

# Protein palmitoylation and dynamic modulation of protein function

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**The function of palmitoylation will depend on the protein that is being considered. Palmitoylation increases the hydrophobicity of proteins and contributes to their membrane association. Progress has been made in our understanding of protein S-palmitoylation as one of a repertoire of dynamic post-translational protein modifications that can control protein trafficking, localization, partitioning into domains, protein–protein interactions and functions. Some light has also been shed on the enzymology of palmitoylation. However, much needs to be learnt about the sequence motifs specific for enzymatic and non-enzymatic palmitoylation and on the battery of proteins that are likely to mediate enzymatic palmitoylation–depalmitoylation cycles. With the development of knowledge, inhibitors of palmitoylation may find use as drugs in the foreseeable future. The purpose of this review will be to overview some of the recent advances in our understanding of the mechanisms and function of protein S-palmitoylation.**

THE covalent attachment of lipid moieties to proteins takes place in many forms best studied in eukaryotic and viral systems. The well recognized forms are: the co-translational amino-terminal myristoylation of cytosolic proteins; the modification of plasma membrane proteins with glycosylphosphatidyl inositol, the carboxyterminal isoprenylation of cytoplasmic proteins and the post-translational addition of palmitic acid to many integral and peripheral membrane proteins<sup>1–3</sup>. S-palmitoylation is the reversible addition of palmitate to proteins on cysteine residues via a thioester linkage. N-palmitoylation is the addition of palmitate in amide linkage to the N-terminal cysteine residues of proteins. It was first reported for the secreted signaling protein sonic hedgehog.

Proteins that are S-palmitoylated can be broadly classified into four groups (Figure 1). One group of transmembrane proteins is palmitoylated on cysteine residues located in close proximity to transmembrane domains. This group is exemplified by a large number of G-protein coupled receptors such as the V2 vasopressin receptor (V2R). The second group of proteins is first myristoylated at an N-terminal glycine residue<sup>4</sup> as in the case of endothelial nitric oxide synthase (e-NOS), certain Src-family tyrosine kinases and certain  $G_{\alpha}$  subunits. A third group of proteins

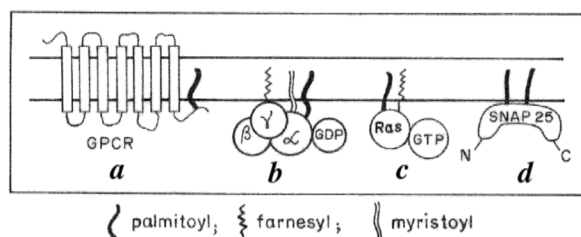
including H-Ras and N-Ras is first farnesylated at a C-terminal CAAX box and then modified at a nearby cysteine residue<sup>5</sup>. Finally, the last group includes peripheral membrane proteins modified with palmitate only, such as the neuronal proteins SNAP25 and GAP43.

The function of palmitoylation will depend on the protein that is being considered. Palmitoylation increases the hydrophobicity of proteins and contributes to their membrane association. Palmitoylation also appears to play an important role in subcellular trafficking of proteins between membrane compartments, as well as in modulating protein–protein interactions<sup>1,2,4,6–8</sup>. The purpose of this review will be to overview some of the recent advances in our understanding of the mechanisms and function of protein S-palmitoylation.

## S-palmitoylation in localization, targeting and trafficking of proteins

In cytosolic proteins exemplified by a group of non-receptor tyrosine kinases and  $G_{\alpha}$  subunits of heterotrimeric G proteins, palmitate is found attached close to myristic acid. Newly synthesized Src family kinases and  $G_{\alpha}$  subunits are co-translationally myristoylated, but they do not stably associate with membranes until palmitoylation has occurred<sup>9–12</sup>. Such dually acylated proteins often have positively charged amino acids around the palmitoylation sites.

Appending N-terminal targeting sequences to green fluorescent protein (GFP), it has been demonstrated that



**Figure 1.** Different classes of palmitoylated proteins. *a*, G-protein coupled receptors (GPCRs) are palmitoylated on cysteine residues close to their transmembrane domains. *b*,  $G_{\alpha}$  subunits are myristoylated at an N-terminal glycine residue and also palmitoylated near their N-terminus. *c*, The Ras family of proteins is farnesylated at a C-terminal CAAX box and also modified near the C-terminus. *d*, Peripheral membrane proteins such as SNAP25 are modified with palmitate only.

in addition to myristoylation, palmitoylation or the presence of a polybasic domain is required for plasma membrane targeting<sup>13</sup>. The precise position of the palmitate can also influence distribution: in Lck, mutation of Cys5 but not Cys3 results in accumulation of the protein in the Golgi region of transfected NIH-3T3 cells<sup>14</sup>.

Ras proteins are prenylated at their C-termini and several, including H-Ras and N-Ras, are also palmitoylated. Palmitoylated H-Ras and N-Ras are associated to some extent with the Golgi apparatus. Brefeldin A (BFA) treatment causes these Ras proteins to accumulate on intracellular membranes without reducing palmitate incorporation<sup>15,16</sup>. By contrast, the nonpalmitoylated K-Ras is not found in the Golgi region. Its transport is unaffected by BFA. Palmitoylation of H-Ras and N-Ras therefore determines Golgi targeting and transport to the plasma membrane. The recent knowledge of palmitoylation in relation to protein transport is summarized in Figure 2.

The targeting of proteins to axons and dendrites in neurons is influenced by changes around protein palmitoylation sites. Post-synaptic density protein 95 (PSD-95) normally localizes to dendrites, but deleting the amino acids between the palmitoylated cysteines allows transport into axons as well<sup>17</sup>.

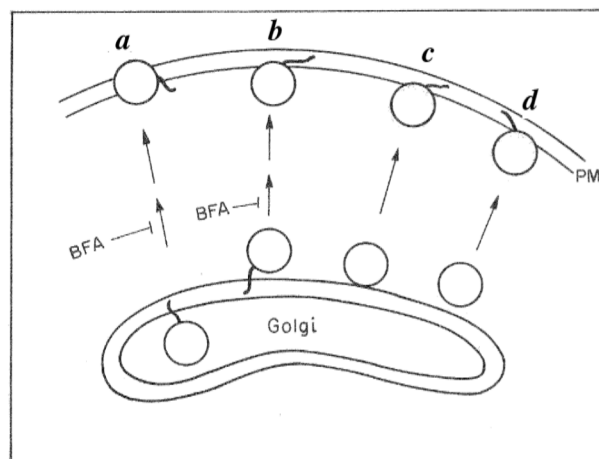
Palmitoylation of signaling proteins has, in several instances, been linked to localization of these proteins into cholesterol and sphingolipid-enriched domains as in the case of Src family kinases and some  $G_{\alpha}$  subunits<sup>2</sup>. It has been suggested that signal transduction could be regulated, in part, by sequestering signaling proteins into different plasma membrane domains until they are brought

together by an activating signal. Engineered forms of Lck that are attached to membranes through a transmembrane domain (TMD), rather than through acylation, show reduced association with detergent-resistant membrane fractions and reduced signaling activity<sup>18</sup>. Similarly, mutation of the palmitoylation sites on LAT, a transmembrane adaptor protein that is essential for TCR signaling, abrogates both raft localization and T-cell activation<sup>19</sup>. The TCR co-receptors CD4 and D8 are palmitoylated and associate with rafts<sup>20-22</sup>. Palmitoylation and raft localization of the Src family kinase Lyn is required for Fc epsilon RI signaling<sup>23,24</sup>.

Palmitoylation has a role in trafficking<sup>25,26</sup>. Mutants of carboxypeptidase D that lack palmitoylation sites have an increased half-life and a slower rate of exit from the Golgi<sup>27</sup>. Palmitoylation facilitates transport of the newly synthesized chemokine receptor CCR5 to the plasma membrane. Non-acylated CCR5 that does reach the cell surface is compromised in its ability to couple to signaling pathways activated by chemokine agonists and in endocytosis through clathrin-coated vesicles<sup>28,29</sup>. Palmitoylation of a cysteine-rich sequence CCCPC of the  $Ca^{2+}$ -dependent phospholipid scramblase is necessary for its trafficking to the plasma membrane<sup>30</sup>. The transport of two neuronal palmitoylated proteins, SNAP25 and GAP43, has been studied using chimeras of green fluorescent protein (GFP) in living cells<sup>31</sup>. SNAP25, (a t-SNARE) functions in the fusion and exocytosis of secretory vesicles. It is palmitoylated on cysteines located in its central domain<sup>32</sup>. GAP43 is palmitoylated at its N-terminus<sup>33</sup>. The transport of both these proteins from their site of accumulation at the *trans*-Golgi network to the plasma membrane is necessary for palmitoylation. BFA inhibits palmitoylation of both proteins underlining the requirement of functional Golgi membrane-dependent trafficking for palmitoylation<sup>34</sup>. The palmitoylation of these proteins thus requires functional Golgi membranes either to deliver the proteins to a specific location or, perhaps, to facilitate the reaction itself. On the other hand, the ER to plasma membrane trafficking of the Ras2p protein of *Saccharomyces cerevisiae* occurs in a non-classical palmitoylation-dependent manner<sup>35</sup>.

### Palmitoylation in protein function

Activation of G-protein-coupled receptors by peptides and hormones catalyses the exchange of GDP with GTP on the  $\alpha$ -subunit of its associated heterotrimeric G protein. The active, GTP-bound form of the  $\alpha$ -subunit interacts with effectors, initiating a signaling cascade. Deactivation of this signaling pathway is mediated by the intrinsic GTPase activity of  $\alpha$ -subunits, which is accelerated by cognate GTPase activating proteins (GAPs) and the regulators of G-protein signaling (RGS proteins). The role of internal palmitoylation in RGS16 localization and GAP activity has been analysed. Enzymatic palmitoylation of RGS16 results in internal palmitoylation on residue Cys-98. Mutation of RGS16 Cys-98 to alanine reduces GAP activity on the 5-



**Figure 2.** Trafficking of palmitoylated proteins. *a*, Some transmembrane proteins are palmitoylated at the Golgi and follow the normal route through the exocytic pathway to the plasma membrane (PM). Transport is blocked by brefeldin A (BFA). *b*, Cytosolic proteins such as Ras are palmitoylated at the Golgi membrane and then transported to the plasma membrane. Transport is blocked by BFA. *c*, Palmitoylation of proteins such as  $G_{\alpha}$  occurs at the plasma membrane after transport from the Golgi. *d*, Cytosolic proteins such as Fyn are palmitoylated at the plasma membrane without the involvement of Golgi membrane trafficking. Pathways *c* and *d* are not inhibited by BFA.

HT<sub>1A</sub>/G<sub>αo1</sub> fusion protein. Palmitoylation of a Cys residue in the RGS box is critical for RGS16 and RGS4 GAP activity and their ability to regulate G<sub>i</sub>-coupled signaling in mammalian cells<sup>36</sup>. The amino-terminal palmitoylation of an RGS protein also promotes its lipid raft targeting that allows palmitoylation of a poorly accessible cysteine residue<sup>37</sup>.

The shape and mechanical stability of the erythrocyte membrane is maintained by proteins constituting a filamentous scaffold, the membrane cytoskeleton, underlying the lipid bilayer. The human erythrocyte has been the best studied model for understanding the molecular mechanisms governing maintenance of membrane deformability and stability. The cytoskeleton is composed mainly of spectrin tetramers held together at their junctions by short actin filaments. The linkage of spectrin to the bilayer is mediated by interactions of ankyrin with β spectrin and the cytoplasmic domain of band 3. In human erythrocytes, band 3 further associates with another 72000 *M<sub>r</sub>* peripheral membrane protein, namely band 4.2. Human erythrocyte protein 4.2 is a major protein in the membrane skeletal network that associates with the cytoplasmic domain of the anion exchanger, band 3. The site of palmitoylation of protein 4.2 has been mapped in our laboratory to cysteine 203. Using recombinant derivatives of protein 4.2 it has been demonstrated that the palmitoylatable cysteine residue resides within a 22-residue domain of the protein which is crucial for its binding to band 3 (ref. 38). Band 3-binding of protein 4.2 has been shown to be modulated by palmitoylation, providing the first evidence of protein palmitoylation as a potential modulator of membrane-cytoskeleton interactions.

The role of palmitoylation in protein-protein interaction is also supported by the report that oligomerization of PSD-95 requires palmitoylation of two cysteine residues within its N-terminal domain. Disrupting palmitoylation disrupts PSD-95/K<sup>+</sup> channel clusters<sup>39</sup>.

The best evidence of S-palmitoylation as a regulator of enzyme activity is the case of the mitochondrial methylmalonyl semialdehyde dehydrogenase (MMSDH). It is acylated by an <sup>125</sup>I-labeled analogue of myristoyl-CoA on an active site cysteine, resulting in enzyme inhibition<sup>40</sup>. This observation together with evidence that palmitoyl-CoA inhibits the activity of several mitochondrial enzymes suggest a regulatory role of S-acylation in metabolism<sup>41-44</sup>. In the case of carbamoyl-phosphate synthetase I, Corvi *et al.*<sup>44</sup> have presented evidence that active site S-palmitoylation occurs spontaneously at physiological concentrations of palmitoyl-CoA. Inhibition of CPSI by long chain fatty acyl-CoAs might serve to reduce the extent of amino acid degradation during starvation.

### Palmitoylation motifs

Palmitoylation motifs are poorly characterized till date. In the case of transmembrane proteins, palmitoylation

occurs either close to the transmembrane domain (TMD)/cytoplasmic domain (CD) boundary, or is located in the CD. In the case of the GPCRs there is a greater proportion of hydrophobic basis residues in the vicinity of the palmitoylated cysteine(s). Systematic substitution of the amino acids flanking the palmitoylated cysteine of peptides derived from the β<sub>2</sub>-adrenergic receptor sequence has demonstrated that basic and hydrophobic amino acids next to the palmitoylated cysteine play a crucial role at least in an *in vitro* acylation process<sup>45</sup>. These residues could presumably favour peptide interactions with the CoA polar head and the acyl chain of the palmitoyl-CoA. These results suggest a possible requirement for specificity in the amino acid sequence around the palmitoylation sites. However, this is not the case for all GPCRs. For the α<sub>2</sub>-AR, the deletion of positively charged residues from the CD has no effect on palmitoylation<sup>46</sup>.

Most palmitoylated cysteines are found within ten residues on either side of the TMD/CD boundary. However, the acylation of CD cysteines that are further from a TMD also occurs in several proteins. Exemplary of this is the cation-independent mannose 6-phosphate receptor (MPR) in which palmitoylation occurs 34 residues from the TMD<sup>47</sup> and the envelope (Env) protein of human immunodeficiency virus<sup>48</sup>.

In cytosolic proteins, palmitate is attached either close to N-terminal myristoyl or C-terminal prenyl groups. Src family kinases and G<sub>α</sub> subunits are co-translationally myristoylated, but can stably associate with membranes only when palmitoylated<sup>9-12</sup>. Such dually acylated proteins often have positively charged amino acid residues around the palmitoylation sites which may be necessary to enhance membrane binding<sup>49</sup>. Hydrophobic residues that neighbour a cysteine can influence palmitoylation in some cytosolic proteins such as eNOS (ref. 50).

The importance of hydrophobic residues around the sites of palmitoylation is also exemplified by the scaffolding protein PSD-95 (refs 51-53).

### Mechanisms of protein palmitoylation

Our understanding of dynamic protein palmitoylation as a cellular control mechanism has been limited by the lack of detailed knowledge about the enzymology of palmitoylation, and by the lack of understanding of principles distinguishing enzymatic versus spontaneous S-acylation. Several recent reports have described novel palmitoyltransferases<sup>54-58</sup>. Protein palmitoyl acyltransferase (PAT) activity has been found in plasma membranes, Golgi and mitochondrial membranes<sup>59</sup>. PAT activity has also been found to be enriched in sphingomyelin and cholesterol rich membrane microdomains.

Our search for the PAT from human erythrocytes was based on our own observations and that of other laboratories that protein-bound palmitate associated with erythrocyte membrane proteins turns over<sup>60,61</sup>. Moreover, palmito-

ylating activity has been demonstrated in human erythrocyte ghosts<sup>62</sup>. The PAT from human erythrocytes was the first enzyme of its class to be purified<sup>63</sup>. Whether the plasma membrane-associated PAT from erythrocytes is identical to, or different from PAT activities associated with other membranes<sup>64,65</sup> needs to be evaluated.

Very recently, a protein complex comprising two proteins Erf2p and Erf4p has been identified as a Ras palmitoyltransferase in yeast<sup>66</sup>. Erf2p is a 41 kDa protein localized to the endoplasmic reticulum and containing a conserved DHHC cysteine-rich domain. Erf4p is necessary for stable expression or solubilization of Erf2p from yeast cells, suggesting that it may act as a chaperone for Erf2p. Erf2p/Erf4p carries out palmitoylation preferentially on Ras substrates. The second DHHC cysteine-rich domain protein to be identified as a palmitoyltransferase is Arlp which palmitoylates the casein kinase Yck2p *in vitro*<sup>67</sup>. The protein has six predicted transmembrane domains and localizes in the Golgi. Many more PAT activities may exist.

Skinny hedgehog is a typical *Drosophila* palmitoylating enzyme which palmitoylates Sonic hedgehog, a *Drosophila* protein attached to the outer leaflet of the plasma membrane. However, it does this through an amide-linked palmitoyl moiety<sup>68</sup>.

The functions of palmitoylation are diverse. Palmitoylation increases the hydrophobicity of proteins or protein domains and contributes to their membrane association. Palmitoylation may modulate protein-protein interactions and also subcellular trafficking of proteins between membrane organelles and within microdomains of the same membrane compartments. In all likelihood distinct palmitoyltransferases recognize distinct palmitoylation motifs in different classes of proteins. Given the diverse nature of palmitoylated proteins, it would not be surprising if there were multiple protein acyltransferases. Two protein palmitoylthioesterases, one a lysosomal hydrolase (PPT1) and the other a cytoplasmic enzyme (APT1), have been identified and characterized. In both cases, crystal structures have been determined, providing insight into the mechanism of the thioesterase reaction. However, there are likely to be other palmitoyl thioesterases at other subcellular locations, controlling cycles of palmitoylation and depalmitoylation.

The cellular site of palmitoylation has not been extensively investigated. Palmitoylation of vesicular stomatitis virus glycoprotein (VSV-G) and sindbis virus glycoprotein E1 occurs early in the exocytic pathway. When cells are incubated at 15°C, these proteins are not transported from the ER and palmitoylation is blocked. Restoration of transport leads to palmitoylation before aspartate-linked oligosaccharides are trimmed, which implicates the *cis*-Golgi as a possible site of palmitoylation<sup>69</sup>.

In the absence of cellular factors, palmitoyl-CoA is capable of spontaneously S-acylating cysteinyl thiols of several proteins. This can occur in the context of short

peptides as well as folded proteins. For example, peptides derived from palmitoylated proteins such as myristoyl-GCG, myristoyl-GCV, and IRYCWLRR undergo spontaneous S-acylation in the presence of palmitoyl-CoA and large unilamellar vesicles<sup>70,71</sup>. Under similar conditions, rhodopsin undergoes spontaneous S-acylation with a  $K_m$  of approximately 40  $\mu$ M<sup>72</sup>. Interestingly, the efficiency of spontaneous palmitoylation varies. A peptide derived from myelin P0 glycoprotein (RYCWLRR) is efficiently acylated using palmitoyl-CoA<sup>71</sup>.

Autoacylation of  $G_{i\alpha 1}$  has been shown to be influenced by the position of basic amino acids which may create a favourable environment for thiol anion formation<sup>73,74</sup>. Autoacylation can also be influenced by the presence of an associated subunit as is seen in the case of the  $G_{\beta\gamma}$  subunit enhancing the spontaneous palmitoylation of  $G_{i\alpha 1}$ <sup>73</sup>. Attempts to autoacylate other known palmitoylated proteins *in vitro*, including SNAP-25, GAP-43, and Fyn kinase, have not met with success.

### Palmitate turnover

For several palmitoylated proteins, the half life of the palmitate moieties is significantly shorter than that of the proteins, indicating that the complex goes through cycles of depalmitoylation and repalmitoylation. The reversibility of palmitoylation suggests that it is a regulated modification much like protein phosphorylation. Given that palmitoylation provides a mechanism for binding cytosolic proteins to membranes, or for segregating proteins to microdomains, or for mediating protein-protein interaction, depalmitoylation and repalmitoylation could provide a mechanism to regulate these processes. For proteins involved in signal transduction, these cycles could be induced by activation and, by controlling access to specific substrates, could regulate signaling. Palmitate cycling on PSD-95 has been proposed to modulate synaptic strength by controlling the postsynaptic density of DL- $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. As an example, palmitoylation allows PSD-95 to cluster in the postsynaptic membrane. Blocking PSD-95 palmitoylation leads to a loss of AMPA receptors from these domains, and the rapid endocytosis of AMPA receptors requires depalmitoylation of PSD-95 (ref. 7). Agonist-induced increase in palmitate turnover has been observed for  $\beta_2$ -AR,  $G_{\alpha s}$  and e-NOS<sup>75-77</sup>.

### Concluding remarks

In summary, progress has been made in our understanding of protein S-palmitoylation as one of a repertoire of dynamic post-translational protein modifications that can control protein trafficking, localization, partitioning into domains, protein-protein interactions and functions. Some light has also been shed on the enzymology of palmitoy-

lation. However, much needs to be learnt about the sequence motifs specific for enzymatic and non-enzymatic palmitoylation and on the battery of proteins that are likely to mediate enzymatic palmitoylation-depalmitoylation cycles. With the development of knowledge, inhibitors of palmitoylation may find use as drugs in the foreseeable future.

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## MEETINGS/SYMPOSIA/SEMINARS

### SERC Winter School on Geological Mapping of Sedimentary Terrain in Cuddapah Basin, Kurnool area, Andhra Pradesh

Date: January 2005  
Place: Hyderabad

The course is for a period of four weeks. It will essentially be a field training with relevant lectures and practical exercises. The course is open to research scholars, lecturers from universities, colleges and professional geologists from Central and State organizations and institutions who have interest in geological mapping of sedimentary terrains.

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### Workshop on Molecular Modelling and Pharmainformatics

Date: 1–5 November 2004  
Place: S.A.S Nagar

Topics include: Molecular modelling, Energy minimization, Conformational analysis, Molecular docking, 3D QSAR, Bioinformatics, Chemoinformatics, Pharmainformatics, etc.

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