FATTY ACYLATION OF PROTEINS

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1. Introduction

The various biosynthetic modifications of proteins, as for instance the addition of oligosaccharides, phosphate groups and fatty acid chains have become an important area of study in the biomedical sciences. Especially in a time where cloned eukaryotic genes are expressed in bacterial (or other alien) systems yielding non-modified protein products for medical application it would be of interest to know what the biological implications are of using such "unnatural" proteins. Accordingly, three lines of investigations are in progress which aim at the structural analysis of a given modification, elucidating its various biological functions and at an understanding of the biosynthetic event of the modification.

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Here we would like to focus on the hydrophobic modification through covalent linkage of fatty acyl chains, mainly palmitic acid, to a polypeptide backbone. In nature this seems to be verified by three biosynthetic mechanisms, palmitoylation, myristilation and through covalent linkage of phosphotidylinositol to an extended carboxyterminus (for reviews see Schmidt, 1983; Sefton and Buss, 1987; Low et al., 1986).

Following the first reports of fatty acids in polypeptides (Folch-Pi, and Lees, 1951; MacLennan et al., 1972) it was generally believed that this modification would be restricted to a highly specialized class of proteins soluble in organic solvents. When fatty acylation was observed also in typical membrane glycoproteins (Schmidt et al., 1979; Schmidt and Schlesinger, 1979) a subsequent quest for such acylation revealed its wide distribution in nature across all levels of development. Myristic acid in a protein was first detected with cAMP-dependent protein kinase (Carr et al., 1982) and later shown to occur also with various tumor virus related proteins (Schultz et al., 1985). It was then also established that the palmitoylation mentioned above differs principally from this latter myristilation since different sets of proteins were acylated with palmitic acid (Pal) or myristic acid (Myr) in a given cell species. Likewise, the linkage properties differed in these two types of acylproteins (Magee and Courtneidge, 1985; McIlhinney et al., 1985; see below). In order to give an idea of the wide distribution of hydrophobic modifications of proteins in nature, such proteins are listed in tables 1 - 5. Especially with the cellular acylproteins the great functional variety of the respective protein species is striking, whereas with the virus specific acylproteins some common features have become apparent (see below).

TABLE 1

CELLULAR PROTEINS ACYLATED WITH PALMITATE

ACYLPROTEIN SPECIES	REFERENCE
VINCULIN	Burn and Burger, 1987
MHC, HLA B7 HEAVY CHAIN	Kaufman et al., 1984
MHC, HLA DR HEAVY CHAIN	Kaufman et al., 1984
Ia INVARIANT CHAIN	Koch and Hammerling, 1985
GOLGI MANNOSIDASE II	Moreman and Touster, 1985
CONTACT SITE A GP, ACTIN (D.discoideum)	Stadler et al., 1984
ANKYRIN	Staufenbiel and Lazarides, 1986
APOLIPOPROTEIN I	Hoeg et al., 1986
INTERPHOTORECEPTOR RETINOID-BINDING PROTEIN	Bazan et al., 1985
MURINE ERYTHROCYTE SIALOGLYCOPROTEINS	Dolci and Palade, 1985
IgE RECEPTOR	Kinet et al., 1985
TRANSFERRIN RECEPTOR	Omary and Trowbridge, 1981
GOLGI GALACTOSYLTRANSFERASE	Strous et al., 1983
SALIVARY MUCOUS GP's	Slomiany et al., 1983
INSULIN RECEPTOR	Magee and Siddle, 1986
$p21^{ras}$	Sefton et al., 1982
PROTEOLIPOPROTEIN (MYELIN)	Agrawal et al, 1982
RHODOPSIN	O'Brien and Zatz, 1984
LIPOPHILIN	Folch-Pi and Lees, 1951
α-FACTOR (YEAST)	Betz et al., 1987
TGF-α PRECURSOR	Bringman et al., 1987
BUTYROPHILIN	Keenan et al., 1982
XANTHINOXIDASE	Keenan et al., 1982
RAS1, RAS2, (YEAST)	Fujiyama and Tamanoi, 1986
Ca^{2+}-ATPáse (SARCOPLASMIC)	Mac Lennan et al., 1982
DM20 (BRAIN MYELIN)	Agrawal et al., 1982
PO (SCIATIC NERVE MYELIN)	Agrawal et al., 1983
ACETYLCHOLINE RECEPTOR	Olson et al., 1984
DEVELOPMENTAL GP`s (SEA URCHIN)	Bolanowski et al., 1984

TABLE 2

VIRAL PROTEINS ACYLATED WITH PALMITATE

VIRUS SPECIES	ACYLPROTEIN SPECIES	REFERENCE
SEMLIKI FOREST VIRUS	E1, E2	Schmidt, 1982
SINDBIS VIRUS	E1, E2	Schmidt et al., 1979
VESICULAR STOMATITIS VIRUS	G	Schmidt et al., 1979
INFLUENZA VIRUSES	HA_2	Schmidt, 1982
SIMIAN VIRUS 40	TAg	Klockman and Deppert, 1983
CORONAVIRUS (MURINE, BOVINE)	E2	Schmidt, 1982
ADENOVIRUS 12 (Ad 12)	Eb 18K	Grand et al., 1985
HERPES SIMPLEX VIRUS	gE	Johnson and Spear, 1983
LA CROSSE VIRUS	G1, G2	Madoff and Lenard, 1982
SPLEEN FOCUS FORMING VIRUS	gp 65	Srinivas and Compans,1983
MOUSE MAMMARY TUMOR VIRUS	gp 78, gp 70, 38	Firestone et al., 1982
VACCINIA VIRUS	p37	Hiller and Weber, 1985
ADENOVIRUS 2 (Ad 2)	72K	Deppert et al., 1985
NEWCASTLE DISEASE VIRUS	F	Schmidt, 1982
MUMPS VIRUS	F (HN)	Merz and Wolinsky, 1983
PARAMYXO VIRUS YUCAIPA	F	Schmidt, unpublished
ROUS SARCOMA VIRUS	gP35	Gebhardt etal., 1984
FRIEND LEUKEMIA VIRUS	p15E	Srinivas and Compans, 1983

TABLE 3

CELLULAR PROTEINS ACYLATED WITH MYRISTATE

$pp60^{c-SRC}$	Buss and Sefton, 1985
p36	Soric and Gordon, 1985
$p56^{tlk}$ (LSTRA MURINE THYOMA)	Voronova et al., 1984
CALCINEURIN B	Aitken et al, 1982
cAMP-DEPENDENT P.K. (c SUBUNIT)	Carr et al., 1982
NADH-CYT B5 REDUCTASE	Ozols et al., 1984
NICOTINIC AChR (α and β SUBUNITS)	Olson et al., 1984
FIBRONECTIN	Magee, unpublished
VINCULIN	Kellie and Wrigglesworth, 1987

TABLE 4

VIRAL PROTEINS ACYLATED WITH MYRISTATE

Protein	Reference
$pp60^{v\text{-}src}$	Sefton et al., 1982
$p120^{gag\text{-}abl}$	Sefton et al., 1982
GAG PROTEINS OF MAMMALIAN RETROVIRUSES (TYPES B, C, D)	Schultz and Oroszlan, 1983
GAG-ONC FUSION PROTEINS	Schultz and Oroszlan, 1984
$p75^{gag\text{-}raf}$	Schultz et al., 1985
$p85^{gag\text{-}fes}$	Schultz and Oroszlan, 1984
$p110^{gag\text{-}fes}$	Schultz and Oroszlan, 1984
$p29^{gag\text{-}ras}$	Schultz and Oroszlan, 1984
POLIO VIRUS, VP4	Chow et al., 1987
FOOT AND MOUTH DISEASE, VP4	Chow et al., 1987
POLYOMA VIRUS, VP2	Streuli and Griffin, 1987
SIMIAN VIRUS 40, VP2	Streuli and Griffin, 1987

TABLE 5

PROTEINS WITH ACYL CHAINS IN THEIR MEMBRANE ANCHOR (PI-TAIL)

Protein	Reference
DELAY ACCELERATING FACTOR	Medof et al., 1986
P.falciparum TFN-R	Haldar et al., 1985
TRYPANOSOME VSG	Ferguson et al., 1985
N-CAM 120	He et al., 1986
Leishmania gp63	Bordier et al., 1986
ALKALINE PHOSPHATASE (RAT KIDNEY)	Low and Zilversmit, 1980
ALKALINE PHOSPHODIESTERASE (RAT KIDNEY)	Nakabayashi and Ikezawa, 1984
5'NUCLEOTIDASE	Nakabayashi and Ikezawa, 1984
TREHALASE	Takesue et al., 1986
ACh 'ASE	Roberts and Rosenberry, 1985
MYELIN BASIC PROTEIN	Yang et al., 1986
Thy-1	Tse et al., 1985
ALKALINE PHOSPHATASE (BRUSHBORDER MEMBRANE)	Nayudu et al., unpublished

2. Structure and Function of Protein Bound Fatty Acyl Chains

Understanding the function of protein linked acylchains in molecular terms requires some knowledge of the overall structure of the acylprotein and of the linkage site in particular. Since the small amounts of

fatty acid (usually 1 - 2 chains per polypeptide; Schmidt, 1986) are not easily detected in low abundance proteins, radiolabeling with ^{3}H-myristic acid or ^{3}H-palmitic acid and subsequent isolation of the protein with various established methods is applied. Figure 1 gives an example for the visualization of the acylated protein of influenza viruses, the small subunit HA_2 of the viral hemagglutinin. Neither the large glycoprotein fragment of HA, HA_1, nor the membrane linked neuraminidase (NA) or the hydrophobic M-protein contain fatty acids.

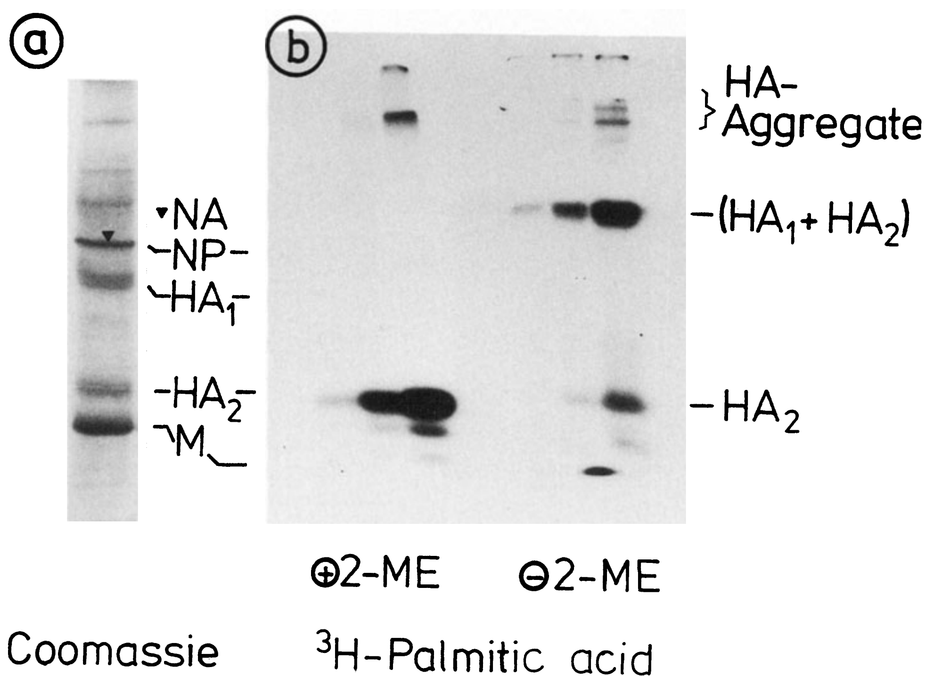

Figure 1: <u>Acylproteins of Influenza Virus</u>

PAGE-analysis of the proteins of ^{3}H-Pal labeled influenza A virus (fowl plague virus) on 12% SDS-polyacrylamide gels with 6 M urea. Panel a shows the Coomassie staining pattern and panel b a fluorogram of viral proteins. In panel b 50 µg, 300 µg or 1 mg viral protein were applied and the gel run under reducing (left side) or non-reducing conditions (right side).

One step towards the topographical identification of acyl linkage sites in acylproteins has been the method of limited proteolysis. Since most of the virus particles with fatty acylated proteins are enveloped by a lipid bilayer, proteases can only digest the exposed peripheral portions of the relevant spike proteins. Thus, if ^{3}H-Pal labeled virus is digested and subsequently centrifuged, supernatants with peripheral spike-peptides and sedimented "naked" virus particles can be analysed separately. Typical results obtained from such experiments are documented in table 6 and Fig. 2. After digesting ^{3}H-acylated and ^{3}H-glycosylated virus particles, the distribution of radioactivity in supernatants and sediments is opposite for ^{3}H-glucosamine labeled and ^{3}H-palmitoylated peptides.

TABLE 6

LIMITED DIGESTION OF ^{3}H-PAL LABELED INFLUENZA VIRUS

		Radioactivity (cpm) in fractions		
Label	Treatment	Sediment	Supernatant	Total
^{3}H-Palmitic acid	Control	263300	20600	283900
	Bromelain	268500	6600	275100
^{3}H-Glucosamine	Control	55100	8000	63100
	Bromelain	15500	48200	63700

About 300 µg of labeled fowl plague virus was digested for 3 min at 37°C in 100 µl phosphate buffered saline (PBS) pH 7.4 in the presence of 5 mM dithiothreitol. After dilution with ice cold PBS the samples were centrifuged for 30 min at 45000 rpm in a Beckman SW 50 Ti-rotor and radioactivity measured in aliquots of supernatants and pellets.

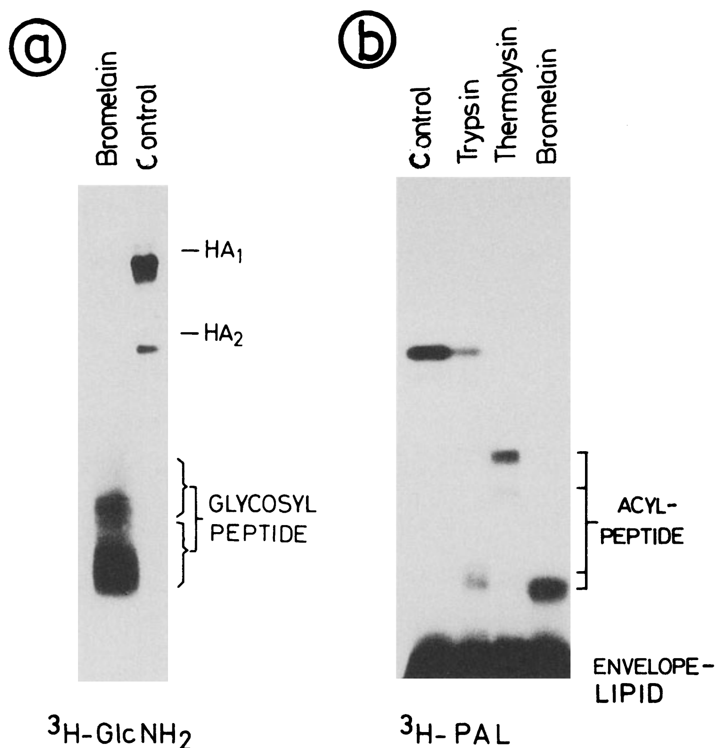

Figure 2: Fatty Acylated Peptides of Influenza HA_2

^{3}H-Glucosamine (GlcNH$_2$) or ^{3}H-palmitic acid (Pal) labeled influenza A virus (fowl plague) was subjected to limited proteolysis as described in the legend of table 6. ^{3}H-GlcNH$_2$ labeled peptides in the supernatant after bromelain digestion (panel a) and ^{3}H-palmitoylated material in the sediments after trypsin, thermolysin and bromelain digestion were analysed on 15% SDS-gels. Fluorograms of the respective gels are shown.

In order to identify the nature of the radioactive material, the relevant fractions were subjected to PAGE-analysis on 15% SDS-polyacrylamide gels. The fluorograms of such gels shown in Fig. 2 reveal, that the ^{3}H-Pal material in the sediments contains indeed defined ^{3}H-acylated peptides beside the ^{3}H-labeled bulk lipid of the viral envelope still present in "naked" particles.

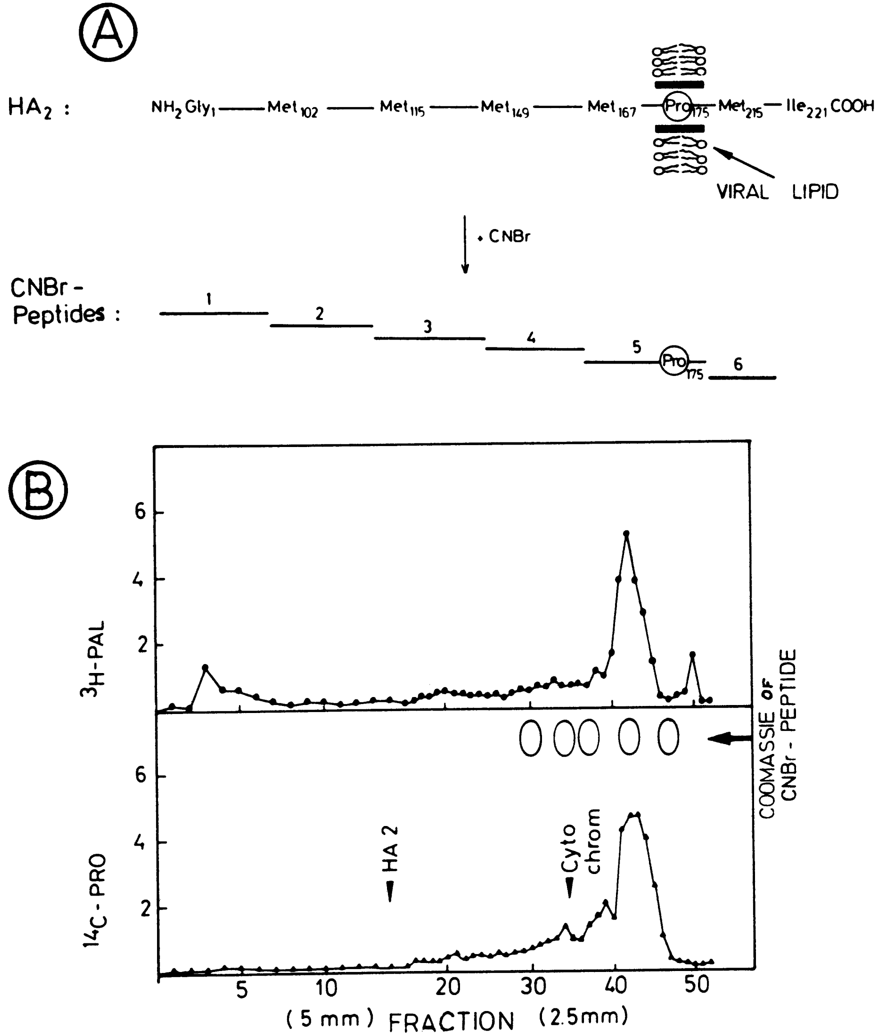

Figure 3: Cyanogen Bromide Cleavage of HA_2

A, Schematic of the expected cleavage pattern of fowl plague virus HA at methionine residues.

B, PAGE-analysis of CNBr-peptides on 15% cylindrical SDS-polyacrylamide gels. The distribution of ^{3}H-palmitic acid (PAL) and ^{14}C-proline (PRO) in gel slices is shown. Prior to fractionation gels were stained with Coomassie and staining pattern is indicated by ovals in lower panel. The positions of non-treated HA_2 and cytochrome C (Cyto) run as references are indicated by arrows.

This can be taken as an indication that the linkage site is close to the region of HA_2 which interacts with the viral lipid envelope.However more is required to prove this. One approach is to start with purified acylproteins, which seems most promising for proteins of which the aminoacid sequence is known. This is the case for the hemagglutinin (HA) of fowl plague virus (Porter et al., 1979) as well as for E1 and E2 of Semliki Forest virus (SFV; Garoff et al., 1980) which are discussed below.

As indicated in panel A of figure 3 fowl plague HA offers a promising fragment pattern if cyanogen bromide (CNBr) is used as the cleavage reagent. If fatty acids are located in the membrane segment, then this same fragment should also contain the only proline residue present in this HA_2. PAGE-analysis of CNBr-fragments generated in such an experiment show that ^{3}H-Pal and ^{14}C-Pro labeled peptides run to the same position, which substantiates the above hypothesis that the acylation site is located near to the membrane. Furthermore, the realistic possibility that more peripheral regions of HA_2 are acylated which potentially could loop back into the viral bilayer (compare ^{3}H-$GlcNH_2$ radioactivity in sediment in table 6) has been excluded by this latter finding (Figure 3, panel B).

All our attempts to cleave the acylated CNBR-peptide of HA_2 further in order to isolate short peptides for sequence studies failed because of apparently adverse properties of such material. For this reason we chose a viral acylprotein, which can be purified to higher yields and the primary structure of which is known, the SFV-E1 (Garoff et al., 1980). E1-protein from ^{3}H-palmitoylated SFV digested with chymotryp-

sin yielded only one ^{3}H-acylated peptide of about 6000 dalton (Figure 4, lanes 1 and 2). Likewise, digestion of the more heavily acylated SFV-E2 with Staph. aureus protease V8 yielded several defined ^{3}H-acylpeptides (Figure 4, lanes 3 and 4).

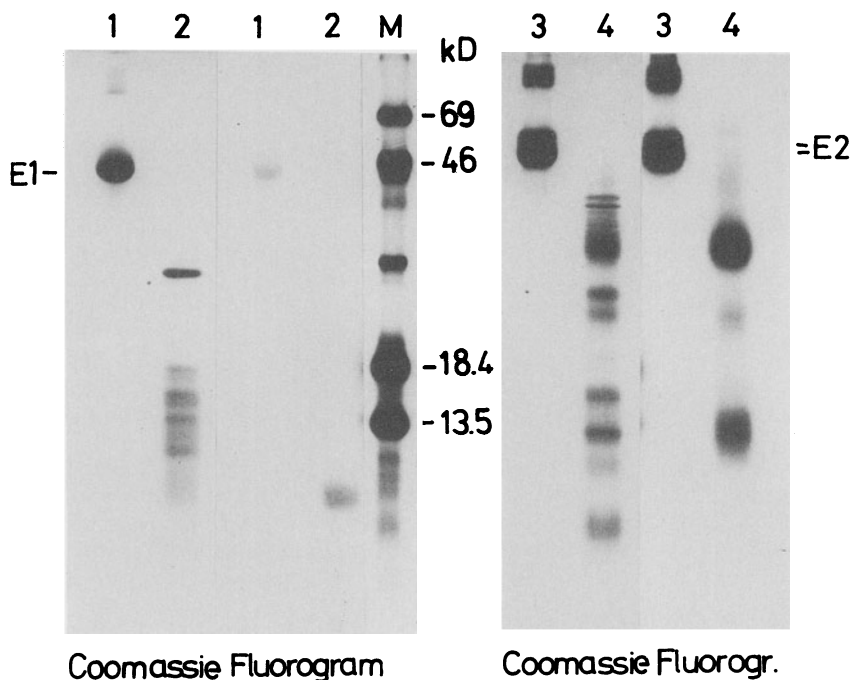

Figure 4: Proteolytic Digestion of ^{3}H-Acyl E1 and -E2 from Semliki Forest Virus

Acylproteins were isolated from purified ^{3}H-palmitic acid labeled SFV and digested with chymotrypsin (E1-protein, lanes 2) or with V8-protease (E2-protein, lanes 4) and run on 15% SDS-polyacrylamide gels. Coomassie staining patterns and fluorograms of the same gels are shown. Nontreated E1 and E2 are shown in lanes 1 and lanes 3, respectively.

Since it was found unsuitable to isolate acylpeptides from polyacrylamide gels (unacceptable losses), separation was attempted by utilizing reversed phase materials. The best results have been achieved with chymotryptic peptides of E1. The digest was loaded onto Sep-Pak cartridges in 60% formic acid and then eluted stepwise with increasing

concentrations of n-propanol. The distribution of ^{3}H-Pal in the various fractions shown in table 7 reveals the expected high solubility of ^{3}H-acylated peptide in fractions with high n-propanol concentration.

TABLE 7

ELUTION OF CHYMOTRYPTIC PEPTIDES FROM ^{3}H-PALMITOYLATED SFV-E1

Fraction	Eluent	^{3}H-Pal (cpm)	Percent of Total
1	SDS (0.1%)	63875	30.3
2	0% n-Propanol	9430	4.5
3	3% "	4980	2.4
4	8% "	3910	1.8
5	25% "	16890	8
6	40% "	55480	26.3
7	80% "	56200	26.7

Table 7: The digest in 0.1% SDS was loaded onto C18 reversed phase Sep-Pak cartridges. After washing several times with water step elution was started with 60% formic acid and increasing concentrations of n-propanol. Radioactivity was measured by scintillation counting of aliquots.

Amino acid analysis of fractions revealed that after elution with 25% n-propanol more than 80% of all peptides had been eluted. Fraction 6 (40% n-propanol in 60% formic acid) was chosen for further purification by HPLC on a C4-wide pore column. As shown in figure 5 the ^{3}H-acylpeptide elutes at a concentration of about 65% 2-propanol in 0.1% trifluoroacetic acid (TFA).

Our attempts to sequence the fraction containing radiolabeled peptides failed because of the extremely hydrophobic properties of this material. To identify the peptide we utilized amino acid analysis by the Pico-Tag method (Waters) by which the phenylthiocarbamyl (PTC) amino

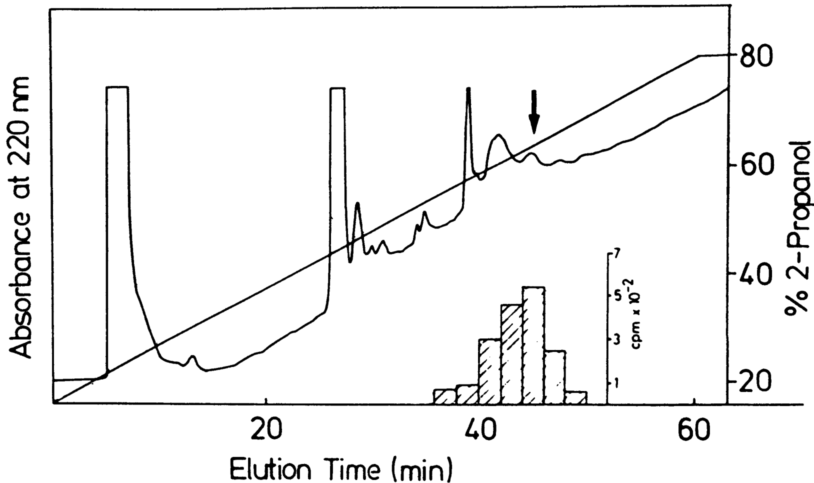

Figure 5: HPLC of Chymotryptic Peptides of ^{3}H-Pal labeled SFV-E1

Prepurified ^{3}H-Acylpeptides from E1 (fraction 6 from elution shown in table 7) were applied to a wide pore reversed phase column (Organogen, Heidelberg). Elution was with a gradient from 20-80% 2-propanol in TFA at a flow rate of 0.6 ml/min. Protein was monitored at 220 nm and radioactivity was measured by scintillation counting.

acids are separated. The results shown in table 8 strongly suggest that, as with the influenza HA, also the ^{3}H-acylated peptide isolated from SFV-E1 originates from the carboxyterminnus of E1 (compare figure 6).

Recently experiments are underway to sequence this material on solid phases. Preliminary results indicated alanin residues both in the first and second step (compare Fig. 5). Thus, we have purified fatty acylated peptide material which upon sequence analysis will eventually yield the precise linkage site of palmitic acid. This type of analysis, the protein chemical study of the "real thing" is tidious and hitherto only in one case has an acyl linkage site for palmitic acid been determined, a threonine residue in lipophilin from bovine brain (Stoffel, 1983).

TABLE 8

AMINO ACID ANALYSIS OF ³H-ACYLPEPTIDE FROM E1

Amino Acid	^{3}H-Acylpeptide	Theoretical Peptide (Pos. 400-438 in Fig. 6)
Asp	1	1
Glu	1 - 2	1
Ser	4	3
Gly	(10?)	6
His	-	-
Arg	1 - 2	2
Thr	2	2
Ala	4	4
Pro	1	1
Tyr	-	-
Val	2 - 3	5
Met	0 - 1	1
Cys	-	1
Ile		4
Leu	4	5
Phe	1	1
Lys	1 - 2	1

Table 8: HPLC-fractions containing the ^{3}H-Pal labeled peptides (arrow in Fig. 5) were hydrolysed for 24 h at 110°C in 6N HCl. After derivation with phenylisothiocyanate according to the PICO-TAG procedure (Waters) the PTC-derivatives were separated by HPLC with a gradient from 6 to 46% acetonitril in 0.14 M sodium acetate with 0.05% (v/v) triethylamin. PTC-amino acids were detected at 254 nm.

NH_2-Tyr-Glu-...-Leu-Gys-Ser-Ala-Arg-Ala-Thr-Cys-Ser-Ala-
1 378
Ser-Cys-Glu-Pro-Pro-Lys-Asp-His-Ile-Val-Pro-Tyr↙-Ala-
391
Ala-Ser-His-Ser-Asn-Val-Val-Phe↙-Pro-Asp-Met-Ser-Gly-
404
Thr-Ala-Leu-Ser-Trp-Val-Gln-Lys-<u>Ile-Ser-Gly-Gly-Leu-</u>
417
<u>Gly-Ala-Phe-Ala-Ile-Gly-Ala-Ile-Leu-Val-Leu-Val-Val-</u>
430
<u>Val-Thr-Cys-Ile-Gly-Leu-</u>Arg-Arg-COOH

Figure 6: Genome derived amino acid sequence of the carboxyterminal region of SFV-1

Only the aminoterminus and the last 70 carboxyterminal amino acids given as determined by Garoff et al. (1980). The potential chymotrypsin cleavage sites are indicated by arrows. The membrane spanning segment is underlined.

However, identification and sequencing of acylpeptides seems advantageous over the gene technological approach. With this latter technique acylation is studied by expresion of genes in which potential acylation sites (Cys, Ser, Thr) have been replaced. In a number of cases Cys has been suggested to be the linkage site, because its replacement by Ser or other amino acids led to a loss of acylation (Willumsen et al., 1984; Koch and Hämmerling, 1986; Rose et al., 1984; Jing and Trowbridge, 1987). Such interpretations are certainly valid but not compelling. Replacement of cystein residues may lead to an alteration of the structure of a protein such that other acylation sites may not be available for the biosynthetic acylation which seems to be a membrane located process involving specific enzymes (Berger and Schmidt, 1984; Schmidt, 1984; Mack et al., 1987). For this reason further results of palmitic acid linkage analyses on the protein level will have to be awaited before any general conclusions can be drawn on consensus sequences for palmitoylation. It should be noted that for myristilation much more structural information has become available due to the stability of the amide linkage between myristic acid and the aminoterminal glycine residue (see Sefton and Buss, 1987). On the basis of this information Glaser and coworkers recently succeeded in isolating a protein N-myristoyl-transferase from yeast (Towler et al., 1987).

Concerning the function of fatty acids in a protein more speculations are available in the literature than hard experimental results, particularly with palmitoylated proteins. The most intrinsing hypothesis for a general function of palmitoylation of cellular proteins was recently put forward by Glick and Rothman (1987). These

authors reported that in an in vitro system protein transport through the Golgi-stack was stimulated by palmitoyl-CoA, wich is the known precursor for palmitoylation of proteins (Schmidt, 1984).

With viral structural acylproteins it was noted that many of these were fusogenic as their most prominent biological feature (Schmidt, 1982). In order to test the hypothesis that acyl chains are in some way involved in fusion induction, virus particles with acylated protein or detergent solubilized acylproteins were compared with their hydroxylamin deacylated counterparts in their fusogenic properties as measured by hemolysis and resonance energy transfer with N-NBD-PE and N-Rho-PE labeled liposomes (Struck et al., 1981). The results of such experiments utilizing influenza virus show clearly, that no fusion occurs between virus particles and cell membranes if the viral hemagglutinin HA_2 is devoid of fatty acids. Similar results have been obtained with Semliki Forest- and Newcastle disease virus (Schmidt and Lambrecht, 1985; Lambrecht and Schmidt, 1986). It is therefore likely that with enveloped viruses protein bound fatty acids may be essential to initiate an infection by triggering the fusion between viral and cellular membranes.

In the myristilated transforming proteins (table 4) fatty acids are essential for anchoring the protein to the cytoplasmic face of the plasma membrane. If no myristic acid is present at their aminoterminus these proteins are found in the cytoplasm. As a consequence, the cells cannot be transformed and retain their normal phenotype (Kamps et al., 1986; Pellman et al., 1985).

3. Conclusion

Although many acylproteins have been identified and many more with as yet unknown identities have been reported, quite little is known on the structure and function of protein bound acyl chains. Thus far, in large the viral acylproteins can be regarded as the "avantgarde class of molecules" in the study of the various aspects of fatty acylation. Hopefully, as perhaps indicated by the recent identification of a protein myristoyl transferase (Towler et al., 1987) more data will become available in the near future on the linkage, enzymology and functional role of acyl chains in the ever increasing number of cellular acylproteins.

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