

The Covalent Modification of Eukaryotic Proteins with Lipid

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A surprisingly large number of proteins in eukaryotic cells are now known to contain covalently bound lipid. Several cell surface proteins, the Thy-1 glycoprotein (27, 55), acetylcholinesterase (14), alkaline phosphatase (28), and the variant surface glycoprotein of trypanosomes (13), among others, are anchored to the outer cell surface by a complex, glycosylated phospholipid (Table I; Fig. 1). A second group of proteins, which are found within the cell, contain the 14 carbon saturated fatty acid myristic acid at their amino-termini (Table I; Fig. 1). Included in this class is the product of the *c-src* proto-oncogene, p60^{c-src} (5); the catalytic subunit of the cAMP-dependent protein kinase (9); the protein phosphatase calcineurin (1); a *gag* internal structural protein of mammalian retroviruses (18); and a number of viral transforming proteins (48). Finally, the surface glycoproteins of several enveloped viruses (45–47), the HLA glycoprotein (24, 50), the transforming protein of Harvey sarcoma virus, p21^{ras} (6, 11, 50), and the transferrin receptor (36) are part of a diverse third class of proteins that contain the 16 carbon saturated fatty acid palmitic acid linked to cysteine (Table I; Fig. 1).

The modification of protein with lipid is not simply a biochemical curiosity. Lipid is, in several cases, clearly required for the binding of proteins to cellular membranes. Treatment of cells with phospholipase C, an enzyme that can cleave the lipid from acetylcholinesterase, alkaline phosphatase, and the Thy-1 glycoprotein, releases a significant fraction of each of these proteins (14, 27, 28). Additionally, point mutations that prevent myristylation of p60^{c-src} and Pr65^{gag} and palmitylation of p21^{ras} render these proteins unable to bind to membranes (8, 22, 40, 57). It has become clear therefore that the signals and sequences necessary for a protein to bind to a membrane need not be present in the primary structure of the protein, but can instead be acquired through co-translational or posttranslational modification.

Myristylation, palmitylation, and the addition of the complex glyco-phospholipid occur by remarkably different mechanisms.

Myristylation

In every case, myristic acid is found linked through an amide bond to the α -amino group of amino-terminal glycine (1, 9, 18, 37, 49) (Fig. 1). The myristyl transferase appears to have an absolute specificity for both amino-terminal glycine and

for myristic acid. Even mutations that convert a myristylated glycine to alanine abolish myristylation completely (22, 40). Additionally, chemical analysis of the lipid that modifies amino-terminal glycines yields only myristic acid (1, 9, 18, 37, 49). These observations are borne out by studies characterizing the myristyl transferase of yeast *in vitro*. This enzyme will myristylate only synthetic peptides that contain an amino-terminal glycine and will not incorporate a fatty acid with a chain length of >14 methylenes (54). The question of whether another lipid could functionally replace myristic acid has not yet been addressed.

Myristylation occurs very early during the life of a protein. It may in fact occur co-translationally, before the synthesis of the polypeptide is complete. Drugs that inhibit protein synthesis inhibit myristylation completely and without a detectable lag (7, 30, 34). The total cessation of myristylation after the inhibition of protein synthesis suggests that amino-terminal myristate does not turn over. Consistent with this is the fact that the half-life of the myristyl group in p60^{c-src} is the same as that of the polypeptide itself (7).

Perhaps surprisingly, a number of myristylated proteins are synthesized on soluble polysomes, rather than on membrane-bound polysomes. Because myristylation occurs during or immediately after the synthesis of a protein, the fact that myristylation can occur on soluble polysomes implies that both the myristyl donor, which is probably myristyl CoA (53), and the myristyl transferase are either soluble or bound to polysomes.

Myristylation requires the recognition of a specific amino acid sequence in the substrate protein by the myristyl transferase. Amino-terminal glycine is obviously an essential component of such a sequence. What else is required is not yet known. A sufficient signal for myristylation is apparently present in the 14 amino-terminal residues of p60^{c-src}. A chimeric protein containing these residues appended to the amino-terminus of globin is myristylated (38). Unexpectedly, the amino-termini of myristylated proteins have little sequence homology, save for the myristylated glycine. An amino-terminal glycine is not in itself a sufficient signal for myristylation. Studies with synthetic peptides have demonstrated that certain penultimate residues inhibit severely the myristylation of peptides containing amino-terminal glycine (54).

Palmitylation

Palmitylation differs significantly from myristylation. Palmitic acid is most often found to be linked through a thioester

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Table I.

	Reference
Proteins containing a phospholipid tail	
Thy-1 glycoprotein	27, 54
Variant surface glycoprotein of trypanosomes	13
TAP glycoprotein	41
Acetylcholine esterase	14
5'-nucleotidase	26
Alkaline phosphatase	28
Neural cell adhesion molecule-120	17
<i>Plasmodium falciparum</i> transferrin receptor	15
<i>Plasmodium falciparum</i> p195 surface antigen	16
Myristylated proteins	
p60 ^{src}	5, 49
Catalytic subunit of cAMP-dependent protein kinase	9
Calcineurin B	1
NADH-cytochrome b ₅ reductase	37
p56 ^{lck}	32, 56
p15 ^{gag} of Moloney murine leukemia virus	18
p120 ^{gag-abl} transforming protein	48
p85 ^{gag-fes} transforming protein	48
p29 ^{gag-ras} transforming protein	48
Palmitylated proteins	
p21 ^{ras}	6, 11, 50
Vesicular stomatitis virus G glycoprotein	45
Alphavirus E2 glycoprotein	47
Influenza virus HA glycoprotein	44
Parainfluenza virus F glycoprotein	44
SV40 virus large T antigen	25
Mammalian transferrin receptor	36
HLA B glycoprotein	24, 50
Apolipoprotein A-I	19
Rhodopsin	33
Ankyrin	52

bond to cysteine (Fig. 1). This has been established quite conclusively in the case of the G glycoprotein of vesicular stomatitis virus (43), p21^{ras} (11), the transferrin receptor (21), and the HLA glycoprotein (24). Early reports of palmitic acid esterified to serine in the viral G protein were

probably erroneous. Unlike myristic acid, which is only found attached to amino-terminal residues, palmitic acid is found to modify cysteines present within the body of a polypeptide. These residues are invariably found near membrane-binding domains of a protein and usually on the cytoplasmic face of a membrane.

The amino acid sequence or sequences specifying palmitylation are not known. The palmitylated cysteine in p21^{ras} is located four residues from the carboxy-terminus of the protein and is followed by two aliphatic residues. A cysteine followed by two aliphatic residues is also found four residues from the carboxy-terminus of *ras* proteins found in yeast (39) and *Dictyostelium* (42), and in several other proteins with partial homology with p21^{ras} (10, 29). It may be that this sequence is sufficient to signal palmitylation. Comparison of this sequence with that flanking the sites of palmitylation in the G glycoprotein of vesicular stomatitis virus and the transferrin receptor reveals no obvious homology.

The palmityl transferase has not been purified. It appears to show less specificity with regard to fatty acid than does the myristyl transferase since fatty acids other than palmitic acid (e.g., stearic acid) can be detected in Sindbis virus glycoproteins (47). The palmityl transferase may be a cytoplasmic protein because palmitic acid is usually found in regions of proteins interacting with the cytoplasmic face of membranes. The donor is likely to be palmityl CoA (3).

In contrast to myristylation, palmitylation is a posttranslational event. The addition of palmitic acid to a number of viral glycoproteins occurs ~20 min after the release of the proteins from the polysome, probably during the transport of the protein through the Golgi apparatus (46). Similarly, newly synthesized p21^{ras} contains no palmitic acid. Only older, processed forms of the protein are palmitylated (50). Palmitylation can apparently occur long after the synthesis of the polypeptide. The palmitylation of the transferrin receptor (35), ankyrin (52), and p21^{ras} (31, and our unpublished results) many hours after the cessation of protein synthesis can be readily observed. Although this could reflect a slow rate of palmitylation, most probably it suggests that the palmityl moieties in some proteins undergo fairly rapid turnover.

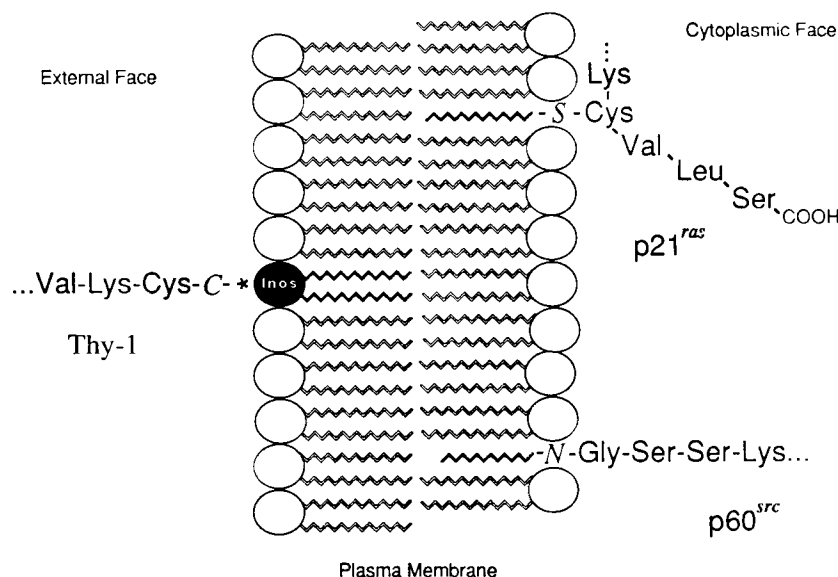


Figure 1. The linkages of the lipids bound to p60^{src}, p21^{ras}, and the Thy-1 glycoprotein and their probable mode of interaction with the cellular plasma membrane. (*) The phosphatidylinositol to which the Thy-1 glycoprotein is bound contains glucosamine, galactosamine, mannose, and ethanolamine, which are not depicted here.

Complex Phospholipid Tails

The third type of lipid modification is the most unusual and unexpected. A number of cell surface glycoproteins contain a large, complex, glycosylated phospholipid at their carboxy-termini (Fig. 1). This lipid serves to anchor these proteins to the lipid bilayer. Incubation of intact cells with phospholipase C leads to the apparent loss of the lipid tail and the release of a significant fraction of these proteins in a form that is much less hydrophobic than is the form of the protein found in membranes (14, 17, 28).

The structure of the lipid bound to these proteins has not been elucidated completely. As expected from its sensitivity to types of phospholipase C that are specific for phosphatidylinositol, it contains this phospholipid as one of its components. In addition, ethanolamine, glucosamine, galactosamine, and mannose are present (13, 20, 55). The variant surface glycoprotein protein of trypanosomes is linked to the glycosylated lipid through an amide bond between the terminal carboxyl group of the polypeptide and the nitrogen of ethanolamine (20). Ethanolamine is probably linked to the head group of phosphatidylinositol. The structure of the carbohydrate portion of the tail is not understood.

Although linked to the carboxy-terminus of both Thy-1 and variant surface glycoprotein, the glycosylated phosphatidylinositol is not in fact bound to the true carboxy-terminus of either protein. DNA sequence analysis shows that the genes for both the Thy-1 glycoprotein and the variant surface glycoprotein of trypanosomes encode proteins that are longer than the protein that is present on the surface of cells; the additional residues being found at the carboxy-terminus of the primary translate (4, 51). Apparently, these proteins undergo proteolytic processing that removes 17–31 carboxy-terminal residues very soon after their synthesis (2). Only then is the carboxyl group to which the lipid is bound exposed. Unlike myristate and palmitate, a glycosylated lipid tail can be linked to a variety of terminal amino acids. Whether the lipid is attached as a complete unit or in individual components is not yet known.

Because the lipid tail is susceptible to digestion when intact cells are incubated with added phospholipase C, it probably resides in the outer leaflet of the plasma membrane (Fig. 1). Transport of the Thy-1 glycoprotein and the variant surface glycoprotein to the plasma membrane almost certainly is initiated by the insertion of the nascent polypeptide into the endoplasmic reticulum. Work with the variant surface glycoprotein suggests that addition of the lipid occurs very soon after synthesis of the protein (2). It is reasonable to suspect therefore that the addition of the lipid tail occurs on the luminal face of the membrane of the endoplasmic reticulum and that both the donor of the lipid and the enzymes catalyzing the reaction are found there.

The Function of Lipid Modification

There is evidence that myristic acid, palmitic acid, and complex phospholipid tails can each anchor proteins to cellular membranes. The function of the myristyl groups in both p60^{src} and Pr65^{gag} has been studied in the most detail. The presence of the myristyl moiety is essential for the binding of these proteins to the inner face of the cellular plasma membrane. Conservative point mutations that prevent myristylation abolish membrane binding (8, 22, 40). In the case

of p60^{src} such a mutation also almost completely inactivates the transforming activity of the protein without affecting its intrinsic protein kinase activity (8, 12, 23). The fact that both p60^{src} and Pr65^{gag} are soluble when not myristylated is compelling evidence that myristylation can confer the ability to bind to membranes on an otherwise soluble protein.

Myristylation could facilitate the binding of a protein to membranes simply because it is hydrophobic in character. This cannot explain however why a myristylated protein like p60^{src} binds predominantly to the plasma membrane. Indeed, if a hydrophobic lipid is all that is required for membrane binding, it is not clear why only this nonabundant fatty acid is used as an amino-terminal anchor. This raises the possibility that p60^{src} is bound to the plasma membrane because only this membrane contains a protein with a selective affinity for myristylated proteins. Myristic acid could therefore facilitate membrane binding not because it is moderately hydrophobic but rather because it provides a signal that promotes transport of the protein to a specific location within the cell.

Myristylation does not induce membrane binding in all cases. Cells contain a number of myristylated proteins that are not bound tightly to cellular membranes (7, 30, 34). Notable among these is the catalytic subunit of the cAMP-dependent protein kinase, which is a soluble protein when not associated with its regulatory subunit. It is possible therefore that myristylation has some other function, or even no function, in soluble proteins. The creation and characterization of a nonmyristylated catalytic subunit should help answer this question.

Palmitylation appears to be essential for the binding of p21^{ras} to cellular membranes. Mutations that replace cysteine 186, the site of palmitylation in p21^{ras}, abolish palmitylation and render the protein unable to bind tightly to membranes (57). It is noteworthy that such mutations also render the protein inactive in cellular transformation. The simplest interpretation of this result may not however be correct. The fraction of the population of p21^{ras} that contains palmitic acid may be low (6) and the possibility exists that the mutation at Cys 186 has a primary effect on membrane binding, and prevents palmitylation only because it prevents membrane binding, rather than preventing membrane binding because it prevents palmitylation.

Palmitylation is apparently not essential for the correct interaction of the viral G glycoprotein with membranes. A site-specific mutation replacing the palmitylated cysteine has no measurable effect on the transport or membrane association of the protein (43). Generalizations as to the function of protein palmitylation are therefore difficult to draw. Indeed, cell fractionation suggests that some as yet unidentified palmitylated proteins behave as soluble proteins (our unpublished results) and at least one secreted protein, Apolipoprotein A-I is palmitylated (19).

The fact that phospholipase C can release proteins possessing phospholipid tails from the surface of cells demonstrates directly that these lipids serve to anchor proteins to membranes. It is certainly not obvious however why a lipid with such an exotic structure is used in this apparently structural role. The possibility exists therefore that the lipid tail has a function in addition to serving as an anchor. This might be manifest after cleavage of the protein by phospholipase C. Digestion by this phospholipase would yield a soluble form

of the protein, containing a tail comprising an oligosaccharide, ethanolamine and inositol phosphate, and, separately, diacylglycerol. Either could be physiologically significant. Diacylglycerol is the natural activator of protein kinase C and its production during release of a protein such as the Thy-1 glycoprotein could allow the cell to respond to the removal of antigen. Clearly much more needs to be learned about the function of phospholipid tails and their constituent parts.

Another obvious and important question is whether the properties of cell surface proteins that are anchored by a glycopospholipid tail are different from those of proteins that are bound to membranes by a hydrophobic polypeptide domain and a cytoplasmic tail. It could be that lateral mobility of proteins with lipid tails is greater and that an ability to diffuse rapidly plays a role in the normal function of this class of protein. Alternatively, a lipid tail would allow the protein to be removed rapidly from the cell surface by a phospholipase C and might obviate the need for internalization as a means to clear the protein from the cell surface.

How common is the modification of protein with lipid? Clearly it is neither extremely rare nor very common. Analysis of cellular protein labeled biosynthetically with either radioactive myristic acid or palmitic acid by one-dimensional SDS PAGE reveals ~50 labeled polypeptides in both cases (7, 30, 34). Only a few of these proteins has been identified and characterized to date. Lipid modified proteins are almost certain to be exceptional. p60^{v-src}, the myristylated transforming protein of Rous sarcoma virus, is readily apparent when total cell protein from RSV-transformed cells, labeled biosynthetically with radioactive myristic acid, is analyzed by gel electrophoresis. Because p60^{v-src} is not an abundant polypeptide and cannot be distinguished by one-dimensional gel analysis of total cellular protein, its easy detection among myristylated proteins suggests that none of the myristylated proteins are particularly abundant.

However, given the surprisingly large number of physiologically important proteins already known to contain lipid, and the fact that lipid is often essential, it can be anticipated that additional examples of proteins which require lipid modification for function will be found.

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