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FATTY ACID ACYLATION OF EUCARYOTIC CELL MEMBRANE PROTEINS

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Contents

I. Summary

Membrane proteins from a number of enveloped viruses and animal cells have been found to contain small amounts of tightly-bound lipid. This lipid consists predominately of long-chain fatty acids and appears to be linked covalently in ester bond directly to hydroxy-amino acids in the polypeptide. The precise location of the fatty acid linkage in the amino acid sequence has not been determined for any acylated protein; however, there is substantial evidence that lipid is bound in a domain of the protein which interacts strongly with the lipid bilayer. Stoichiometry of fatty acid has been measured for a few of these proteins and, in most cases, there are on the order of two lipids on each polypeptide chain. Fatty acid binding is a post-translational event in virus glycoprotein biosynthesis and intracellular membranes associated with the Golgi apparatus have been implicated as the site of acylation. No functions have yet been found for covalent-lipids. They do not seem to be essential for the intracellular transport of mem-

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Abbreviations: VSV, vesicular stomatitis virus; G, glycoprotein of VSV; CoA, coenzyme A; E1, (P)E2, glycoproteins of Sindbis virus.

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brane proteins or for anchoring these proteins to the membrane although they may facilitate both activities. Preliminary data suggest that virus budding from infected cells requires fatty acid acylated proteins.

II. Introduction

The primary structure of many proteins in their 'mature' functional state includes various kinds of chemical substituents that have been added onto the protein's polypeptide backbone. Among these are oligosaccharides, phosphate, methyl groups, and nucleosides and their addition to the polypeptide occurs both during nascent chain biosynthesis and later as the protein becomes integrated into cellular metabolic activity. It is clear that a protein's sequence of amino acids and the gene encoding them is the primary determining factor in controlling such modifications, but precisely how these modifications affect function is known in only a few cases. In this review, we discuss a modification that appears to influence the interaction of some proteins with membranes in eukaryotic cells. The substituents here are fatty acids and, until recently, knowledge of their covalent attachment to proteins was limited to a small number of highly specialized bacterial proteins (reviewed in Ref. 1). The discovery by Braun [2] of a 'lipoprotein' in the *Escherichia coli* cell wall led to detailed structural studies which showed that fatty acids were bound in both ester and amide linkages to the protein [3]. The esterified fatty acids were attached to a glycerol moiety that was in thioether linkage to the amino terminal cysteine while a third fatty acid was bound to the terminal α amino group. More recently, the membrane penicillinase of *Bacillus licheniformis* has been shown to contain fatty acids and glycerol in linkages analogous to the *E. coli* protein [4–7].

The possible covalent attachment of lipid to proteins in higher organisms was first reported over 30 years ago by Folch-Pi and Lees in their analysis of an organic-solvent extractable protein from brain myelin [8,9]. About 3 years ago, studies of two glycoproteins from an enveloped animal virus suggested that these membrane-associated proteins also contained covalently-bound fatty acids [10,13]. Presently, there are a substantial number of membrane proteins from enveloped viruses, tissue culture cells, and animal cells that are reported to contain covalent lipid (Table I), and it is likely that the list will grow considerably in the coming years. It is the purpose of this review to discuss the data which led to the discovery of this latter class of proteins and to attempt a rationalization of their possible functions.

TABLE I

MAMMALIAN MEMBRANE-ASSOCIATED PROTEINS ACYLATED WITH FATTY ACID
Sindbis virus glycoproteins, E2 (.Y.Y.; .Y.T.); E1 (.S.K.T.) [13]
Semliki forest virus glycoproteins E2 (.Y.Y.;.T.V.S.); E1 (.S.G.T.) [16]
Vesicular stomatitis virus glycoprotein G [14]
Influenza virus hemagglutinin HA_2 (.S.G.Y.) [16]
Fowl-plague virus hemagglutinin HA_2 (.S.G.Y.) [16]
Newcastle disease virus fusion glycoprotein F1 [16]
Corona virus glycoprotein E2 [16,17]
Brain myelin proteolipoprotein [8,9,26,48]
Human transferrin receptors [20]
Membrane-associated tissue-culture cell proteins [18]
Butyrophilin and xanthine oxidase of milk fat globule membrane [47]
Preliminary Data:
Human histocompatability antigen, HLA B7
Fc receptor encoded by Herpes virus
p15E of murine leukemia virus
Transforming proteins of Rous sarcoma virus ($p60^{src}$), Harvey sarcoma virus (p21) and Abelson virus (p120).
MEMBRANE PROTEINS NOT ACYLATED WITH FATTY ACID
NA (neuraminidase) influenza virus [16]
HN (hemagglutinin, neuraminadase) Newcastle disease virus [16]
Corona virus glycoprotein E1 [16,17]
Two major glycoproteins of Herpes virus.
Human homologue of murine T200 glycoprotein [20]

Hydroxy amino acid-containing sequences shown in brackets are conserved between equivalent proteins from related viruses. Underlined residues indicate identity. The single letter code for amino acids is used.

III. Fatty acid acylation of viral membrane glycoproteins

Enveloped RNA viruses have proved to be invaluable tools in the investigation of co- and post-translational processing of membrane-associated proteins. These viruses have a number of unique features which contribute to this utility:

(1) Viral proteins are made in numbers often

10^2–10^3 fold higher than normal cellular proteins; (2) frequently host cell protein synthesis is shut off allowing easy identification of viral proteins; (3) viral structural components are relatively easily purified facilitating the preparation of specific antisera and the gathering of structural and sequence data; (4) the genomes of these viruses are small and code for a limited number of essential proteins. Thus the co- and post-translational processing of these proteins is dependent on host cell enzymes.

These factors have enabled the extensive investigation of the assembly of membrane structures including co-translational insertion and glycosylation, proteolytic and carbohydrate processing, intracellular transport and topology of membrane proteins (reviewed in Refs. 11 and 12). The detection of fatty acid acylated proteins was undoubtedly dependent on these factors.

IIIA Sindbis virus glycoproteins

Fatty acid labelling of Sindbis virus membrane glycoproteins was first reported by Schmidt et al. in 1979 [13]. The E1 and E2 glycoproteins of Sindbis virus purified from the extracellular fluid of [^{3}H]palmitate-labelled infected cells contained fatty acid in a ratio of approx. 1:3 although there are equal numbers of each protein per virion. No labelling of the capsid protein, which is present in equimolar amounts, was detected thus arguing against metabolic conversion of the label into amino acids. In addition, no radioactivity was detected in the glycoproteins from virus grown in ^{32}P-labelled cells. The ^{3}H-labelled fatty acid was not removed from protein by boiling in concentrated detergents before gel electrophoresis nor by extraction with organic solvents and thus was present in a tightly-bound form. The label could, however, be rapidly and quantitatively released from the glycoproteins by alcoholic KOH, a reaction characteristic of esters. The methanolysis products were analyzed by gas-liquid chromatography, and most of the label was in methyl palmitate, with lesser amounts in methyl stearate and oleate. Quantitation of unlabelled fatty acids bound to E1 after extensive delipidation with organic solvents provided an estimate of the tightly-bound fatty acid of 1–2 mol/mol protein. When [^{3}H]palmitate-labelled E1 was treated with a mixture of pepsin, thermolysin, and pronase low molecular weight products were obtained that could be resolved from free palmitic acid by thin layer chromatography, and the ^{3}H-label in these fragments was released by alcoholic KOH. All these data indicated that fatty acids were linked covalently to the polypeptide chain.

Intracellular E1 and E2, as well as the PE2 precursor to E2, also contain fatty acids. However, when the initial glycosylation of these proteins was blocked with tunicamycin, their synthesis continued but fatty acid acylation was not detected. Artefactual labelling of glycoproteins during their isolation was ruled out, since mixing extracts of labelled mock-infected cells with unlabelled infected cells produced no labelling of viral glycoproteins.

IIIB. Vesicular stomatitis virus glycoprotein

Subsequent studies with vesicular stomatitis virus glycoprotein (G) confirmed and extended the early results with Sindbis virus [14]. G was the only structural protein labelled when virus was

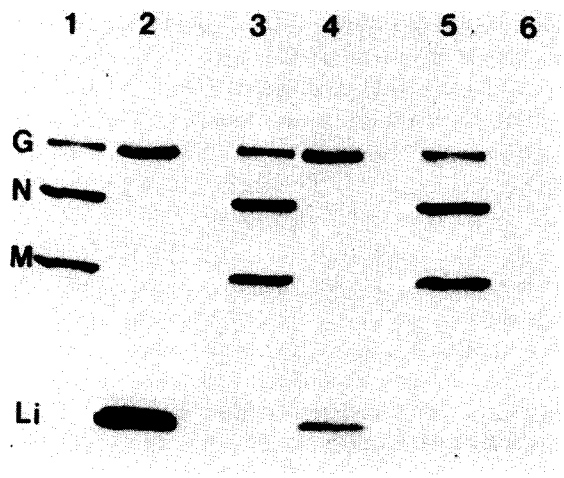

Fig. 1. Binding of [^{3}H]palmitate to VSV-G protein and its release by alkali. Viral particles labelled with [^{3}H]palmitate (lanes 2, 4 and 6) or [^{35}S]methionine (lanes 1,3 and 5) were pretreated and then analyzed on a 10% SDS-polyacrylamide slab gel. (1 and 2) No pretreatment; (3 and 4) extracted 3-times with chloroform-methanol (2:1); (5 and 6) incubated with 0.1 N KOH in methanol at 23°C for 20 min. 25000 cpm of ^{35}S and 700000 cpm of ^{3}H were added. Fluorography was used to reveal positions of the labelled bands. (G, N and M) refer to VSV glycoprotein, nucleoprotein and matrix protein, respectively; (Li) is viral lipid [14].

grown in chicken embryo fibroblasts given [^{3}H]palmitate (Fig. 1, lane 2). This fatty acid was resistant to release by SDS or organic solvents (lane 4) but could be released by transesterification (lane 6) as the methyl esters of palmitate, stearate and oleate. 1–2 mol of fatty acid were detected per mol protein, but no phosphate and little label from [2 –^{3}H]glycerol could be detected. The latter appeared to result from metabolic conversion to amino acids, since the other viral proteins contained label in proportion to the amounts of protein present in virus. Proteolytic digestion of ^{3}H-labelled G produced discrete fatty acid-containing fragments. Among these was one fragment heavily enriched in serine.

Important additional information about fatty acid acylation was obtained from studies with temperature-sensitive mutants of vesicular stomatitis virus and with different virus strains grown in cells treated with tunicamycin to block glycosylation. In all but one of the latter experiments, non-glycosylated G failed to move to the infected cell plasma membrane and failed to become acylated with fatty acid. In one case, the Orsay strain grown at 30°C, a non-glycosylated G was made which did move to the cell surface and was incorporated into infectious virus [15]. This non-glycosylated G was acylated with fatty acids. Most mutants with a temperature-sensitive G are unable at the non-permissive temperature to produce a glycoprotein that can be either acylated with fatty acid or processed for oligosaccharide alterations. Under these conditions, G is not detected on cell surfaces. There is one *ts* mutant in which the oligosaccharides on G are processed but no fatty acid acylation occurs [34]. This G also is not detected on the cell surface and it was proposed that fatty acid acylation might be required for intracellular transport to the plasma membrane, although more recent data indicate that transport is not dependent on acylation (see below). It is clear from all of the studies noted above that oligosaccharide processing and fatty acid acylation are independent events.

IIIC. Acylation of other viral glycoproteins

Since the initial observations of fatty acid acylation of viral membrane glycoproteins Schmidt [16] as well as our own laboratory have investigated several other virus systems and found at least one acylated membrane protein in each example studied. Three strains of avian influenza virus were labelled in chicken embryos or chicken embryo fibroblasts given [^{3}H]palmitate and label was associated with the uncleaved haemagglutinin (HA) as well as the small HA_2 subunit of the cleaved haemagglutinin. Neither the neuraminidase nor the hydrophobic matrix protein were labelled. The H0, H1, H2 and H3 haemagglutinin subtypes of human influenza viruses were the only labelled proteins in these viruses. The fatty acid was labile to methanolic KOH and digestion of intact particles with bromelain released the glycoprotein spikes but left the [^{3}H]palmitate bound to a peptide which could be repurified with the spikeless particles. The F (fusion) protein of Newcastle Disease Virus, a paramyxovirus, contained covalently attached fatty acid both in its uncleaved (F_0) and cleaved (F_1) forms. Semliki Forest Virus, an alphavirus related to Sindbis virus, contained fatty acid in its E1 and E2 glycoproteins when grown in a variety of cell types of arthropod, avian, hamster and human origin. Finally the E2 glycoprotein of coronavirus also labelled with [^{3}H]palmitate. This last result has been confirmed by Neimann and Klenk [17]. Recently, fatty acid has been found linked to the Fc receptor glycoprotein of Herpes virus (D.C. Johnson, personal communication) and to the transforming proteins of Rous sarcoma, Harvey sarcoma and Abelson virus (B.M. Sefton, I.S. Trowbridge, J.A. Cooper and E.M. Scolnick, personal communication).

It is clear from the data presented above that every enveloped virus which has been studied thus far contains at least one acylated glycoprotein. However, many of these viruses also contain non-acylated membrane glycoproteins which speaks for a specialized function of acylation in membrane glycoproteins.

IV. Fatty acid acylation of non-viral membrane proteins

The finding of a wide range of fatty acid acylated proteins in viruses prompted a search for similar proteins in uninfected cells. Chicken embryo fibroblasts labelled with 10–20-times more [^{3}H]palmitate than had been used in the viral

studies revealed the presence of more than 20 labelled bands resolved in a one-dimensional SDS-polyacrylamide gel [18]. All these proteins were associated with cellular membranes, although further localization was hampered by the difficulty of obtaining purified membrane fractions from cultured cells. The pattern of labelling with fatty acid was distinct from that with [^{3}H]leucine or [^{3}H]mannose. To confirm that the fatty acid label was esterified to protein whole gels were treated with 1 M hydroxylamine before fluorography. All the fatty acid label was removed from the proteins by this reagent and the released label co-chromatographed with authentic palmitoyl hydroxamic acid. The amino acid and carbohydrate labelling patterns were unaffected by this treatment. Cycloheximide, which blocks de novo protein synthesis, also blocked fatty acid addition in these cells. A similar number of acylated proteins were observed in human KB cells and murine myeloma cells after [^{3}H]palmitate labelling. In particular all three cell systems contained a major acylated protein of an approximate molecular weight of 20000.

In the study described above, none of the proteins were identified. However, a number of normal cell proteins of known identity have now been demonstrated to contain covalently attached fatty acid. As mentioned earlier, the proteolipoprotein of myelin contains approx. 2% by weight of covalently bound fatty acid. This has been confirmed by intracranial injection of [^{3}H]palmitate in young rats and subsequent resolution of labelled myelin proteolipoprotein on SDS-polyacrylamide gel (M.F.G. Schmidt, M.J. Schlesinger and H. Agrawal, unpublished data; Ref. 48). The proteolipid subunit of the ($Mg^{2+} + Ca^{2+}$)-ATPase of sarcoplasmic reticulum appears to be acylated with fatty acid [19]. In a recent report, Omary and Trowbridge [20] detected fatty acid labelling of the human transferrin receptor from a leukemic T-cell line. The linkage was labile to hydroxylamine, releasing palmitoyl hydroxamic acid. As with the acylated viral proteins, the fatty acid appears to be located in the portion of the protein protected by the membrane. These workers were unable to detect [^{3}H]palmitate labelling of the human HLA antigen and the human homologue of the murine T200 glycoprotein in this study although subsequent work has identified covalently attached fatty acid in the former (I.S. Trowbridge, personal communication). Covalent attachment of fatty acids to membrane proteins thus appears to be quite a common phenomenon which may have structural and functional consequences for a group of specialized proteins.

Recently Marinetti and Cattieu [21] have reported a covalent attachment of fatty acids to cell membrane proteins, but the characteristics of this attachment are somewhat different from those reported above. In several rat tissues, human red cells, and polymorphonuclear cells these workers found predominantly unsaturated fatty acids bound to proteins which were resistant to SDS and detergent extraction but labile to methanolic HCl or mild alkaline hydrolysis. Surprisingly, liver cytosol contained as much protein-bound fatty acid as the microsomal fraction. Incorporation of labelled palmitate into protein-bound lipid was stimulated by ATP and coenzyme A (CoA) both in intact cells and red cell ghosts. [^{14}C]Palmitoyl-CoA was also an effective donor of fatty acids particularly in ghosts. SDS-polyacrylamide electrophoresis of [^{3}H]palmitate-labelled red cell ghosts resolved a number of labelled bands, the major one of which ran between bands 4.2 and 5. This was a minor band on the Coomassie blue stained gel. Neither actinomycin D nor cycloheximide reduced the labelling of polymorphonuclear cell proteins by [^{3}H]palmitate. It is not clear at this time whether the acylation seen in these experiments is related to that noted above. Keenan et al. [47] have recently reported covalently attached fatty acids on rat erythrocyte membrane proteins, as well as on butyrophilin and xanthine oxidase, the two major proteins of the milk fat globule membrane.

V. Nature of the fatty acid protein linkage

Two lines of evidence have led to the postulate that the fatty acids are linked covalently to the protein by simple ester bonds, probably to the side-chains of hydroxyl-containing amino acids (Ser, Thr, Tyr). Firstly, the fatty acid is labile to transesterification and hydroxylaminolysis, both reactions characterisitic of ester linkages. Methanol-KOH treatment is a fairly rigorous process which subjects the proteins to high pH and organic solvent while hydroxylamine treatment can be performed under mild conditions at neutral pH. We have used hydroxylamine to study the acyl-protein linkages in Sindbis E1, E2, PE2 and VSV G protein. The fatty acid was released rapidly from

totally denatured PE2, E2 and G ($t_{1/2} = < 10$ min at 23°C) while most of the fatty acid bound to denatured E1 was highly resistant to this treatment (A.I. Magee, D.Z. Wen and M.J. Schlesinger, unpublished data). This suggests that the substituted amino acid may be different in E1; for example, Lenard has found that serine esters are much more labile to hydroxylamine than threonine esters [22]. Secondly, there are convincing data from several laboratories that the fatty acid is bound to a restricted region in the protein's primary amino acid sequence. Most of the proteins with covalent fatty acid have a major portion of their structure "outside" (extracytoplasmic) of the lipid bilayer; a region imbedded in the membrane, which consists of a strongly hydrophobic segment that is probably helical; and a carboxyterminal hydrophilic domain. The hydrophobic "helix" is postulated to span the bilayer and the hydrophilic portion is positioned "inside" (cytoplasmic). Proteolytic removal of the extracytoplasmic regions of the Sindbis, VSV, and influenza virus membrane proteins and human transferrin receptor leaves the proteins' membrane portion attached to virus or cell. This membrane-associated fragment has the bound fatty acid residues [23,24]. Capone et al. [25] have further characterized a thermolysin peptide of the VSV G protein that contains fatty acid and they have confirmed by peptide mapping and sequencing that it contains the membrane-spanning hydrophobic sequence. A tryptic peptide from the proteolipoprotein of myelin which appears to contain fatty acid [26] has the sequence Thr-Ser-Ala-Ser-Ile-Gly-Ser-Leu-Cys-Ala-Asp-Ala-Arg. However, no investigator has thus far definitively located the fatty acid-containing amino acid by direct sequence analysis, mainly due to the technical difficulties of working with these extremely hydrophobic peptides. Further attempts at direct sequencing are currently under way in this laboratory.

Despite the lack of absolute information about the site of acylation a number of possibilities can be envisioned in the light of the above data and the known sequences of many of these membrane glycoproteins as determined directly or from their nucleotide sequences [27–29]. A number of hydroxy amino acids which are potential acylation sites can be found both within the postulated membrane-spanning segments and close to this region on both the amino and carboxy-terminal sides. However, comparison of the sequences of related proteins, such as the Sindbis and Semliki Forest virus E1 and E2, the influenza haemagglutinins and the human histocompatibility antigens, reveals a concentration of hydroxyamino acids occurring within ten residues of the N-terminal side of the membrane-spanning segments (Table I). These residues are adjoining each other or separated by a single amino acid. A similar sequence (Thr-Ser-Ala-Ser) exists in the acylated tryptic peptide from myelin proteolipoprotein. This suggests that these residues may be good candidates for acylation and that acylation sites may occur on the luminal (extracytoplasmic) side of intracellular membranes. Hydroxyamino acid residues within or C-terminal to the membrane-spanning segments are, in general, less well con-

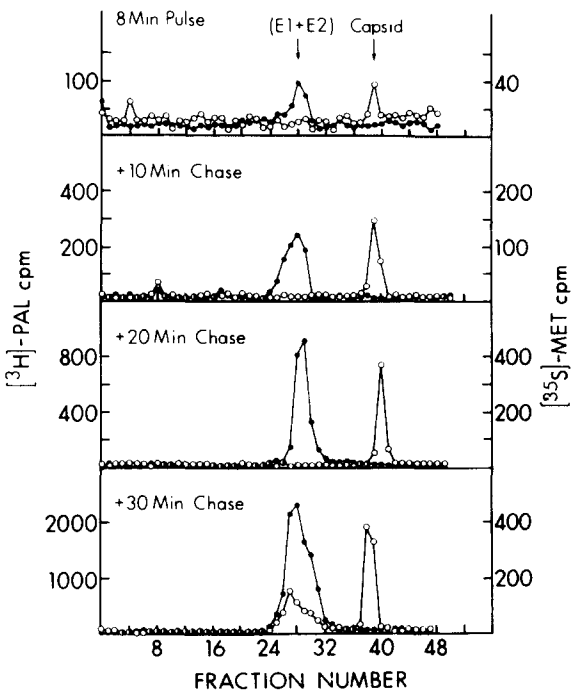

Fig. 2. SDS-polyacrylamide cylindrical gel profiles of Sindbis virus particles relased from cells double-labelled with [^{3}H]palmitic acid (PAL; ●———●) and [^{35}S]methionine (MET, ○———○). Infected cells were pulsed at 6 h postinfection for 8 min and chased for 10, 20 and 30 min. Released virus particles were harvested from the medium purified by ultracentrifugation, and disrupted with SDS for gel analysis [30].

served but these cannot be ruled out as fatty acid acceptors. Insufficient information is available about the sequences of membrane proteins known not to be acylated to allow any useful comparison.

VI. Biosynthesis of acylated proteins

The biosynthesis of fatty acid acylated proteins was first studied in Sindbis and VSV infected chicken embryo fibroblasts [30]. Very short (1–3 min) pulses of [^{3}H]palmitate were rapidly incorporated into cell lipid and, in Sindbis virus-infected cells, glycoproteins PE_2 and E1 were labelled. After only 6 min of chase 50% of the PE_2 label had been converted to E2, and all the label was in E2 by 15 min of chase. In contrast, PE_2 pulse-labelled for 3 min with [^{35}S]methionine was not chased into E2 for at least 20 min, in agreement with earlier observations which identified PE2 cleavage as a late event in virus maturation [11]. When cells pulse-labelled with both [^{3}H]palmitate and [^{35}S]methionine were chased with unlabelled medium the fatty acid-labelled glycoproteins appeared in extracellular viral particles after as little as 8 min whereas amino acid-labelled glycoproteins took 30 min to be chased into virions (Fig. 2). Similar results were obtained with VSV G protein and it was possible to estimate the time of first addition of fatty acid as approx. 15 min after initiation of protein synthesis. In other experiments, cycloheximide was used to block protein synthesis and incorporation of [^{35}S]methionine into protein fell immediately. However, the incorporation of [^{3}H]palmitate into G protein continued for 10–15 min after which it too fell. These data place the time of addition of fatty acid to protein in these cells approx. 10–20 min after synthesis, a time when viral glycoproteins are considered to be passing through the Golgi complex of the cell. To confirm this, fatty acid addition was related to oligosaccharide processing events which are known to occur in the Golgi complex [31]. The enzyme endo-β-*N*-acetylglucosaminidase-H which removes high-mannose carbohydrate chains from proteins but is ineffective against chains which have been processed could be used since the deglycosylated endo-β-*N*-acetylglucosaminidase-H-sensitive proteins have faster mobility on SDS-polyacrylamide

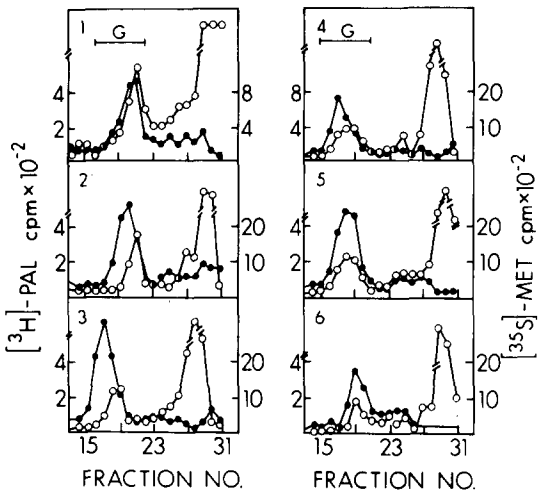

Fig. 3. SDS-polyacrylamide gel analysis of cell lysates from VSV-infected cells after treatment with endo-β-*N*-acetylglucosaminidase-H. The cells were double-labelled with [^{3}H]-palmitic acid (PAL, ●———●) and [^{35}S]methionine (MET, ○——— ○) at 5.5 h postinfection for 6 min and subsequently chased for 3, 6, 14, 24 and 54 min. After the various time points cell lysates were prepared and treated with endo-β-*N*-acetylglucosaminidase-H for 18 h at 37°C. Samples were prepared for electrophoresis and separated on cylindrical 10% SDS gels. The gel fractions containing the G protein are indicated by the bar in panels 1 and 4. The N and NS proteins are in fractions 27 to 31. Panel 1, 6-min pulse, no chase; panel 2, 6-min pulse plus 3-min chase; panel 3, 6-min pulse plus 6-min chase; panel 4, 6-min pulse plus 14-min chase; panel 5, 6-min pulse plus 24-min chase; panel 6, 6-min pulse plus 54-min chase [30].

gels. VSV-infected cells were pulsed with [^{3}H]palmitate and [^{35}S]methionine and chased for increasing times, followed by treatment of the cell lysates with endo-β-*N*-acetylglucosaminidase-H (Fig. 3). After a 6 min pulse, both fatty acid- and amino acid-labelled G protein were sensitive to endo-β-*N*-acetylglucosaminidase-H (panel 1). After only 3 min of chase some of the fatty acid-labelled G became resistant to endo-β-*N*-acetylglucosaminidase-H (panel 2) and all G was resistant after a 6 min chase (panel 3). In contrast, amino acid-labelled G required 30 min to become totally resistant to endo-β-*N*-acetylglucosaminidase-H (panel 5). These data confirm that, in this system, fatty acid addition occurs just before or at the same time as trimming of oligosaccharide chains is occurring. In further studies, the effect of the ionophore monensin on the intracellular process-

ing of VSV G protein and the Sindbis glycoproteins was investigated [32]. Other work had suggested that the mode of action of this drug was to block intracellular membrane movement from the Golgi apparatus to the cell surface, resulting in the accumulation of protein in distended smooth membrane sacs [33]. Monensin at 10^{-6} M prevented virus formation without affecting viral protein synthesis, but movement of viral proteins to the cell surface was inhibited as was Sindbis virus PE_2 cleavage, a step required for budding. The incorporation of [^{3}H]palmitate into viral proteins was essentially unaffected in the monensin-treated cells, and VSV G protein became endo-β-*N*-acetylglucosaminidase-H-resistant indicating that the early stages of oligosaccharide processing had occurred. Thus, the interpretation that acylation occurs before these proteins leave the Golgi complex is well supported.

Rothman and co-workers, in an elegant series of experiments, have partially reconstructed in vitro the pathway of intracellular processing of membrane glycoproteins [35–39]. They used extracts of [^{35}S]methionine-labelled VSV-infected CHO 15B cells, which lack the ability to process oligosaccharide chains to endo-β-*N*-acetylglucosaminidase-H resistance, as donors of newly synthesized G protein. The G protein oligosaccharides could be processed in vitro on addition of membrane fractions from wild-type cells or purified rat liver Golgi membranes. Transport between the donor and acceptor membranes is energy-dependent and appears to be mediated by coated vesicles. In a recent publication [40] these workers examined fatty acid acylation of G protein in their in vitro system, and found that a smooth membrane fraction which contains oligosaccharide trimming activity acts as a 'donor' of glycoprotein to another smooth membrane fraction separable by density gradient centrifugation and containing the terminal glycosylating enzymes. They propose that these 'donor' membranes are equivalent to the *cis*- or early stacks of the Golgi complex observed morphologically. G protein pulse-labelled with [^{3}H]palmitate was found in 'donor' membranes and was present on glycoprotein whose oligosaccharide chains had been partially or fully trimmed. Partially trimmed G protein was also observed in rough endoplasmic reticulum which led these authors to propose that the acylating activity may be present in transitional elements of the endoplasmic reticulum or in early acting Golgi membranes.

Studies on the biosynthesis of normal cellular acylated proteins have been limited to the transferrin receptor [41]. All acylated receptors in a human leukemic T cell line CCRF-CEM were in the mature form suggesting that addition of fatty acid was a late event. Interestingly, tunicamycin did not block acylation, but most of the [^{3}H]palmitate-labelled receptor was of the mature form, suggesting that fatty acid was being added to pre-formed receptor. This addition of fatty acid could still occur in cells treated with tunicamycin for as long as 48 h. Inhibition of protein synthesis also did not inhibit acylation of pre-synthesized receptor. The turnover rate of the receptor, based on stability of [^{35}S]methionine-labelled receptor was quite slow, ($t_{1/2}$ approx. 60 h); however, 50% of the [^{3}H]palmitate label associated with the receptor was lost at a much faster rate ($t_{1/2}$ approx. 10 h), followed by a rate of turnover paralleling that of the amino acid-labelled protein. These results will be discussed in the next section in the context of a possible function for covalently attached fatty acid. However, it should be stated that these measurements of fatty acid turnover should be interpreted with caution since the label given in the initial pulse is incorporated into cellular lipids and is not effectively chased, unlike an amino acid label.

In conclusion it seems that the intracellular site of acylation is probably a Golgi-like membrane but that the actual location and kinetics of acylation may vary considerably between different cell types. To this time no enzyme capable of transferring fatty acid to protein has been isolated. The ability to generate large amounts of deacylated, but otherwise apparently intact G protein using hydroxylamine may aid in providing a suitable acceptor substrate but there is also the need to identify the fatty acid donor. An activated ester such as palmitoyl-CoA would seem to be a logical candidate particularly in light of the work of Marinetti and Cattieu previously described [21]. Alternatively, phospholipid may be a donor, as has been postulated for the acyl moieties of the murein lipoprotein of *E. coli* [42].

VII. Possible roles for covalently attached fatty acid

As with other post-translational processing events, the investigation of the functional significance of covalent fatty acid attachment has lagged behind the structural studies of this interesting phenomenon. It is clear from the data presented here that not all membrane proteins undergo this post-translational modification and it therefore seems that acylated proteins must have specific functions. A number of interesting possibilities, which are not mutually exclusive, have been put forward, but none have significant experimental support at this time. As a result, the suggestions made here are essentially speculative.

Schmidt et al. [13] initially proposed that the fatty acid may function to anchor the protein in the lipid bilayer by providing an additional hydrophobic moiety. This suggestion has been reiterated by other workers [20,21]. In support of this idea, it has been shown that immunoglobulin chemically acylated with fatty acids acquires the ability to bind to liposomes [43]. Also, the transforming protein of Rous sarcoma virus, which is initially synthesized as a soluble protein, associates with cell membranes possibly facilitated by acylation near its amino-terminus (Sefton et al., personal communication). Since this protein presumably is never exposed to the luminal side of intracellular membranes one might speculate that the acylating enzyme activity could be an integral membrane protein whose active site can orient either cytoplasmically or luminally. Butyrophilin, which is glycosylated, and non-glycosylated xanthine oxidase are associated with phospholipid and triglyceride in the milk fat globule membrane, possibly by virtue of covalently attached fatty acids [47]. However, most of the proteins known to be acylated undergo this modification some time after they have been inserted into cellular membranes [21,30,40], and examination of the sequences of the putative membrane-spanning segments of acylated proteins indicates that this part of the protein would not be inherently unstable in the hydrophobic interior of a lipid bilayer. Furthermore, the neuraminidase of influenza virus contains a membrane-spanning hydrophobic segment, does not contain bound lipid and appears to be stably bound to the virus envelope as do a number of other membrane proteins (Table I).

Another role for covalently attached fatty acid could be in membrane fusion, and it is interesting that all the viral proteins which are known to be involved in fusion are acylated, such as the haemagglutinin of influenza, the F protein of Fowl Plague virus, the VSV G, and Sindbis virus E1 and E2. Covalently-bound fatty acids should influence the distribution of lipid in regions of the bilayer enriched in proteins and potentially destabilize membranes; for example when membranes are brought into close proximity by a virus-receptor interaction. Petri et al. [44] have shown that a fluorescently-labelled fatty acid attached to VSV G protein inserted in a liposome appears to be embedded in the bilayer but does not detect the gel to liquid-crystalline phase transition. This may be due to the binding of relatively immobile lipid around the membrane-spanning peptide.

Related to fusion activity is the process of membrane budding. If the fatty acids are concentrated asymmetrically in the outer leaflet of the bilayer, as suggested by the conservation of hydroxy amino acids in this region, then these "excess" chains could contribute an outward curvature which may be needed for viral budding. Acylated cellular proteins could perform analogous functions in the many intracellular budding events as well as membrane budding from the cell surface [47]. The transferrin receptor is thought to be internalized and recycled to the surface continuously, possibly passing through the Golgi complex at each cycle. At least two budding and two fusion events would have to occur in this process. If attachment and removal of fatty acid were occurring during cycling then this may explain why fatty acid can be added to preformed receptor up to 48 h after synthesis, as well as the rapid turnover of the acyl moiety [41].

Finally, addition of fatty acid could modify the local conformation of a polypeptide and thereby establish new sites for protein-protein interaction, either within or outside the bilayer. For a protein already inserted into and spanning the membrane, the new sites might be established in the cytoplasmic tail segment and serve as "receptors" for cytoplasmic proteins. For enveloped viruses, the latter consist of nucleoproteins or matrix proteins

with which glycoproteins are known to interact.

None of the above hypotheses have substantial experimental support but recent data obtained in this laboratory offer evidence for a role of acylation in membrane-protein interaction. The antibiotic cerulenin, which inhibits de novo fatty acid biosynthesis, was found to severely inhibit virus release from infected cells under conditions where its main effect was to block addition of fatty acid to viral glycoproteins [49]. The non-acylated virus glycoproteins were transported to the cell's plasma membrane but not budded into virus particles; thus, it is unlikely that the fatty acid moiety is essential for intracellular transport and sorting of proteins into a particular membrane, although it might facilitate such activity. As noted earlier, there are non-acylated proteins that can move to the cell surface.

VIII. Conclusion

Historically, the structure of post-translational modifications of proteins has been elucidated long before any function has been found. This is particularly so in the field of covalent carbohydrate attachment, which has been known for several decades but for which a role in protein folding [45] and in signalling have been reported only in recent years [46]. The post-translational fatty acid acylation of mammalian membrane proteins seems likely to gain increasing attention as its functional significance becomes clear. The groundwork summarized here, which has been expanding rapidly in the last 2 years will provide a firm basis for further studies which will eventually elucidate the function or functions of this potentially important phenomenon in cell biology.

Note added in proof (Received September 1st, 1982)

Recent data have shown fatty acid (myristic) acylation of the amino terminal glycine in murine leukemia virus *gag* protein precursor (Henderson, L.E., Krutzch, H.C. and Oroszlan, S., Proc. Natl. Acad. Sci., in the press). This type of peptide bond linkage is postulated to enhance membrane binding for this cytoplasmic protein during its role in virus assembly. Amino terminal modification by long chain fatty acid acylation has been found in some bacteria proteins but this is the first report for eucaryotic cells.

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