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.

S-palmitoylation in localization, targeting and trafficking of proteins

In cytosolic proteins exemplified by a group of non-receptor tyrosine kinases and Gα subunits of heterotrimeric G proteins, palmitate is found attached close to myristic acid. Newly synthesized Src family kinases and Gα subunits are co-translationally myristoylated, but they do not stably associate with membranes until palmitoylation has occurred. 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...
in addition to myristoylation, palmitoylation or the presence of a polybasic domain is required for plasma membrane targeting\textsuperscript{13}. 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\textsuperscript{14}. 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\textsuperscript{15,16}. 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\textsuperscript{17}. 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\_a subunits\textsuperscript{2}. 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\textsuperscript{18}. 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\textsuperscript{19}. The TCR co-receptors CD4 and D8 are palmitoylated and associate with rafts\textsuperscript{20-22}. Palmitoylation and raft localization of the Src family kinase Lyn is required for Fc epsilon RI signaling\textsuperscript{23,24}.

Palmitoylation has a role in trafficking\textsuperscript{25,26}. Mutants of carboxypeptidase D that lack palmitoylation sites have an increased half-life and a slower rate of exit from the Golgi\textsuperscript{27}. 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\textsuperscript{28,29}. Palmitoylation of a cysteine-rich sequence CCCCPC of the Ca\textsuperscript{2+}-dependent phospholipid scramblase is necessary for its trafficking to the plasma membrane\textsuperscript{30}. The transport of two neuronal palmitoylated proteins, SNAP25 and GAP43, has been studied using chimeras of green fluorescent protein (GFP) in living cells\textsuperscript{31}. SNAP25, (a t-SNARE) functions in the fusion and exocytosis of secretory vesicles. It is palmitoylated on cysteines located in its central domain\textsuperscript{32}. GAP43 is palmitoylated at its N-terminus\textsuperscript{33}. 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\textsuperscript{34}. 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\textsuperscript{35}.

**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-
HT1a/Gα01 fusion protein. Palmitoylation of a Cys residue in the RGS box is critical for RGS16 and RGS4 GAP activity and their ability to regulate Gαi-coupled signaling in mammalian cells. The amino-terminal palmitoylation of an RGS protein also promotes its lipid raft targeting that allows palmitoylation of a poorly accessible cysteine residue.

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, 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+ channel clusters.

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 125I-labeled analogue of myristoyl-CoA on an active site cysteine, resulting in enzyme inhibition. This observation together with evidence that palmitoyl-CoA inhibits the activity of several mitochondrial enzymes suggest a regulatory role of S-acylation in metabolism. In the case of carbamoyl-phosphate synthetase I, Corvi et al. 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 β2-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. 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 α2–AR, the deletion of positively charged residues from the CD has no effect on palmitoylation.

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 and the envelope (Env) protein of human immunodeficiency virus.

In cytosolic proteins, palmitate is attached either close to N-terminal myristoyl or C-terminal prenyl groups. Src family kinases and Gα subunits are co-translationally myristoylated, but can stably associate with membranes only when palmitoylated. Such dual acylated proteins often have positively charged amino acid residues around the palmitoylation sites which may be necessary to enhance membrane binding. 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. Protein palmitoyl acyltransferase (PAT) activity has been found in plasma membranes, Golgi and mitochondrial membranes. 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. Moreover, palmito-
yling activity has been demonstrated in human erythro- 
cyte ghosts. The PAT from human erythrocytes was the 
first enzyme of its class to be purified. Whether the 
plasma membrane-associated PAT from erythrocytes is 
identical to, or different from, PAT activities associated 
with other membranes needs to be evaluated.

Very recently, a protein complex comprising two pro- 
teins Erf2p and Erf4p has been identified as a Ras palmi- 

toyltransferase in yeast. Erf2p is a 41 kDa protein 
localized to the endoplasmic reticulum and containing a 
conserved DHHC cysteine-rich domain. Erf4p is neces- 
sary 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 preferen- 
tially on Ras substrates. The second DHHC cysteine-rich 
domain protein to be identified as a palmitoyltransferase 
is Ar1p which palmitoylates the casein kinase Yck2p in vitro. The protein has six predicted transmembrane do- 

mains and localizes in the Golgi. Many more PAT activi- 
ties 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 mem- 

brane. However, it does this through an amide-linked pal- 

mitoyl moiety.

The functions of palmitoylation are diverse. Palmitoyla- 
tion 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 mem- 
brane organelles and within microdomains of the same 
membrane compartments. In all likelihood distinct palmi-

toyltransferases 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 (APTI), have been 

identified and characterized. In both cases, crystal struc-

tures have been determined, providing insight into the 

mechanism of the thioesterase reaction. However, there 
are likely to be other palmitoyl thioesterases at other sub-

cellular locations, controlling cycles of palmitoylation and 
depalmitoylation.

The cellular site of palmitoylation has not been exten- 
sively investigated. Palmitoylation of vesicular stomatitis 

virus glycoprotein (VSV-G) and sindbis virus glycopro- 
tein 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.

In the absence of cellular factors, palmitoyl-CoA is capa-

ble of spontaneously S-acylating cysteinyl thiols of sev-

eral 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 sponta-

neous S-acylation in the presence of palmitoyl-CoA and 
large unilamellar vesicles. Under similar conditions, 
rhodopsin undergoes spontaneous S-acylation with a Kₘ 
of approximately 40 μM. Interestingly, the efficiency of 
skeletal muscle myosin heavy chain and depalmitoylation 

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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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