Fatty Acid Binding: A New Kind of Posttranslational Modification of Membrane Proteins

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1 Introduction

Many cellular functions are carried out by proteins which are in close association with lipid bilayers. The key structure for regulating many of the cell's activities as a function of the various environmental stimuli is the cell surface membrane. In this regulation, membrane proteins of the cell surface are of utmost importance for receiving extracellular signals as, for instance, through the binding of antigens, hormones, neurotransmitters, lectins, antibodies, neighboring cells, or viruses. The receptors themselves, or other membrane proteins, then transduce information to the appropriate intracellular sites where specific biochemical responses are induced, often including the participation of internal membranes.

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While many ligands, such as antigens, hormones, or viruses, which in a given cell induce specific biochemical reactions have been identified and characterized on a molecular level, their counterparts in the cell surface, the receptors, are usually undefined entities. However, the available data indicate that in many cases surface receptors are structurally represented by glycoproteins (*Kathan* and *Winzler* 1963; *Cuatrecasas* 1974; *Ashwell* and *Morell* 1974; *Cuatrecasas* et al. 1975; *Heidmann* and *Changeux* 1978; *Morgan* 1981; *Lonberg-Holm* and *Philipson* 1981).

To gain more insight into the biosynthesis and properties of membrane glycoproteins, enveloped viruses are increasingly used as model systems. Since these viruses, in contrast to cells, contain only a small number of membrane proteins, it is easy to isolate specific membrane glycoproteins from virus particles in pure form (for review see *Schmidt* 1982c). As will be evident below, the virus system has been widely applied to the study of the biosynthesis and function of membrane glycoproteins in general. The switch-off of host-specific protein synthesis after infection with certain enveloped viruses allows one to observe the biosynthesis of specific viral glycoproteins. It is thus possible to evaluate the various states of modification of these proteins during their maturation to the functional end product (for reviews see *Klenk* and *Rott* 1980; *Gibson* et al. 1980; *Schwarz* and *Schmidt* 1982; *Schlesinger* and *Kääriäinen* 1980; *Simons* and *Garoff* 1980).

In the course of studying the biosynthesis of Sindbis-virus-specific glycoproteins, a new type of posttranslational modification was discovered (*Bracha* et al. 1977b; *Schmidt* et al. 1979). This modification involves the covalent attachment of fatty acids to the polypeptide backbone of the glycoprotein. Since acyl chains are transferred onto the polypeptide, this event is termed "acylation" (*Schmidt* et al. 1979).

It is the purpose of this article to summarize all data available up to July 1982 on fatty acid acylation of membrane proteins. This includes information on the occurrence of fatty acid binding among viral and cellular membrane proteins, the chemical nature of the bond between fatty acid and polypeptide, and finally, the biosynthetic process of fatty acid attachment in relation to other well-characterized co- and posttranslational modifications of these macromolecules. In the discussion, an attempt will be made to explain the implications of fatty acid binding to proteins with respect to the interactions with the lipid bilayer, and to the possible biological functions of the protein-bound acyl chains.

1.1 Modification of Membrane Proteins During Their Biosynthesis

1.1.1 Types of Modifications

During or after their translation on ribosomes many membrane proteins are modified. The main types of modifications are (a) the covalent addition of molecules other than amino acids to the polypeptide, and (b) the proteolytic cleavage of the polypeptide chain at specific sites. The prominent feature of the first type of modification of membrane proteins is the presence of carbohydrate in such proteins, which are therefore, termed glycoproteins. Although known for about 100 years (see *Gottschalk* 1966; *Hughes* 1976), information on the biosynthesis of glycoproteins and the biological functions of their carbohydrate is only recently accumulating (for reviews see *Parodi* and *Leloir* 1979; *Kornfeld* and *Kornfeld* 1980: *Klenk* and *Rott* 1980; *Hubbard* and *Ivatt* 1981; *Schwarz* and *Datema*

1982). Other molecules found covalently linked to amino acids of the polypeptide backbone of proteins are, for instance, acetyl, formyl, and phosphate groups (see *Wold* 1981 for review). However, these kinds of modifications are much less common with membrane proteins than with cytoplasmic or nuclear proteins and by orders of magnitude less frequent than covalently bound carbohydrate. In contrast, the modification described in this article, acylation through the covalent binding of fatty acids to proteins, seems to be very frequent with membrane proteins. The biological significance of acylation, therefore, may well be compared to glycosylation.

The second type of modification of membrane proteins is the proteolytical cleavage of the polypeptide backbone at specific sites. This proteolytical maturation is very widespread and represents a feature quite common with proteins in general (*Reich* et al. 1975; *Holzer* and *Heinrich* 1980; *Wold* 1981).

Proteolytic cleavages occur at different times and sites during or after the translation of a polypeptide. The *cotranslational cleavage* of a common signal peptide has been proven to occur with many membrane and secreted proteins of plant, animal, bacterial, and viral origin (*Wickner* 1979; *Blobel* 1979; *Bonatti* and *Blobel* 1979; *Bar-Nun* et al. 1980; *Nelson* and *Ryan* 1980; *Movva* et al. 1980; *Suchanek* et al. 1980; *Sabatini* et al. 1982; *Lingappa* et al. 1978; *Wirth* et al. 1977; *Bonatti* et al. 1979). Whereas this cotranslational proteolysis seems confined to the rough endoplasmic reticulum, the *posttranslational cleavages* may occur at the endoplasmic reticulum, the Golgi complex, the plasma membrane, or even extracellularly (*Klenk* et al. 1974, 1975; *Klemenz* and *Diggelmann* 1979; *Shapiro* and *August* 1976; *Smith* and *Brown* 1977; *Schlesinger* and *Schlesinger* 1972; *Bracha* and *Schlesinger* 1976; *Lazarowitz* and *Choppin* 1975).

1.1.2 Biosynthesis of Viral Glycoproteins

The use of enveloped animal viruses has proved extremely helpful in correlating biosynthetic modifications of membrane-bound glycoproteins with specific biological functions (*Gibson* et al. 1980; *Rott* 1977, 1979; *Bosch* et al. 1979; *Klenk* et al. 1975, 1978; *Nagai* et al. 1976; *Kääriäinen* and *Söderlund* 1978; *Scheid* and *Choppin* 1976; *Kaluza* et al. 1980; *Schlesinger* and *Kääriäinen* 1980; *Schwarz* and *Schmidt* 1982; *Nakamura* and *Compans* 1978; *Simons* and *Garoff* 1980; *Gething* and *Sambrook* 1981). Likewise, viral systems are indispensable for the study of structural features of membrane proteins with respect to the sites of modification of the polypeptide backbone, i.e. the cleavage and glycosylation sites (*Ward* 1981; *Keil* et al. 1979; *Robertson* et al. 1976; *Bosch* et al. 1981; *Garten* et al. 1981; *Wilson* et al. 1981; *Ward* and *Dopheide* 1979).

Viral glycoproteins are translated like membrane glycoproteins on membrane-bound ribosomes from a specific messenger RNA. In addition to the code for the mature proteins, this mRNA also contains information for a short, hydrophobic amino acid sequence, which in most cases is located at the aminoterminus of the respective protein (*Blobel* and *Dobberstein* 1975; *Rothman* and *Lodish* 1977; *Porter* et al. 1979; *Blobel* and *Sabatini* 1970). This hydrophobic stretch of amino acids (a "signal sequence") aids the insertion of the nascent polypeptide chain into the membrane of the endoplasmic reticulum (*Blobel* and *Dobberstein* 1975; *Wickner* 1979; *Engelman* and *Steitz* 1981; *Sabatini* et al. 1982). During translation the polypeptide is translocated deeper into the lumen of the endoplasmic reticulum and primary glycosylation commences with the transfer of high-mannose-type oligosaccharides from a dolichol-lipid intermediate (*Hemming* 1977;

Parodi and *Leloir* 1979; *Waechter* and *Lennarz* 1976; *Schwarz* et al. 1978) onto specific sites within the primary sequence of the polypeptide (*Nakamura* et al. 1980; *Keil* et al. 1979; *Ward* 1981). Transfer of the nascent polypeptide into the cisternae of the endoplasmic reticulum then proceeds until a hydrophobic region close to the carboxyterminus becomes wedged in the lipid bilayer, thereby anchoring the polypeptide and exposing a few, partially basic amino acids and the carboxyterminus on the cytoplasmic side (*Porter* et al. 1979; *Ward* 1981; *Garoff* et al. 1978; *Simons* and *Garoff* 1980). By an unknown mechanism, the glycoproteins are then transported through the smooth endoplasmic reticulum towards the Golgi complex. During this vectorial intracellular transport the high-mannose oligosaccharides may be "trimmed" to yield complex sugar side chains (*Robbins* et al. 1977; *Hunt* et al. 1978; *Tabas* et al. 1978; *Kornfeld* et al. 1978). From the Golgi complex viral glycoproteins usually move to the plasma membrane where they perform their functions in the assembly of progeny virus particles.

While the intracellular transport is proceeding, many virus-specific glycoproteins are proteolytically cleaved, yielding two or more fragments which may still be linked through disulfide bonds. Although at least with togavirus, influenza, and Rous sarcoma viral glycoproteins, the intracellular and the protein substrate's cleavage sites are defined to some extent, near to nothing is known about the proteolytical enzymes involved in this type of modification (for review see *Klenk* and *Rott* 1980; *Simons* and *Garoff* 1980; *Shapiro* and *August* 1976; *Schlesinger* and *Kääriäinen* 1980).

This route of biosynthesis seems to hold true not only for membrane-bound glycoproteins, but is also principally valid for secreted and lysosomal glycoproteins, which in their mature state function extracellular or in lysosomes. The difference in the final destination of these glycoproteins requires specific recognition mechanisms to provide the proper targeting. Although the Golgi complex is believed to be the cellular organelle in which the "addressing" of glycoproteins occurs, the molecular basis for this process remains to be elucidated (*Palade* 1975; *Tartakoff* 1980; *Bergman* et al. 1981; *Jokinen* et al. 1979; *Hasilik* 1980; *Rothman* 1981).

1.2 Some Properties of Membrane Proteins

One aspect of research on cell surfaces concerns the interaction between proteins and lipids, both of which represent the main chemical components of cellular membranes. To introduce an important distinction, a few words on some properties of membrane proteins seem necessary since interactions between these membrane components may be of an entirely different nature.

Proteins are sometimes associated with lipid bilayers relatively loosely, in which case they are termed peripheral membrane proteins. They can also be inserted deeply or even span the lipid bilayer and are then termed integral membrane proteins. These latter proteins are often closely associated with certain lipids called boundary lipids. In many instances boundary lipids seem essential for the expression of specific biological activities. An example is provided by the mitochondrial cytochrome *c* oxidase, which requires closely associated diphosphatidyl glycerol (cardiolipin) for maximal enzymatic activity (*Awasthi* et al. 1971; *Vik* and *Capaldi* 1977; *Downer* et al. 1976; *Robinson* and *Capaldi* 1977). Similarly, β-hydroxybutyrate dehydrogenase expresses highest enzymatic activity only in the presence of lecithin (*Grover* et al. 1975; *Bock* and *Fleischer* 1975; *Gazotti* et al. 1975).

It is important to note that the interaction between boundary lipid and membrane proteins is of a *noncovalent* nature. Therefore it must be distinguished from the biosynthetic modification of proteins through acylation, which represents the topic of the present article. This novel form of modification results in a *covalent* attachment of fatty acids to the respective polypeptide. As will become apparent below, most protein substrates of acylation that have been characterized to date represent glycosylated membrane proteins.

2 Acylated Proteins

2.1 Viral Glycoproteins with Covalently Linked Fatty Acids

In the course of studying the action of p-nitrophenyl-guanidinium benzoate (NPGB) on viral multiplication (*Bracha* et al. 1977a), the question arose whether lipids might be bound to viral spike glycoprotein. To answer this question, Sindbis virus was grown in chick embryo fibroblasts that were labeled with ^{3}H-palmitic acid. While it had been expected that the viral envelope would be heavily labeled with fatty acids, some ^{3}H-radioactivity was also found to comigrate with viral glycoprotein during the centrifugation of disrupted particles through a detergent-containing sucrose gradient (*Bracha* et al. 1977b). This observation was followed up by the analysis of fatty-acid-labeled Sindbis virus on polyacrylamide gels. The resulting fluorograms revealed that mainly the E2 glycoprotein contained labeled lipid, whereas the E1 glycoprotein, which in virions is present in equimolar amounts, showed only about one-fourth of the radioactivity found in E2 (*Schmidt* et al. 1979).

In order to ascertain and identify the lipid nature of the protein-bound radioactivity, the individual viral glycoproteins were isolated from fatty-acid-labeled Sindbis virus by a variety of methods, including detergent extraction, immunoprecipitation, and preparative polyacrylamide-gel electrophoresis. It was not possible to extract the protein-bound radioactivity with organic solvents in any combination, from either the labeled glycoprotein preparations or from whole virus particles indicating that the lipid was very tightly associated with Sindbis virus glycoproteins. Also pretreatment of virus particles or purified glycoprotein with various detergents failed to render the bound radioactivity susceptible to organic extraction (*Schmidt* et al. 1979). Only through the application of mild alkaline solutions could the tritiated material be cleaved, to be subsequently identified as fatty acid by gas-liquid chromatography (GLC). To establish the molar content of fatty acid in Sindbis virus E1 and E2, these two glycoproteins were isolated from batches of 10–20 mg of *nonlabeled* purified Sindbis virus.

After a sequence of organic extractions to eliminate contaminating envelope lipids the remaining protein-bound lipid radioactivity was cleaved by mild alkali (0.1 *M* potassium hydroxide in methanol, 20 min at room temperature) and quantitated by GLC. This analysis revealed that 1–2 mol fatty acids were bound to 1 mol of Sindbis E1 glycoprotein, while the Sindbis E2 spike glycoprotein contained 5–6 mol fatty acids per mole polypeptide.

In addition to stoichiometric information, these experiments demonstrated that palmitic acid represented the main species of fatty acid bound to these glycoproteins. However, significant amounts of stearic acid and oleic acid were also detected by GLC

(*Schmidt* et al. 1979). Accordingly, viral glycoprotein could be labeled with radioactive oleic and stearic acids, although 9,10-^{3}H-(*N*)-palmitic acid gave the most efficient and most specific labeling (*Schmidt* and *Schlesinger,* unpublished). The fatty acids appear to be bound directly to the polypeptide, since no specific labeling of the acylated glycoproteins could be achieved through the incorporation of ^{32}P-phosphate or 2-^{3}H-glycerol.

From these and other data given below a number of characteristic chemical features can be summarized, which leads to the conclusion that the acyl chains must be covalently linked to the respective protein:

1. Fatty acids are associated with the protein in a linkage which is resistant to detergents and organic extractions with chloroform, methanol, acetone, hexane, ether, and other solvents. The linkage is also resistant to boiling in SDS and during SDS-polyacrylamide-gel electrophoresis (*Schmidt* et al. 1979; *Rice* et al. 1982; *Capone* et al. 1982; *Marinetti* and *Cattieu* 1982).
2. Proteolytic or chemical cleavage of the acyl protein does not liberate the bound fatty acids but instead yields acylated peptides (*Schmidt* and *Schlesinger* 1979; *Petri* and *Wagner* 1980; *Schmidt* 1982a; *Schlesinger* et al. 1981; *Rice* et al. 1982; *Capone* et al. 1982).
3. Fatty acids are released from acyl proteins through treatment with mild alkali in an aqueous or methanolic environment. The latter alkaline hydrolysis yields fatty acid methyl esters via transesterification. These methylesters were identified through GLC (*Schmidt* et al. 1979; *Roßmann* and *Schmidt,* unpublished).
4. Fatty acids are cleaved from acylated proteins during incubation with hydroxylamin at neutral pH. In this case the cleavage products were identified as the respective hydroxamate esters (*Schlesinger* et al. 1980; *Omary* and *Trowbridge* 1981b; *Lambrecht* and *Schmidt,* unpublished).

Meanwhile a series of other enveloped RNA viruses have been analyzed for fatty acid binding in their glycoproteins. These studies revealed a differential distribution of fatty acids among the different species of spike glycoproteins in a given virus (Table 1). In various strains of human and avian influenza viruses labeled with tritiated fatty acid only the hemagglutinin (HA) carries fatty acid, whereas no label could be detected in their second spike glycoprotein, the neuraminidase (NA) (Fig. 1; *Schmidt* 1982b). Very similar results have been obtained with paramyxoviruses. Sendai virus and three different strains of Newcastle disease virus (NDV) which differ in their pathogenicity afford fatty acid binding to their fusion proteins (designated F protein). The other spike glycoprotein of NDV the hemagglutinin-neuraminidase (HN), is virtually free of fatty acid (*Schmidt* 1982b; *Chatis* and *Morrison* 1982; *Schmidt,* unpublished). Vesicular stomatitis virus (VSV), a rhabdovirus, contains 1–2 mol fatty acid per mole of its only glycoprotein, which is designated G protein (*Schmidt* and *Schlesinger* 1979). Also the large glycoproteins (G1 and G2) of LaCrosse virus, a bunyavirus, have recently been reported to be acylated (*Madoff* and *Lenard* 1982). Furthermore, two members of the coronavirus family were found to contain fatty acid in E2, which probably represents the fusogenic one of its two spike glycoproteins (*Schmidt* 1982b; *Niemann* and *Klenk* 1981; *Sturman* et al. 1980). Studying acylation of the glycoproteins of Semliki Forest virus give results almost identical to those with Sindbis virus (both are togaviruses, see above). As seen in Table 1 both Semliki Forest virus glycoproteins contain fatty acid, with E2 being more strongly acylated than E1.

To answer the question whether acylation is host dependent, Semliki Forest virus,

Table 1. Acylated and fatty-acid-free glycoproteins of enveloped viruses

Virus	Cells	Spike glycoproteins		Stoichiometry (mol fatty acid per mol protein)	Reference
		Nonacylated	Acylated		
Rhabdovirus:					
Vesicular stomatitis virus	CEF, CHO	–	G protein	1–2	*Schmidt* and *Schlesinger* (1979)
Togavirus:					
Semliki Forest virus	CEF, BHK, Eveline cells, human lymphoma, insect cells (*Aedes albopictus*)	–	E1, E2	n. d.	*Schmidt* (1982b)
Sindbis virus	CEF, BHK, L cells, insect cells (*Aedes albopictus*)	–	E1, E2	1–2, 5–6	*Schmidt* et al. (1979)
Myxovirus:					
Influenza virus (several strains)	CEF, MDBK, Eveline cells, human lymphoma	NA	HA, HA_2	n. d.	*Schmidt* (1982b)
Newcastle disease virus (several strains)	CEF, MDBK, CAM, CHO	HN	F protein	n. d.	*Schmidt* (1982b) *Chatis* and *Morrison* (1982)
Sendai virus	CEF, CAM	HN (?)	F protein	n. d.	*Roßmann* and *Schmidt* (unpublished)
Coronavirus:					
Bovine coronavirus L9	Bovine fetal thyroid cells	E1	E2	n. d.	*Schmidt* (1982b)
Murine coronavirus (mouse hepatitis virus A59)	Balb C3T3 cells	E1	E2	n. d.	*Niemann* and *Klenk* (1981)
Bunyavirus:					
LaCrosse virus	BHK	–	G1, G2	n. d.	*Madoff* and *Lenard* (1982)

Abbreviations:
CEF – chick embryo fibroblasts; CHO – Chinese hamster ovary cells; BHK – baby hamster kidney cells; MDBK – Madin Darby bovine kidney cells; CAM – chorio allantoic membrane; n. d. – not determined

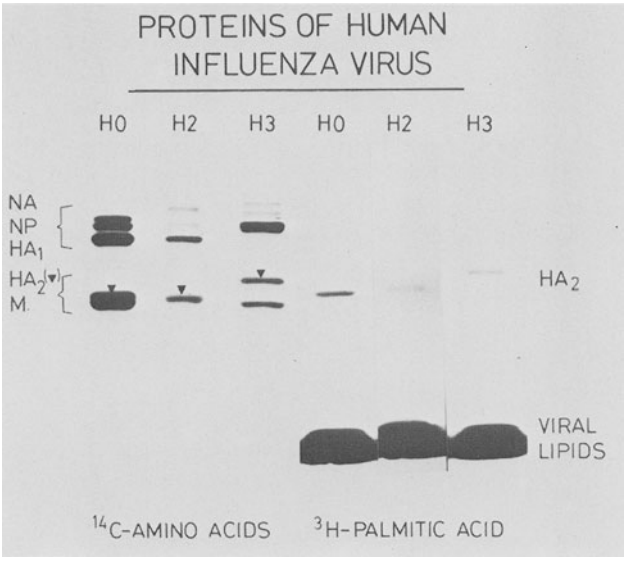

Fig. 1. Acylated polypeptides of human influenza viruses with different hemagglutinin subtypes (*HO, H2,* and *H3*). Influenza virus was grown in chick embryo cells labeled with ^{14}C-protein hydrolysate or ^{3}H-palmitic acid in the presence of 0.001% trypsin in the medium (*Klenk* et al. 1975; *Lazarowitz* and *Choppin* 1975). Released virus particles were purified from the culture fluid and run on a 12% acrylamide gel containing 6 *M* urea. The depicted fluorogram shows viral polypeptides labeled with ^{14}C-amino acids (*left panel*) and with ^{3}H-palmitic acid (*right panel*). Only the smaller cleavage product of the hemagglutinin, designated HA_2, is acylated with fatty acid. The heavily ^{3}H-labeled lipids of the viral envelope are seen as thick black ovals at the bottom of each lane

which has a wide host range, was grown in a number of different cell types including insect, avian, and mammalian cells. In all host cells utilized, the acylation pattern of the virus-specific glycoproteins was found to be the same (*Schmidt* 1982b). In addition to the viruses listed above, preliminary results show fatty acid binding also with glycoproteins coded by murine sarcoma virus (*Schmidt, Schneider, Hunsmann,* unpublished) and with herpesvirus (*Magee* and *Schlesinger*, to be published). Likewise, recent reports indicate that the transforming proteins p60^{src}, p21, and P120 of Rous sarcoma, Harvey sarcoma, and Abelson virus, respectively, are acylated with fatty acids (*Magee* and *Schlesinger*, to be published).

Although not all enveloped viruses have been analyzed for acylation, it seems justified to draw a few conclusions. Firstly, fatty acid binding occurs with enveloped viruses of totally different taxonomic groups including RNA and DNA viruses, whether they are oncogenic or not. Secondly, acylation does not depend on the use of specific host cells, since different cell types were used to propagate the different viruses under study. Thirdly, it is obvious that acylation is restricted to certain species of the specific proteins of a given virus, which may indicate specialized structural or functional features.

In the past many features of membrane glycoproteins have been first detected with viral glycoproteins (see above). It is therefore reasonable to suspect that acylation may also be a structural component of membrane glycoproteins of nonviral origin. And this is indeed the case, as will become apparent in the following section.

2.2 Acylated Membrane Proteins from Noninfected Cells

2.2.1 Classical Proteolipids

Although there is no precedent for covalently bound fatty acids in typical membrane glycoproteins of eukaryotic cells (*Hughes* 1976), tightly bound fatty acids have been reported for a few nonglycosylated bacterial proteins over several years (Table 2). The only protein which by rigorous chemical analysis has been shown to contain covalently bound lipid is the lipoprotein of *E. coli*, which ties the bacterial outer membrane to the peptidoglycan layer (*Braun* and *Rehn* 1969; *Braun* and *Bosch* 1972; *Hantke* and *Braun* 1973; *Chattopad-*

Table 2. Acylated and fatty-acid-free cellular membrane proteins

Membrane protein	Cell type	Acylated	Reference
Lipoprotein	*E. coli*	+	*Hantke* and *Braun* (1973)
Penicillinase	Bacillus species, *S. aureus*	+	*Nielsen* and *Lampen* (1982); *Lai* et al. (1981); *Smith* et al. (1981)
P20	CEF, BHK, KB cells, mouse myeloma cells	+	*Schlesinger* et al. (1980) *Berger* and *Schmidt* (unpublished)
Numerous undefined	CEF, BHK, KB cells, mouse myeloma cells	+	*Schlesinger* et al. (1980) *Berger* and *Schmidt* (unpublished)
Myelin proteolipoprotein	Rat brain	+	*Folch-Pi* and *Lees* (1951); *Schmidt, Schlesinger* and *Agrawal* (unpublished); *Agrawal* et al. (1982)
DM 20	Rat brain	+	*Agrawal* et al. (1982)
Butyrophilin	Milk fat globule membrane	+	*Keenan* et al. (1982)
Xanthine oxidase	Milk fat globule membrane	+	*Keenan* et al. (1982)
F_c receptor	Cells infected with herpesvirus	+	*Magee* and *Schlesinger* (to be published)
$p60^{src}$, p21, p120 transforming proteins	Cells infected with Rous sarcoma, Harvey sarcoma, and Abelson virus	+	*Magee* and *Schlesinger* (to be published)
Ca^{2+}-ATPase	Sarcoplasmic reticulum	+	*MacLennan* et al. (1972)
Transferrin receptor	Human leukemic T-cell lines CCRF-CEM and RPMI 8402	+	*Omary* and *Trowbridge* (1981b)
Histocompatibility antigen HLA	Human leukemic T-cell lines CCRF-CEM and RPMI 8402	−	*Omary* and *Trowbridge* (1981b)
Human T200	Human leukemic T-cell lines CCRF-CEM and RPMI 8402	−	*Omary* and *Trowbridge* (1981b)
Membrane-bound IgM	Human leukemic T-cell lines Ramos and Daudi	−	*Schmidt* and *Fleischer* (unpublished)

hyay and *Wu* 1977). More recent reports describe covalent lipid binding also with membrane penicillinase of *Bacillus licheniformis* and related bacteria (*Sawai* and *Lampen* 1974; *Yamamoto* and *Lampen* 1976a, b; *Smith* et al. 1981; *Lai* et al. 1981; *Nielsen* et al. 1981; *Nielsen* and *Lampen* 1982).

For eukaryotic cells two classes of highly specialized membrane-bound nonglycosylated protein complexes have been reported to contain peptide components with tightly linked fatty acids: the myelin and the sarcoplasmic proteolipids. The first fatty-acid-carrying protein ever described is the protein complex of the myelin membrane (*Folch-Pi* and *Lees* 1951; *Stoffyn* and *Folch-Pi* 1971). In the course of developing the now widely applied procedures for the extraction of lipids from tissue (*Folch* et al. 1951), these investigators discovered a protein complex in lipid extracts from brain tissue. This protein was found to be soluble in chloroform/methanol (2:1, v/v), but insoluble in water. To indicate its lipidlike nature this type of protein was termed "proteolipid" (*Folch-Pi* and *Lees* 1951). Structural analysis of the protein components isolated from the myelin membrane revealed that myelin proteolipid contains 2 mol esterified fatty acid per mole of polypeptide (*Gagnon* et al. 1971; *Moscarello* et al. 1973; *Jolles* et al. 1977; *Lees* et al. 1979). This finding was recently confirmed by in vivo labeling experiments during which tritiated fatty acid coupled to bovine serum albumin was injected into the brains of rats. It was found that radioactivity was specifically incorporated into proteolipid of the myelin membrane (*Schmidt, Schlesinger* and *Agrawal,* unpublished; *Agrawal* et al. 1982). During the course of these studies a second myelin protein, designated DM 20 (*Agrawal* et al. 1972), was also detected in fatty-acid-labeled form (*Agrawal* et al. 1982).

The second example of a proteolipid with covalently linked fatty acids is provided by the Ca^{2+}-dependent ATPase of the sacroplasmic reticulum. This enzyme complex contains a protein component which carries 1–2 fatty acid moieties in covalent linkage (*MacLennan* et al. 1972; *MacLennan* 1975).

Studies following *Folch-Pi* and *Lees'* original reports on myelin proteins revealed that proteolipids were also present in a variety of other tissues of plant or animal origin and were frequently located in mitochondria of the respective cells (*Folch-Pi* and *Stoffyn* 1972; *Zill* and *Harmon* 1962; *Lapetina* et al. 1968; *Murakami* et al. 1962; *Folch-Pi* and *Sakura* 1976; *Lees* et al. 1979). More recently hydrophobic proteins have been shown to be functional constituents of the mitochondrial ATPase complex. Since these proteins bind dicyclohexylcarbodiimide (DCCD), they are often referred to as DCCD-binding proteins (*Tzagloff* and *Meagher* 1972; *Nelson* et al. 1977; *Criddle* et al. 1977; *Sebald* et al. 1979). Although soluble in organic solvents, which qualifies DCCD-binding proteins as proteolipids, no evidence for covalent attachment of any lipids or fatty acids in DCCD-binding proteins has as yet been reported.

In a recent review article *Schlesinger* (1981) suggested altering the original operational definition for the term "proteolipid" (*Folch-Pi* and *Lees* 1951). He suggested the term be used for proteins that contain a lipid moiety as part of their primary structure. Thus proteolipids defined by this new criterion would be analogous to glycoproteins, phosphoproteins, etc., which are terms based on structural features of the respective entities. From my point of view this alteration might lead to the confusion of old and new definitions of the term proteolipid in the literature. Therefore I suggest that we retain the definition originally introduced by *Folch-Pi* and *Lees* (1951) for proteins soluble in organic solvents, and to introduce the new term *acylprotein* for all proteins whose primary structure is modified through covalently bound fatty acids whether they are soluble in organic

solvents or not. Thus, for example, myelin proteolipid would be an acylprotein whereas DCCD-binding proteins would not belong to this new category.

2.2.2 Acylproteins from Cells in Tissue Culture

Since membrane proteins of noninfected tissue culture cells usually represent only a small fraction of total cellular protein, it has been more difficult to study their biosynthesis and properties compared with viral membrane proteins. However, the limitation due to the relative scarcity of specific membrane proteins has been overcome by the development of powerful isolation techniques such as cell fractionation, affinity chromatography, immunological procedures, or their combinations (see *Azzi* et al. 1981). A number of cellular acylproteins have been identified using these techniques (see Table 2).

Accordingly, acylation with *nonviral* proteins was first detected when membrane fractions from cells labeled with ^{3}H-palmitic acid were analyzed (*Schlesinger* et al. 1980). Approximately 20 different protein bands with the ^{3}H-label were detected after polyacrylamide-gel electrophoresis of chloroform/methanol-extracted fractions. Since the proteins could not be labeled in the presence of cycloheximide, fatty acid incorporation must be dependent on protein biosynthesis and is not due to any kind of strong lipid affinity to certain proteins as, for instance, described for bovine serum albumin (*Spector* 1975). Furthermore, no ^{32}P-phosphate was incorporated into the proteins which could be labeled with ^{3}H-palmitic acid and, therefore, the presence of phospholipid, as in the boundary lipids of certain membrane proteins (see above), is most unlikely (*Schlesinger* et al. 1980; *Schmidt*, unpublished). It is noteworthy that acylproteins of about 20 000 daltons molecular weight were found in a number of different cell types (KB cells, chick embryo fibroblasts, mouse myeloma cells, and babyhamster kidney cells) (*Schlesinger* et al. 1980; *Berger* and *Schmidt*, unpublished). Unfortunately no information is yet available on the precise intracellular origin or the structural and functional characteristics of this common acylprotein.

By applying monoclonal antibodies for immunoprecipitation of cell lysates, *Omary* and *Trowbridge* (1981b) demonstrated fatty acid binding with a defined surface glycoprotein, the transferrin receptor of the plasma membrane of human lymphoma cells. In the same report the authors described the lack of fatty acids in two other cell surface glycoproteins, the T200 glycoprotein and the major histocompatibility antigen (HLA). Our own results are compatible with these findings and lend support to the hypothesis that only certain membrane glycoproteins are acylated (*Schmidt* and *Fleischer*, unpublished). As mentioned above, this feature had already become apparent from the study of acylproteins of viral origin (*Schmidt* 1982a, b).

Very recently fatty acid binding has been detected in a number of membrane proteins in rat tissues, human red cells, and in polymorphonuclear cells (*Marinetti* and *Cattieu* 1982). Furthermore, specific labeling with ^{3}H-fatty acids was also achieved with rat erythrocyte membrane proteins and with the two major proteins of the milk fat globule membrane butyrophilin and xanthine oxidase (*Keenan* et al. 1982).

3 Structure of the Linkage Between Fatty Acids and the Polypeptide

3.1 Linkage in Acylproteins of Bacterial Origin

Of all proteins which have been reported to contain tightly bound lipid (for recent reviews see *Schlesinger* 1981 and *Lees* et al. 1979), the murein lipoprotein of the outer membrane of *E. coli* is the only one for which the precise chemical structure of the lipid-protein linkage has been characterized (*Braun* and *Radin* 1969). By analyzing peptides of the lipoprotein, *Hantke* and *Braun* (1973) proved the presence of diglyceride linked to the aminoterminal cysteine residue through a thioether linkage. The same amino acid, in addition to this lipid moiety, also contained one acyl chain in amide linkage, thereby blocking the aminoterminus.

Contrasting results have been reported on the structure of lipid bound to membrane penicillinase of *Bacillus licheniformis*. From a series of investigations *Yamamoto* and *Lampen* (1975, 1976a, b) concluded that the protein was covalently modified through the binding of phosphatidic acid to serine. These results were later disputed by *Simons* et al. (1978), who failed in their attempt to prepare peptides from penicillinase from the same organism with covalently linked lipid. However, the case does not seem to be closed yet, because researchers from three different laboratories have since reported that membrane penicillinase can be biosynthetically labeled with ^{32}P-phosphate, 2-^{3}H-glycerol, and ^{3}H-palmitic acid. The label can be released from membrane penicillinase when the aminoterminus is cleaved through proteolytic digestion (*Lai* et al. 1981; *Nielsen* et al. 1981; *Smith* et al. 1981). Both findings support the original reports by *Yamamoto* and *Lampen* (1975, 1976a, b), but they do not add information with regard to the type of linkage between lipid and polypeptide. However, in their most recent report, *Nielsen* and *Lampen* (1982) demonstrate that covalent lipid binding also applies to β-lactamases of other gram-positive organisms, e.g., *Bacillus cereus* and *Staphylococcus aureus*. After performic acid oxidation and hydrolysis of the membrane forms of penicillinases from *Bacillus licheniformis* and from the above-mentioned species, these authors isolated glyceryl cysteine sulfone (*Nielsen* and *Lampen* 1982). Their results suggested that membrane penicillinases are covalently modified through lipids in a manner very similar to the above-mentioned well-known lipoprotein of the outer membrane of *E. coli* (*Hantke* and *Braun* 1973).

3.2 Linkage in Acylproteins Generated in Tissue Culture Cells

Although the data summarized in Sect. 2 on the properties of protein-bound fatty acids are regarded as very strong evidence for an ester linkage between fatty acids and hydroxy-amino acids of the polypeptide, more definitive chemical analysis of the acylation site has been sought by a number of laboratories. The isolation of small fatty-acid-containing peptides through proteolytical degradation of purified viral or cellular acylproteins was attempted. Acylated peptides were initially obtained through pronase digestion of the VSV G protein and subsequently isolated through extraction with organic solvents (*Schmidt* and *Schlesinger* 1979). In the course of purifying fatty-acid-carrying peptides for amino acid and sequence analyses, unfavorable properties of this material became apparent. Extreme stickiness, tendency to aggregate, and undefined losses of material continue to make it extremely difficult to generate conclusive sequence data on the

attachment site. However, despite these problems in handling acylpeptides, initial amino acid data on fragments of the VSV G protein revealed a relative enrichment of serine residues which, through their hydroxyl groups, could represent the potential binding partners for fatty acid in an ester linkage (*Schmidt* and *Schlesinger* 1979). Similar results were also obtained for acylpeptides derived from the influenza hemagglutinin, and the glycoproteins of Semliki Forest virus (*Schmidt,* unpublished).

While the exact binding sites for fatty acids in acylproteins have not yet been determined, the attempts to localize their topographical position have been more successful. By making use of the protective function of the lipid bilayer during controlled proteolytical digestion of the external portions of membrane-inserted glycoproteins, particles containing membrane-bound spike fragments were generated (Fig. 2). Through such types of experiments it was established that the protein-bound fatty acids are located in polypeptide regions which are not accessible to proteolytic enzymes (*Schlesinger* et al. 1981; *Petri* and *Wagner* 1980; *Rice* et al. 1982; *Schmidt* 1982b; *Capone* et al. 1982; *Omary* and *Trowbridge* 1981b).

Although these data strongly suggested that fatty acids were bound in the carboxy-terminal region of the respective acylprotein, direct proof for such a topographical position was only recently provided through the analysis of large acylated protein fragments.

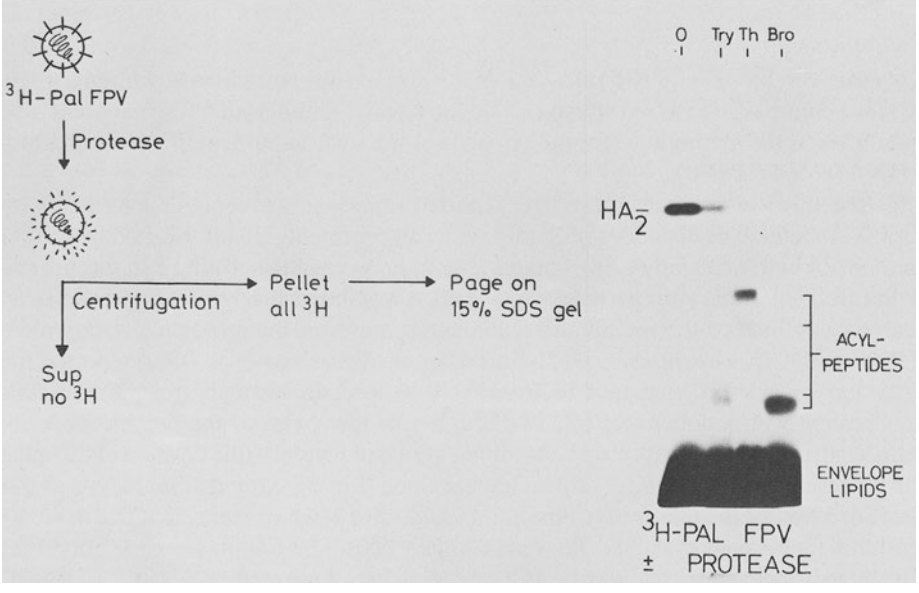

Fig. 2. Analysis on polyacrylamide-gel electrophoresis of membrane-bound acylpeptides after controlled proteolysis of ^{3}H-palmitate-labeled virus particles. The schematic illustration on the *left half* of the figure depicts the experimental procedure. On the *right* a fluorogram of influenza viral acylproteins before and after treatment of virus particles with proteases is shown (*O*, control; *Try,* trypsin; *Th*, thermolysin; *Bro,* bromelain). The enzymes were present at a concentration of 2 µg/ml. Incubations were for 3 min at 37 °C (*Schmidt* 1982b). Application of this experimental setup yielded similar results with a number of other RNA-envelope viruses, for instance vesicular stomatitis virus (*Schlesinger* et al. 1981; *Petri* and *Wagner* 1980; *Capone* et al. 1982), Sindbis virus (*Schmidt,* unpublished; *Rice* et al. 1982), and Semliki Forest virus (*Schmidt,* unpublished)

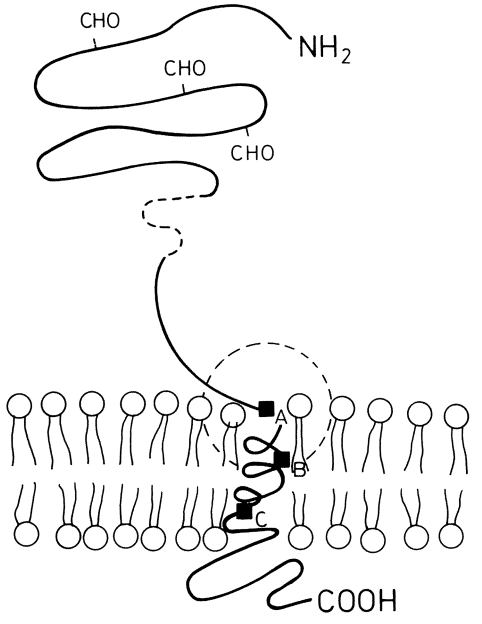

Fig. 3. Model of membrane glycoprotein with potential acylation sites. Fatty acids of fusogenic viral glycoproteins are probably bound to hydroxyamino acids inside or close to the membrane-spanning fragment of the respective polypeptide. If the acylation site is located in the outer leaflet of the lipid bilayer (designated *A*), the fatty acids could potentially be involved in the induction of fusion with heterologous membranes (*Schmidt* 1982a)

By cyanogen bromide (CNBr) cleavage of the purified influenza hemagglutinin a single CNBr peptide was obtained which carried fatty acids. This acylated fragment could be identified as the membrane-spanning peptide of the small subunit of the hemagglutinin (HA_2) (*Schmidt* 1982b).

Recently two research groups have reported amino acid sequence data on the membrane-embedded acylpeptides of Sindbis virus glycoproteins E1 and E2, and of VSV G protein. In both cases fatty-acid-containing peptides were obtained after external proteolytic attack on whole virus particles (see Fig. 2). It was shown that fatty acids were quantitatively confined to the membrane-spanning segments of the respective glycoprotein (*Rice* et al. 1982; *Capone* et al. 1982). Furthermore, *Schlesinger* et al. (1981) reported the isolation of a short oligopeptide from VSV G protein through treatment of this acylprotein with iodobenzoic acid. Although only low yields of the peptide were obtained, after purification it could be confined to an extracytoplasmic position of the spike just "outside" the lipid bilayer of the viral envelope (Fig. 3). Amino acid analysis of this peptide revealed the presence of nine amino acids, five of which were identified as serine residues (*Schlesinger* et al. 1981; *Rose* and *Gallione* 1981). This finding lends support to the hypothesis that serine might serve as the binding partner for fatty acids in acylproteins (*Schmidt* and *Schlesinger* 1979).

4 The Biosynthetic Event of Acylation

While a large body of information has accumulated on the biosynthetic modification of proteins through glycosylation and proteolytic cleavages (*Wold* 1981) our knowledge of the biosynthetic process of fatty acid addition to proteins is very limited. The available data have been derived mainly from pulse-chase experiments with virus-infected tissue

culture cells. Very few data have been reported for acylation in noninfected cells. Some useful information has been obtained through the use of viral glycoprotein mutants and through the application of various metabolic inhibitors.

4.1 Acylation in Virus-Infected Cells

Experimental data on the process of covalent fatty acid addition to membrane proteins were initially obtained through the study of the biosynthesis of VSV and Sindbis virus glycoproteins in infected chick embryo fibroblasts. The incorporation of ^{3}H-palmitic acid into virus-specific glycoproteins during short pulses was related to other well-documented modifications of these polypeptides. It was revealed that in cells infected with Sindbis virus, not the mature glycoprotein E2, but its immediate precursor, PE2, represented the acceptor for fatty acids (Fig. 4). Short-pulse-labeled PE2 was subse-

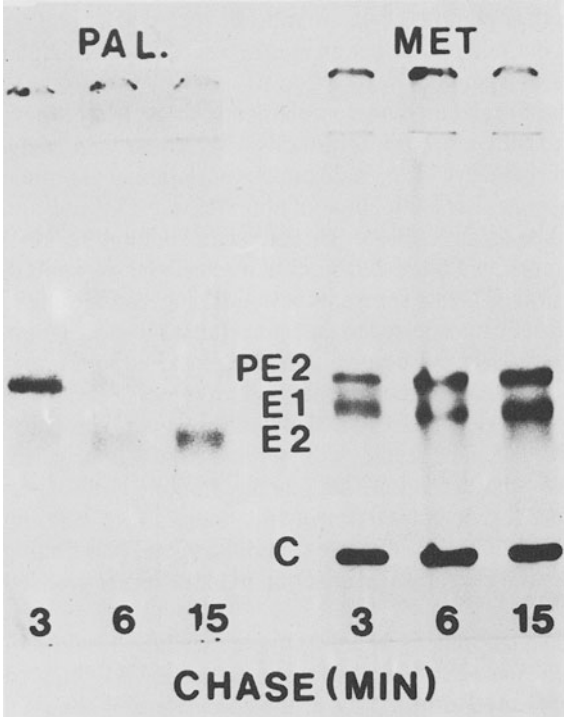

Fig. 4. Cleavage of short-pulse-labeled acylated Sindbis virus precursor glycoprotein *PE2* during a chase. A fluorogram of radiolabeled proteins from Sindbis virus-infected chick embryo cells is shown. The infected cells were labeled at 4.5 h postinfection for 3 min with ^{3}H-palmitic acid (*PAL*) and ^{35}S-methionine (*MET*). Pulse media were replaced by media containing nonlabeled palmitic acid or methionine, and cell lysates were prepared for polyacrylamide-gel electrophoresis after chase periods of 3, 6, and 15 min. While fatty-acid-labeled *PE2* is cleaved into *E2* after 6 min of chase, cleavage of the ^{35}S-methionine-labeled species of *PE2* only starts after 15 min of chase. This indicates that newly synthesized *PE2* takes significantly longer to reach the intracellular cleavage site than freshly acylated *PE2* (*Schmidt* and *Schlesinger* 1980). Almost identical results were obtained with BHK cells infected with Semliki Forest virus (*Berger* and *Schmidt,* unpublished)

quently cleaved very rapidly into E2 during a chase period of only 3–6 min. Since newly synthesized Sindbis PE2 pulse labeled with ^{35}S-methionine took at least 20–30 min to be cleaved, it was concluded that fatty acid addition occurs after the translation of PE2 and just prior to the cleavage of this precursor glycoprotein (*Schmidt* and *Schlesinger* 1980). Acylation must thus be regarded as a posttranslational event (see Fig. 4). The same type of results were obtained with BHK cells infected with Semliki Forest virus (SFV). Again it was the precursor glycoprotein p62 which was acylated first and which then became cleaved into E2 and E3 (*Berger* and *Schmidt,* unpublished).

In addition to defining acylation as a posttranslational process, this type of experiment provides another important piece of information. At the time of pulse labeling (4 h postinfection), precursors and the mature forms of the respective virus-specific proteins are abundant in the infected cells (*Klenk* and *Rott* 1980; *Schlesinger* and *Kääriäinen* 1980; *Schmidt,* unpublished). If acylation represented a nonspecific adhesion of fatty acids to the glycoproteins, one would expect all the different forms of this protein to be labeled during the short pulse of the infected cells with ^{3}H-fatty acid. Since only the precursor glycoproteins are labeled under such conditions acylation must be regarded as a highly specific event which depends on a defined conformation and/or a specific intracellular location of the respective acceptor polypeptide.

The posttranslational character of acylation is also emphasized by data from experiments using cycloheximide and tunicamycin, or virus mutants with defects in their glycoprotein. It was shown, for instance, that cycloheximide inhibits acylation of G protein and of Sindbis virus glycoproteins only after a lag phase of about 15 min (*Schmidt* and *Schlesinger* 1980), which can be taken as direct proof that fatty acid addition does not occur during translation of the polypeptide. Likewise, a block of intracellular transport of G protein by tunicamycin (*Gibson* et al. 1979) also prevents acylation, suggesting that the newly synthesized glycoproteins must be transported to the intracellular site of acylation. Furthermore, when virus-infected cells were pulse labeled with both ^{35}S-methionine and ^{3}H-palmitic acid and the released virus particles were analyzed after increasing chase periods, ^{3}H-labeled glycoproteins preceded the ^{35}S-labeled species in the virions by 15–20 min (*Schmidt* and *Schlesinger* 1980). The studies with ts-mutants of Sindbis virus, VSV, and influenza viruses revealed that acylation was severely inhibited at nonpermissive temperature while allowing for unrestricted protein synthesis (*Schmidt* and *Schlesinger* 1980; *Zilberstein* et al. 1980; *Schmidt* and *Klenk,* unpublished). This finding again stresses the posttranslational nature of fatty acid attachment to the respective viral glycoproteins.

Extending the information on acylation with regard to the intracellular location of the acylating enzymes, *Schmidt* and *Schlesinger* (1980) demonstrated that in VSV-infected chick embryo cells acylation occurs only 3–5 min prior to the completion of oligosaccharide trimming of the G protein. Since it is known that the last steps of this trimming process are conducted by mannosidases present in the Golgi apparatus (*Kornfeld* and *Kornfeld* 1980; *Hubbard* and *Ivatt* 1981; *Tabas* and *Kornfeld* 1979; *Grinna* and *Robbins* 1979, 1980; *Rothman* 1981), it was concluded that acylation occurs in the immediate vicinity of the Golgi apparatus, possibly during late stages of the transit between the endoplasmic reticulum and the Golgi apparatus (*Schmidt* and *Schlesinger* 1980). This hypothesis was recently confirmed through experimental data reported by *Dunphy* et al. (1981). Through cell fractionation studies with Chinese hamster ovary cells (CHO cells) infected with VSV, these authors found that G protein labeled with ^{3}H-palmitic acid in a

short pulse sedimented with the same membrane fraction that also carried α-1,2-mannosidase activity. From their knowledge of the precise location of this glucosidase they concluded that acylation of the G protein must have occurred very close to the cis-portion of the Golgi apparatus (*Dunphy* et al. 1981).

That acylation does not function at a location between the Golgi apparatus and the plasma membrane has been established, at least for viral acylproteins. By growing Sindbis virus and VSV in the presence of the ionophore monensin, *Johnson* and *Schlesinger* (1980) found that intracellular transport of viral glycoproteins functions normally between the endoplasmic reticulum and the Golgi apparatus, but that any further transport towards the plasma membrane of the cells was inhibited. When the glycoproteins were labeled with ^{3}H-palmitic acid in the presence of monensin no effect at all was recorded with regard to acylation of Sindbis virus and VSV glycoproteins (*Johnson* and *Schlesinger* 1980).

Few attempts have yet been made to block acylation of viral glycoproteins by cerulenin (*Omura* 1976) and nafenopin (*Schwarz,* personal communication), drugs which interfere with lipid metabolism. It was found in our laboratory that during in vivo experiments with Semliki Forest virus in BHK cells, both of these drugs severely inhibited both ^{14}C-amino *and* ^{3}H-palmitic acid incorporation into viral glycoprotein, indicating a generalized toxic effect on the cell metabolism. These observations are at variance with a recent report by *Schlesinger* and *Malfer* (1982), who under certain conditions observed a more selective inhibitory effect of cerulenin. These authors found that less tritiated fatty acid was incorporated into VSV and Sindbis virus glycoproteins when virus-infected chick cells had been treated with cerulenin prior to pulse labeling with ^{3}H-palmitic acid. Since cerulenin inhibits the de novo synthesis of fatty acids (*Omura* 1976), it is presently not clear how it influences the incorporation of exogenously applied ^{3}H-palmitic acid into glycoproteins. Although suggested by the above data, a direct inhibitory effect on the acyltransferase is unlikely since cerulenin, even at extremely high concentrations (up to 150 μg/ml), had no effect on the transfer of acyl chains onto lipid acceptors in vitro (*Berger* and *Schmidt,* unpublished). However, different enzymes may catalyze the transfer of fatty acids onto the polypeptide and lipid acceptors.

4.2 Acylation in Noninfected Cells

Information about acylation in noninfected cells is scarce since for eukaryotic cells acylation of nonviral proteins has been described for only a few membrane proteins, most extensively for the transferrin receptor. However, since viral glycoproteins are synthesized by cellular enzymes, it is to be expected that the acylation of viral and nonviral membrane proteins is catalyzed by the same cellular enzymes.

Schlesinger et al. (1980) reported that acylation of cellular membrane proteins, as with viral species, can be inhibited by inhibitors of protein biosynthesis. This demonstrates the specific nature of the event, or at least excludes the possibility of acylating proteins through adsorptive effects. In a more recent study of the biosynthesis of the transferrin receptor, *Omary* and *Trowbridge* (1981a) reported that acylation continued to function even after glycosylation had been blocked through tunicamycin. This result is in accordance with the findings of *Schmidt* and *Schlesinger* (1979), who had shown that the G protein of VSV strain Orsay (*Gibson* et al. 1978, 1979), grown at 30 °C, can be acylated even if

no carbohydrates are bound to the polypeptide backbone of this protein. Both these results represent evidence that oligosaccharides are neither involved as acceptors for fatty acids, nor are they required for allowing the acyltransferases to operate. The only prerequisite for acylation seems to be that the acceptor polypeptide reach a certain intracellular location, probably that of the protein-acyltransferases.

While showing the same chemical binding characteristics, the transferrin receptor seems to differ from viral acylproteins in a few respects. In their recent communication, *Omary* and *Trowbridge* (1981a) demonstrate that this surface protein becomes acylated after oligosaccharide processing is completed. This conclusion is based on the finding that transferrin receptor labeled with ^{3}H-fatty acid during a short pulse is completely resistant to β-endo-glucosaminidase H. Furthermore, by comparing the half-lives of receptor labeled with either ^{35}S-methionine or with ^{3}H-palmitic acid, the above authors found that tritiated fatty acids were lost from the protein at a faster rate than explainable by a mere protein turnover. It was concluded from these data that the transferrin receptor may be subject to deacylation, thereby making this protein available for the addition of new fatty acids during its internalization cycle (*Omary* and *Trowbridge* 1981a; *Morré* et al. 1979; *Karin* and *Mintz* 1981; *Octave* et al. 1981). It was also shown that in this system acylation continued after protein synthesis inhibition by emetine; this emphasizes the authors' hypothesis of de- and reacylation reactions (*Omary* and *Trowbridge* 1981a). The reasons for the discrepancy between the findings with the receptor glycoprotein and the data available for the acylation of viral glycoproteins are presently not understood. However, the transferrin receptor must afford some special features because, in order to function in iron transport, it is subject to internalization (*Morré* et al. 1979; *Karin* and *Mintz* 1981; *Octave* et al. 1981). This process is unlikely to occur with viral glycoproteins, because once at the plasma membrane the mature glycoproteins are subject to homo- and heterologous interactions with viral structural components. This leads to the budding of progeny virus particles (*Simons* and *Garoff* 1980). Also, it is noteworthy that glycosylation of the transferrin differs from that of viral glycoproteins. While the oligosaccharide processing of VSV G protein takes about 15–30 min, the same process with the receptor protein requires a period of at least 4 h (*Omary* and *Trowbridge* 1981a; *Kornfeld* and *Kornfeld* 1980).

Presently, no protein-acyltransferases have been isolated, or even localized, intracellularly. Also the lipid donor for the acyl moities transferred onto the protein has not yet been identified. Since palmitoyl-CoA functions as the acylation precursor during the biosynthesis of phospholipids, its involvement in the transfer of acyl chains onto protein is to be expected (*Bell* and *Coleman* 1980). On the other hand, phospholipids or neutral lipids cannot be excluded as potential acyl donors. Circumstantial evidence for the donor function of phospholipids in the acylation of the bacterial murein lipoprotein has been obtained by *Lai* et al. (1980) and *Chattopadhyay* and *Wu* (1977). Our own in vivo experiments with eukaryotic membrane glycoproteins also indicate the potential for general cellular lipid to act as donor in the acylation of protein. After a long period of labeling of BHK cells with ^{3}H-palmitic acid the pulse medium was replaced with nonlabeled culture fluid and cells were infected with Semliki Forest virus. Four to six hours later cell lysates were prepared and analyzed for protein-bound tritiated fatty acids. Although after long-term labeling no labeled palmitoyl-CoA could be detected in extracts of the cells, acylation of glycoprotein had occurred quite effectively (*Berger* and *Schmidt,* unpublished). However, these experiments do not exclude palmitoyl-CoA as the acyl donor. Because of the very rapid turnover of the intracellular pool of this metabolite, the amount present

may have been too small to be detected by thin-layer chromatography and subsequent radiochromatogram scanning. It thus becomes obvious that an in vitro system of acylation is required to test the suitability of different lipid species for the transfer of acyl chains into polypeptides.

5 Functional Role of Protein-Bound Fatty Acids

Myelin proteolipid was the first acylated protein described more than 30 years ago (*Folch-Pi* and *Lees* 1951). However, the wide occurrence of acylation as a posttranslational modification of membrane proteins has been revealed only during the last 3 years (*Schmidt* et al. 1979; *Schmidt* 1982a, b; *Magee* and *Schlesinger* to be published; *Schlesinger* 1981; *Omary* and *Trowbridge* 1981b; *Madoff* and *Lenard* 1982; *Marinetti* and *Cattieu* 1982; *Keenan* et al. 1982; *Agrawal* et al. 1982; *Nielsen* et al. 1981). It seems natural that to date little experimental evidence for the functional role of this new phenomenon of acylation in cell biology is available, given our limited knowledge about the biological significance of glycosylation, a long-known posttranslational modification of proteins. However, it is not too early to attempt to compare various speculative models that have been discussed in this field.

5.1 The Anchor Hypothesis

Adding hydrophobic fatty acid residues to a protein will certainly increase the hydrophobic affinity of the respective stretch of the polypeptide. It therefore is logical to assume that acylation will contribute to the anchorage of membrane proteins to the lipid bilayer of biological membranes (*Schmidt* et al. 1979), a hypothesis which was followed by other authors (*Omary* and *Trowbridge* 1981a; *Marinetti* and *Cattieu* 1982; *Keenan* et al. 1982; *Agrawal* et al. 1982; *Petri* and *Wagner* 1980). Experimental support for this theoretically based hypothesis has been provided mainly by experiments by *Huang* et al. (1980), who chemically acylated soluble immunoglobulins with fatty acids. Only after such chemical modifications were those glycoproteins suitable for incorporation into liposomes. Furthermore, the possibility was discussed that deacylation of the transferrin receptor at the cell surface may be related to its internalization (*Bretscher* 1977; *Morré* et al. 1979; *Omary* and *Trowbridge* 1981a). This seems an attractive idea, although no data were given that would explain the molecular mechanism of this trigger for receptor recycling. It is known that receptor glycoproteins remain membrane-bound during their internalization. Triggering this process through deacylation, therefore, certainly does not lead to the release of the respective protein from the bilayer of the plasma membrane or from internalized membrane vesicles.

The only data that more directly indicate an anchor function for protein-bound fatty acids come from recent reports on the acylation of transforming proteins and on the fluorescence anisotropy of fatty acids in VSV G protein (*Magee* and *Schlesinger*, to be published; *Petri* et al. 1981). Transforming proteins coded for by certain tumor viruses are synthesized on cytoplasmic ribosomes before they are inserted into membranes (*Levison* et al. 1981). This posttranslational membrane insertion, or anchoring process, is possibly facilitated through the covalent attachment of fatty acids. In a more direct approach, *Petri* et al. (1981) specifically labeled VSV G protein with 16-(9-anthroyloxy)-palmitate and reconstituted it into dipalmitoylphosphatidylcholine vesicles. With such liposomes the mobility of protein-bound fatty acids as a function of temperature could be determined

by fluorescence measurements. The authors found a strong interaction between acyl chains of the G protein and lipids of the artificial vesicles which led to the removal of bilayer lipid from the phase transition during temperature shift experiments (*Petri* et al. 1981). This experiment is important in several respects. It shows that fatty acids bound to VSV G protein are accessible to the environment and thus are not hidden in any clefts or pockets which may be present in the native configuration of the glycoprotein. The data furthermore confirm the biochemical evidence for a membrane location of the acylation site (see Sect. 3.2) through physicochemical methods.

The fact that quite a few fatty-acid-free membrane proteins do exist (*Omary* and *Trowbridge* 1981b; *Schmidt* 1982a, b) does not necessarily exclude the validity of the anchor hypothesis. Similar to glycosylation, where mutations in the polypeptide chain of VSV G protein render the glycoprotein completely functional even without any oligosaccharide chains (*Gibson* et al. 1978, 1979, 1980, 1981), conformational features of nonacylated membrane proteins may provide the required membrane affinity. This may apply, for instance, to the influenza neuraminidase, to the HN glycoprotein of paramyxoviruses, to coronavirus glycoprotein, E1 (*Schmidt* 1982a), to human and murine T200 glycoprotein, and to the histocompatibility antigen, HLA (*Omary* and *Trowbridge* 1981b; *Schmidt* and *Fleischer,* unpublished). One of these nonacylated species deserves special attention, the μ chain of the membrane form of human immunoglobulin (IgM) (*Schmidt* and *Fleischer,* unpublished). This membrane glycoprotein has a surprisingly high proportion of hydrophilic hydroxyamino acids (10 out of a total of 26) within the membrane-associated segment (*Rogers* et al. 1980). Thus acylation, which potentially would convert these hydroxyl-containing residues into hydrophobic moieties thereby fixing the macromolecule into the bilayer, does not seem to be necessary to hold the μ chain in the membrane. Since the hydrophobicity index of the membrane-spanning fragment of IgM is fairly low, one must assume a three-dimensional configuration of this molecule which prevents the exposure of too many hydroxyamino acids to the lipid environment. With acylated membrane proteins, more "configurational freedom" of the membrane-attached region would be provided since "unwanted" hydroxyl groups would be hydrophobically masked.

At first glance the above hypothesis is seriously challenged by certain features of the biosynthesis of membrane proteins. It is widely accepted that initial membrane insertion and subsequent anchorage are processes operating during the translation of the respective molecules (see Sect. 1.1.2), but the attachment of fatty acids, at least with membrane glycoproteins, occurs in the "early" Golgi complex (*Schmidt* and *Schlesinger* 1980; *Dunphy* et al. 1981; *Rothman* 1981), or possibly even later (*Omary* and *Trowbridge* 1981a). A reasonable explanation for this discrepancy is based on the assumption that different stabilities of membrane anchorage may be required during biosynthesis. At early stages membrane affinity of the nascent polypeptide may of necessity be relatively weak, allowing for some flexibility to facilitate the action of contranslationally modifying enzymes. Reaching a higher degree of maturity, the glycoprotein may become anchored more tightly in the lipid bilayer when it becomes modified through fatty acid addition at the site of acylation.

5.2 The Transport Hypothesis

Schmidt and *Schlesinger* (1979) and *Zilberstein* et al. (1980) investigated a number of ts mutants of VSV which carried defects in the G protein. In both reports the lack of acy-

lation at the restrictive temperature is described. With most of the mutants of VSV, the G protein failed to reach the Golgi complex during its biosynthesis, and thus lacked fatty acids. However, *Zilberstein* et al. (1980) detected one mutant, VSV ts L 511 (V), which in Vero cells leads to the synthesis of almost fully glycosylated, but still nonacylated, G protein. Since this species of G protein did not appear on the cell surface, the authors claimed that acylation may be required for the transport of membrane glycoproteins from the Golgi complex to the plasma membrane. However, they did not provide any information on the possible molecular mechanism for this transport function of protein-bound acyl chains.

Meanwhile, more virus mutants of VSV and influenza virus with defects in their glycoproteins have been studied (*Scholtissek* and *Bowles* 1975; *Lohmeyer* and *Klenk* 1979). With these mutants the above hypothesis could not be substantiated, because with none of them was it possible to exclude defects other than in acylation (*Schmidt,* unpublished). With the data available it therefore seems impossible to directly correlate intracellular transport with the presence of fatty acids in the glycoprotein under study at the present time. However, it certainly would be of great interest if a viral ts mutant could be identified with a defect strictly limited to the acylation site of the respective glycoprotein. Obviously, such mutants would facilitate research on the functional relevance of protein-bound fatty acids.

5.3 The Fusion Trigger Hypothesis

Since membrane proteins are modified through fatty acid addition, it seems reasonable to look for common features of the acylated species. It is striking that almost all viral membrane glycoproteins which are acylated with fatty acids are also believed to be involved in fusion induction (*Schmidt* 1982a). The best-documented examples for this property are represented by the F protein of paramyxoviruses (e.g., Newcastle disease virus, Sendai virus); the hemagglutinin, HA_2, of human and avian influenza viruses; and the E2 protein of murine and bovine coronaviruses (*Klenk* and *Rott* 1980; *Schmidt* 1982b; *Scheid* and *Choppin* 1974; *Huang* et al. 1980a, b; *Sturman* et al. 1980). Although no direct experimental evidence has yet been provided for the involvement of protein-bound acyl chains in membrane fusion, a number of findings indicate their potential participation. Oleic acid has long been known to be fusogenic (*Ahkong* et al. 1973) and, among others, this fatty acid has been shown to be a component of acylproteins (*Schmidt* et al. 1979; *Keenan* et al. 1982). Furthermore, using an in vitro system of fusion between artificial membranes and erythrocytes it was recently found that specific lipids inhibit the fusion process (*Huang*, to be published). Similar results had been obtained by *Sands* et al. (1979), who reported the inhibition of penetration of bacteriophage PR4 into *E. coli* through certain fatty acid derivatives. Although neither report offered a molecular mechanism for the observed specific inhibition of fusion, it has been proposed that certain lipid structures in the cellular plasma membranes may function as "fusion receptors." Through an excess of exogenous lipids, these "receptors" could be rendered nonfunctional for their interaction with fusogenic acylproteins which may lead to the observed inhibition of fusion and penetration. The potential function of protein-bound acyl chains in fusion induction is also supported by our knowledge of the topographical location of fatty acids within the polypeptide backbone of viral glycoproteins. Structural analysis of the acylation site in the G

protein of VSV strongly indicates that the acyl chains are bound to amino acids located at the external boundary of the viral lipid bilayer (position A in Fig. 3; *Schlesinger* et al. 1981) where they could function best in triggering fusion with a closely associated membrane.

Clearly, this fusion trigger hypothesis is derived exclusively from data on acylated viral spike glycoproteins, and certainly more structural and functional data are required to substantiate it. Whether this hypothesis has any relevance to acylated membrane proteins in general remains to be seen, since the nonviral proteins found thus far to be acylated serve receptor or enzymatic functions (see Table 2). However, since fusion is a high-frequency process during membrane traffic in any cell (*Morré* et al. 1979; *Bretscher* 1977; *Singer* and *Nicolson* 1975), it must be expected that the cells have some means of controlling these processes. Analogous to the properties of viral glycoproteins, it is anticipated that membrane proteins found to be acylated in noninfected cells may be fusogenic, in addition to their enzymatic or receptor functions. Certainly more experimental data will be necessary to substantiate this hypothesis.

6 Conclusions

Acylproteins must be regarded as a class of proteins involved with many cellular activities operating in a membrane environment. The fact that proteins modified through fatty acids have been found in viral, bacterial, plant, and animal membranes emphasizes their potential importance in cell biology. Obviously, at a time in which the crucial role of cell membranes in cellular function becomes more and more apparent, information on the functional role of this novel kind of polypeptide substituent is of major interest.

Despite the lack of direct evidence for fatty acid function in acylproteins, various models have been discussed based on distribution and structure. However, it will not be surprising if none of the given hypotheses alone suffices to describe the function of acyl chains in proteins. It is more likely that combinations of the individual theories operate with a given acylprotein. Furthermore, as with the oligosaccharides in glycoproteins, the acyl chains in different acylproteins may well serve different functions. Once it is possible to generate deacylated forms of a given acylprotein in the native conformation, meaningful experiments can then be conducted to assess the contribution of acyl chains to the biological activity of these membrane proteins.

In addition to the determination of the functional significance of acylation, there are two more major questions, the answers to which would strongly contribute to an understanding of the function of acylproteins. First of all, the precise chemical structure of the acylation site needs to be elucidated. Secondly, the intracellular location and general identification of the polypeptide acyltransferases need to be determined with certainty.

Acylproteins, through their classical form of "proteolipids," have been known for some 30 years (*Folch-Pi* and *Lees* 1951), but to date neither with these - except for bacterial lipoprotein (*Hantke* and *Braun* 1973) - nor with the recently detected acylproteins (*Schmidt* et al. 1979) has it yet been possible to identify the chemical linkage between fatty acid and polypeptide. However, with all the data on acylation summarized in this article, any initial doubt about the covalent nature of this linkage should be dissipated. Nevertheless, protein chemical analysis of short acylated peptides from various acylproteins (*Jolles* et al. 1981; *Schmidt* 1982a; *Gorski* and *Schmidt*, unpublished) needs to be taken further despite the unfavorable properties of fatty-acid-containing hydrophobic peptides.

This is necessary in order to verify our hypothesis that in most acylproteins fatty acids are linked to hydroxyl-containing amino acids. With regard to the intracellular location of the acylation event, the evidence presented points to the cis-Golgi complex as the acylating organelle. Clearly this conclusion is based on knowledge about the intracellular location of certain other enzymes that operate during glycosylation. Therefore, cell fractionation studies are necessary to prove this hypothesis in a more direct way. Once a precise localization of the cellular acylation site has been achieved, the modificational step of fatty acid addition would be suitable to serve as a marker for specific intracellular membranes during cell biology experiments. Through the establishment of an in vitro system of acylation additional questions could be addressed which eventually would lead to the biochemical characterization of protein-acyltransferases.

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