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Fatty Acid Acylation of Eukaryotic Cell Proteins

By Milton J. Schlesinger

There are numerous examples of proteins and enzymes whose structure and function involve an intimate association with lipid. For most of these proteins, the lipid fraction can be dissociated relatively easily and protein obtained lipid-free. For others, the lipid moiety is tightly complexed and strong denaturants are needed to remove lipid, leading in many cases to denatured enzymes. There is a third group of proteins for which the lipid moiety can be removed only under conditions that cleave covalent bonds. The latter proteins can be divided into two general types. The first type has long-chain fatty acids esterified to glycerol that is linked in a thioether bond to a cysteinyl residue of the protein. Proteins of this kind are found, thus far, only in prokaryotic organisms and are localized to the outer membranes of cells. The second type has long-chain fatty acids esterified to amino acids that are part of the polypeptide backbone of the protein. Proteins of this kind are localized to the membranes of eukaryotic cells or their viruses. It is the latter group that is to be described in some detail in this section.

Many of the data pertaining to fatty acid acylation of eukaryotic cell membrane proteins come from studies with those glycoproteins that form the external spikes on enveloped RNA animal viruses. This is because virus-infected tissue culture cells provide a particularly advantageous system for examining events that occur during synthesis, transport, and maturation of membrane-bound glycoproteins. Infection of cells with many of these viruses establishes within a few hours a virtually synchronous synthesis of millions of molecules of a very few virus-coded proteins that are acted upon by cellular enzymes involved in the normal processing and transporting of cellular membrane proteins (see this volume [36]). There is abundant evidence that the events required to synthesize and translocate virus-specified membrane-bound glycoprotein are identical to those occurring when uninfected cells synthesize glycoproteins and translocate them through intracellular organelles to the plasma membrane. But, when compared to events during normal cell growth in which there might be ~100 different membrane proteins undergoing processing asynchron-

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1 M. lnouye, *Biomembranes* 10, 141 (1979).
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FATTY ACID ACRYLATED PROTEINS

| Proteina | Referenceb |
|----------|------------|
| Sindbis virus glycoproteins PE2*, E1*, E2* | 4 |
| Semliki Forest virus glycoproteins E1*, E2* | 5 |
| Vesicular stomatitis virus glycoprotein G* | 6 |
| Influenza virus hemagglutinin HA‡ | 6 |
| Fowl-plague virus hemagglutinin HA; | 5 |
| New Castle disease virus fusion glycoprotein F1 | 5 |
| Corona virus glycoprotein E2 | 5 |
| Brain myelin proteolipoprotein | 7, 2 |
| Human transferrin receptor | 8 |
| Human histocompatibility antigen* | 9 |
| Membrane-associated tissue-culture cell proteins | 10 |
| Avian and murine virus "sarc" proteins | 28 |
| Butyrophilin and xanthine oxidase of milk lipid globule | 29 |
| Human red blood cells and rat tissues | 30 |
| Catalytic subunits cAMP-dependent protein kinasec | 26 |
| Murine retrovirus proteinsd | 27 |

a Asterisks indicate proteins having characteristics described in detail in the section Site and Mechanism of Fatty Acid Acylation.
b Numbers refer to text footnotes.
c Identified as myristic acid acylated in peptide bond to the protein's amino terminus.

distributions through a cell growth cycle of 24 hr, the one or two "synchronously" made virus-membrane proteins constitute about a 100- to 1000-fold increase in "sensitivity" for detecting processing events. It is, undoubtedly, this feature of the virus tissue culture system that has permitted the relatively straightforward study of fatty acid acylated proteins.

Distribution of Fatty Acid Acrilated Proteins

The proteins that have been reported to contain covalently bound fatty acid are listed in the table.2-4,10 Most are virus-associated, but it is noteworthy that an important cell surface receptor (for transferrin) is among those listed. The evidence for fatty acid bound to the human histocompa-

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tability antigen has not been published in detail. In my laboratory, we detected covalently bound fatty acid in about 30–40 different proteins extracted from membranes of chicken embryo fibroblasts. These membranes were from intracellular organelles as well as the cell surface. A few of these protein bands appear to be common to several other kinds of tissue culture cells, but none have been assigned to a protein of known function (this volume [30]). The covalently bound fatty acids in brain myelin proteolipidoprotein were first reported some 30 years ago and subsequently carefully quantitated and detected in tryptic peptide fragments of the protein.

Detection of Fatty Acid Acylated Proteins

The procedures for detecting and identifying protein-bound fatty acids have relied primarily on incorporating high specific radioactive labeled fatty acids into growing tissue culture cells and analyzing proteins from these cells by electrophoresis in sodium dodecyl sulfate (SDS)–polyacrylamide gels. In a typical experiment, 50–100 μCi of fatty acid ([9, 10(n)-3H]palmitic acid, 10–30 Ci/mmol, available from Research Products International Corp., Mt. Prospect, Illinois, stored in absolute ethanol at 4 °C at 5 μCi/μl; note that the isotope should be discarded after 6 weeks in storage) are added to a 35 mm2 to 100 mm2 tissue culture dish (106 to 107 cells) for 4–6 hr at 37°C. Serum that contains large amounts of fatty acid should be delipidated. Medium is removed, and cells are washed twice with cold sterile saline solution, once with cold sterile H2O, and scraped with 0.5 ml of cold sterile H2O into conical tubes, then centrifuged to form a pellet. The supernatant solution is removed and the cells are stored at −70°C. Extracts are prepared by resuspending cells in 0.5 ml of water and sonifying for 30 sec. These extracts are centrifuged for 5 min at 180,000 g in a Beckman airfuge or an equivalent rotor. The supernatant fraction is discarded, and the pellets are solubilized in a buffer consisting of 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1 M Tris-HCl, pH 6.8, and 0.1% Bromphenol Blue.

Radioactivity is determined on a 2-μl sample and 1 to 10 × 10^6 cpm are loaded onto a single lane of a slab gel consisting of 10% polyacrylamide in 0.1% SDS and Tris buffers. Electrophoresis is generally performed at 120 V (constant) for about 3 hr or until the tracking dye has run out the

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bottom of the gel. The gel is processed directly for fluorography using DMSO-PPO. Attempts to stain the gel for protein or fix gels in methanol–acetic acid prior to fluorography may lead to loss of bound fatty acid. Use of other reagents for fluorography also decreases the amounts of bound fatty acid. Of the order of 100–200 cpm of $^3$H-labeled fatty acid in a single protein band can be detected after a 4- to 5-day exposure of the film in cassettes stored at $-70^\circ$. Detection of low levels of radioactivity can be increased by flashing the film prior to exposure. Accurate quantitation (to ±5%) of the radioactivity in protein bands is obtained by orienting the fluorogram over the dried gel and carefully excising the gel with a scalpel. The paper is removed from the gel, and the latter is placed in a vial with scintillation fluid, allowed to swell for 6 hr, and counted. Even a double-labeled band can be measured by this procedure.

Staining of proteins in acrylamide gels previously impregnated with PPO and fluorographed can be carried out as follows.

The dried gel is rehydrated by soaking for 5–10 min in 10% methanol–5% acetic acid. The paper backing is removed, and the gel is transferred to a solution of 0.001% Coomassie Brilliant Blue R250 in 10% methanol–5% acetic acid. The protein bands preferentially and rapidly take up the dye. After 30–60 min the gel is destained briefly in 10% methanol–5% acetic acid and washed twice in water for 30 min each time.

Electrophoresis of proteins in gels effectively separates the small amount of labeled protein-bound fatty acid (estimated for a single protein to range from 0.005% to 0.1% of the total lipid label) from the bulk lipid. Because of the extremely low yield of radioactivity into protein-bound lipid and the limited capacity of the gels for protein mass, it is useful to remove nonmembrane proteins (cytoplasmic material) by differential centrifugation prior to gel analysis. In some cases, it may be helpful to perform a partial membrane fractionation by sucrose gradient centrifugation and analyze the membrane fraction only.

If appropriate antibodies are available, one can remove most of the extraneous proteins and a substantial fraction of bulk lipid by preparing antigen–antibody complexes. The procedures are described as follows: Cells previously labeled (as above) with fatty acid are washed and lysed with small volumes (~0.5 ml) of buffer consisting of 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, and 0.05 M Tris-Cl, pH 7.2. A general protease inhibitor, e.g., phenylmethanesulfonyl fluoride (0.1 mM), is generally included in the lysis buffer. Antibody is added and, after 1-hr incubation at 4°, the antigen–antibody complex is precipitated by adding a preparation of washed Staphylococcus aureus (Cowan) ac-

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according to procedures described by Cullen and Schwartz. The bacteria-Ab-Ag complex is washed twice by vigorous vortexing with the lysis buffer and once with lysis buffer containing $M$ NaCl. A brief (15 sec) centrifugation (15,000 g in a microfuge) is used to repel lent the material after the washes. The final pellet is suspended in 50–100 µl of a gel loading buffer consisting of 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.1% Bromphenol Blue, and 0.6 $M$ Tris, pH 6.8, and boiled for 10 min. Insoluble material is removed by microfuge centrifugation and the protein analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described above.

Analysis of the Fatty Acid

Fatty acid can be rapidly released from protein by treating the sample with mild alkali (0.2 $N$ KOH) in absolute methanol at ambient temperatures for 15–30 min (depending on the amounts of material analyzed). When performing this hydrolysis on material separated in gels, it is important to treat samples before the gels have been dried or heated. For analyzing protein not well separated from bulk lipid, (i.e., a membrane fraction or a virus preparation) the protein should be thoroughly delipidated by organic solvent extraction, for example, using sequentially chloroform–methanol (2:1), chloroform-methanol (1:2), chloroform-methanol, $H_2O$ (1:1:0.3), and acetone. After delipidation, the hydrolysis reaction is carried out. The released fatty acid is extracted with heptane or pentane and analyzed quantitatively by gas chromatography or qualitatively by thin-layer chromatography. The reaction with methanolic KOH converts the fatty acid to the methyl ester, and it can be analyzed without further conversion to an ester with diazomethane.

Protein-bound fatty acid can also be released by incubating protein with $M$ hydroxylamine (pH 8.0). The time required (at 23°) for complete release of the fatty acid to the hydroxamate ester depends upon the particular protein and can range from minutes to hours.

Site and Mechanism of Fatty Acid Acylation

The virus glycoproteins that contain fatty acids form external spikes on virions. For many of these proteins, a portion of the carboxyl-terminal region spans the lipid bilayer and the actual terminus is inside the lipid bilayer (this volume [40]). The major part of the glycoprotein that is external to the membranes of vesicular stomatitis virus (VSV), Sindbis

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virus, and fowl plague virus can be removed quantitatively by appropriate proteases under conditions that leave the membrane-embedded portion of the protein (called the root) still associated with the virion. This root portion of the glycoprotein contains the covalent bound fatty acid.\textsuperscript{5,17-20} Small peptide fragments of VSV-G with esterified fatty acid have been purified in low yields and shown to be enriched in serine.\textsuperscript{6} We do not know yet precisely which amino acid(s) in the protein sequence have acylated fatty acids. There are, however, certain common features in the amino acid sequences of the membrane-spanning regions of several proteins known to contain acylated fatty acids. All these proteins (noted by asterisks in the table) contain a positively charged amino acid (lysine or arginine) on that part of the chain that lies on the internal (cytoplasmic) side of the membrane; all have a sequence of 20–24 hydrophobic residues in the region spanning the bilayer, and all have within 10–12 residues of the membrane spanning segment amino acids that could be acylated.

No information is yet available on the enzyme(s) responsible for acylating the membrane proteins. \textit{In vivo} studies carried out with VSV- and Sindbis virus-infected cells (this volume [40]) show that the glycoproteins are acylated \textit{after} synthesis of the polypeptide chains has been completed, but before substantial processing occurs to the protein-bound oligosaccharides.\textsuperscript{21,22} Based on these data, a site in the cell’s smooth endoplasmic reticulum or “early” (cis) Golgi complex has been suggested as the cellular site of acylation (this volume [36]). The donor for fatty acid could be newly synthesized long-chain fatty acyl-CoA, fatty acylcarnitine, or fatty acid from neutral or phospholipid. There are data indicating that exchange of fatty acid can occur between membrane lipid and protein.\textsuperscript{23}

The function of fatty acid in membrane proteins is unknown. It has been suggested that fatty acid acylation might be important for proper intracellular transport of proteins destined to be localized to the plasma membrane. There are, however, several virus membrane proteins\textsuperscript{5} (the neuraminidase of influenza virus, the HN protein of Newcastle disease

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\textsuperscript{23} M. B. Omary and I. S. Trowbridge, \textit{J. Biol. Chem.} \textbf{256}, 12888 (1981).
virus, and the E1 protein of corona virus) that do appear at the cell surface membrane but are not acylated. Results of studies using the antibiotic cerulenin showed that VSV G protein acylation can be blocked, yet transport of G to the cell surface is normal.\textsuperscript{24} In addition, the G protein from a VSV New Jersey serotype (Ogden) does not become acylated, yet is incorporated efficiently into biologically active virions.\textsuperscript{25}

The fatty acid acylated VSV-G (from an Indiana serotype) differs from the nonacylated New Jersey serotype G form in several amino acids in that part of the polypeptide embedded in the lipid bilayer.\textsuperscript{25} Thus, appropriate modifications to the protein's structure can circumvent any possible essential need for fatty acid.

All of the fatty acid-bound proteins described thus far are postulated to contain O-acyl ester linkages, based on their lability to alkali and hydroxylamine. There is now unambiguous evidence, however, that long-chain fatty acids are found also in peptide bonds of eukaryotic cell proteins—acylating the amino terminus of two proteins.\textsuperscript{26,27} The proteins with this kind of amino terminal modification are considered to be cytoplasmic but are localized in function to cellular membranes. For these polypeptides, fatty acid would facilitate binding to the lipid bilayer. A similar role can be envisaged for fatty acid bound to various tumor virus "sarc" proteins.\textsuperscript{28} Indeed, even for the transmembranal proteins the bound lipid could enhance stability between lipid and proteins. But, in addition to providing an anchor, fatty acid should influence the kinds of lipid in the bilayer surrounding the protein and significantly affect protein–lipid interactions.

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