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Acylation of proteins — a new type of modification of membrane glycoproteins

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Fatty acids are covalently attached to many membrane glycoproteins during their biosynthesis.

Proteins are of utmost importance for the functioning of biological membranes. This is especially evident for the cell surface membranes of eukaryotic cells in which glycoproteins aid the regulation of many cellular activities in response to various extracellular stimuli. In order to function the hydrophobic regions of the glycoprotein must interact specifically with the lipids of the membrane bilayer. Although information on the molecular basis of these interactions is still very limited one important aspect may be concerned with the covalent modification of membrane-bound glycoproteins through the attachment of fatty acids during biosynthesis.

**Biosynthetic modifications of proteins**

During the biosynthesis of functional proteins stepwise modifications of the primary translation products often occur. Two such modifications have been studied: the covalent addition of oligosaccharide residues and their subsequent maturation and the proteolytic cleavage of the polypeptide backbone of a protein or glycoprotein.

Enveloped viruses played an important role in the study of both modifications and also enabled the recent discovery and study of protein acylation — a post-translational modification as important as glycosylation — by attaching fatty acid to proteins.

**Acylation of proteins**

**Acylation of proteolipids**

The first indication that fatty acid bound to proteins came from Folch and Lees. They discovered a novel class of proteins (proteolipids) in the white matter of the brain, which were soluble in chloroform-methanol (2:1) and insoluble in water. More recent analyses of the chemical composition of rat myelin proteolipid have shown that 2–3 mol of ester-linked fatty acid is present for every mol of protein. It is thought that the acyl chains are probably attached to specific sites in the polypeptide as only two out of twelve soluble tryptic peptides carry fatty acid.

Although many other proteolipids have been isolated from bacteria, plants and animals, only the Ca²⁺-ATPase from sarcoplasmic reticulum has been shown to contain a proteolipid component with covalently linked fatty acids. Of other proteins, only two bacterial species have been reported to contain lipids; *Escherichia coli* and *Bacillus licheniformis*. The murine lipoprotein of the outer membrane of *E. coli* contains a glycerylcysteine with two ester-bound fatty acids and one acyl-chain in an amide linkage at the amino-terminus and there is conflicting evidence concerning the presence of covalently bound lipid in the membrane penicillinase of *B. licheniformis*. All the proteins mentioned in this section are of relatively low molecular weight (10,000–25,000) and represent non-glycosylated protein species, possibly of a highly specialized nature.

**Acylation of viral glycoproteins**

Protein acylation with typical membrane glycoproteins was first observed in Sindbis virus. The fatty acids bound to the two spike glycoproteins of Sindbis virus (E1 and E2) were found to be resistant to organic and/or detergent extractions. However, 1–2 and 5–6 mol of fatty acid could be released from E1 and E2 respectively by treating the purified glycoproteins with potassium hydroxide.

Analysis of the different species of enveloped virus revealed that glycoprotein acylation occurs, whether mammalian, avian or insect cells are used for virus multiplication (Table 1). In addition, the fatty acids appear to be linked to the various viral glycoprotein species with the same binding characteristics as those in the E1 and E2 of Sindbis virus.

It is noteworthy that with all viruses containing more than one glycoprotein in their lipid envelope, acylation was much enhanced in or even restricted to one protein species (see Table 1). Interestingly, the only common feature of the fatty-acid-containing viral glycoproteins is their ability to induce membrane fusion. This activity has been well documented especially for the F-protein of Sendai virus and the hemagglutinin of the influenza viruses.

Since membrane proteins of non-infected cells represent only a small fraction of the total cellular protein, it has been difficult to study their biosynthesis and properties. Immunological and cell fractionation techniques must be used to analyse them. Accordingly, acylation with non-viral proteins was first detected when membrane preparations from chicken-, KB- and mouse myeloma cells were analysed after labeling with tritiated palmitic acid. Although at least 20 different protein bands showed specific labeling in acylamide gels, one major protein species of about

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**TABLE 1. Acylated and fatty acid free glycoproteins of enveloped viruses**

| Virus                             | Cells | Spike-glycoproteins | Acylated | Stoichiometry (Mol fatty acid per mol protein) |
|-----------------------------------|-------|---------------------|----------|-----------------------------------------------|
| Vesicular stomatitis virus (VSV)  | CEF, CHO | -                   | G-protein | 1–2                                           |
| Semliki Forest virus (SFV)        | CEF, BHK, Eveline cells, human lymphoma, insect cells (Aedes albopictus) | -       | E1 – E2 | n.d.                                           |
| Sindbis virus (SIN)              | CEF, BHK, L cells, insect cells (Aedes albopictus) | -       | E1 – E2 | 1–2, 5–6                                      |
| Influenza viruses (human and avian) | CEF, MDBK, Eveline cells, human lymphoma | NA | HA (HA) | n.d.                                           |
| Newcastle disease virus (NDV)    | CEF, MDBK, CAM | -   | F-protein | n.d.                                           |
| Sendai virus                     | CEF, MDBK, CAM | -                   | E1 – E2 | n.d.                                           |
| Bovine Coronavirus               | CEF, Eveline cells, human lymphoma | -   | -       |                                               |
| Mouse hepatitis virus A 99       | CEF, Eveline cells, human lymphoma | -   | -       |                                               |

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20,000 mol wt was acylated in all three cell types. Unfortunately, this protein has not been identified.

By using monoclonal antibodies, Bishr Omary and Trowbridge recently demonstrated the specific binding of fatty acid to the transferrin receptor from human lymphoma cells. No fatty acids could be detected in two other transmembrane glycoproteins in the same cell, the human T200 glycoprotein and the major histocompatibility antigen (HLA)19. Our own results indicate that neither HLA, nor membrane bound immunoglobulins (lgM) from various human lymphoma lines are acylated (Schmidt and Fleischer, unpublished).

Because viral glycoproteins are synthesized by the same enzyme systems that produce cellular membrane glycoproteins, one would expect more acylated glycoproteins of non-viral origin to be described in the near future. However, as with viral glycoproteins, it becomes apparent that acylation is also restricted to certain species of cellular membrane glycoproteins.

What is known about the process of acylation?

Experiments with vesicular stomatitis virus (VSV) and Sindbis virus in chick embryo cells indicate that acylation occurs at a specific intracellular site on the transport pathway20. When the intracellular transport of VSV G-protein is impaired by treating cells with tunicamycin, the attachment of fatty acid is blocked although synthesis of the non-glycosylated polypeptide backbone proceeds normally. Likewise, ts-mutants of VSV, that lead to synthesis of transport-defective G-protein at non-permissive temperatures, fail to incorporate labelled fatty acids44. These results strongly indicate that protein acylation is a post translational event.

The site at which fatty acids are bound can be determined by comparison with other well established post-translational modifications of viral glycoproteins. Data were obtained that place the acylation of both the VSV G-protein and the transferrin receptor to a site reached shortly before the protein-bound high-mannose oligosaccharides are trimmed to an endo H (endo-β - N - acetylglucosaminidase) - resistant form20,21, a process known to occur near the Golgi complex1. Furthermore, by analysing cell fractions after a short pulse with [3H]palmitic acid, Dunphy et al.22 recently detected acylated G-protein in Golgi-like membranes that also carried α-1,2-mannosidase activity – an enzyme involved in oligosaccharide trimming.

This strongly indicates that fatty acids are attached to the polypeptide in the immediate neighbourhood of the Golgi complex. However, for a more definite localization and characterization of this enzymatic activity, cell fractionation studies and an in vitro system of acylation will be essential.

Structure of the acylation site

The structural analyses of acylated membrane glycoproteins are essential for two reasons. Despite the circumstantial evidence for the covalent nature of the linkage between fatty acid and protein the binding partners have yet to be identified. Secondly, to understand the functions of protein-bound fatty acids the fatty acid attachment sites must be determined.

No-one has yet purified and identified acylated peptides or even acylated amino acids from viral glycoproteins which carry fatty acids. The main obstacles arise from the unusual properties of acylated peptides which tend to aggregate, stick to the walls of test tubes and be trapped irreversibly on various matrices (Refs 14, 23, 24; Schlesinger, personal communication; and Schmidt, unpublished). However, one common feature of a number of different preparations of acylpeptides from various glycoproteins is the presence of significant amounts of serine. Since the acylated glycoproteins are sensitive to mild alkaline conditions or treatment with hydroxylamine (Refs 18 and 19; Schmidt, unpublished) the results of amino acid analysis indicate an ester-linkage between the fatty acids and serine residues of the polypeptide. To verify this hypothesis, more powerful separation techniques for acylated peptides will have to be developed.

Work from several laboratories has shown that membrane glycoproteins are acylated in a region of the protein which is closely associated with the lipid bilayer. This has been demonstrated through limited proteolysis of native influenza-, VSV-, Semliki Forest- and Sindbis virus particles as well as glycoprotein-loaded lipid vesicles. While the distal and hydrophilic portions of the spike glycoproteins are digested, the carboxyterminal portions are retained within the 'protective' lipid bilayer. Peptides originating from the distal regions of the respective glycoproteins are free of fatty acid, whereas acylated protein fragments can be recovered only from the proteolytically 'shaved' virus particles or lipid vesicles19,20–25. Recently, these results were confirmed for the small subunit of the influenza hemagglutinin (HA). Chemical fragmentation of this glycoprotein with cyanogen bromide showed that the acylation site was located at its membrane-spanning fragment26.

What could be the function of protein-bound fatty acids?

Our limited knowledge of acylation prevents any definite conclusions concerning the functional role of fatty acids in acylated proteins. However, the data obtained during the last 3 years allows certain possibilities to be explored.

If fatty acids are linked to the membrane spanning regions of acylated glycoproteins this may aid in the stable anchorage of the protein molecule in the cellular or viral lipid bilayer24,25,28. This view is supported by amino acid sequence data which reveal a surprisingly high proportion of hydrophilic hydroxy-amino acids (mainly serine) within the membrane-associated portion of a number of glycoproteins. Acylation would convert these hydrophilic residues to...
Fatty Acid Attachment Sites in Membrane Glycoproteins

Fig. 1. Fatty acids of viral glycoproteins are probably bound to hydroxyamino acids in region A. This location would potentially allow interaction of acyl chains with neighbouring lipid bilayers that could trigger membrane fusion.

hydrophobic ones thereby fixing the macromolecule more tightly to the lipid bilayer. Furthermore, the process of acylation and deacylation, recently reported by Bishr Omary and Trowbridge for the transferrin receptor 2α, would modulate the affinity of a glycoprotein for the membrane. This would offer a means of regulating certain biological activities expressed on the cell surface. Though this 'anchor hypothesis' seems attractive at first glance it is not clear why many cellular and viral membrane-bound glycoproteins do not become acylated during their biosynthesis 21. One of these non-acylated species is the µ-chain of the membrane-form of IgM (Schmidt and Fleischer, unpublished) which should contain fatty acids if the above hypothesis is correct since it is known to be tightly bound to the plasma membrane and contains 10 hydroxyamino acids, out of a total of 26 amino acids, within the membrane spanning segment 20.

Another hypothesis suggests a functional link between protein-bound acyl chains and intracellular transport and/or sorting processes 25,27. It is based on the observation that a defect in acylation prevents intracellular transport from the Golgi complex to the plasma membrane (Ref. 27 and Schmidt and Klenk, unpublished). Temperature-sensitive mutants of VSV and influenza virus which synthesize acylation-defective glycoprotein at the non-permissive temperature were used. Unfortunately, the mutant-glycoproteins are also slightly defective in glycosylation (lack of terminal sugars in the complex type oligosaccharides) and other structural differences could not be excluded 28. In addition, if acylation is the signal for plasma membrane destination, why are not all the glycoproteins acylated which are transported and inserted in the cell surface? It seems that it is necessary to look for features exclusive to acylated proteins.

Fusion activity represents the most prominent common feature of acylated glycoproteins of viral origin. The best documented examples for this property are the F-protein of paramyxoviruses (e.g. Sendai virus), the hemagglutinin (HA2) of influenza viruses and the E-protein of a murine Corona-virus (Refs 3, 15-17 and Table I). In addition to this restriction of acylation to the fusogenic viral glycoproteins, two other findings indicate the potential participation of fatty acids in the induction of fusion. One is the presence of oleic acid in the fatty acid fraction released from Sindbis virus glycoprotein E1, a residue long known for its fusogenic properties 29. Secondly, proviral data on the fatty acid binding site in the VSV G-protein 20,26 locates the acyl chains at the external boundary or just outside the viral lipid bilayer (see position A in Fig. 1). In such a position protein-bound oleic acid could aid in perturbing closely associated lipid bilayers and thereby trigger the fusion between the two membranes.

Certainly more data on the exact site of acylation as well as functional tests are required to further substantiate this hypothesis. If acylation sites were found in the centre of the viral lipid bilayer (position B in Fig. 1) or inside the cytoplasmic leaflet (position C in Fig. 1) it would indicate that the protein-bound acyl chains are probably not involved in membrane fusion. They would be too deeply embedded in the lipid bilayer to reach into the external space for interaction with other membranes.

This fusion hypothesis, which is derived exclusively from data on acylated viral glycoproteins, may be verified with more experimental data. Whether it will explain the function of fatty acids in non-viral glycoproteins remains to be seen; many more cellular membrane proteins must be characterized. However, the chances are that this provocative hypothesis may be valid for all acylated glycoproteins if we remember two well known facts. First, viral glycoproteins have in many instances been excellent models for normal cellular glycoproteins. Second, membrane fusion is an important process in cellular function and thus requires an effective means of regulation.