Published in final edited form as:
Sci China Chem. 2011 December ; 54(12): 1888–1897. doi:10.1007/s11426-011-4428-2.

Understanding Protein Palmitoylation: Biological Significance and Enzymology

Xiaomu GUAN and **Carol A FIERKE***
Departments of Chemistry and Biological Chemistry, University of Michigan, 930 N University, Ann Arbor, MI 48109-1055, USA

Abstract

Protein palmitoylation is a widespread lipid modification in which one or more cysteine thiols on a substrate protein are modified to form a thioester with a palmitoyl group. This lipid modification is readily reversible; a feature of protein palmitoylation that allows for rapid regulation of the function of many cellular proteins. Mutations in palmitoyltransferases (PATs), the enzymes that catalyze the formation of this modification, are associated with a number of neurological diseases and cancer progression. This review summarizes the crucial role of palmitoylation in biological systems, the discovery of the DHHC protein family that catalyzes protein palmitoylation, and the development of methods for investigating the catalytic mechanism of PATs.

Keywords

protein palmitoylation; palmitoyltransferase (PAT); DHHC protein family; catalytic mechanism; palmitoylation assay

1 Introduction

As many as 25–40% of eukaryotic cellular proteins are estimated to be membrane-associated proteins (1). Proteins can interact with the hydrophobic lipid bilayer of cell membranes via a hydrophobic surface formed by structures such as α-helices or β-sheets. In addition, a protein can be modified with a lipid anchor under enzymatic control that interacts with the lipid bilayer and localizes proteins to the membrane surface. Lipid modifications are increasingly recognized as important mechanisms for both targeting proteins to the membrane and subcellular protein trafficking. To date hundreds of lipid-modified proteins have been identified and, in many cases, the modification has been demonstrated to be important for the cellular function of these proteins. For example, the lipidated forms of receptors, monomeric and trimeric G-proteins (2–5) and protein tyrosine kinases (6–11) play crucial roles in a variety of cell signaling events.

Palmitoylation (12, 13), prenylation (14–17), and myristoylation (18, 19) represent three types of common lipid modifications that occur in the cell (Table 1). During the past decade, the enzymes responsible for prenylation and myristoylation have been extensively studied,

*Corresponding author (fierke@umich.edu).

and the target sequences for these modifications have been identified. In recent years, palmitoylation, which involves the formation of a thioester bond between a cysteine thiol side chain and a saturated 16-carbon fatty acid (20–28), has attracted great attention. Compared to the other lipid modifications, palmitoylation is readily reversible due to the lability of the thioester bond. Therefore rapid cycles of palmitoylation and depalmitoylation allow proteins to be facilely shuttled between the plasma membrane and the Golgi apparatus to regulate many cellular functions (29–35). Palmitoylation is catalyzed by protein palmitoyltransferases (PATs). PATs were first identified in yeast more than a decade ago using a genetic screen against palmitoylation-dependent Ras proteins (36). Since then many PATs have been identified using sequence homology; however the recognition motifs in the substrate proteins are still poorly defined. The structures, recognition signals, and the corresponding enzymes that catalyze lipid modifications are summarized in Table 1. Studies conducted over the past decade have substantially advanced our understanding of not only the molecular mechanisms of PATs and the functional consequences of protein palmitoylation, but have also begun to disclose important roles of these enzymes in human health. This review focuses on recent discoveries demonstrating the biological significance of protein palmitoylation and PATs and the development of methods to investigate the catalytic mechanism of these enzymes.

2 Biological Significance of Protein Palmitoylation

Protein palmitoylation is an important reversible lipid modification (29, 30, 32–35). However, in the cell palmitoylation does not function solely as a lipid anchor to localize proteins to the membrane. The reversible cycles of palmitoylation and depalmitoylation play an important function in shuttling modified proteins between cellular compartments, allowing relocalization of the protein in the cell or within different regions of the membrane (13). A scheme of this process is shown in Figure 1. Therefore, in addition to enhancing the membrane association of proteins, palmitoylation also helps target proteins in neurons, recruit proteins into lipid rafts, regulate cell signaling processes, etc. (32–34, 37). Identification of the enzymes responsible for catalyzing the formation and hydrolysis of specific palmitoylated proteins is a key step towards understanding the biological significance and function of this modification.

One major function of protein palmitoylation is to promote protein membrane association. Proteins can either contain a single palmitoyl modification or be dually modified with one or more palmitoyl groups and either a prenyl group or a myristoyl group. Palmitoylated proteins can be divided into 4 distinct types (38–44), shown in Table 2, including (1) a single palmitoyl modification frequently near the end of a protein; (2) palmitoylation proximal to a transmembrane domain; (3) dual palmitoylation and prenylation; and (4) dual palmitoylation and myristoylation.

Examples of proteins that are only modified with a palmitoyl group include the G-protein α subunits. These subunits are palmitoylated to enhance the interaction with $G_{\beta\gamma}$ subunits, an essential step in cell signaling cycles (27). Additionally, regulators of G-protein signaling (RGS) are also palmitoylated, regulating membrane localization and inactivation of G proteins to turn off G protein-coupled receptor signaling pathways (27, 35, 45). In contrast,

mammalian Ras proteins may be dually lipidated. Ras proteins are modified with a farnesyl moiety to target the protein to the cell membrane. However, additional interactions are needed to enhance membrane localization; H-Ras and N-Ras contain both farnesyl and palmitoyl modifications, while K-Ras membrane association is enhanced by the electrostatic interaction between a positively charged basic sequence and the negatively charged plasma membrane (46–49). For dually lipid modified proteins, the first modification (*e.g.* prenylation or myristoylation) provides substrate proteins with weak membrane interaction, while the subsequent palmitoylation generates sufficient hydrophobicity for strong membrane affinity (50, 51).

A large number of neuronal proteins are palmitoylated, including synaptic scaffolding proteins, G-protein-coupled receptors (GPCRs) and synaptic vesicle proteins (35). Palmitoylation of these proteins is vital for regulating proper neuronal development and function, including neuronal differentiation, neurotransmission, and neurotransmitter release (52–54). One of the first neuronal proteins that was demonstrated to be palmitoylated was rhodopsin, in which two cysteines proximal to the seventh transmembrane domain are modified with palmitoyl groups (55). Similarly, many GPCRs are palmitoylated on analogous cysteines (53). A second example is the dendritic postsynaptic density protein 95 (PSD-95) that requires palmitoylation to enhance clustering of AMPA receptors at excitatory synapses; furthermore, palmitate cycling of PSD-95 also regulates synaptic plasticity (56–59). Palmitoylation of the SNARE protein (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor) SNAP-25 (25 kDa synaptosome-associated protein) is also important for neuronal function (60). SNAP-25 is located at presynaptic axon terminals, and is essential for neurotransmitter release (61). Palmitoylation of SNAP-25 is proposed to help regulate aspects of synaptic vesicle fusion, and to target synaptic vesicles to the site of transmitter release (62).

Another important function of palmitoylation is to target modified proteins to lipid rafts. Lipid rafts are dynamic nanoscale assemblies in the cell membrane that are enriched in sphingolipid, cholesterol, GPI-anchored proteins and palmitoylated proteins (63, 64). When palmitoylation is blocked by either mutagenesis or inhibition of PATs, the unmodified proteins no longer localize to lipid rafts, implicating the palmitoyl modification as an important localization determinant (65–69). Localization to lipid rafts is especially important for signaling proteins. The best-studied examples are nonreceptor tyrosine kinases (NRTKs) and linker for activation of T cells (LAT) which are involved in T cell signaling events; blocking palmitoylation in these proteins by mutation of the palmitoylated cysteine residue disrupts the lipid raft structure, and leads to attenuated T-cell responses (70–72). These studies assert the important role that palmitoylation plays in localizing proteins to functional membrane subdomains, such as lipid rafts.

3 DHHC Protein Family and Palmitoyltransferases (PATs)

Protein thiols can be palmitoylated nonenzymatically *in vitro* upon incubation with palmitoyl-CoA within a few hours at neutral pH (73). However, in a cell protein palmitoylation occurs much more rapidly and is catalyzed by palmitoylating enzymes, namely, protein palmitoyltransferases (PATs) (47). PATs catalyze the thioesterification of

cysteines in the substrate protein with a palmitoyl group using palmitoyl-CoA as a co-substrate. One of the first identified PATs was the *Saccharomyces cerevisiae* Ras palmitoyltransferase Erf2p/Erf4p (effectors of Ras function) (74). After the CaaX sequence in Ras is farnesylated to enhance localization to membranes, the Erf2p/Erf4p PAT catalyzes palmitoylation of an upstream cysteine to further stabilize membrane interactions. The two proteins in the Erf2p/Erf4p complex co-purify and are both required for Ras palmitoylation activity. Deletion of either the *ERF2* or *ERF4* gene in yeast leads to a decrease in palmitoylation and mislocalization of Ras in the cell (36). A second palmitoyltransferase, Akr1p (ankyrin-repeat-containing protein 1), was identified in yeast by phenotypic analysis (75). This enzyme catalyzes palmitoylation of Yck2p (yeast casein kinase 2) both *in vivo* and *in vitro*, as well as other proteins (76). In the *AKR1*Δ strain, Yck2p localizes diffusely in the cytosol rather than associating with the membrane (77).

Both Erf2p and Akr1p are integral membrane proteins that contain several transmembrane domains (TMDs), and share a common DHH/YC-CRD (Asp-His-His/Tyr-Cys-cysteine rich domain) positioned between two TMDs (47, 78). The majority of PATs contain a consensus DHHC sequence and a conserved cysteine-rich domain (CRD), although Akr1p contains a DHYC sequence and an attenuated CRD with fewer cysteines conserved (47). For both PATs, mutations in the DHH(Y)C motif abolish the palmitoyltransferase activity (77, 79), indicating that this motif is important for palmitoylation activity.

Based on the prediction that the DHHC motif may be the active site of PATs, a search of the yeast genome for DHHC-containing proteins led to the discovery of five other putative PATs, including Akr2p, Swf1p, Pfa3p, Pfa4p, and Pfa5p (41, 76, 80, 81). All of these proteins catalyze palmitoylation with varying substrate recognition, confirming the importance of the DHHC-CRD sequence for PAT activity. Similarly, 23 mammalian DHHC proteins have been identified and most possess palmitoyltransferase activity (47). In addition, 15 genes from *Caenorhabditis elegans* and 22 genes from *Drosophila melanogaster* are also predicted to have PAT activity from their conserved DHHC sequence (82).

Among the 23 mammalian PATs, several are suggested to have biomedical relevance as their palmitoylated substrates are involved in the pathogenesis of neurological disorders, such as Huntington's disease (DHHC17) (83), schizophrenia (DHHC8) (84), and X-linked mental retardation (DHHC9 and DHHC15) (85). One of the most notable examples is DHHC17, also known as HIP14 (huntingtin interacting protein 14) (83, 86, 87), which catalyzes the palmitoylation of several neuronal proteins, including huntingtin, PSD-95 (56, 57, 88, 89), and SNAP-25 (60). Furthermore, HIP14 activity regulates the subcellular trafficking and function of huntingtin, leading to the suggestion that it is involved in the pathogenesis of Huntington's disease (87). Consistent with this, studies have indicated that the N-terminal polyglutamine (polyQ) expansion of the huntingtin protein prevents interaction with HIP14, and therefore leads to a decrease in palmitoylation and enhanced aggregation (87). Further studies on the palmitoylation mechanism of HIP14 as well as other DHHC proteins may disclose their exact pathophysiological roles in neurological disorders (90).

4 Akr1p as a Protein Palmitoyltransferase

Akr1p, the yeast homologue of HIP14, is an 86-kDa integral membrane protein with six transmembrane domains. The N-terminal hydrophilic domain contains six ankyrin repeat sequences. The topology of Akr1p in the membrane indicates that the conserved DHHC-CRD sequence is located on the cytosolic side of the membrane between transmembrane domains four and five (91). A scheme of the Akr1p domains and topology is shown in Figure 2.

Davis and colleagues have carried out multiple studies demonstrating that Akr1p is a palmitoyltransferase that catalyzes the palmitoylation of yeast casein kinase (Yck2p) both *in vivo* and *in vitro* (77). In wild-type (*AKR1*$^{+}$) yeast cells Yck2p is exclusively trafficked to the cell membrane, whereas in *akr1*Δ cells Yck2p is diffusely localized in the cytoplasm (77). This finding is consistent with palmitoylation playing an important role in tethering proteins to the membrane. In addition, the C-terminus of Yck2p contains two cysteines that are hypothesized as the palmitoylation site(s). Consistent with this, mutation of the two C-terminal cysteine residues (-CC) to serine (-SS), leads to the relocalization of Yck2p from the membrane to the cytoplasm, as observed for WT Yck2p in *akr1*Δ cells (77). Furthermore, the DHYC motif of Akr1p is important for catalytic activity, as also observed for Erf2p (79), mutation of DH to AA, or C to A in Akr1p disables catalysis of Yck2p palmitoylation both *in vivo* and *in vitro* (77).

To search for palmitoylated proteins in yeast, the Davis lab carried out a global analysis of yeast membrane proteins using MudPIT (multi-dimensional protein identification technology) tandem-MS-based proteomic methodology coupled with methods to enrich palmitoylated proteins (76). Forty-seven palmitoylated proteins, including Akr1p, were identified by MS/MS data. To identify the substrate proteins that are palmitoylated by each of the DHHC-containing palmitoyltransferases, this proteomic method was applied to mutant yeast strains deficient in one of more DHHC proteins. Decreased representation in the MudPIT analysis of proteins isolated from the *akr1*Δ strain led to the identification of six yeast proteins as potential Akr1p substrates: Yck1p, Yck2p, Akr1p, Ypl199c, Ykl047w, and Meh1p.

Akr1p is palmitoylated upon incubation with palmitoyl-CoA, which is known as auto-palmitoylation (77). Interestingly, this property is shared with many other DHHC protein palmitoyltransferases, including Erf2p (79). In Akr1p alanine mutations for DH or C in the conserved DHYC sequence abolish both the auto-palmitoylation and transpalmitoylation activity both *in vivo* and *in vitro* (77). Furthermore, mutations in this conserved motif in other PATs, including C203S in Erf2p (79) and C468S in HIP14 (83), also abolish both palmitoylation activities. These data led to the hypothesis that auto-palmitoylation is enzyme-catalyzed and, further, that the auto-palmitoylated enzyme could be a covalent intermediate that occurs during the catalytic cycle. This palmitoylated intermediate is cleaved upon addition of hydroxylamine, suggesting a thioester linkage (75). In this proposed mechanism (Figure 3), a cysteine thiol at the active site of the PAT reacts with palmitoyl-CoA to form a covalent palmitoyl thioester enzyme intermediate. In a second step, the palmitoyl group is transferred from the enzyme to the substrate protein. Support for this

mechanism is provided by kinetic measurements of the Erf2p/Erf4p PAT that show a “burst” of CoA formation, consistent with the formation of a reaction intermediate (92). In comparison to the activation of thiol nucleophiles in thiol proteases (93) and N-acetyltransferases (94–96), the conserved Asp and/or His in the DHHC domain may function to stabilize a nucleophilic thiolate anion in both steps of the reaction.

5 Methods Used for Studying PATs

Palmitoyltransferases are integral membrane proteins that contain several transmembrane domains. Therefore these proteins are difficult to overexpress and purify in large quantities, presenting a significant challenge to the characterization of PATs. Recently methods have been developed to enhance the expression, purification, and characterization of yeast PATs which are reviewed in the next section.

5.1 Expression and purification of yeast PATs

The largest quantities of active yeast PATs have been obtained using galactose inducible yeast expression systems. No high recombinant expression of a PAT in *E. coli* has yet been described, although small amounts of active HIP14 (DHHC17) (97) and Erf2p/Erf4p (79) were observed. This difficulty is likely due to both the problems associated with expression of eukaryotic membrane proteins in bacteria and the lack of necessary posttranslational modifications.

Deschenes and colleagues have optimized the expression of yeast PATs, including Erf2p, Akr1p, Pfa3p, Pfa4p, and Pfa5p (98) while the Davis lab has developed methods to overexpress Akr1p (77). The FLAG-tagged and/or His-tagged versions of these protein genes encoded on a yeast plasmid were transformed into yeast strain, and the cells were grown on selective media. The culture was then induced by addition of galactose, harvested by centrifugation, lysed by vigorous vortexing with glass beads and the membrane fractions were collected using ultracentifugation. The PAT activity in these membrane fractions was sufficient to catalyze palmitoylation of their corresponding protein substrates.

The Erf2p/Erf4p purification method serves as the prototypical method for other PATs. The first step is solubilization of the membrane fractions by addition of 0.75% (w/v) detergent. Dodecylmaltoside (DDM), deoxycholic acid (DCA), and Triton X-100 were all efficient at solubilizing the PAT Erf2p/Erf4p; however, DDM was most effective in maintaining the palmitoylation activity (98). Following protein solubilization, His_6-Erf2p/Flag-Erf4p was then purified using nickel affinity and anion exchange chromatography, with DDM present in all wash and elution buffers. This procedure provides purified active protein based on absorbance, SDS-PAGE analysis, and PAT activity assay.

5.2 Palmitoylation assays

To study catalysis of palmitoylation and obtain a better understanding of its biological significance, a robust palmitoylation assay is required. Several palmitoylation assays have been developed and used to study PATs; each assay has both advantages and disadvantages (99).

The first developed and most frequently used assay is the *in vivo* metabolic labeling of proteins using radiolabeled palmitate, most commonly [^{3}H]palmitate (57, 74, 100–102). The radiolabeled palmitate is added to the cell culture during growth, where the palmitoyl moiety is incorporated onto target proteins. The labeling of proteins is evaluated by fractionation of the proteins by SDS-PAGE of the cell lysate followed by exposing the dried gel to high-sensitivity films. This method provides an efficient way to identify palmitoylated proteins and to assess the palmitoylation level of a certain protein in living cells. In a pulse-chase format this assay can also be used to measure both the palmitoylation and depalmitoylation rates. Disadvantages of this method are: low sensitivity requiring long hours of labeling and exposure time for detection of the radioactive signal and difficulty with accurately quantifying the result, since the *in vivo* labeling process is affected by many cellular factors. Therefore, this method is useful for the initial identification of PATs but is not generally suitable for further mechanistic characterization of the enzymes.

A similar assay labels palmitoylated proteins with ω-azido-fatty acids. In this method synthetic ω-azido-fatty acid is added to the cell culture during growth, so the target proteins are labeled with a palmitoyl group containing an azido group at the end of the fatty acid chain (103). Following *in vivo* labeling, proteins can be extracted and subjected to click chemistry (104) to add a detectable chemical probe, such as biotin or a fluorophore, to quantify the palmitoylated proteins. This method is both safer and more sensitive than labeling with radiolabeled palmitate; however, it requires multiple steps and presents challenges for quantification and measurement of the time-dependence of the reactions.

Another commonly used assay is the *in vitro* palmitoylation assay, in which PATs catalyze palmitoylation of a substrate (either whole proteins or peptides mimicking the protein palmitoylation motif) upon addition of exogenous palmitoyl-CoA (105). When using peptides as substrates, fluorescent labeling enhances the sensitivity of the assay and the palmitoylated peptides can be separated from the non-palmitoylated peptides by high performance liquid chromatography (HPLC) (106). However, not all PATs react readily with peptide substrates. When using whole proteins as substrates, radiolabeled palmitoyl-CoA is used so that palmitoylation is detected by autoradiography after separation by SDS-PAGE. This method is more quantitative, requires less reaction handling for signal detection, and can be used to measure time-dependent reactions. This assay has also been applied in a cell-based assay format, in which intact cells are incubated with fluorescently-labeled peptides and endogenous palmitoyl-CoA (105). This assay has the advantage of speed and simplicity and can be used to study the intracellular trafficking of the palmitoylated peptides. The *in vitro* palmitoylation assay has been widely used to study the catalytic mechanism of PATs.

In a recent kinetic study of Erf2p/Erf4p, the Ras PAT, Deschenes and colleagues introduced a coupled fluorescence-based assay measuring NADH production to monitor the rate of CoASH production from palmitoyl-CoA in the palmitoylation reaction (92). This assay does not require using radioactive material, and can be performed in a 96-well plate making it useful for high-throughput inhibitor screening. However, this assay is not as sensitive as the radioactive assay, and therefore larger quantities of active enzyme and substrate protein are required to generate a detectable signal.

Besides the traditional biochemical assays, mass spectrometry has become an important tool for studying the posttranslational modification of proteins, including lipidation (102, 107, 108). In this case, the palmitoylated proteins are subjected to protease digestion and the resulting peptides are separated by chromatography. By comparing the sample spectrum with the control (no modification), the peptides with the palmitoyl group are identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) based on the 272 Da mass shift for the palmitoyl group (109). This method is not quantitative but is a versatile tool to identify palmitoylated proteins. However, this method has some limitations. For example, the thioester linkage could be cleaved during sample preparation, especially at high or low pH in the presence of exogenous thiols. In addition, peptides containing a hydrophobic fatty acyl chain, like palmitoyl, have low solubility, can stick to the separation column, and may have poor ionization efficiency. Based on these difficulties, this method was optimized by performing an additional step, called fatty acyl exchange chemistry, before mass spectral analysis to replace the labile thioester bond with a more stable thioether linkage (62, 76, 110–114). In this method first all of the unlabeled free cysteine thiols are blocked by reaction with N-ethylmaleimide (NEM) after reduction of disulfide bonds by incubation with tris(2-carboxyethyl)phosphine (TCEP). Next, the thioester bond with the palmitoyl group is cleaved by the addition of hydroxylamine, exposing the palmitoylated cysteine. Last, the exposed cysteine thiols are reacted with thiol-specific reagents with detectable functional groups, such as Btn-BMCC (1-biotinamido-4-[4′-(maleimidomethyl) cyclohexanecarboxamido] butane) and biotin-HPDP (N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionami de). Finally, the labeled peptide is purified and analyzed by mass spectrometry. This optimized method has been successfully used to identify palmitoylated proteins in several biological systems. For example, Davis and colleagues identified a host of palmitoylated proteins in yeast (76), and Freeman and colleagues identified palmitoylated proteins and their sites of modification in lipid raft-enriched and non-raft membranes of mammalian cells (112). This method can generate false positives, due to the non-specific nature of the reactions; therefore, careful controls and confirmation of the results using other biological tools are essential. Overall, this method has great advantages for discovering novel palmitoylated proteins but may not be suitable for detailed mechanistic studies of the enzymatic reactions.

To sum up, there are a number of assays that have been developed to study protein palmitoylation, with each having their own advantages and disadvantages. Using these assays, many novel palmitoylated proteins have been identified, some of which have been further mechanistically characterized. However, to obtain an in-depth picture of the palmitoylation mechanism, faster, more sensitive, and higher-throughput assays are desired.

6 Conclusions and Perspectives

This review summarized recent advances in the analysis of protein palmitoylation and PATs, from the roles of protein palmitoylation in nature and the discovery of the conserved residues in PATs, to the development of methods to characterize protein palmitoylation. To unravel the catalytic mechanism of PATs, a number of useful methods have been developed, including approaches for expressing and purifying PATs and assays for measuring palmitoylation activity both *in vitro* and *in vivo*. The discovery of the large DHHC protein

family has led to the prediction that the DHHC motif is the active site, providing insight into the catalytic mechanism of PATs.

Even though progress has been made toward understanding various aspects of protein palmitoylation, several critical issues still remain unclear, including the detailed catalytic mechanism of protein palmitoylation, substrate specificity of PATs, and the development of possible inhibitors. Furthermore, the current data suggest that protein palmitoylation proceeds with two steps: auto-palmitoylation of the enzyme followed by trans-palmitoylation of the substrate protein. However, mechanistic information about these steps remains to be obtained, including identification of the site of auto-palmitoylation in these enzymes. Although both trans- and auto-palmitoylation disappear when mutations are made in the DHHC domain of PATs, there is not yet any direct evidence that the DHHC cysteine is the site of auto-palmitoylation. Furthermore, even though a number of palmitoylated proteins have been discovered both *in vivo* and *in vitro*, there is no consensus signal for protein palmitoylation. Hence, analysis of the substrate specificity determinants of PATs will provide important information about molecular recognition of the enzymes, and will help produce reagents that will facilitate mechanistic biochemistry on PATs. These results will eventually lead to the development of robust high-throughput screens for PAT inhibitors and, potentially, specific PAT inhibitors that have the potential to be used to treat a variety of diseases.

Acknowledgments

This work was financially supported by the NIH R01 grant GM040602 (CAF).

List of Abbreviations

Biotin-HPDP	N-[6-(biotinamido)hexyl]-3′-(2′-pyridyl dithio)propionamide
Btn-BMCC	1-biotinamido-4-[4′-(maleimidomethyl) cyclohexanecarboxamido] butane
DCA	deoxycholic acid
DDM	dodecylmaltoside
DHHC-CRD	Asp-His-His-Cys cysteine rich domain
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
HIP14	huntingtin interacting protein 14
LAT	linker for activation of T cells
MudPIT	multi-dimensional protein identification technology
NEM	*N*-ethylmaleimide
NRTK	nonreceptor tyrosine kinase
PAT	palmitoyl acyltransferase
PPT	palmitoyl protein thioesterase

PSD-95	dendritic postsynaptic density protein 95
RGS	regulator of G protein signaling
SNAP-25	25 kDa synaptosome-associated protein
SNARE	soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor
TCEP	tris(2-carboxyethyl)phosphine
TMD	transmembrane domain

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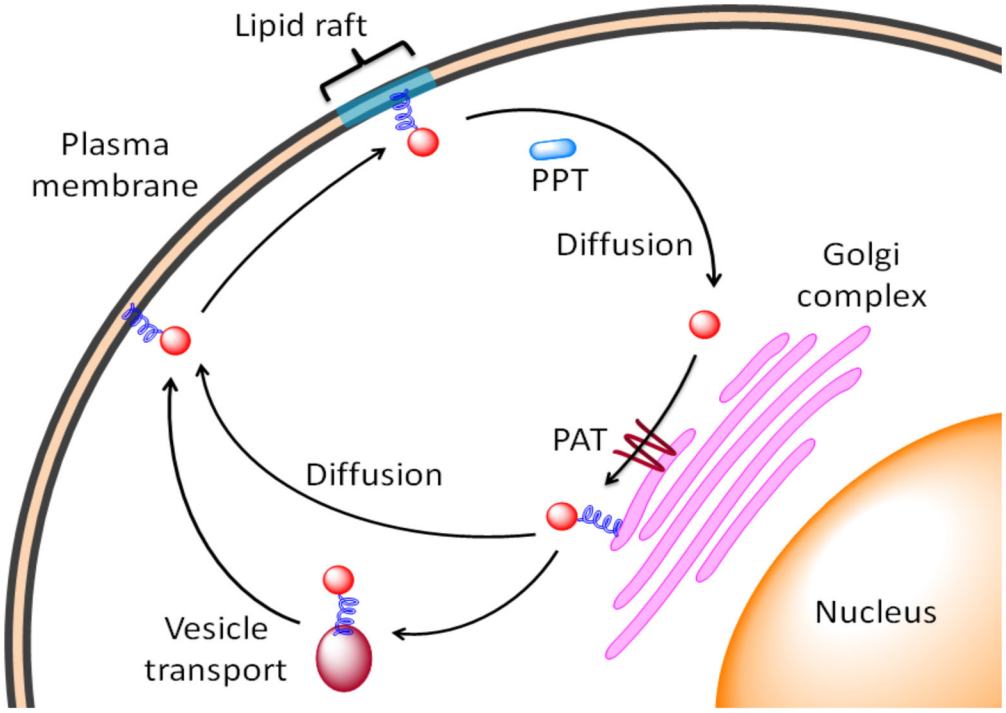


Figure 1. Inter-compartment protein shuttling through palmitoylation-depalmitoylation cycles

Palmitoylated proteins (shown as a red circle with an attached blue fatty acid chain) is trafficked to the plasma membrane though either diffusion or vesicle transport, and is then targeted to lipid rafts. Depalmitoylation is catalyzed by a putative palmitoyl protein thioesterase (PPT, shown as a blue oval), leading to a palmitoylated half-time estimated at 0.3 to 2 h. The depalmitoylated protein diffuses back to the Golgi complex where it can be palmitoylated for another cycle.

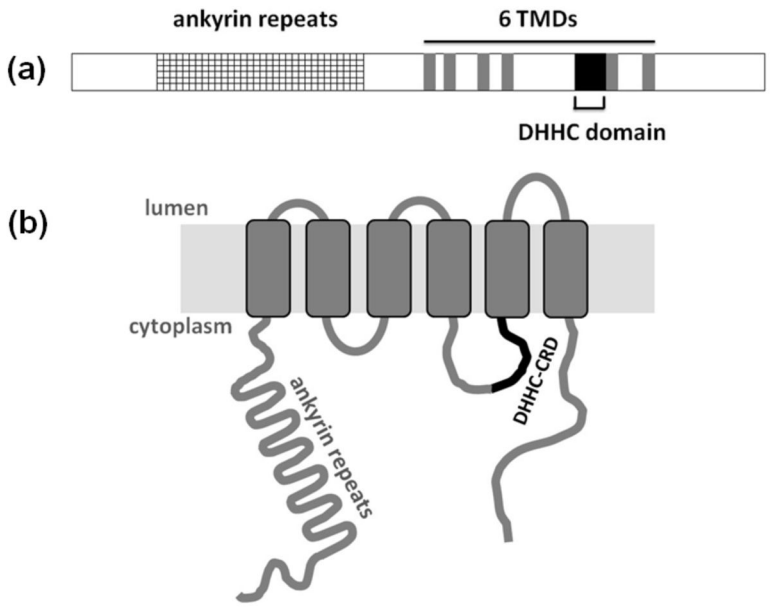


Figure 2.

(a) Schematic representation of the identified domains in the Akr1p sequence. Ankyrin repeats are shown in the grid region; the six transmembrane domains are indicated in grey and the DHHC domain is highlighted in black; **(b) Schematic representation of Akr1p membrane topology.** Both ankyrin repeats and the DHHC-cysteine rich domain (CRD) are positioned on the cytoplasmic side of the membrane.

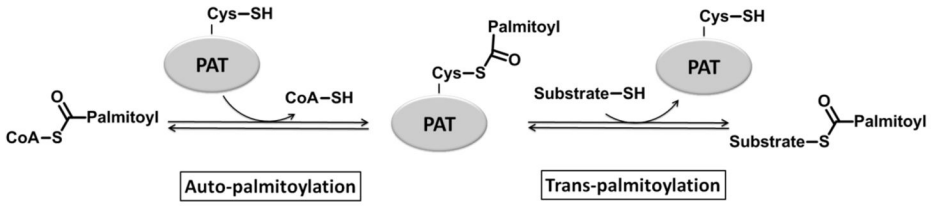


Figure 3. Scheme of the proposed two-step palmitoylation mechanism of PATs

A covalent palmitoyl thioester enzyme intermediate is formed in the first auto-palmitoylation step, followed by the transfer of the palmitoyl moiety from PAT to the substrate protein in the second trans-palmitoylation step.

Table 1

Structures, signals, and corresponding enzymes that catalyze three types of lipid modifications

Common recognition signals in substrate proteins for both prenylation and N-myristoylation have been identified, while a consensus signal for palmitoylation has not yet been defined. In the signal sequences of prenylation and N-myristoylation, “a” refers to any aliphatic amino acid, and “X” could be a subset of amino acids.

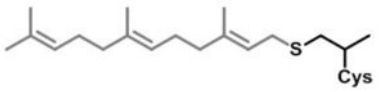
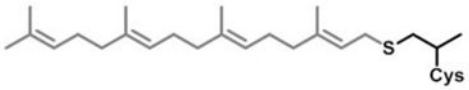
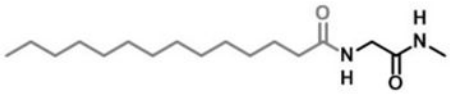
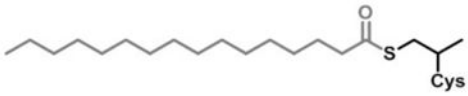
	Lipid Modifications	Signals	Enzymes
Prenylation		–CaaX	Farnesyltransferase Geranylgeranyltransferase 1
		–CC or –CXC (Rab proteins only)	Geranylgeranyltransferase II
N-myristoylation		MGxxS—	N-myristoyltransferase
Palmitoylation		Poorly defined	Palmitoyltransferase

Table 2

Four different types of substrate proteins that are palmitoylated

Although many palmitoylated proteins have been identified, the recognition signals in these proteins remain poorly defined. In addition to the palmitoyl modification, proteins may contain other membrane interactions, such as a transmembrane domain or a second lipid modification (prenyl or myristoyl group). Palmitoylation sites are identified with a *.

palmitoylation type	substrate proteins
palmitoylation + prenylation −**C**XC*aaX	Ras proteins, Rho Proteins, G_{γ} subunits of *S. cerevisiae*
palmitoylation + myristoylation MG**C***−	G_{α} subunits, Src family kinases (Yes, Fyn, Lck)
proximal to TMDs	GPCRs, viral-envelope proteins (HIV, influenza)
palmitoylation alone	G_{α} subunits, GRKs, RGS, SNAP-25, PSD-95