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Structure, Function, and Biosynthesis of Fatty Acid-Acylated Proteins

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The covalent attachment of long chain fatty acids to proteins was first described for brain myelin proteolipoprotein (15). The major membrane lipoprotein in the *Escherichia coli* cell wall was later shown to contain fatty acids attached through both ester and amide linkages (6, 19). This covalent modification has been subsequently shown to be common to a variety of eukaryotic, bacterial, and viral membrane glycoproteins. However, fatty acid acylation is a highly selective modification, even among membrane glycoproteins, since only a subset of membrane-associated proteins contain lipids. Because fatty acylation is such a recently identified covalent modification, the characteristics of a protein that specify whether it becomes acylated, as well as the biosynthetic events involved in the attachment of fatty acids to proteins, are only beginning to be explored. This review will summarize current knowledge regarding this unique covalent modification and will discuss possible avenues of future research that might provide a clearer understanding of the structure, function, and biosynthesis of fatty acid-acylated proteins.

Proteins containing covalently bound fatty acid are generally referred to as "acylproteins" (51). In order for a protein to be classified as an acylprotein, the fatty acid moiety must be resistant to removal with organic solvents, resistant to denaturing conditions, and resistant to removal with proteases. The covalent fatty acid must also be identified following its release from the polypeptide by chemical cleavage of the protein-lipid linkage. A variety of lipid moieties other than fatty acids (*e.g.*, phospholipid, diglyceride, mevalonate) has also been shown to be covalently associated with cellular proteins. However, this review will focus only on proteins that contain covalent fatty acid.

A. W. Strauss et al. (eds.), *Protein Compartmentalization*

Nature of the Protein-Lipid Linkage

The majority of the acylproteins described to date have been shown to contain fatty acid linked to the polypeptide by an O ester or thiol ester bond. Proteins containing esterified fatty acids are listed in Table 6.1. All of these proteins, with the exception of myelin proteolipoprotein, contain covalent fatty acid linked through a bond that exhibits the characteristics of a thiol ester. Thiol ester linkages are extremely labile and are rapidly broken by treatment with hydroxylamine at neutral pH. The linkage of the fatty acid moiety to myelin proteolipoprotein is considerably more stabile than a typical thiol ester, but it can be broken by hydroxylamine treatment at alkaline pH. As will be discussed below, this protein-lipid linkage has been identified as an O ester.

Recently, a small number of proteins has also been reported to contain covalent fatty acid attached through a linkage that exhibits the characteristics of an amide bond. The fatty acid moiety on these acylproteins is highly resistant to removal with hydroxylamine and requires acid hydrolysis for release from the polypeptide. Many of these proteins are soluble, nonglycosylated proteins that transiently associate with membranes (Table 6.2). In those cases in which the amide-linked fatty acid has been identified, it has been shown to be the 14-carbon fatty acid, myristate.

Despite the identification of this wide range of acylproteins, the actual amino acid residues that serve as acylation sites have been determined in only a few cases. The difficulties in identifying acylated amino acids are due, at least in part, to the extremely hydrophobic nature of acylated protein domains, in addition to

Table 6.1. Proteins Containing Fatty Acid Linked by an Ester-Type Bond

Protein	References
Semliki Forest virus glycoproteins	52
Sindbis virus glycoproteins	51
Vesicular stomatitis virus glycoprotein G	50
Influenza virus hemagglutinin HA_2	52
Fowl-plague virus hemagglutinin H_2	52
Newcastle disease virus fusion glycoprotein F1	52
Corona virus glycoprotein E2	32, 52
LaCross virus glycoprotein G1 and G2	34
Simian virus 40 large T antigen	21
Brain myelin proteolipoprotein	2, 15
The transferrin receptor	38
Milk fat globule membrane butyrophilin and xanthine oxidase	24
Bacteriorhodopsin	33
The major histocompability complex antigens	23
Ca^{2+} ATPase	28
p21-Transforming proteins	54
Human gastric mucus glycoprotein	55, 56
Unidentified cellular proteins	5, 31, 34, 46

Table 6.2. Proteins Containing Fatty Acid Linked by an Amide Bond

Protein	References
$p60^{src}$	12, 53
p15	20
p120	54
Catalytic subunit of cAMP-dependent protein kinase	9
Calcineurin b	1
Nicotinic acetylcholine receptor	35
Escherichia coli lipoprotein	19
NADH cytochrome b_5 reductase	39
Unidentified cellular proteins	34, 36

the low level of expression of most cellular acylproteins. For the latter reason, the initial discovery by Schlesinger and coworkers (48, 49) that Sindbis virus E1 and E2 and vesicular stomatitis virus G glycoproteins contain covalent fatty acid led to major advances in the understanding of the structure and biosynthesis of fatty-acylated proteins. The envelope virus glycoproteins are amenable for the study of fatty acylation, because following virus infection, host cell protein synthesis is inhibited and viral proteins are synthesized at levels up to 10^3-fold higher than normal cellular proteins (30). The study of fatty acylation of envelope virus glycoproteins has now been extended to a wide range of viruses, and it appears to be a modification common to at least one membrane glycoprotein of every enveloped RNA virus (48, 51).

Amino acid acylation sites within the envelope virus glycoproteins have been localized to hydrophobic membrane domains of these polypeptides. By protease digestion of membranes from cells infected with Sindbis and vesicular stomatis virus, the fatty acid on E1, E2, and G glycoproteins has been shown to be contained within a carboxy terminal domain (29, 49, 44). In the case of G glycoprotein, this domain contains 64 amino acids and includes the cytoplasmic domain, the transmembrane domain of 20 amino acids, and 14 amino acids on the extracellular side of the membrane-spanning domain. By constructing mutagenized cDNA clones for G protein and studying their expression in eukaryotic cells, Rose *et al.* (45) demonstrated that palmitate was linked to an amino acid within the first 14 residues on the carboxy terminal side of the membrane. This sequence contained a cysteine residue that, when changed to a serine by oligonucleotide-directed mutagenesis, resulted in the expression of nonacylated G protein. Thus, this cysteine residue appears to be the acylation site. However, a direct demonstration that this residue is acylated in the normal protein has not yet been possible.

Identification of acylation sites within cellular acylproteins has been considerably more difficult. Some progress has been made, however, toward localizing acylated amino acids to specific regions of acylproteins. For example, [^{3}H]palmitate is incorporated into the HLA-B and HLA-DR heavy chains of the human B lymphoblastoid cells Jy and T51 (23). Protease digestions localize the acylation

sites in these proteins to transmembrane hydrophobic domains, each of which contain a single cysteine residue and no serine or threonine residues. Because palmitate appears to be attached to these proteins via a thiol ester linkage, these cysteines appear to be the acylation sites. Similar protease digestion experiments have localized the acylation site on the transferrin receptor to a region of the molecule that is closely associated with the plasma membrane (38). To date, the only cellular protein, containing esterified fatty acid, in which the acylated amino acid residue has been absolutely identified is brain myelin proteolipoprotein (lipophilin). In this case, fatty acid was shown to be attached to Thr-198, which exists within a hydrophilic segment of the polypeptide (59).

Attempts to identify amino acids that contain amide-linked fatty acid have been considerably more successful, due at least in part to the more hydrophilic nature of this class of proteins. N-acylated amino acid residues have been identified for the catalytic subunit of the cAMP-dependent protein kinase (9), calcineurin b (1), the retrovirus-transforming proteins p15 (20) and $p60^{src}$ (53), and NADH cytochrome b_5 reductase (39). In each of these cases, an amino terminal glycine residue was shown to be acylated with myristate. Olson *et al.* (35) reported recently that the α- and β-subunits of the nicotinic acetylcholine (ACh) receptor also contain covalent fatty acid attached through a linkage that exhibits the characteristics of an amide bond. In the case of these polypeptides, however, the acylation sites are probably not located at the amino terminal, since these amino acids have been identified by amino acid sequencing (10); and covalent fatty acid would have prevented their identification. An ϵ amino group on a lysine residue in a domain closely associated with the membrane would appear to be a likely site for N-linked acylation of the receptor subunits. It remains to be determined, however, whether fatty acids are linked to proteins through amide bonds both at the amino terminus and at other regions of the polypeptide chain.

The fact that not all proteins containing glycine at the amino terminus are acylated suggests that there is specificity with respect to proteins that can serve as acceptors for myristate. Currently, there is not enough information available on the identities of myristate-containing proteins to permit identification of common structural features or possible amino acid recognition sequences that might

Table 6.3. Amino Acid Sequences at the Amino Termini of Myristylated Proteins

Protein	Sequence	References
Catalytic subunit of cAMP-dependent protein kinase	Myristyl-Gly-Asn-Ala-Ala-Ala-Ala-Lys	9
Calcineurin b	Myristyl-Gly-Asn-Gln-Ala-Ser-Thr-Pro	1
p15	Myristyl-Gly-Gln-Thr-Val-Thr-Thr-Pro	20
$p60^{src}$	Myristyl-Gly-Ser-Ser-Lys-Ser-Lys-Pro	53
NADH cytochrome b_5 reductase	Myristyl-Gly-Ala-Gln-Leu-Ser-Thr-Leu	39

be required for myristylation. The amino acid sequences adjacent to the myristylated glycine residue in the catalytic subunit of cAMP-dependent protein kinase, calcineurin b, p15, $p60^{src}$, and NADH cytochrome b_5 reductase are shown in Table 6.3. While these sequences do share some similarities in amino acid residues or in types of residues at a given location, there is no obvious concensus sequence common to the four acylproteins; *e.g.*, such as that required for N glycosylation. It will be important in the future to identify the recognition sequence(s) that are required for myristylation and to determine whether myristate is attached to proteins only at amino terminal glycine residues.

Acyl Chain Specificity of Acylation

Until recently, the possibility that fatty acylation might be highly specific with respect to acyl chain length of fatty acids had not been thoroughly examined. Using the mouse muscle cell line, BC3H-1, Olson *et al.* (34) addressed this issue by labeling cultures with [^{3}H]palmitate and [^{3}H]myristate and by examining on $NaDodSO_4$ polyacrylamide gels the spectrum of proteins that were acylated with these fatty acids. As shown in Figure 6.1, palmitate and myristate were incorporated into distinct sets of proteins that appear to be minor protein species, since they do not correspond to major methionine-labeled polypeptides. The most highly labeled palmitate-containing proteins exhibited apparent Mrs ~18,000 and 20,000. A number of other cell types have also been shown to contain major [^{3}H]palmitate-labeled proteins of Mr ~20,000 (46, 51). The identities of these acylated proteins are unknown.

In BC3H-1 cells, the pattern of proteins that was acylated with myristate was very different from the palmitate-labeling pattern. Myristate was also incorporated into acylproteins to a much greater degree than palmitate. The large number of myristate-containing proteins is surprising, considering that myristate comprises less than 2% of the fatty acids in most cells (25, 26). It is tempting to speculate that the specific cellular function of myristate is as a substrate for acylation of proteins. Recent studies indicate that fatty acids other than palmitate and myristate are also incorporated in a highly specific manner into different cellular proteins (E. Olson, unpublished results).

To determine whether this broad spectrum of acylproteins was common to other cell types, Olson *et al.* (34) also examined the patterns of palmitate- and myristate-labeled proteins in 3T3 mouse fibroblasts in the PC12 cells—a rat pheochromocytoma cell line. As was observed with BC3H-1 cells, palmitate and myristate were incorporated into distinct sets of proteins. The palmitate-labeling pattern was similar in the different cell types. Many of the myristate-containing proteins also appeared to be expressed in all of the cells examined, while others were cell-type-specific, suggesting that they may serve specialized functions.

The dramatic acyl chain specificity observed in the experiments described above suggested that additional specificity might reside within the proteins that serve as substrates for acylation. To further investigate the acyl chain specificity

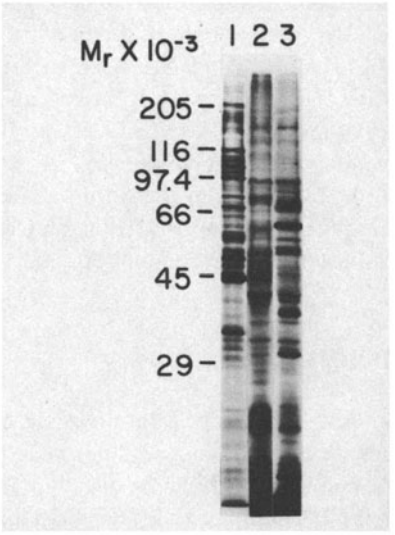

Fig. 6.1. $NaDodSO_4$ polyacrylamide gel electrophoresis of metabolically labeled proteins. BC3H-1 cells were labeled for 4 h with [^{35}S]methionine (Lane 1), [^{3}H]palmitate (Lane 2), or [^{3}H]myristate (Lane 3). At the end of the labeling period, cell extracts were prepared and analyzed by electrophoresis on a 10% polyacrylamide gel followed by fluorography (34).

of acylation, Olson *et al.* (34) examined the sensitivity of palmitate- and myristate-containing proteins in BC3H-1 cells to alkaline methanolysis and hydroxaminolysis. Treatment of [^{3}H]palmitate-labeled proteins with 1 *M* hydroxylamine, pH 7, resulted in the rapid release of covalent fatty acid, indicating that palmitate was linked to proteins primarily through thiol ester or extremely labile O ester bonds. In contrast to the lability of the palmitate linkage, the bond through which myristate was linked to proteins was resistant to hydroxylamine at pHs 7 and 10, and it required acid hydrolysis for release of fatty acid. These data suggest that myristate is highly specific for amide linkages to proteins. To determine whether palmitate and myristate were the actual fatty acid species attached through ester and amide linkages, respectively, protein-bound lipids were released with methanolic HCl and analyzed by high-pressure liquid chromatography. Analysis of [^{3}H]palmitate-labeled proteins indicated that palmitate was the fatty acid that was incorporated into proteins, and that no interconversion to other fatty acids occurred during the labeling period. In contrast, the fatty acids released from myristate-labeled proteins were primarily myristate, with small quantities of palmitate. The degree of chain elongation of myristate to palmitate varied between experiments, but did not exceed 20% of the protein-bound fatty acid in BC3H-1 cells. A small fraction of the protein-linked myristate in BC3H-1 cells was present in an ester linkage. This fraction varied between different cell types, but in all cells examined, greater than 70% of the myristate was linked to

proteins by an amide linkage. The degree of chain elongation of myristate, as well as its conversion into amino acids, also varied significantly between cell types. These studies indicate that metabolic labeling of cellular proteins with fatty acids, followed by $NaDodSO_4$ polyacrylamide gel electrophoresis, is not sufficient for the identification of protein-bound fatty acids—and that chemical characterization of the radioactivity associated with a particular protein is essential. Together, these results also demonstrate that fatty acylation is a very common covalent modification that exhibits a high degree of specificity with respect to fatty acyl donor and acceptor.

Subcellular Distribution of Acylproteins

Until recently, it was generally thought that fatty acid acylation occurred within a membrane subcompartment and was restricted to membrane glycoproteins. The studies of the envelope virus glycoproteins, which led to this conclusion, are discussed in detail in a later section. The demonstration of covalent fatty acid on retrovirus-transforming proteins that are synthesized on free polysomes (27, 54) provided a preliminary indication that acylation might be a modification common to cytoplasmic as well as membrane proteins. To examine the subcellular distribution of cellular acylproteins, Olson *et al.* (34) labeled BC3H-1 cells separately with [^{3}H]palmitate, and [^{3}H]myristate, and they isolated cytosolic and membrane fractions. Analysis of these fractions by electrophoresis on $NaDodSO_4$ polyacrylamide gels indicated that virtually all of the proteins acylated with palmitate were localized in the membrane fraction. In contrast, myristate-containing proteins were found in both the membrane and cytosolic fractions. Many of the myristate-containing proteins appeared to be distributed exclusively in one or the other of the two fractions, while others were found in both fractions. Similar results for the subcellular distribution of myristylated proteins in chicken embryo fibroblasts were also reported by Buss *et al.* (8).

The large number of myristate-labeled proteins in the soluble fraction is striking and suggests that fatty acylation is not restricted to proteins that are tightly bound to membranes. It is not known yet whether some of the myristate-containing proteins associated with membranes are synthesized in the cytosol and bind to membranes following acylation, as in the case of the retrovirus transforming proteins (8, 12, 20, 54), or whether these proteins are integral membrane glycoproteins. It is possible that some of the soluble acylproteins may be loosely attached to membranes and are released during the cell fractionation procedure. It also remains to be determined whether the membrane-associated acylproteins are found exclusively in the plasma membrane or, alternatively, are localized to specific intracellular membrane systems or organelles.

The finding that many myristate-containing proteins are localized to the cytosol is surprising, since these proteins would be expected to be extremely hydrophobic and insoluble in an aqueous milieu. The solubility of these cytosolic acylproteins also might be maintained by protein folding, so that the lipid moiety

is buried within a cleft or pocket in the native structure of the polypeptide. Alternatively, these soluble acylproteins might be complexed with carrier proteins that maintain their solubility in the cytoplasm. In this regard, studies of $p60^{src}$ have shown that this acylprotein is synthesized on free polysomes, released into the cytosol, and reaches the plasma membrane 5 to 15 minutes thereafter (27). During its brief transit through the cytosol, $p60^{src}$ is associated with two carrier proteins: p89 cell and p50 cell (7, 11). This mechanism for transit of myristate-containing proteins to the plasma membrane may represent a pathway followed by other acylproteins.

Biosynthesis of Acylproteins

The high degree of specificity of palmitate and myristate for ester and amide linkages, respectively, suggests the existence of at least two distinct classes of protein acyltransferases: A class that esterifies palmitate to cysteines and a class that attaches myristate to free amino groups on proteins. Currently, no protein acyltransferases have been isolated or localized intracellularly. The acyl donor and acceptor specificities of the enzymes involved in the acylation of proteins have also not been thoroughly examined. Using a cell-free system for acylation, Slomiany and coworkers (57, 58) have demonstrated a fatty acyltransferase activity that catalyzes the transfer of palmitic acid from palmitoyl CoA to deacylated mucus glycoprotein from gastric mucosa. Subcellular fractionation of rat gastric mucosa revealed that the highest specific activity of the enzyme was in the Golgi-rich fraction. Berger and Schmidt (3) have also demonstrated recently that palmitoyl CoA serves as the acyl donor for acylation of deacylated Semliki Forest virus *in vitro*. The significance of these studies is unclear, however, since the cell-free acylation exhibited no specificity, with respect to fatty acyl chain length or degree of saturation of the acyl chain used as substrate—in striking contrast to the remarkable degree of specificity observed in studies of acylation in BC3H-1 cells (40).

The majority of studies that have examined the biogenesis of acylproteins have focused on the viral membrane glycoproteins due to their high level of expression in virus-infected cells. Using virus-infected chicken embryo fibroblasts, Schmidt and Schlesinger (48) demonstrated that palmitate incorporation into Sindbis virus E2 and VSV-G glycoproteins continued for 10 to 20 minutes following inhibition of protein synthesis with cycloheximide, indicating that acylation of these glycoproteins is a relatively early posttranslational modification. To determine the approximate subcellular site of acylation, the timing of fatty acid incorporation into viral glycoproteins was compared to the processing of glycoprotein oligosaccharide side chains. These studies indicated that acylation of VSV-G protein occurs immediately prior to the completion of oligosaccharide trimming, which is known to occur within the Golgi apparatus. Dunphy *et al.* (14) and Quinn *et al.* (43) have also demonstrated that fatty acids are added to G glycoprotein in a smooth membrane fraction that contains oligosaccharide-

trimming activity, suggesting that the acylating activity is located in the cis-Golgi or transitional elements of the endoplasmic reticulum (ER). These results are supported by the studies of Johnson and Schlesinger (22). Using the ionophore monensin, which allows transport of membrane proteins from the ER to the Golgi, but which blocks further transport to the cell surface, these investigators demonstrated that acylation of VSV-G occurred normally. In the presence of tunicamycin, however, nonglycosylated VSV-G protein does not move to the Golgi and is not acylated (48), providing additional evidence that acylation of viral envelope glycoproteins occurs early in the pathway of glycoprotein maturation.

Studies on the biosynthesis of normal cellular acylproteins have been limited primarily to the transferrin receptor (37, 38). In this case, acylation appears to be a late posttranslational event that occurs after oligosaccharide processing is complete. Unlike the viral glycoproteins, palmitate attachment to the receptor occurs normally in the presence of tunicamycin or cycloheximide. The possibility that the receptor also undergoes deacylation during its lifetime is suggested by pulse chase experiments, in which the fatty acid moiety on the receptor has been shown to turnover at a rate greater than that of receptor degradation. Thus, the kinetics of acylation of this cellular membrane glycoprotein clearly differ from those of the viral membrane glycoproteins. Further work is necessary in order to determine whether other cellular acylproteins undergo acylation (and possibly deacylation) by mechanisms similar to the transferrin receptor, or whether this glycoprotein is unique in its posttranslational processing.

Because acylproteins containing amide-linked fatty acids have only recently been identified, virtually no information is available regarding the biosynthesis of this class of acylproteins. The fact that many myristate-containing proteins are soluble (8, 34) and are synthesized on free polysomes (27) suggests that at least some myristoyl N-acyltransferase activity may be localized to the cytosol. The subcellular distribution of the enzymes involved in myristylation of proteins has not been examined, nor has the time of addition of this fatty acid to newly synthesized proteins been established. It also remains to be determined whether all of the membrane-associated proteins containing myristate are peripheral membrane proteins or whether some are also integral membrane glycoproteins. Because of the remarkable fatty acyl chain specificity of protein acylation, it seems reasonable to postulate that both the time of fatty acid addition as well as the subcellular site(s) of palmitate and myristate acylation may be very different.

Recent studies on myristate attachment to newly synthesized acylproteins in chicken embryo fibroblasts and BC3H-1 cells indicate that this is a very early covalent modification (8, 36). Simultaneous addition of cycloheximide and [^{3}H]myristate to cells results in a complete block in acylation of all the major cellular acylproteins, indicating that myristate is added to newly synthesized acylproteins during, or very soon after, translation. In future studies, it will be important to determine more precisely as to when myristate is attached to acylproteins, and to investigate whether soluble and membrane-associated myristate-containing proteins are acylated with the same kinetics and by the same mechanisms.

Several characteristics of myristate-containing proteins suggest the possibility that the attachment of this fatty acid actually might occur cotranslationally. First, the labeling experiments described above indicate that myristylation occurs during or very soon after translation, which in the case of integral membrane proteins would restrict this modification to the ER or cis-Golgi complex. Second, the findings that many myristate-containing proteins are soluble (34) and are synthesized on free polysomes (27) indicate that myristylation does not require transit of newly synthesized acylproteins through a membrane compartment that contains the acylating enzymes. The myristylation of soluble proteins also suggests that at least some of the enzymes involved in myristate attachment to proteins may be localized to the cytosol, where they might have access to nascent polypeptide chains. Third, calcineurin b (1), the catalytic subunit of the cAMP-dependent protein kinase (9), $p60^{src}$ (12), and p15 (20)—the only myristate-containing proteins to be studied in detail—have each been found to be acylated on an amino terminal glycine. Attachment of myristate to a glycine residue at the amino terminus requires the removal of the initiator methionine and possibly other amino acid residues. Cleavage of the initiator methionine is a common modification that has been shown to occur for a wide range of proteins after synthesis of the first 30 to 40 amino acids, while the nascent polypeptide is attached to the ribosome (40, 41). In a number of proteins, the new amino terminus is then rapidly acetylated. Attachment of acetate to the amino terminus of newly synthesized proteins has been shown to occur *in vivo* and *in vitro* by a ribosome-associated protein acetyltransferase that uses acetyl CoA as an acetyl donor (4, 60). It is tempting to speculate that a myristoyl N-acyltransferase and an acetyltransferase, both of which modify amino terminal amino acids, might have similarities in their mechanisms. If the myristoyl N-acyltransferase were associated with ribosomes in a manner similar to the acetyltransferases (60), it would provide a mechanism whereby one enzyme could acylate both soluble proteins and integral membrane glycoproteins. Obviously, a number of other possible mechanisms for myristylation can be postulated; however, the possibility that myristylation occurs cotranslationally is relatively easy to test; and if it is shown to be the mechanism for acylation, it would have broad implications for the role of this covalent modification. Moreover, if myristylation is shown to be cotranslational, this would represent an important first step toward the eventual purification of the protein acyltransferases, since one would know the approximate subcellular location of these enzymes.

A complete understanding of the biosynthetic events involved in the attachment of fatty acids to proteins will require the development of cell-free systems that exhibit the same acyl donor and acceptor specificities observed *in vivo*. The enzymology of palmitate incorporation into proteins is amenable to these types of studies, because palmitate can be easily removed from acylproteins by treatment with hydroxylamine and the deacylated proteins can then be used as substrates for acylation. The development of a similar system for *in vitro* acylation of proteins with myristate presents more difficulties, because there are no current methods for specifically releasing myristate from proteins. Therefore, the pro-

duction of a substrate for *in vitro* myristylation will require the identification of the amino acid sequence that serves as the specific recognition signal for myristate attachment. Synthetic peptide substrates can then be prepared and used as artificial acceptors for myristylation *in vitro*. A similar approach has been used for studying N-linked glycosylation (61) and acetylation of proteins (18).

Based on the studies discussed above, a number of possible pathways for synthesis, processing, and intracellular transport of acylproteins can be envisioned. Some of these pathways are illustrated in Figure 6.2. It is clear that palmitate is esterified to a number of integral membrane glycoproteins as they are transported from the rough endoplasmic reticulum (RER) through the Golgi apparatus, en route to the cell surface. It is not yet known whether the enzymes involved in the attachment of palmitate to proteins are also located in other regions of the cell, or whether palmitate-containing proteins are found in membranes other than the plasma membrane. It is also well established that many myristate-containing proteins are synthesized and acylated in the cytosol. Can myristate also be added to integral membrane glycoproteins; and, if so, in what subcellular compartments do the myristoyl N-acyltransferases involved in this modification reside? Are myristylated proteins targeted to specific subcellular organelles or membrane systems? The answers to these questions must await further studies.

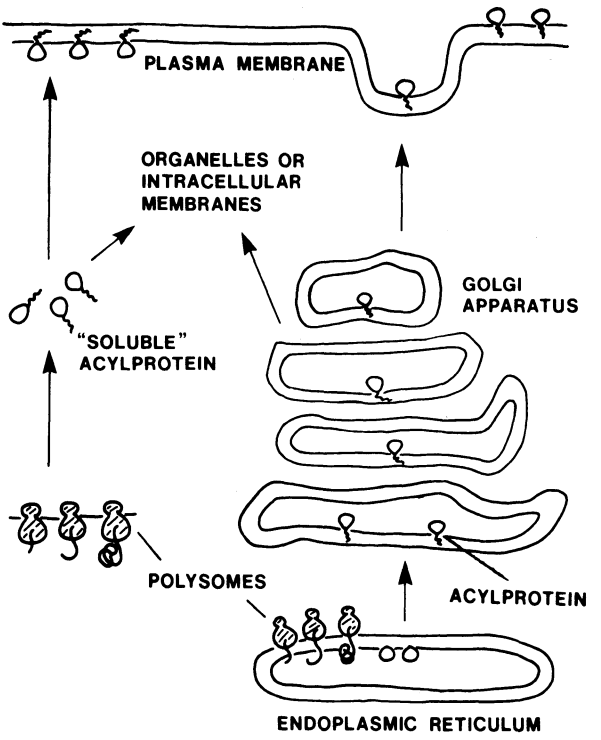

Fig. 6.2. Possible pathways for processing and intracellular transport of acylproteins.

Functions of Covalent Fatty Acid

Despite the wide range of fatty-acylated proteins that have been identified, the precise function of this covalent modification has not been determined and will no doubt be one of the most difficult questions to address in the study of protein acylation. The fact that many cell-surface proteins are not acylated indicates that covalent fatty acid is not required for intracellular transport to the plasma membrane. However, it is possible that covalent fatty acid might play a role in targeting acylproteins to specific subcellular membrane systems or organelles. The subcellular distribution of acylproteins has not yet been examined, and it will be an interesting subject for future studies.

Studies of the retrovirus-transforming proteins suggest that fatty acid may serve as an anchor for binding otherwise soluble proteins to membranes. Using deletion mutants in the N terminal region of $p60^{src}$, Cross *et al.* (12) demonstrated that the site of myristate attachment is contained in amino acids 1 to 14. Deletion of this region prevents myristylation. Under these conditions, $p60^{src}$ does not associate with membranes and is transformation-defective. Similar studies have been carried out with $p21^{ras}$, which contains covalent palmitate near the C terminus (63). Mutants containing deletions located at or near the C terminus of p21 were not acylated and did not associate with membranes. These mutants were also transformation-defective. Thus, in the case of retrovirus-transforming proteins, acylation not only appears to be responsible for mediating the interaction of "soluble" proteins with membranes, but it also appears to be the key step required for cell transformation. These results should be interpreted with some caution, however, since it is unclear as to what effects these relatively large amino acid deletions might have on the secondary structure and/or function of the protein.

A role for covalent fatty acid in influencing the properties of the lipid bilayer surrounding acylproteins has been suggested by Petri *et al.* (42). These investigators fluorescently labeled VSV-G protein by growing virus-infected hamster kidney cells in the presence of 16(9-anthroyloxy)palmitate. Labeled G protein was then reconstituted into dipalmitoylphosphatidylcholine vesicles, and the mobility of protein-bound fatty acid was examined as a function of temperature by fluorescence measurements. These studies indicated that the covalent lipid moiety strongly interacted with the surrounding bilayer, resulting in the removal of phospholipid in the region of G protein from the phase transition. These data also demonstrate that the acylation site on G protein is located within a protein domain that interacts with the membrane, and that the covalent fatty acid is associated within the bilayer, rather than buried within the secondary structure of the polypeptide.

Mycoplasma capricolum, a prokaryotic sterol and fatty acid auxotroph, has been shown to contain a number of membrane proteins that are specifically acylated with palmitate and oleate (13). Acylation of the oleate-containing proteins is stimulated in the presence of cholesterol. This is the only example described to date in which acylation has been shown to be modulated, suggest-

ing the interesting possibility that membrane fluidity may influence protein acylation.

Slomiany and coworkers (55–58) have examined the function of covalent fatty acid on the human gastric mucus glycoprotein. Purified mucus glycoprotein is resistant to digestion with pronase, whereas following removal of covalent fatty acid by treatment with hydroxylamine, the glycoprotein becomes susceptible to pronase. Mucus glycoprotein, isolated from patients with cystic fibrosis, contains two- to threefold more covalent fatty acid per molecule and is highly resistant to pronase. These data suggest that covalent fatty acid protects this acylprotein from proteolytic digestion, and that the increased acylation of mucus glycoprotein in cystic fibrosis may prevent normal turnover of this protein and contribute to the abnormal accumulation of poorly soluble secretions associated with the disease.

In an effort to determine the role of acylation in the expression of envelope virus glycoproteins, Schlesinger and Malfer (47) treated virus-infected chicken embryo fibroblasts with cerulenin (2,3 epoxy-4-oxo-7,10 dodecadienoylamide), which inhibits *de novo* fatty acid synthesis by binding irreversibly to the β-ketoacyl acyl carrier protein synthetase. In the presence of cerulenin, G glycoprotein from the Indiana serotype of vesicular stomatitis virus (VSV_{Ind}) was not acylated, and assembly and budding of virions was blocked. However, transport of VSV-G to the cell surface occurred normally in cells treated with the drug. Cerulenin also inhibits ACh receptor expression in BC3H-1 cells by blocking assembly of receptor subunits in a multisubunit receptor complex—a process that normally occurs in the Golgi apparatus (35). Together, these studies suggest that acylation might play a role in protein-protein or protein-lipid interactions within membranes. The mechanism by which cerulenin inhibits acylation of envelope virus glycoproteins with exogenous [^{3}H]palmitate is unclear. One possibility is that cerulenin, which is a 12-carbon fatty acid amide, may act as a fatty acid analog blocking the active site of the acyltransferase involved in the acylation reaction.

In contrast to the apparent role of covalent fatty acid in the assembly of G protein from VSV_{Ind} into virions, studies by Gallione and Rose (16) demonstrate that acylation is not an absolute prerequisite for virion assembly. Using the New Jersey serotype of VSV—which contains a number of amino acid substitutions in the domain cytoplasmic tail domain, where the acylation site normally appears to reside—these investigators demonstrated that this G protein is nonacylated. However, virions are normally formed in cells infected with this strain of the virus. As in the studies of $p60^{src}$ described above, it is somewhat difficult to interpret results of studies such as these, in which proteins have undergone large changes in their primary structure. These changes in amino acid sequence could result in alterations in the secondary structure of the proteins that eliminate the need for a fatty acid moiety.

Currently, there are no inhibitors of fatty acylation of cellular proteins, which have been thoroughly characterized. The identification of specific inhibitors of N-linked and SH-linked acylation would be of major importance for an under-

standing of the function of this covalent modification. The development of temperature-sensitive mutants that exhibit defects specifically in acylation of proteins would also contribute to our knowledge of the significance of acylation.

Summary and Future Directions in the Study of Protein Acylation

Acylproteins represent a major class of cellular proteins whose identities and functions are only beginning to be understood. As was found for glycosylation, multiple functions as well as biosynthetic pathways for fatty acylation will probably be discovered in the future. From the limited work that has been done on protein acylation, a number of important questions regarding both the role of fatty acid acylation and the biosynthetic events involved in this covalent modification of protein can now begin to be addressed. For example, when are the palmitate and myristate attached to newly synthesized acylproteins (*i.e.*, cotranslationally or posttranslationally)? And where are the protein acyltransferases localized within the cell? Are membrane-bound acylproteins localized to specific membrane systems? And, if so, how do these proteins make their way from the site of synthesis to their destination within a specific membrane system? What is the function of the fatty acid moiety on acylproteins? What are the characteristics of a protein that specify whether or not it becomes acylated? What are the identities of the amino acid residues that serve as acylation sites? With the continued identification of cellular acylproteins and the development of *in vitro* systems for studying the biosynthetic steps involved in the attachment of fatty acids to proteins, many of these questions will be answered in the near future.

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