutants produced as many virus particles as the wild type. The mutant at 973 showed a very low infectivity, while the others were fully infectious. However, at 41°C, the mutations at positions 973 and 1075 inhibited virus particle release by a factor of 20 and abolished infectivity. Interestingly, both linker insertions were near proline rich regions of the protein. Intracellular viral protein analysis showed that the mutants synthesized $Pr76^{gag}$ at a rate similar to that of the wild type. Together, the data support a role for p10 in budding processes as well as in an early step on infection. We are now constructing cell lines that would constitutively shed these defective thermosensitive retrovirus particles. Further characterization of these p10 thermosensitive mutants is under way, and results will be presented.

500

EXPRESSION OF THE MOUSE MAMMARY TUMOR VIRUS ORF PROTEIN IN MAMMALIAN CELLS

C. Krummenacher and H. Diggelmann

Swiss Institute for Experimental Cancer Research 1066 Epalinges

The Mouse Mammary Tumor Virus (MMTV) long terminal repeat contains an open reading frame (orf) of 960 base pairs encoding a 36 kDa polypeptide with a putative transmembrane sequence and five N-glycosylation sites. The product of this gene has never been detected in MMTV infected cells. In vivo, the presence of the GR-orf gene is correlated with the clonal deletion of $V\beta^{14}$ bearing T cells.

Recombinant vaccinia viruses containing complete or truncated GR-orf sequences under the control of an early vaccinia promoter have been made. The ORF protein expressed in mammalian cells is a glycoprotein of 47 kDa. In this system, its half-life time is relatively short. In vivo experiments suggest that the GR-ORF expressed in recombinant vaccinia virus can act as superantigen and delete $V\beta^{14}$ bearing T cells in infected mice.

501

OVEREXPRESSION OF THE PB2 PROTEIN OF INFLUENZA VIRUS ABROGATES THE ANTIVIRAL ACTIVITY OF THE MURINE MX1 PROTEIN

M. Stranden and J. Pavlovic. Institute for Medical Virology, University of Zürich, CH-8028 Zürich, Switzerland

The interferon-induced murine Mx1 protein possesses an intrinsic antiviral activity with selectivity for influenza viruses. Mx1 accumulates in the nucleus and inhibits the replication of influenza virus at the level of primary transcription.
To examine, whether viral proteins interact with Mx1, we stably transfected a murine cell line derived from a A2G mouse with expression-vectors carrying cDNAs of the virus polymerase subunits PB1 and PB2 or of the nucleoprotein (NP) of the viral transcription-complex. A2G cells (Mx+) are resistant to influenza virus infection when pretreated with interferon-a/ß. Interferon treatment of A2G cells expressing PB1, PB2 and NP induced Mx1 synthesis to high levels. Stable expression of PB1 and NP in A2G cells had no influence on the antiviral activity of Mx1. However, the PB2 expressing cells remained partially sensitive to influenza virus infection.
Neutralization of the Mx1 protein by expression of PB2 protein, suggest that Mx1 interferes with the normal function of the influenza virus RNA polymerase either by removing the PB2 subunit from the functional complex or by competing with PB2 for the relevant partners in the polymerase complex. The nature of this interaction is now under investigation.

502

Expression of the Mouse Mammary Tumor Virus env proteins from a recombinant vaccinia virus vector.

Fuchs, R. and Diggelmann, H.
Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges, Switzerland

A recombinant vaccinia virus has been made with the MMTV env gene. The foreign gene is expressed under the control of the vaccinia 11K late promoter. In CV1 and in COMMA cells, the expression of the precursor Pr73 env and of the glycoprotein gp52 has been demonstated by immunoprecipitation with a polyclonal anti-gp52 antibody after ^{35}S-methionine labelling. This antibody also detected these proteins by immunostaining of a western blot. Immunofluorescence staining of CV1 and COMMA cells with a monoclonal IgA anti-gp52 antibody demonstrates the presence of MMTV specific proteins in the cytoplasm and on the surface of infected cells. The recombinant vaccinia viruses will be used to immunize mice against MMTV.

503

PURIFICATION AND CHARACTERISATION OF THE GTPASE ACTIVITY OF RECOMBINANT Mx1 AND MxA PROTEIN

A. Schröder, P. Hüssy, C. di Paolo, F. Pitossi and J. Pavlovic, Institut für Medizinische Virologie der Universität Zürich, CH-8028 Zürich

Human MxA and murine Mx1 are closely related interferon-induced proteins with intrinsic antiviral activities. Both proteins inhibit influenza virus multiplication, although at different steps of the replication cycle. Mx proteins are members of a new family of GTP-binding proteins and possess an intrinsic GTPase activity. GTP-binding or GTPase activity of Mx proteins appears to be necessary for their antiviral function.
To further elucidate the molecular mechanism of influenza virus inhibition by Mx proteins, we attempt to test the function of purified Mx proteins in *in vitro* transcription and translation systems of influenza virus. First, we expressed recombinant Mx1 and MxA proteins, with a His-tag at their N-terminus, in *E. coli* or in Swiss 3T3 cells and purified them under nondenaturing conditions by Ni-chelate affinity chromatography. Mx proteins carrying mutations in the GTP-binding site were used as controls. Mx1 and MxA but not the mutant proteins hydrolysed GTP to GDP. The Km values for the GTPase activity of recombinant Mx1 and MxA were in the range of 0.1 to 1.0. The hydrolysing activity of Mx proteins was specific for GTP. We currently test whether the recombinant Mx proteins are able to inhibit distinct influenza virus replication steps using *in vitro*-systems.

504

MODULATION OF STRESS RESPONSE, THERMOPROTECTION, AND TRANSLATIONAL TOLERANCE BY SEMLIKI FOREST VIRUS (SFV) CAPSID (C) PROTEIN

Michel, M.R., Studer, E., Favre, D. and Arrigo, A.-P.*, Institute of Medical Microbiology, University of Berne, CH-3010 Berne, Switzerland; *Molecular and Cellular Genetics, CNRS UMR-106, Claude Bernard University, Lyon, France.

It is well documented that transiently, thermotolerant cells will survive otherwise lethal heat shock challenges. Biologically active C protein of SFV acts in the target cell as a complex pleiotropic regulator of host cellular protein synthesis. Here we show that low amounts of C protein (10^3-10^4 molecules per cell) transferred by electroporation conferred heat resistance to the target cell. In contrast, high amounts (10^5-10^6 molecules per cell) resulted in a significant thermosensitivity. Moreover, in a cell-free translation system, low concentrations of added C protein led to an increased translational efficiency at 30°C and a translational tolerance at the supra-optimal temperature of 43°C. In contrast, high concentrations were responsible for the shut-off of protein synthesis at 30°C, but did not abolish translational tolerance at 43°C.

505

FIRST DIRECT EVIDENCE FOR CIRCULAR AND LINEAR GENOMES OF A NEUROTROPIC HERPESVIRUS DURING LATENCY IN THE TRIGEMINAL GANGLIA

Wirth, U.V. Dept. of Biol. Chemistry, KZO F. Glattal, CH - 8600 Dübendorf

Bovine herpesvirus 1 (BHV-1) is an important pathogen of cattle. Like human herpesvirus HSV-1, BHV-1 establishes a latent state in the peripheral nervous system. For HSV-1 the viral DNA genome was shown to be linear in virions, a rolling circle mechanismn was postulated during lytic infection and some evidence for joined genome ends during latency is available.

Here I will present some data about the genome configuration of BHV-1 analyzed by pulsed field gelelectrophoresis (PFG) and Southern blot technique. To examine the latent DNA in the bovine trigeminal ganglia (a tissue with high percentage of connective and nervous fibers) a novel tissue preparation technique was developed without any shearing of DNA for characterization by PFG.

As expected BHV-1 DNA in virions was shown to be linear with size of about 140 kb. BHV-1 DNA obtained from lytically infected bovine tissue culture cells exhibited mainly a linear unit size of 140 kb,in addition a smear above and below this band was observed. BHV-1 DNA was detected in one of three ganglia from seropositve cattle. In this ganglia of a latent infected animal, most of the DNA was found to be circular and a small amount linear unit size. Most interestingly the circular DNA appeared smaller than unit size. Southern blot analysis using genomic subfragments of BHV-1 revealed some evidence for deletion of certain BHV-1 DNA sequences in the circular genomes during latency. The coexistence of a high amount of circular genomes with deletions and a low amount of linear unit size genomes gives a new perspective for regulation of neurotropic herpesvirus latency!

506

SEQUENCE ANALYSIS OF THE PORCINE EPIDEMIC DIARRHOEA VIRUS MEMBRANE AND NUCLEOCAPSID GENES

Tobler, K., Bridgen, A. and Ackermann, M.
Institut für Virologie, Winterthurerstrasse 266a, 8057 Zürich.

We cloned 4 kbp of the porcine epidemic diarrhoea virus (PEDV) genome following polymerase chain amplification of the cDNA using degenerate primers based on conserved coronaviral sequences. Sequence analysis of this cloned DNA revealed two major open reading frames. The translated products showed good homology to other coronavirus membrane and nucleocapsid proteins, particularly to those of human coronavirus 229E (HCV 229E) and porcine transmissible enteritis virus (TGEV). The PEDV membrane protein showed greater homology to those of other coronaviruses than did the nucleocapsid protein, which was longer than the corresponding HCV 229E or TGEV proteins. A second, smaller, open reading frame was present within the PEDV N gene, similar to the I gene of bovine coronavirus. The PEDV genome also contained RNA motifs typical of coronaviruses including a 7 base intergenic sequence and an 11 base sequence near the poly A tail. These results confirm the previous, provisional, classification of PEDV as a coronavirus.

507

GENOMIC HETEROGENEITY OF BOVINE PESTIVIRUSES

Hertig, C.; Stalder HP. and Peterhans, E., Institute of Veterinary Virology, University of Bern, Länggass-Strasse 122, CH-3012 Bern, Switzerland

We have amplified and sequenced parts of the genomes of 12 laboratory strains of bovine viral diarrhoea/mucosal disease virus. The virus strains originated from North America and Europe.The cumulative sequence heterogeneity of the amplified fragments located in the region of the nonstructural protein p80 was 24% as compared to 47% in the gp53 region. The nucleotide changes did not alter the deduced amino acid sequence in p80. In contrast, the nucleotide changes located in gp53 resulted in mutations of 42% of deduced amino acids. These results indicate that the viral surface glycoprotein gp53 is under considerable immunological pressure while conservation in p80 indicates that this protein is subject to constraints related to its function in the replication of the virus.

Analysis of the genome of virus from persistently infected animals immunotolerant to their "own" virus revealed that both the p80 and gp53 fragments remained identical over a period of 11 months. Although restricted to selected regions of the viral genome, our analysis suggests that immunological tolerance may limit or even "freeze" the evolution of BVD/MD viruses despite the high mutation rate typical of RNA viruses.

508

COMPARISON OF CORONAVIRAL CONSERVED SEQUENCES

Bridgen, A., Tobler, K. and Ackermann, M.
Institut für Virologie, Winterthurerstrasse 266a, 8057 Zürich.

Our work with the coronavirus porcine epidemic diarrhoea virus (PEDV) has led us to look more closely at RNA and protein sequences and sequence motifs which are conserved between different coronaviral genomes. Many of these are specific to coronaviruses, while others are shared with other positive stranded RNA viruses, for example Berne virus and equine arteritis virus. Functions can be assigned to some of these conserved regions, for example the spike and membrane protein trans-membrane regions or the intergenic "leader RNA" binding site. Other well conserved domains, including regions within the membrane and nucleocapsid proteins, have as yet no defined role. The study of such conserved sequences has two purposes 1) the identification of functionally important regions of the viral RNA and proteins and 2) application to genome amplification by polymerase chain reaction as an aid to cloning. In this poster we compare some of these conserved regions. We also describe the use of the latter technique for the cloning of 4 kbp of the PEDV genome, including the membrane and nucleocapsid protein genes, at a time when nothing was known of the PEDV sequence or of its relationship to other coronaviruses. This technique is therefore applicable to the cloning of other coronaviruses since it is fast, efficient and avoids extensive initial clone screening.

509

INDIRECT AND DIRECT IN-SITU POLYMERASE CHAIN REACTION (IN-SITU PCR) FOR VIRAL DNA DETECTION

Komminoth P., Long A.A.and Wolfe H.J., Dept. of Pathology and Medicine, University of CH-8091 Zurich and Tufts University, Boston MA, USA.

We recently reported the detection of rearranged genomic and viral DNA by indirect in-situ PCR in cell preparations (Diagn Mol Pathol 1: 85-97, 1992) using single primer pairs for in-situ DNA amplification and oligonucleotide probes for subsequent detection of PCR-products by in-situ hybridization (ISH). In this study we tested the feasability of different in-situ PCR techniques in detection of CMV and HBV DNA in tissue sections. We compared 1. indirect in-situ PCR with single primer pairs and oligo-nucleotide probes, 2. indirect in-situ PCR with multiple primer pairs and genomic probes, and 3. direct in-situ PCR incorporating labeled nucleotides into PCR-products and immunohistochemical detection without ISH.

In-situ DNA amplification using multiple primer pairs followed by ISH with genomic probes provided clear evidence of amplification on tissue sections. In-situ PCR with single primer pairs and an oligonucleotide probe yielded negative results. Direct in-situ PCR resulted in non-specific nuclear signals, which were thermal cycling and Taq-polymerase dependent and were also observed in experiments with omission of primers in the PCR reaction mixture, excluding mis-priming as a possible cause. These non-specific nuclear signals may be related to internal priming and incorporation of labeled nucleotides through DNA repair. Indirect in-situ PCR provides more specific results and can be applied to tissue sections when modified to include multiple primer pairs and genomic probes. Direct in-situ PCR is not suitable for tissue sections with presently used protocols.

510

POLYDNAVIRUS OF A PARASITIC WASP: CHARACTERIZATION AND ANALYSIS OF REPLICATION

U. Albrecht, E. Kurt, T. Wyler, R. Pfister, D. Schümperli and B.Lanzrein, Abt. Entwicklungsbiologie, Baltzerstr. 4, Universität Bern, CH-3012 Bern

Parasitc wasps of the genus Chelonus inject their eggs into eggs of various lepidopterous species. The parasitoid larvae develop within the developing host larvae; parasitisation causes hosts to enter metamorphosis one instar earlier than non-parasitised larvae. The wasp females inject, together with the parasitoid egg, venom and calyx fluid which contains polydnavirus. This virus appears to be essential for successful parasitoid development. We observed that the viral genome consists of at least 10 different classes of circular double-stranded DNA molecules with a length of 7 - 31 kb. Various circles were cloned and characterized by restriction analysis. Southern blot experiments revealed that viral DNA exists in the wasp in two forms, either integrated in the genome or extrachromosomally. Both forms exist even in tissues from males which are not known to produce polydnavirus particles. Replication of extrachromosomal viral DNA was seen to begin in females at a specific stage of pupal development. We are currently analysing the structure of integration sites of individual viral DNAs in the wasp genome.

511

THE ROLE OF THE HOST FACTOR IN THE INITIATION OF RNA SYNTHESIS BY Qβ REPLICASE ON Qβ PLUS AND MINUS STRAND RNA TEMPLATES

Imma Barrera, Daniel Schuppli, José M. Sogo+ and Hans Weber
Institut für Molekularbiologie I, Universität, 8093 Zürich, and +Institut für Zellbiologie, ETH-Hönggerberg, 8093 Zürich

Our earlier work on the recognition of bacteriophage Qβ plus strand RNA by Qβ replicase had shown that specific binding occurred at two internal RNA sites, the S- and the M-site, but that binding at the 3'-end (the site of initiation of synthesis) was not detectable. We now found that double-looped structures resulting from simultaneous binding at the 3'-end and at the two internal sites could be demonstrated by electron microscopy after initiation of RNA synthesis in presence of host factor, GTP and ATP. Unexpectedly, similar structures were also found for complexes consisting of plus strand RNA and host factor without replicase. This suggests that the role of the host factor on the plus strand template is to bring the 3'-end into the proximity of the S-site/M-site domain, where replicase can initiate on it. Kinetic studies of initiation by replicase showed that the plus strand, even in presence of host factor, was a far slower and less efficient template than the minus strand; for the latter, host factor had a substantial inhibitory activity. Our findings are easily explained in the context of a template specificity model based on steric exclusion developed earlier.

512

REPEATED DNA SEQUENCES ON CIRCULAR AND LINEAR PLASMIDS OF THE SPIROCHETE *BORRELIA BURGDORFERI*

Zückert, W.R., Filipuzzi-Jenny, E., and Meyer, J.
Zahnärztliches Institut, Abt. PZMOM, CH-4051 Basel

Like other *Borrelia* species, *B. burgdorferi* displays a quite unique feature among prokaryotes: Its genome consists of a linear chromosome complemented by circular and linear plasmids. We cloned a 3.8 kb *Eco*RI fragment from the 29 kb circular plasmid of the type strain B31, which hybridized to several additional restriction fragments of the 29 kb circular (1.4, 6.4, 7.0 kb *Eco*RI) and the 50 kb linear plasmid (4.5 kb *Hind*III) of this strain. These were also cloned and their crosshybridizing segments identified on subclones. Partial DNA sequence analysis revealed > 90 % homology over several hundred bp. Whether a second region of homology occurs on some of these fragments is still under investigation. Signals with linear and circular plasmids of other Swiss and American *B. burgdorferi* isolates were also detected. The repeated DNA seems to be species-specific and therefore may be useful in DNA diagnostics of Lyme disease.

513

RESEARCH OF *CHLAMYDIA TRACHOMATIS* (CT) rRNA AND DNA IN SYNOVIAL FLUID CELLS (SFC)

S. BAS and T.L. VISCHER, Division de Rhumatologie, Hôpital cantonal universitaire, Geneva, Switzerland.

Reactive arthritis may follow urethritis or cervicitis caused by CT but the process leading to joint inflammation from a distant infection is not known. An important question concern the presence or not of viable CT in the joint. To resolve whether whole bacteria or merely antigenic fragments were present in reactive arthritis synovial fluid, we tried to detect bacterial rRNA or DNA.

The presence of CT rRNA was tested on SFC of 9 suspect patients and 55 controls patients. This was performed by hybridization with a chemiluminescent labeled, single-stranded DNA probe complementary to the rRNA of CT (GEN-PROBE PACE 2 SYSTEM, San Diego, California) in a liquid reaction mixture [1]. Without cells, the sensitivity of the probe was of 1000 elementary bodies. In presence of 28.5 to 60.10^6 SFC, the sensitivity was of 10 000 elementary bodies. The RLU (relative light units) values were comparable for both groups.
The presence of CT DNA was searched in SFC of 14 suspects patients and 13 controls patients. The polymerase chain reaction (PCR) was used to detect a sequence of CT plasmid DNA considered to be essential for chlamydial growth. After amplification by PCR in presence of specific oligonucleotide primers amplifying a sequence of 201 bp [2] and hybridization with an internal digoxigenin labelled DNA probe of 114 bp, the sensitivity of the method in presence of 5 µg of SFC DNA was of 10 elementary bodies. In these conditions we found 7 positive results from the suspected patients and none from the controls. The working conditions have been established with this system and we are trying now to confirm these results with two other sequences (one for plasmid DNA and one for genomic DNA).

1. Urdea, et al., Clinical Chemistry, 1989, **35**, 1571-1575.
2. Griffais, R. and Thibon, M.,, Res. Microbiol., 1989, **140**, 139-141.

514

Antigenic variation in *Borrelia burgdorferi* after passage through *Ixodes ricinus* and *Ixodes hexagonus* ticks.

Hu C.M., Gern L., Toutoungi L.A., Aeschlimann A. Institut de Zoologie de l'Université, 2000 Neuchâtel.

In Europe, *Borrelia burgdorferi (Bb)*, the causative agent of Lyme borreliosis is transmitted to humans and/or animals by *Ixodes ricinus (Ir)* and *I. hexagonus (Ih)*.This means that Bb spend part of its life cycle within the arthropod, an environment completely different from that found within the vertebrate hosts. In this study , 9 *in vitro* grown Bb isolates were artificially reintroduced into Ir and Ih and afterwards recultivated to determine wether the passage through ticks influences the protein and antigen profiles of Bb. All isolates and reisolates were examined by SDS-Page and Western Blot using poly- and monoclonal antibodies. Comparing initial isolates with the reisolates, 7/9 presented modifications of the protein and antigen profiles with respect to outer surface proteins A, B and the 22kD protein after passage through Ir. The same modifications were observed after passage through Ir and Ih using a cloned isolate. The fact that the phenotype of Bb changed during residence in the tick suggests that ticks provide the environment which leads to the variation of one or the other epitope of Bb antigens.

515

Ω2005 (*fem*C::Tn*551*) Inactivates a Putative Regulatory Element in the *Staphylococcus aureus* Glutamine Synthetase Operon? J. E. Gustafson*, B. Berger-Bächi and F. H. Kayser. Institute for Medical Microbiology, University of Zürich, Zürich, Switzerland.

We have recently reported the existence of four distinct chromosomally located sites *fem*AB, *fem*C and *fem*D, by Tn*551*-mutagenesis, lowering the level of resistance in methicillin resistant *Staphylococcus aureus* strain BB270 (*mec*). We now report the cloning and sequencing of a 2.6 kb *Hind*III fragment containing the 1.0 kb right end of Tn*551* and an adjacent 1.6 kb chromosomal DNA junction fragment of the *fem*C region from strain BB589 (BB270, Ω2005 *fem*C::Tn*551*). Tn*551* has integrated upstream of an open reading frame that has 76.5% identity at the amino acid level with the *Bacillus cereus* glutamine synthetase (GS). By location and analogy with the *B. cereus* GS operon, we suspect the insertion has occurred in a possible *S. aureus* GS repressor. Northern blots probed with the putative GS coding region revealed a 1.9 kb transcript in BB270 that was replaced by a weak, approximately 7.0 kb transcript in BB589. Ω2005 (femC::Tn551) thus alters the size and relative amount of the putative GS-transcript, suggesting that the lowering of methicillin resistance observed in the *fem*C mutant is due to this alteration.

516

FUNCTIONAL INTERACTION OF HUMAN NADPH-CYTOCHROME P450 REDUCTASE AND HUMAN CYTOCHROME P450 1A1 IN *S. CEREVISIAE*

C. Sengstag, H.-P. Eugster, S. Bärtsch and F. E. Würgler

Institute of Toxicology, Swiss Federal Institute of Technology, Schorenstrasse 16, 8603 Schwerzenbach near Zurich, Switzerland

For studies of human specific activation of promutagenic compounds, a cDNA coding for the human cytochrome P450 enzyme CYP1A1 was functionally expressed in *Saccharomyces cerevisiae* . With the idea of further increasing the heterologous enzyme activity, a human NADPH-cytochrome P450 reductase (hOR) cDNA was coexpressed in the strain expressing human CYP1A1. Furthermore, to characterize the hOR enzyme in more detail the hOR expression plasmid was introduced into a strain where the yeast homolog *CPR1* had previously been disrupted. From these experiments it was concluded that: (1) The *CPR1* gene is not essential for growth. (2) The *cpr1::URA3* disrupted strain exhibits no cytochrome-*c*-reductase activity. (3) The *cpr1::URA3* disruption confers sensitivity towards ketoconazole. (4) Heterologous hOR expression complements the *cpr1::URA3* disruption. (5) The hOR enzyme exhibits a four times higher affinity than the yeast enzyme for the substrate NADPH. (6) The hOR enzyme can functionally interact with human CYP1A1 enzyme resulting in a more than 16 fold increase in ethoxyresorufin-deeethylase activity.
Ref: Eugster et al. (1992) Biochem. Biophys. Res. Com. 185, 641-647

517

ADENINE REGULATED GENES IN *SCHIZOSACCHAROMYCES POMBE*

K. Stockmaier, A. Zurlinden, L. Edenharter, and M.E. Schweingruber
Institut f. allg.Mikrobiologie, Universität Bern, Baltzerstr. 4, 3012 Bern

Expression of *pho1* encoded acid phosphatase of *S.pombe* has been reported to be regulated by phosphate. Here we show that in addition it is regulated by adenine.
Starving adenine auxotrophs for adenine, we find a drastic increase of *pho1* encoded acid phosphatase activity. This derepression of *pho1* occurs at the mRNA level and is dependent on non limiting phosphate concentrations in the media.
In order to find genes, that are involved in this adenine-dependent regulation, we screened for mutants exhibiting high aPase activity in adenine excess. The mutants define four complementations groups (*anr1, anr2, anr3, anr5*) and they also display defective phosphate regulation of *pho1*.
Adenine as well as phosphate are necessary to repress *pho1* expression. We speculate, that the regulating signal is a compound of the adenine nucleotide pool.
To learn more about genes regulated by the adenine nucleotide pool, we screened a cDNA gene library by differential hybridization and found a gene, which is also repressed by adenine as well as dependent on the *anr* genes. A characterization of this gene will be given.

518

FURTHER CHARACTERIZATION OF THE *S.CEREVISIAE* MUTATION *TRP5A*

Ehrenhofer-Murray, A., Sengstag, Ch. and Würgler, F.E., Inst. für Toxikologie der ETH und Univ. Zürich, CH-8603 Schwerzenbach

Oncogene activation and tumor suppressor gene inactivation can be attributed to genetic changes such as point mutations, chromosome losses, mitotic recombinations and gene conversions, which take place within the future tumor cell and eventually lead to the development of a tumor. The ability of a chemical to induce such genetic changes can be easily tested in the *S.cerevisiae* test strain YHE2, a derivative of strain D7.
In order to detect test substance induced gene conversions, we work with two mutant alleles of the *TRP5* locus. One of these, the allele *trp5a*, carries a stable mutation (reversion rate $<10^{-7}$). Therefore we were interested to characterize it in order to gain further insight into the observed gene conversion events.
Our analysis revealed four mutations in the *trp5a* allele. In contradiction to the Kozak rule, the transition A to G at position -3 before ATG has no influence on gene expression. The transition C to T at position 168 after ATG results in an amber stop codon which abolishes gene expression. Two further mutations in the 3' untranslated region of the *trp5a* gene, transitions G to A lying 182 and 413 bp downstream of the open reading frame, also contribute to the phenotype of yeast strain YHE1 *(trp5a)*, since its tryptophane prototrophy is not amber-suppressable.The extent of these contributions remains to be determined.

Pharmacology and Toxicology

519

BENZODIAZEPINE RECEPTOR LIGAND ACTIVITY (BZRLA) IS INCREASED IN RATS WITH HEPATIC ENCEPHALOPATHY (HE) ONLY AFTER DIAZEPAM P.O.

P. Widler*, H.U. Fisch*, P. Schoch**, A. Zimmermann***, J. Reichen*. Depts of Psychiatry, Clinical Pharmacology* and Pathology***, University of Berne, Switzerland, F. Hoffmann-La Roche**, Basel, Switzerland.

Increased BZRLA has been related to the mechanism of HE. Acute liver failure was induced in adult male Sprague-Dawley rats by intraperitoneal injections of thioacetamide (TAA, 600mg/kg/day for 3 days). All 17 TAA-rats developed HE stage III-IV (behavior, activity monitor, ASAT (1898±1359 U/l), bilirubin (36±27 µmol/l) and centrolobular necrosis in the liver). Respective values for the 14 control rats were: ASAT 45±5 U/l, blilirubin 1.5±0.5 µmol/l. Whole brain BZRLA activity measured with a 3H-Flumazenil binding competition assay was similar in both TAA (52.7±34.1 ng/g brain weight) and control rats (44.3±18.2 ng/g, n.s.). In a control experiment with the same model diazepam (1mg/kg/day p.o.) was given for 3 days. BZRLA activity in TAA-rats was 223±65ng/g, (n=5), in controls 103±23ng/g, n=5, p=0.002. We conclude that the increased BZRLA activity in brain of HE rats is neither necessary nor sufficient to explain the pathogenesis of HE.

520

GAMMA-GLU-GLN AND EXCITATORY AMINO ACIDS (EAA) IN CEREBROSPINAL FLUID (CSF) OF UNTREATED SCHIZOPHRENIC PATIENTS.

K.Q. Do[1], C. Lauer[2], W. Schreiber[2], M. Cuenod[1] and F. Holsboer[2]. [1]Brain Res. Inst., Univ. of Zürich, Zürich, Switzerland; [2]Max-Planck-Inst. for Psychiatry, Munich, FRG

Pathophysiological theories of schizophrenia have emphasized dysfunctions in dopaminergic (DA) systems, although a role of EAA systems has also been proposed. Lumbar CSF of 19 patients with schizophrenic disorders (according to DSM-III-R; all patients were off drugs for at least 1 year) and of 16 age- and sex-matched controls were analyzed by optimized precolumn o-phtalaldehyde derivatization HPLC. Performing an analysis of covariance with age and sex as covariates, taurine and tyrosine were found to be significantly decreased in the patients. The remaining amino acids evaluated (e.g., aspartate, glutamate and sulfur containing EAA) as well as the catecholamines metabolites did not differ between patients and controls. However, the level of a compound (P15.5) which we recently identified as γ-glutamyl-glutamine (as it coeluted with the dipeptide at 3 different pH) was significantly decreased by 13-18% in the patients. The results rather do support a possible role of γ-Glu dipeptides in the pathogenesis of schizophrenic disorders than is the case for DA or EAA systems.

521

ANALYSIS OF THE ENANTIOMERS OF CITALOPRAM (CIT) AND ITS METABOLITES IN DEPRESSIVE PATIENTS

B. Rochat, M. Amey, * H. van Gelderen, P. Baumann

Unité de biochimie et psychopharmacologie clinique, DUPA, CH-1008 Prilly-Lausanne, * ICT, Ecole de pharmacie, Université, 1005 Lausanne-Dorigny/Switzerland

The pharmacological activity of the racemic drug CIT, a selective serotonergic reuptake inhibiting antidepressant resides probably rather in the S-(+)-CIT than in the R-(-)-enantiomer (Hyttel et al., J N Transm 88 (1992) 157).
An HPLC-method has been developed for the quantitative analysis of the enantiomers of CIT, its demethylated metabolites and of the propionic acid derivative (PROP) in the plasma of depressive patients treated with CIT. The basic and acidic compounds were isolated from 2 ml plasma samples using liquid-liquid or solid phase extraction and derivatised with HFBA or methyl iodide, respectively. The extracts were submitted to HPLC (Chiracel OD; fluorescence detection). Identification of the enantiomers was performed with an optical rotation detector. Preliminary results about the stereoselectivity in the metabolism and steady-state kinetics of CIT in patients will be presented.
Financed by FNRS (32-27579.89) and Cost B1.

522

FACTORS INFLUENCING BINDING OF (+)-S- AND (-)-(R)-1-METHYL-5-PHENYL-5-PROPYL-BARBITURATE TO SERUM ALBUMIN

Büch H.P., Altmayer P.* and Drumm R., Institut für Pharmakologie und Toxikologie, Klinik für Anaesthesiologie und Intensivmedizin*, Universität des Saarlandes, W-6650 Homburg/Saar, Germany

(-)-(R)-1-methyl-5-phenyl-5-propyl-barbiturate [(-)-(R)-C] is preferentially bound to human serum albumin [HSA]: its total binding constant is nearly 3-fold higher than that of (+)-(S)-C. Question arose whether this stereoselective difference is dependent on the temperature (range: 4 - 48°C), pH-value (range: 5 - 10.2) and serum albumin species (bovine, rabbit and rat). In all experiments equilibrium dialysis was performed in order to determine % binding of the enantiomers. Using HSA it is shown that the stereoselectively caused binding difference depends not on the pH-value, yet decreases markedly with increasing temperature. Species specificity of the stereoselective different binding becomes obvious when other serum albumins are used: e.g. bovine serum albumin binds - at the opposite to HSA - (+)-(S)-C to a much higher extent than (-)-(R)-C.

523

EFFECTIVENESS OF A PHENYTOIN DOSING REGIMEN USING BAYESIAN FORECASTING FOR DOSAGE INDIVIDUALIZATION IN HOSPITALIZED PATIENTS

E. Martinelli, S. Mühlebach, Dept. of Pharmacy, Kantonsspital Aarau, Switzerland

More than 40 hospital-admitted patients with acute cerebral disorders (aneurisms, polytrauma, cerebral surgery, epilepsy and others) running a high risk for posttraumatic seizures, were treated prophylactically with phenytoin. Drug dosage and serum level monitoring was prospectively followed up over 17 ± 20 days (mean ± SD). The drug regimen consisted in a rapid i.v. loading dose (15 mg/kg infused over 4 hours), followed by 175 mg (≤ 70 kg) or 200 mg (> 70 kg) every 12 hours, for 5 days. Thereafter, individual dose adapatation (i.v. or p.o.) was based on calculation of Michaelis-Menten kinetics. Using Bayesian forecasting three serum level determinations (one prior to the second dose, the others prior to the morning doses of day 4 and 5) allowed a first dose recommendation. Dose adaptation was given after each additional serum level determination (once to twice a week). 16 hours after initiation of therapy more than 85% of the patients showed a plasma trough level of 9-20 mg/L; before starting individualized dosing more than 60% of patients remained within this range. The serum level prediction showed an over all accuracy of 4.6 mg/L ± 0.35 (mean of absolute error ± SEM) and a bias of -0.21 mg/L ± 0.6 (mean ± SEM). Dose range was between 100 and 650 mg/day (i.v.). About 85% of the first maintainance doses had to be adapted. In the patients studied neither posttraumatic seizures nor major intoxications occurred. Therefore, independent of comedication (drug interaction potential), the results of this study indicate a clear patient benefit when using the phenytoin dosage regimen presented. Bayesian forecasting allows rational dose individualization of compounds showing more complex pharmacokinetics and small therapeutic indices. Thereby safety and effectiveness of drug therapy may be greatly enhanced.

524

THE INFLUENCE OF NICOTINE AND TAR YIELD ON COMPENSATORY CIGARETTE SMOKING

Beatrice Baldinger, Markus Hasenfratz and Karl Bättig, Behavioral Biology Laboratory, Swiss Federal Institute of Technology, ETH-Zentrum, 8092 Zurich, Switzerland

As found in an earlier study with cigarettes of varying nicotine and tar yields, tar yield seemed to be more important for regulating smoke intake than nicotine yield, since the smokers compensated only while smoking low tar/low nicotine cigarettes (ultra-light) but not medium tar/low nicotine cigarettes (test cigarette). Hypothesizing compensatory intensification of puffing and inhaling, the present study examined puffing behavior. Twelve female smokers participated in the experiment smoking cigarettes with tar and nicotine yields of 11.4 and 0.9 (habitual); 2.0 and 0.21 (ultra-light); and 9.3 and 0.08 mg/cigarette (test), respectively, resulting in tar/nicotine ratios of 12.6, 9.5, and 116.25. Physiological parameters and subjective ratings confirmed the results of a former switching study. Whereas the test cigarettes produced only weak physiological effects and low taste scores, the effects of the habitual cigarettes were in the expected range and those of the ultra-light cigarettes about halfway in between. The present puffing behavior measurements revealed a greater number of puffs and greater total puff volumes for ultra-light than for habitual and test cigarettes while puff interval was longest for the habitual cigarettes, shorter and similar for the test and the ultra-light cigarettes. These results support the earlier conclusion that ultra-light cigarettes were smoked compensatorily whereas the test cigarettes with a very high tar/nicotine ratio did not produce a comparable behavior.

525

CIGARETTE SMOKING RELATED VARIATIONS OF PULSE AND ACTIVITY UNDER FIELD CONDITIONS

Albert Jacober, Markus Hasenfratz & Karl Bättig
Behavioural Biology Laboratory, Swiss Federal Institute of Technology, ETH-Zentrum, CH-8092 Zurich, Switzerland

Small portable devices were used to record 30-sec averages of heart rate and motor activity over 2 consecutive days and nights. On smoking days, subjects had to press a marker button whenever they lit a cigarette. On abstinence days, when smoking was not allowed, the moments of strong desire to smoke had to be marked. Smoking related pulse (SRP) and activity (SRA) were computed by averaging the 30-sec data across all cigarettes for the 10 minutes preceding and the 10 minutes following the lighting of the cigarettes. The grand means of SRP and SRA started to increase 5 minutes before and reached a maximum with lighting the cigarettes, dropped immediately after lighting to an absolute minimum and while SRA remained at prelighting levels, SRP recovered continuously from 5 minutes after lighting onwards to a maximum. Qualitatively equal curves were obtained for both sexes, for workdays and days off and for subjects with different professions, suggesting an ubiquitous presence of the phenomenon. In contrast to the SRP and SRA of the first 5 cigarettes of the day, which showed the same pattern with even more pronounced increases of SRP 5 minutes after lighting, the SRP of the last 5 cigarettes of the day remained at prelighting levels after cigarette lighting. These findings support the concept of nicotine induced heart rate increases which disappear during the day, and the SRP and SRA of the markings of smoking desire on abstinence days confirmed this view since no SRP increases after these markings occurred. The pronounced increases of SRP and SRA before the lighting of the cigarettes might be interpreted as conditioned unrest, preceding the lighting of a cigarette.

526

Prodrugs of DESFERAL:
An Approach Towards Better Iron Chelation

Jin, Y., Moerker, Th., Flammer, R., Egli-Karmakka, M., Nick, Hp., Schnebli, H.P. and Peter, H.H., Pharmaceuticals Research Department, **Ciba** AG, 4002 Basel, Switzerland

In the search for an orally active form of Desferal® for the treatment of iron overload, we have substituted the 3 hydroxamates and the amine group of desferrioxamine B. A considerable number of modified desferrioxamine B derivatives (prodrugs) have been synthesized and their *in vivo* activity evaluated in animal models. The substitution of the charged and polar functions by hydrophobic groups increases the lipophilicity of the molecules and should improve the absorption of drugs when given orally. Furthermore the modifications of hydroxamates which are the Fe-binding sites of the molecule prevent possible chelation of Fe from food in the GI tract. Many of the tetrasubstituted prodrugs induced significant Fe excretion in both rats and monkeys, indicating that once absorbed, prodrugs are hydrolysed to the active parent compound desferrioxamine B. Several of them were also orally active. Most of the prodrugs, when given subcutaneously have shown long duration of action. This observation has led us to consider these prodrugs as long acting "depot" forms of Desferal. Desferrioxamine B derivatives which were monosubstituted at the amino group have shown similar deferralizing properties and in addition improved chemical stability.

527

PURIFICATION OF N-ACETYLTRANSFERASES IN DROSOPHILA MELANOGASTER

Hintermann E., and Meyer U. A., Department of Pharmacology, Biocenter of the University, CH-4056 Basel

Amine acetylation plays important roles in the regulation of physiological processes as well as in the metabolism of drugs and environmental chemicals. In vertebrates, for example, an N-Acetyltransferase (NAT) which acetylates serotonin, is involved the in seasonal regulation of reproduction and photo-periodism. In insects, dopamine is acetylated, forming an intermediate necessary for sclerotization of the insect cuticle. In addition, NATs may be involved in neurotransmitter inactivation. It is so far unknown whether a single or several different NATs are responsible for all these activities.
Using tryptamine acetylation as activity assay, we have fractionated D. melanogaster homogenate by conventional chromatographic procedures. Two activities could be separated on a MonoQ column by elution with a pH gradient. The electrophoretically almost homogenous enzymes (800x increase of specific activity) have a rel. mol. weight of approximately 30kD. The purified proteins will be used to raise antibodies and to develop probes for molecular cloning. With these tools, we would like to investigate their functional roles and possible relationships to vertebrate NATs.

528

SELECTIVE INHIBITION OF MAJOR HUMAN DRUG METABOLIZING P450 ISOZYMES BY CARBON MONOXIDE

Bonnabry, P., Leemann, T., and Dayer, P., Division de Pharmacologie clinique, Hôpital Cantonal Universitaire, CH-1211 Genève 14

Carbon monoxide (CO) binding to protoporphyrin IX is a typical feature of all cytochrome P450 enzymes and inhibition of monooxygenation reactions by CO is often used to identify the catalyst as a P450. The selectivity of CO binding to specific P450 isozymes was investigated by studying the inhibition by CO of prototype reactions for 3 major drug metabolizing P450s in microsomes from 6 different human livers: dextromethorphan (DM) O-demethylation (P450DB1, CYP2D6), diclofenac (DC) 4'-hydroxylation (P450TB, CYP2C subfamily) and midazolam (MZ) 1'-hydroxylation (P450NF, CYP3A subfamily). Substrates were incubated under identical conditions (10 min, 20 µg microsomal protein in 200 µl). Reactions were monitored (metabolite production - HPLC) in 2 different conditions: CO saturation 1) only before starting the reaction (8ml/min bubbling for 2 min), and 2) continuously (before and throughout incubation). Inhibition was virtually complete (> 94 %) for all 3 reactions under continuous saturation. When saturation was not maintained throughout CO displayed different affinity for P450DB1, P450TB and P450NF: 82 ± sd5, 61 ± 7 and 46 ± 5 % inhibition, respectively ($p < 0.01$, ANOVA). Neither substrate concentration, K_m, V_{max} nor CL_{int} (V_{max}/K_m) appeared to have a major influence on CO inhibition.
Selective inhibition by carbon monoxide may be a useful marker of specific human cytochrome P450 isozymes functions.

529

CLONING OF *DROSOPHILA MELANOGASTER* CYTOCHROME P450 GENES

C. Saner, C. Sengstag and F. E. Würgler

Institute of Toxicology, Swiss Federal Institute of Technology, Schorenstrasse 16, 8603 Schwerzenbach near Zurich, Switzerland

Cytochrome P450 genes (CYP450) of insects are involved in the biosynthesis of steroids and the metabolism of other compounds which makes them necessary in development and physiology. In the field of toxicology they are crucial for chemotoxicity and genotoxicity. First they are associated with insecticide resistance (e.g. DDT). Second there is evidence that they are metabolizing genotoxic substances which can be detected in the somatic mutation and recombination test (SMART).
In order to characterize CYP450 genes of *Drosophila melanogaster* we started a cloning project. A first approach, where we screened a third instar larval cDNA library with various human CYP450 cDNAs, failed. This can be explained as follows: either the over all sequence homology is not conserved enough between human and *Drosophila*, or homologous genes are not expressed at this developmental stage. Therefore, we have been using oligonucleotides as hybridization probes, coding for the conserved heme-binding domain, for screening a genomic library. Independent clones obtained were further characterized by sequencing. Prelimanary results will be shown.

530

HPLC AND GC-MS DETERMINATIONS OF TWO ANTI-VIRAL SUBSTANCES IN OCULAR FLUIDS AFTER TRANSSCLERAL APPLICATION.

M. Saugy[1], J.J. Tritten[2] and L. Rivier[1] 1 Unité d'Analyse du Dopage, IUML, 1005 Lausanne 2 Hôpital ophtalmique Jules Gonin , 1004 Lausanne

Cytomegalovirus retinitis is the most frequent infectious ocular complication among AIDS patients. Ganciclovir and foscarnet are widely used virostatic drugs, which have been proven to be effective in stopping the progression of the retinitis. A new way of application these compounds was tested using transscleral iontophoresis in pigmented rabbits.
The analytical work consisted in the quantification of these drugs in different ocular tissues and fluids.
The procedure for the quantification of gancyclovir involves the use of acyclovir, an antiviral drug structurally related to ganciclovir as the internal standard. After a two steps sample preparation, the extract was injected on a HPLC-UV system (lichrosorb RP 18 , 5 µ, 200 x 4.6 mm) using an isocratic program with a phosphate buffer:acetonitrile mobile phase (1 ml/mn). The effluent was monitored at the UV maximum (254 nm) and the method was specific and sufficiently sensitive to allow quantification in a range of concentration from 0.1 µg to 30 µg/ml.
For GC-MS confirmation, after derivatization with MSTFA, TMS derivatives were obtained allowing a very specific detection of ganciclovir and acyclovir.
The same strategy is now used for the quantification and the confirmation of foscarnet.

531

TOXICITY EVALUATION WITH THE COMPUTERIZED "ArtTox" SYSTEM.

Portmann, R. and Holzer, R., Laboratory Spiez, CH-3700 Spiez

Toxicity of a substance is only a relative and not an absolute value. In many circumstances it is therefore satisfactory to be able to evaluate toxicity with a reproducible method. Since Toxicity may also be dependent on metabolic turnover, easily available only in intact organism as e.g. Artemia salina, being no warmblooded animal, no vertebrate, easy to handle etc and allowing a low cost bioassay for toxicity. But there are almost no comparable data available. We therefore decided to standardize the assay condition and use microtiterplates for the test. To speed up the evaluation of different test substances a computer guided program was developped controlling and calculating the needed dilutions and pipetting. An image analyzing unit is recording at preselected time intervals automatically the number of total organism per cavity as well as the swimming velocity. This allows us to evaluate easily the lethal concentration and the effective concentration (Lc 50 / Ec 50) with the special designed analysis program. Data so far obtained, show that the sensibility towards pesticides is comparable to results obtained with rats or mice. A description and photographs of the apparatus as well as a table of over 50 substances tested so far will be presented.

532

Analysis of waste water: Relation between genotoxicity measured in the umuC test and the umuC plasmid copy number

Giuliani, F.,Sahrey, L., Koller, Th. and Widmer, R.M.
Institut für Zellbiologie, ETH-Hönggerberg, 8093 Zürich
Kantonsapotheke Zürich, 8006 Zürich

The waste water of a hospital is monitored for genotoxic activity by the umuC test, a bacterial test which measures the activation of the SOS repair system. Salmonella thyphimurium TA1535 carrying the plasmid pSK1002 with a umuC-lacZ fusion gene is used. The induction of the fusion gene is assayed colorimetrically. Since a change in the plasmid copy number would influence the number of inducible genes and by this the amount of gene product measured, we performed minipreparation of the plasmid after incubating bacteria with different waste waters and known drugs. With chloramphenicol an amplification of the plasmid could be shown after 16 hours, but not yet after 2 hours, the standard incubation time for the umuC test. With mitomycin C, a known genotoxic compound (Albertini et al, Env.Mol.Mut. 12:353-363 ,1988), no influence on the copy number was found. Interestingly, waste water samples, which produced a significant induction in the umuC test, showed a reduced yield of the plasmid. This effect could be due either to the isolation procedure influenced by waste water components or by a particular cytotoxic mechanism.
In addition selected waste water samples were analysed for their mutagenic potential in the Ames test and for their potential to induce the formation of micronuclei in a eukaryotic cell line.

533

Oxidatively modified proteins in iron overloaded mice

Wieland, P., Schranz, Chr. and Lauterburg, B.H., Institute of Clinical Pharmacology, University of Berne, Switzerland

Iron overload in humans is associated with hepatic injury and a high incidence of hepatocellular carcinoma. The toxicity of iron has been attributed to iron dependent Fenton reactions generating highly reactive oxygen species. Yet, the evidence for oxidative damage in chronic iron overload is scarce. We therefore looked for oxidative alterations in the liver of a hemochromatosis model. Mice were fed regular chow containing 1% carbonyl iron for five months. Liver homogenates and isolated mitochondria were incubated with 2,4-dinitrophenyl hydrazine (DNPH). The proteins were washed extensively and analyzed spectrophotometrically after solubilization in 6M guanidine. Carbonyl functions of oxidatively altered amino acids react with DNPH to form a hydrazone absorbing at 370 nm. Sections of liver from iron fed mice showed grade 2-3 stainable iron in macrophages, Kupffer cells and hepatocytes (preferential in peri-portal zone) but no structural damage by light microscopy. In iron fed mice the concentration of protein carbonyls was 5.4 ± 0.6 compared to 2.4 ± 0.2 nmol/mg protein in the controls. In mitochondria the concentration of protein carbonyls was 1.4 ± 0.2 and 0.8 ± 0.1 nmol/mg protein in iron-loaded and control mice, respectively. The present data indicate that iron overload results in a significant increase in the steady state concentration of oxidatively modified proteins in the liver. Oxidatively altered proteins may eventually result in functional and structural cell injury.

534

ELECTRON MICROSCOPY OF LIVER FROM RATS FED PCB CONGENER #126

Singh, A., MacLellan, K., Chu*, I. and Villeneuve*, D.C.
Atlantic Veterinary College, Charlottetown, PE, and *Environmental Health Directorate, Ottawa, Canada.

Groups of 20 weanling Sprague-Dawley rats, divided equally between sexes were given PCB congener #126 (3,3',4,4',5-pentachlorobiphenyl) in their diets at 0, 0.1, 1.0, 10.0 or 100 ppb for 13 weeks. Many liver parenchymal cells from animals fed the congener contained proliferated smooth reticulum, increased number of lipid droplets, and mitochondria with abnormalities. In addition, lipofuscin particles were numerous in the cells from animals of 100 ppb group. Sex differences in lesions were not discernible except in the highest dose group females where lipofuscin particles were relatively higher in number in the hepatocytes. Our results indicate that the congener causes liver alterations in rats at even 0.1 ppb level.

K.M. is an NSERC Undergraduate Research Awardee.

535

CONTROL OF PSEUDOMONAS AERUGINOSA INFECTION IN MICE BY CHLORINE TREATMENT OF DRINKING WATER

F.R. Homberger, Z. Pataki and P.E. Thomann
Institut für Labortierkunde, Universität Zürich

Pseudomonas aeruginosa as a hospitalism agent is a common contaminant in laboratory animal facilities and is transmitted to mice through drinking water. It can cause septicemia and death in experimentaly immunosupressed mice and is therefore undesirable in SPF animals. Different regimes of treating drinking water are currently in use to prevent infection of mice with P. aeruginosa. This study evaluated the effect of different chlorine concentrations (0, 2, 4, 8, and 10 ppm) in drinking water on existing infections or constant reinfections with P. aeruginosa in mice. Animals that were initially infected and recieved water containing 6 or 8 ppm chlorinated water cleared the infection within one week and remained free for the duration of the study. All other animals stayed infected. Mice that recieved 6 ppm chlorinated drinking water and were weekly reinfected with P. aeruginosa withstood the infection for three weeks. All other animals were infected within one or two weeks.