

Trafficking and signaling by fatty-acylated and prenylated proteins

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A wide variety of signaling proteins are modified by covalently linked fatty acids and/or prenyl groups. These hydrophobic moieties, which include myristate, palmitate, farnesyl and geranylgeranyl, are more than just fat: they provide distinct information that modulates the specificity and efficiency of signal transduction. Recent studies show that lipid modification influences the movement of a signaling protein within the cell and its final destination. Protein lipidation can also confer reversible association with membranes and other signaling proteins. These findings provide new insights into the biochemical and biophysical mechanisms that regulate membrane targeting, trafficking and signaling by lipid-modified proteins.

Several hundred proteins have been shown to contain covalently bound lipid groups. The three most common types of lipids that are attached to proteins are fatty acids, isoprenoids and glycosylphosphatidylinositol (GPI) anchors. This review will focus on fatty acylation and prenylation of signaling proteins, with an emphasis on how modification by these lipophilic moieties regulates protein interaction with membranes and with other proteins, as well as signaling function. For general reviews of lipid modification, the reader is referred to refs. 1–5.

Within the past two years, a number of new advances in the field make a review of trafficking and signaling by lipid-modified proteins particularly timely. New enzymes that catalyze palmitate attachment to signaling proteins as well as new substrates have been identified. We now have new insights into how lipid modification regulates endocytosis, trafficking and signaling of modified proteins. In addition, reversible association of lipidated proteins with membranes has been shown to occur through regulated cycles of depalmitoylation and repalmitoylation and through myristoyl, farnesyl or electrostatic switches. Finally, new studies reveal how lipid modification of secreted ligands influences their cell-to-cell movement and signaling range.

Protein fatty acylation and prenylation

Fatty acylation involves the covalent attachment of the 14-carbon saturated fatty acid myristate (N-myristoylation) or the 16-carbon fatty acid palmitate (palmitoylation) to proteins (Table 1). Some proteins are modified by myristate or palmitate alone, whereas others contain both fatty acids.

N-myristoylation. Approximately 0.5% of all eukaryotic proteins are N-myristoylated. These proteins begin with the sequence Met-Gly- and often contain a serine or threonine at position 5. An internet-based algorithm can predict with high accuracy whether

a particular protein sequence will be N-myristoylated⁶ (Table 2). N-myristoylation generally occurs during protein synthesis. After the initiating Met is removed by methionine aminopeptidase, myristate is linked through amide bond to the N-terminal glycine. This reaction is catalyzed by N-myristoyltransferase (NMT)⁵. In fungi, NMT is a single-copy gene that is essential for viability. Vertebrates have two NMT genes with partially overlapping functions. NMT-1 is essential for early development as well as cell replication and tumor-cell growth^{7,8}.

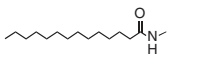
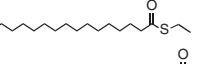
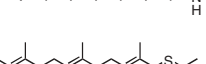
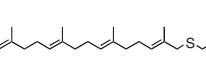
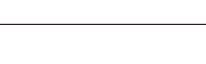
Palmitoylation. Nearly all palmitoylated proteins contain palmitate linked through thioester bond to one or more cysteine residues. Because other medium- and long-chain fatty acids can also be attached to 'palmitoylated' proteins, the term S-acylation is often used^{9,10}. There is no consensus sequence for protein palmitoylation, although some modified sites can be predicted¹¹ (Table 2). Several recent advances have expanded our knowledge of the enzymology of palmitoylation. First, members of the DHHC-CRD (Asp-His-His-Cys cysteine-rich domain) family of proteins have been shown to be palmitoyl acyltransferases (PATs) for yeast and mammalian proteins¹². Second, a comprehensive proteomic approach has led to the identification of a 'palmitoyl proteome': all the palmitoylated proteins in an organism (50 in yeast)¹³. Third, N-palmitoylated proteins with amide-linked palmitate have been identified. These include the G α_s subunit and two secreted signaling proteins, Hedgehog and Spitz¹⁴. Hedgehog and Spitz are N-palmitoylated by Rasp, a member of the membrane-bound O-acyltransferases (MBOAT) family^{15,16}.

Prenylation. Protein prenylation is a posttranslational reaction that occurs in the cytosol. A 15-carbon (farnesyl) or 20-carbon (geranylgeranyl) isoprenoid is linked through thioether bond to one or more cysteine residues at or near the C terminus of the protein (Tables 1,2). Many prenylated proteins contain a C-terminal 'CAAX box' (Cys-aliphatic-aliphatic-X)¹⁷. The 'X' amino acid determines whether the cysteine within the CAAX box is farnesylated by farnesyltransferase or geranylgeranylated by geranylgeranyltransferase I. The three C-terminal amino acids (AAX) are then cleaved, and the C-terminal prenylated

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Table 1 Facts about fats

Modifying group	Chemical structure	Enzyme	Linkage	Sequence
Myristate		NMT	Amide to N-Gly	MGXXXS/T
S-Palmitate		PATs	Thioester to Cys	See text
N-Palmitate		Rasp	Amide to N-Cys	CGPGR
Farnesyl		FTase	Thioether to Cys	CAAX
Geranylgeranyl		GGTase I GGTase II	Thioether to Cys	CAAL CC, CXC or CC(X) _{n=1-3}

cysteine residue is carboxymethylated in the endoplasmic reticulum (ER). Rab proteins are prenylated on cysteines at or near the C terminus by geranylgeranyltransferase II. To be prenylated, Rab proteins must first be complexed with Rab escort protein (REP).

Membrane binding affinity and lipid modifications

Nearly all lipid-modified signaling proteins need to be membrane bound to signal efficiently. Because fatty acids and prenyl groups are hydrophobic, it has been assumed that attachment of these moieties is sufficient to direct membrane binding of the modified protein. However, this simplistic assumption is not correct. In a classic study, Peitzsch and McLaughlin established that the apparent K_d for binding of a myristoylated peptide to a phospholipid bilayer is approximately 10^{-4} M (ref. 18). This binding energy is not sufficient to provide stable membrane binding inside a cell. Likewise, peptides and proteins modified by a single farnesyl dissociate from membranes within 1–2 min (refs. 18,19). Thus, neither myristate nor farnesyl alone provide sufficient hydrophobicity to stably anchor proteins to a lipid bilayer.

How do N-myristoylated and farnesylated proteins achieve stable membrane binding? According to the 'two-signal hypothesis', a second signal, either palmitate or a polybasic domain, is required. For example, membrane binding of Src is mediated by an N-terminal 'myristate + basic' motif^{20–22}. Myristate inserts hydrophobically into the lipid bilayer: 10 of the 14 methylene carbons penetrate the bilayer²³. The basic amino acids in Src associate electrostatically with negatively charged phospholipids that are enriched on the cytoplasmic leaflet of the plasma membrane. Neither the myristate nor the basic residues alone are sufficient for strong binding to membranes. However, when both are present within the same protein, the hydrophobic and electrostatic forces synergize and membrane binding is increased several orders of magnitude²⁴. Myristoylated alanine-rich C kinase substrate (MARCKS), hisactophilin, and retroviral Gag proteins also use a myristate + basic mechanism^{25,26}, whereas K-Ras4B and lamin B3 use prenyl + basic motifs^{27–29} to bind to membranes.

The second signal can also be provided by attachment of palmitate. In this case, two lipids are better than one, and membrane binding is increased significantly^{28,30}. For example, Src family kinases (SFKs), G α subunits and endothelial nitric oxide synthase (eNOS) use myristate + palmitate, whereas H-Ras and N-Ras use farnesyl + palmitate to achieve membrane binding. Mutation of palmitoylation sites within these proteins or inhibition of protein palmitoylation, for example, with 2-bromopalmitate, results in reduced membrane association and decreased signaling.

In a side-by-side contest, which signal provides stronger membrane binding: palmitate or a polybasic motif? Biophysical data reveal that the prenyl + palmitate motif of H-Ras provides a stronger interaction with the bilayer compared to the prenyl + polybasic motif in K-Ras4B. Model studies with peptides that mimic the H-Ras tail indicate that both lipid chains are inserted deeply into the bilayer interior and that hydrophobic side chains from the peptide also contribute to membrane binding³¹. In contrast, K-Ras4B peptides and proteins exhibit rapid, reversible membrane binding. Translocation between intracellular membranes occurs with a half-time of 7–10 min (ref. 32). These findings suggest that cellular strategies may have evolved to promote K-Ras4B release from the plasma membrane.

Regulated, reversible membrane binding

Many fatty-acylated and prenylated proteins undergo reversible association with membranes. For palmitoylated proteins, this is achieved by depalmitoylation. However, the amide and thioether linkages of myristate and prenyl groups to proteins are stable bonds that are generally not broken during a protein's lifetime *in vivo*. If demyristoylation or de-farnesylation reactions do not occur, how are these proteins released from the lipid bilayer?

Myristoyl switches. Structural studies of a number of N-myristoylated proteins have established that the myristate moiety can adopt two distinct conformations: exposed and available to promote membrane binding, or sequestered inside a hydrophobic binding pocket within the N-myristoylated protein. The transition between these two states is regulated by a 'myristoyl switch.' At least one dozen proteins undergo regulated membrane binding through a myristoyl switch (Fig. 1 and Table 3). The switch is usually triggered by ligand binding. A conformational change causes myristate to flip inside the protein, resulting in release of the N-myristoyl protein from the membrane^{33–36}. A variation on this theme is the myristoyl electrostatic switch. Phosphorylation within the polybasic motif introduces negative charge and reduces the electrostatic component of bilayer interaction (Fig. 1). For example, phosphorylation of the MARCKS protein by protein kinase C triggers release of the myristoylated protein from the membrane³⁷.

Farnesyl and geranylgeranyl switches. In theory, reversible sequestration of farnesyl or geranylgeranyl moieties within a prenylated protein could generate a prenyl switch. Although there are no known examples of a prenylated protein sequestering its own prenyl group, prenylated proteins can be extracted from the membrane by binding partners that sequester the prenyl groups (Fig. 1). This prenyl switch mechanism serves to regulate the signaling activities of small GTPases

Table 2 Web resources for fatty acylation and prenylation of proteins

Myristoylation site predictor	http://mendel.imp.ac.at/myristate/
Palmitoylation site predictor	http://bioinformatics.lcd-ustc.org/css_palm/
Prenylation site predictor	http://mendel.imp.ac.at/sat/PrePS/
NMT molecule page	http://www.signaling-gateway.org/molecule/query?afcsid=A000095
Pfa3 yeast PAT	http://www.expasy.org/uniprot/P42836
Akr1p yeast PAT	http://www.expasy.org/uniprot/P39010

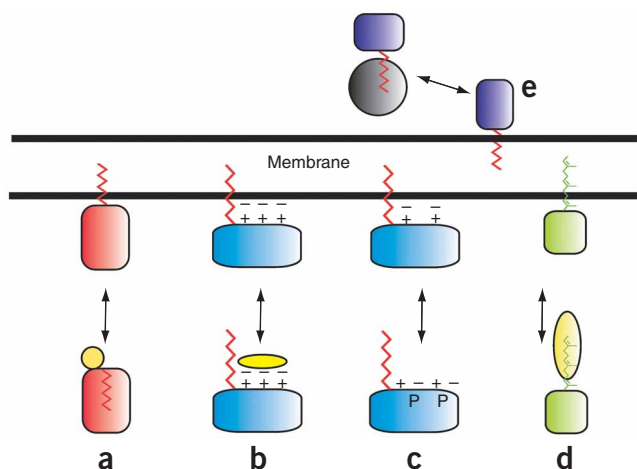


Figure 1 Reversible membrane binding of lipid-modified proteins. Five different schemes for lipidated proteins to associate and dissociate from a lipid bilayer are illustrated. Fatty acyl groups are red; prenyl groups are green. (a) Binding of a ligand (yellow) to an N-myristoylated protein (red) induces a myristoyl switch. (b) Binding of a ligand (yellow) to the basic motif of a myristate + basic-containing protein (blue) induces an electrostatic switch. (c) Phosphorylation within the basic motif induces an electrostatic switch. (d) Sequestration of a prenyl group by a binding partner (yellow) induces release of the prenylated protein (green) from the membrane. (e) The lipid group(s) of a secreted protein (purple) bind to lipoprotein particles (black), allowing the complex to travel from one cell to another.

such as Rho and Rab proteins. For example, binding of the C-terminal geranylgeranyl moiety of Rho GTPases to a hydrophobic pocket within RhoGDI leads to release of the Rho(GDP)–RhoGDI complex from the plasma membrane to the cytosol, where it is maintained in an inactive form³⁸. Likewise, Rab proteins, which are dually geranylgeranylated at two C-terminal cysteines, are regulated by binding to RabGDIs. Binding of one Rab geranylgeranyl group to a shallow hydrophobic groove within RabGDI triggers a conformational change that releases Rab:RabGDI from the membrane³⁹. In the case of the yeast Rab, Ypt1, binding of Ypt1 to its GDI induces a conformational change that opens up a geranylgeranyl binding site within a deep hydrophobic cavity in GDI⁴⁰. The location of the binding site for the second prenyl group has not been determined, but modeling predicts that it will bind to an adjacent hydrophobic groove.

Two new studies indicate that K-Ras4B can undergo signaling-induced translocation from the plasma membrane through farnesyl electrostatic switches. As a result of CaM binding to the K-Ras4B polybasic motif, K-Ras4B is released from the plasma membrane and translocates to Golgi and endosomal membranes⁴¹. Alternatively, phosphorylation of a serine residue (Ser181) within the polybasic

motif by protein kinase C leads to dissociation of phosphorylated K-Ras4B from the plasma membrane⁴². K-Ras4B translocates to mitochondria, where it interacts with Bcl-XL and induces apoptosis. It will be interesting to determine if mutation of Ser181 occurs in human cancers, as this would be expected to prolong signaling of activated K-Ras.

Regulated cycles of palmitoylation and depalmitoylation. The reversible nature of thioester bonds makes palmitoylation a dynamic modification. The relative activities of PATs and palmitoyl thioesterases determine the stoichiometry of protein palmitoylation at steady state. Many palmitoylated proteins undergo regulated membrane binding through cycles of palmitoylation and depalmitoylation. For example, H-Ras is palmitoylated in the Golgi and targeted to the plasma membrane (Fig. 2). H-Ras can then be depalmitoylated at the plasma membrane and internalized through a nonvesicular transport mechanism back to the Golgi^{43,44}. As a consequence, pools of H-Ras can be maintained at and actively signal from two different subcellular localizations^{45,46}.

In many cases, palmitoylation cycles are regulated by agonist stimulation. For example, α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors are members of the family of glutamate receptors that bind neurotransmitters on the postsynaptic cell. To signal efficiently, AMPA receptors are concentrated within a region of the synapse termed the postsynaptic density (PSD). Receptor recruitment to the PSD is mediated by a palmitoylated protein, PSD-95. Palmitate turnover on PSD-95 is dynamic and regulated by the activity of glutamate receptors⁴⁷. Inhibition of PSD-95 palmitoylation prevents synaptic targeting of PSD-95 and synaptic clustering of receptors, thereby inhibiting synaptic signaling⁴⁸.

Three other examples of agonist-induced alterations in palmitoylation come from studies of G protein-coupled receptors (GPCRs), their heterotrimeric G-protein signaling effectors, and regulators of G proteins. Many of the members of the GPCR superfamily are palmitoylated at one or several cysteines within their C-terminal cytoplasmic tails, including the β_2 -adrenergic, α_{2A} -adrenergic, A₁ adenosine, CCR5, H₂ histamine and D₁ dopamine receptors. Agonist stimulation of palmitate turnover has been documented for several of these GPCRs⁴⁹. In some cases, this results in decreased levels of palmitate on the GPCR, leading to alterations in receptor trafficking and/or signaling.

Downstream signals are transmitted from GPCRs through heterotrimeric ($\alpha\beta\gamma$) G proteins, which signal from the cytoplasmic side of the plasma membrane. Localization of G proteins to the plasma membrane is dictated by prenylation of the γ subunit and myristoylation and/or palmitoylation of the α subunits⁵⁰. Agonist activation of the β_2 -adrenergic receptor induces increased palmitate turnover on G α_s . Surprisingly, the stoichiometry of α_s palmitoylation at steady state

Table 3 Proteins that undergo myristoyl or farnesyl switches

Myristoyl switch	Trigger
Calcium sensor proteins	Ca ²⁺ /calmodulin
Recoverin	
Hippocalcin	
Visinin-like proteins	
Neurocalcins	
Frequenin	
Arf	GTP
MARCKS	PKC phosphorylation
MARCKS	Ca ²⁺ /calmodulin
PKA catalytic subunit	PKA regulatory subunit
Hisactophilin	pH
c-Abl	SH2/pTyr
HIV-1 Gag	Entropic (trimerization)
Farnesyl switch	Trigger
K-Ras4B	Ca ²⁺ /CaM
K-Ras4B	PKC phosphorylation

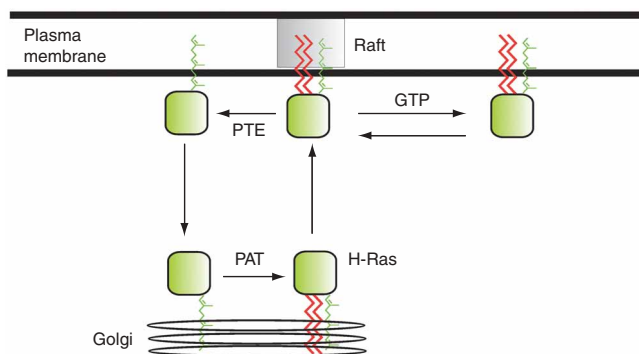


Figure 2 Reversible palmitoylation of H-Ras dictates localization and signaling. H-Ras (green) is palmitoylated by a palmitoyl acyltransferase (PAT) in the Golgi and then transported to the plasma membrane through secretory vesicles. A subpopulation of H-Ras is localized in membrane rafts, but occupancy in rafts is transient. GTP loading of H-Ras induces its egress into non-raft regions of the plasma membrane. H-Ras can be de-palmitoylated at the plasma membrane by one or more palmitoyl thioesterases (PTE). The deacylated protein is released from the plasma membrane and returns to the Golgi through nonvesicular transport, where it is re-palmitoylated. Signaling emanates from both Golgi and plasma membrane associated H-Ras. Palmitoyl groups are in red; the prenyl group is in green.

does not change, implying that depalmitoylated α_s only exists transiently⁵¹. Moreover, the functional consequences of α_s depalmitoylation are in dispute. One study showed that depalmitoylated α_s is released into the cytosol, thereby turning off signaling⁵², whereas others reported that depalmitoylated α_s remains at the plasma membrane but redistributes into a different membrane subdomain⁵³.

The GTPase activity of G proteins is activated by RGS (regulator of G protein signaling) proteins, a family of membrane-bound proteins that turn off signaling. One of the RGS family members, RGS7, shuttles between the plasma membrane and the nucleus. This is accomplished by RGS7 binding to R7BP, a palmitoylated shuttle protein⁵⁴. When R7BP is depalmitoylated, the RGS7–R7BP complex is released from the plasma membrane and translocates to the nucleus. Shuttling enables the complex to signal from two different intracellular locations.

Fatty acylation/prenylation and membrane targeting

Signal transduction efficiency adheres to the classic real-estate mantra: location, location, location. Signaling proteins need to associate with a specific membrane where they can access target proteins and effectors. The next section will consider how lipid modification confers membrane targeting specificity.

Targeting through kinetic trapping. Peptides modified with myristate or farnesyl alone exhibit nonspecific association with intracellular membranes. However, if a palmitoylation site is present, specific localization to the plasma membrane or the Golgi can occur^{30,55}. These findings are best explained by a ‘kinetic trapping’ mechanism. Take the case of a myristoylated protein that is palmitoylated by a PAT residing in the plasma membrane (Fig. 3a). The increased hydrophobicity from the two fatty acids reduces the kinetic ‘off’ rate, and the dually acylated protein becomes trapped at the plasma membrane. Thus, the site of palmitoylation (localization of the PAT) determines the localization of the palmitoylated protein. This mechanism nicely accounts for the plasma membrane and Golgi localizations of H-Ras, N-Ras and yeast Ras2p (refs. 43,44,56) as well as dually fatty-acylated Src family kinases and G α subunits^{3,57}.

Targeting of dually fatty-acylated proteins to rafts. Lipid rafts are functionally defined as membrane subdomains that are highly enriched in cholesterol, glycosphingolipids and phospholipids containing saturated fatty acid chains⁵⁸. The ability of these lipids to pack tightly into a ‘liquid-ordered’ subdomain leads to lateral segregation in the plane of the bilayer. Rafts were originally identified by their ability to withstand extraction by cold, nonionic detergent and to float to the top of density gradients. More recently, fluorescence-based techniques have verified the existence of rafts in biological membranes *in vitro* as

well as in living cells. However, the size, shape and dynamics of rafts remain highly contested issues. Despite this cautionary note, it is clear that association of signaling proteins with lipid rafts enhances the protein-protein and protein-lipid interactions that are important for efficient signal transduction.

One would predict that proteins containing saturated fatty acid chains would prefer to insert into the liquid-ordered domain of the raft, rather than the more disordered, bulk plasma membrane (Fig. 3b). This supposition was verified by the finding that SFKs modified with saturated fatty acids, such as palmitate, preferentially associate with rafts. In contrast, SFKs acylated with unsaturated fatty acids that contain a kinked, *cis* double bond are excluded from rafts and exhibit defective signaling^{10,59,60}.

Protein lipidation and membrane trafficking

The final destination of a lipid-modified protein can be influenced by its intracellular trafficking pathway. The examples provided below illustrate how lipid modification can alter protein trafficking, and conversely, how membrane trafficking regulates how and where lipid modification occurs.

Regulation of ER exit. NADH-cytochrome b5 reductase (b5R) is a myristoylated protein that is localized to two different cellular compartments: the ER and the mitochondrial outer membrane. Because this protein contains a hydrophobic, signal-like sequence at its N terminus, N-myristoylation is not required for b5R membrane association. Rather, N-myristoylation interferes with the binding of the b5R N terminus to signal recognition particle (SRP). As a result, some of the N-myristoylated b5R escapes from the SRP-mediated ER insertion pathway and instead targets the mitochondrial membrane⁶¹. Thus, competition between two cotranslational events, N-myristoylation and SRP binding, regulates protein targeting.

Palmitoylation has also been shown to be important for ER quality control. For example, palmitoylation of Chs3, a yeast chitin synthase, is required for its export from the ER; nonpalmitoylated Ch3 forms aggregates in the ER⁶². A broader role for palmitate in regulating protein folding can also be inferred from a recent finding showing that huntingtin, the protein whose mutation causes Huntington disease, needs to be palmitoylated to avoid forming neurotoxic aggregates⁶³.

Trafficking through the secretory pathway and other routes. Lipidated proteins can use the secretory pathway to achieve delivery to the plasma membrane. This is the case for the SFKs Lck and Lyn, and for inducible nitric oxide synthase^{64–66}. H-Ras and N-Ras also traffic through the secretory pathway. Vesicular transport carries H-Ras and

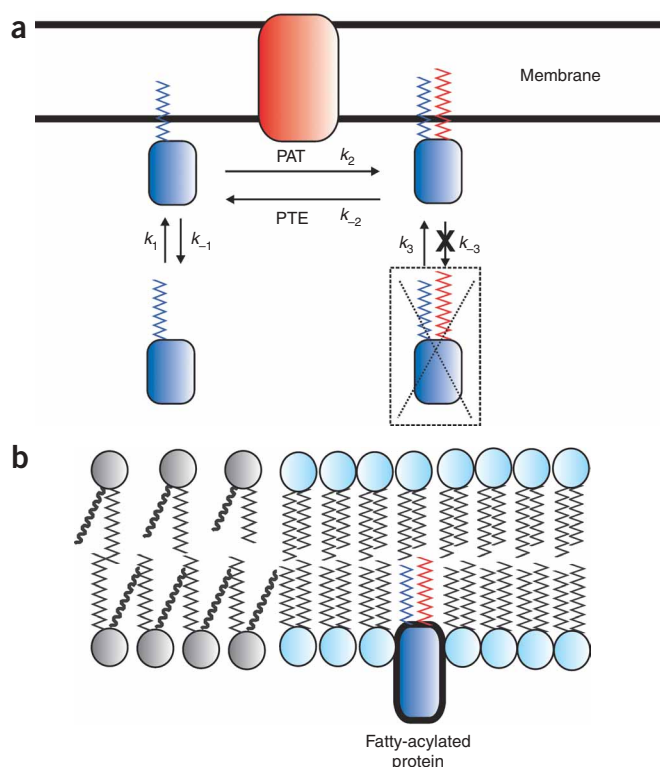


Figure 3 Membrane targeting mechanisms for acylated proteins. The myristoyl group is in blue; the palmitoyl group is in red. **(a)** Kinetic bilayer trapping. An N-myristoylated protein (blue) interacts with and is palmitoylated by a membrane-bound PAT (red). The dually fatty-acylated protein is sufficiently hydrophobic so that its kinetic 'off' rate (k_{-3}) is extremely slow. As a result, the dually fatty-acylated protein is essentially 'trapped' at the membrane where it was palmitoylated. **(b)** Targeting by association with membrane rafts. The saturated fatty acid chains attached to a fatty-acylated protein allow the modified protein to pack within the tightly organized liquid-ordered domain of a lipid raft (blue head groups). This promotes partitioning of the acylated protein into lipid rafts.

N-Ras from the ER to the Golgi, where they are palmitoylated, and then to the plasma membrane^{67,68}. Additional lateral segregation occurs within the plane of the plasma membrane (**Fig. 2**). Inactive H-Ras is partially localized to lipid rafts but upon activation relocates to non-raft regions of the plasma membrane where signal transduction occurs^{69–71}.

Alternative mechanisms can be used for trafficking of lipid-modified proteins. Neither Fyn, a dually fatty-acylated SFK, nor di-geranylgeranylated Rab proteins utilize the secretory pathway for delivery to the plasma membrane^{57,72}. For small G proteins, the number of prenyl groups as well as their identity influence protein targeting. RhoB exists in farnesylated and geranylgeranylated forms; farnesylated RhoB is targeted to the plasma membrane, whereas geranylgeranylated RhoB localizes to late endosomes⁷³. Rab proteins that are di-geranylgeranylated localize to endosomes, whereas monogeranylgeranylated Rabs are in the ER⁷². It is likely that a combination of other factors, such as binding to GDIs or REP proteins, influences where these prenylated proteins ultimately reside within the cell.

Palmitoylation regulates endocytosis, recycling and sorting. Inhibition of protein palmitoylation can have pleiotropic effects on protein

trafficking. Nonpalmitoylated GPCRs such as CCR5 and the delta opioid receptor do not reach the cell surface efficiently because they are either retained in the ER or degraded^{74–77}. Palmitoylation can also regulate various steps of the endocytic pathway. For example, nonpalmitoylated asialoglycoprotein receptors are defective in internalization and lysosomal sorting, whereas recycling of the mucin-like protein MUC1 to the cell surface is blocked when MUC1 is not palmitoylated^{78–80}.

New insights into lipidated protein signaling

Three new areas of investigation merit attention as they illustrate new concepts for signaling by lipidated proteins. First, proapoptotic proteins can be post-translationally N-myristoylated. Second, changes in protein palmitoylation can regulate T-cell signaling. Third, secreted proteins can also be palmitoylated.

Morbid myristoylation. N-myristoylation has generally been considered to be a co-translational modification, occurring as the N terminus on the nascent polypeptide chain emerges from the ribosome. However, it is now apparent that this modification can also occur post-translationally during apoptotic cell death, a process termed 'morbid myristoylation'⁸¹. Post-translational N-myristoylation of the proapoptotic protein BID was first reported by Korsmeyer and colleagues⁸². At least three other proteins, actin, gelsolin and p21Pak2, have since been shown to be cleaved by caspase during apoptosis and post-translationally N-myristoylated on a newly generated N-terminal glycine^{83–85}. The N-myristoylated protein fragments are then targeted to mitochondria, where they activate apoptotic signaling and induce programmed cell death.

T-cell anergy. Signaling mediated by the T-cell receptor relies on fatty acylation of CD4 and CD8 co-receptors, SFKs Lck and Fyn, and adaptor proteins such as LAT. However, T cells can become non-responsive to antigen, a condition known as anergy. A recent study showed that a specific defect in LAT palmitoylation contributes to the development of anergy⁸⁶.

Palmitoylation of secreted ligands: Hedgehog, Spitz and Wingless.

One of the big surprises in the fatty acylation field came with the discovery that secreted signaling molecules are palmitoylated⁸⁷. For example, the Wingless proteins Wnt-1 and Wnt-3a are palmitoylated by Porcupine, an MBOAT-family acyltransferase^{88,89}. Palmitoylation is required for Wnt protein secretion and/or signaling activities^{88,89}.

Hedgehog family proteins, secreted morphogens that regulate growth and differentiation, undergo multiple lipid modifications. The mature Hedgehog signaling protein is generated by autocleavage of a precursor polypeptide. Cholesterol is covalently attached to the C terminus of mature Hedgehog, whereas the N-terminal Cys is N-palmitoylated by Rasp^{15,90}. Both lipid modifications are required for long-range signaling^{91,92}. How can attachment of two sticky anchors regulate cell-to-cell movement? It has been proposed that Hedgehog and Wnt proteins associate with lipoprotein particles, large extracellular complexes composed of a lipid monolayer surrounding a core of triglycerides and cholesterol⁹³. Reversible binding of lipidated signaling proteins to lipoprotein particles allows these morphogens to travel from cell to cell within a tissue and thereby mediate long-range signaling (**Fig. 1e**).

An additional surprise came with the finding that the *Drosophila melanogaster* EGF ligand Spitz is also N-palmitoylated by Rasp; this modification is required for EGF receptor-mediated signaling in flies¹⁶. N-palmitoylation promotes association of Spitz with the

plasma membrane of the producing cell and decreases its diffusion. As a result, Spitz can achieve the high local concentration that is necessary for effective short-range signaling.

Protein-protein interactions and enzyme activity

Finally, there are three examples where lipid modification influences protein-protein interactions and protein activity. (1) Palmitoylation of the RGS protein RGS16 directly increases its GAP activity and thereby regulates its signaling output⁹⁴. (2) The retinal protein RPE65, which participates in the visual cycle, exists in two forms, a palmitoylated, membrane-bound form and a soluble form. Not only do the two forms have different ligand-binding specificities, but the transition between the two forms is regulated during visual transduction⁹⁵. (3) Palmitoylation of the yeast vacuolar protein, Vac8, does more than just target the protein to the vacuolar membrane. A chimeric Vac8 containing the myristate + basic motif of Src instead of the myristate + palmitate motif of Vac8 localizes correctly to the vacuolar membrane but is functionally defective⁹⁶. Taken together, these findings illustrate the pleiotropic effects that lipid modifications have on protein function.

Implications and future directions

In the past few years, our understanding of lipid modification of proteins has gone way beyond that of simple hydrophobic anchors. It is now appreciated that each distinct fatty acid or prenyl moiety provides specific information to assist proteins in finding their correct destination within the cell. Although we now recognize how palmitoylated and prenylated proteins are trafficked and targeted to specific membranes, the mechanism(s) whereby some N-myristoylated proteins find their way to a particular membrane remains a mystery. In particular, we need a better understanding of how myristate + basic or prenyl + basic motif-containing proteins such as Src or K-Ras4B localize specifically to the plasma membrane. Do these proteins exploit a preferential trafficking pathway, or travel down an electrostatic gradient, or interact with a membrane-binding partner? The recent findings showing reversible membrane association of lipidated proteins lead to additional questions. Which thioesterases are involved in removing palmitate from proteins, and how are they regulated? Are there unidentified binding partners or binding sites for fatty-acylated and prenylated proteins that modulate their protein-lipid and protein-protein interactions? To this end, it is encouraging to see more three-dimensional crystal structures of lipid-modified proteins revealing specific binding sites for fatty acids and prenyl groups.

The fact that lipid modification alters signaling-protein function has made the enzymes that catalyze these modifications attractive targets for drug development. For example, prenylation is required for Ras-mediated transformation, and Ras is frequently mutated in human cancers. Thus, several farnesyl transferase inhibitors are currently in clinical trials as antitumor agents. Because NMT is essential for viability in fungi, drugs that target NMT have been developed as antifungal agents. As more PATs and palmitoylated proteins are identified, it is likely that these enzymes and substrates will also prove to be attractive targets for pharmaceutical intervention.

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COMPETING INTERESTS STATEMENT

The author declares that she has no competing financial interests.

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