What does S-palmitoylation do to membrane proteins?

Blaskovic, Sanja; Blanc, Mathieu; van der Goot, F Gisou

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<td>Blaskovic, Sanja; EPFL Lausanne, Inst. of Global Health Blanc, Mathieu; EPFL Lausanne, Inst. of Global Health van der Goot, François Gisou; EPFL Lausanne, Inst. of Global Health</td>
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What does S-palmitoylation do to membrane proteins?

Blaskovic Sanja, Blanc Mathieu, F. Gisou van der Goot

Global Health Institute, Ecole Polytechnique Fédérale de Lausanne (EPFL), Station 19, CH-1015 Lausanne, Switzerland

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* Corresponding author
Ecole Polytechnique Fédérale de Lausanne
Institute of Global Health
Station 15
1015 Lausanne
Switzerland
Tel: (41) 021 693 1791
Fax: (41) 021 693 9538

Contact: gisou.vandergoot@epfl.ch
Abstract

S-Palmitoylation is post-translational modification, which consists in the addition of a C16 acyl chain to cytosolic cysteines and which is unique amongst lipid modifications in that it is reversible. It can thus, as phosphorylation or ubiquitination, act as a switch. While palmitoylation of soluble proteins allows them to interact with membranes, the consequences of palmitoylation for transmembrane proteins are more enigmatic. We will briefly review the current knowledge regarding the enzymes responsible for palmitate addition and removal. We will then describe various observed consequences of membrane protein palmitoylation. We propose that the direct effects of palmitoylation on transmembrane proteins might however be limited to four non-mutually exclusive mechanistic consequences: alterations in the conformation of transmembrane domains, association with specific membrane domains, controlled interactions with other proteins and controlled interplay with other post-translational modifications.

Keywords: membrane proteins, protein conformation, rafts, protein complexes, DHHC, palmitoylation

Abbreviations: Endoplasmic Reticulum: ER; transmembrane domain: TMD.
Introduction

S-palmitoylation is a post-translational modification of proteins, which consists in the attachment of a saturated C16 fatty acid chain to specific cysteines via a thio-ester bond [1]. As opposed to other lipid modifications, S-palmitoylation is reversible. Since S-palmitoylation impacts on protein function, as discussed below, its reversibility allows regulation, or fine-tuning, of protein function, in a manner similar to protein phosphorylation or ubiquitination.

Recent large-scale profiling studies indicated that the number of S-palmitoylated proteins varies from ≈50 in yeast [2] to several hundreds in mammals [3, 4]. Palmitoylation of soluble proteins allows their controlled association with membranes, trafficking and domain localization, as shown for the small Rat sarcoma (Ras) GTPase at the cell surface [5] or phospholipid scramblase 3-mediated apoptosis at the mitochondria [6]. Palmitoylation however also occurs on transmembrane proteins, which will be the focus of this review. These proteins are by definition membrane anchored and thus the effect of attaching an acyl chain to them is less intuitive, specially considering that acylation frequently occurs on cysteines adjacent to, or even within, the transmembrane domain (TMD). The cytosolic domains of membrane proteins can also undergo S-palmitoylation.

The list of membrane proteins that undergo palmitoylation is ever growing and spans a bewildering variety of key cellular functions. Amongst the studied palmitoylated proteins are cell adhesion molecules [7] such as integrins [8] and claudins [9], tetraspanins such as CD81 [10], CD36 [11] and CD151 [12], many G-protein coupled receptors (GPCRs) such as rhodopsin [13], a great variety of channels and transporters (epithelial sodium channel [14], aquaporins [15], BK channel [16],...
dopamine or glutamate transporters,…), multiple receptors (acetylcholine, cannabinoid, opioid, β-adrenergic, dopamine, prostacyclin, estrogen, progesterone, androgen, NMDA, AMPA receptors,… [17, 18] [19] [20]; [21]), receptor ligands such as Fas Ligand [22], but also enzymes such as β and γ-secretase [23, 24], the E3 ligase gp78 [25], the protein disulfide isomerase TMX [26], chaperones (calnexin [27]) or numerous viral glycoproteins (influenza hemagglutinin, vesicular stomatitis virus G protein, SARS CoV S protein,… for review see [28]). In each study documenting protein palmitoylation, a cellular and/or functional consequence was observed such as altered signaling capacity [6, 8, 20], reduced activity [29], modified trafficking [30, 31], differential stability [13, 32]. Many of the reported effects are however likely to be secondary consequence. The direct, mechanistic consequences of palmitoylation of membrane proteins are however difficult to identify. Four consequences, that we will review here through selected examples and which could be either direct or early events, have however emerged over time and are not mutually exclusive: alterations in protein conformation, association with specific membrane domains, controlled interactions with other proteins and controlled interplay with other post-translational modifications (Fig. 1).

**Palmitoyltransferases and thioesterases**

Palmitate is available in the cell in the form of palmitoyl-CoA at nanomolar concentrations [33]. Unless palmitoyl-CoA is enriched in specific membrane microdomains [34], cellular concentrations are insufficient for spontaneous palmitoylation of proteins to occur. S-palmitoylation is thus mostly enzymatic and mediated by a family of DHHHC (Asp-His-His-Cys) motif containing palmitoyltransferases, of which there are 23 in humans [1], some 20 in worms and
flies, and 7 in yeast [2]. It is at present largely unclear why cells express multiple DHHC proteins and what the substrate specificity of these enzymes is [35, 36]. So far no real consensus sequence surrounding the cysteine residues has been identified [2] even though some relatively successful prediction algorithms are available (for example: http://csspalm.biocuckoo.org/).

DHHC proteins are membrane proteins that span the bilayer at least 4 times and harbor the conserved DHHC_cysteine-rich catalytic domain on the cytosolic face. They differ in their subcellular localization: many localize to the early secretory pathway (endoplasmic reticulum (ER) and Golgi) while others can be found at the plasma membrane or in endocytic vesicles [37]. DHHC proteins greatly vary in their N-terminal and C-terminal cytosolic domains which may carry protein-protein interaction domains such as ankyrin repeats in DHHC13 and DHHC17 or a predicted src Homology 3 domain in DHHC6. These extensions are likely involved in their localization and in substrate specificity [38, 39] since the DHHC-containing catalytic domain is not sufficient to convey specificity [39, 40].

What determines the specificity of a given DHHC protein for its substrates is currently unclear. In yeast, it was proposed that certain DHHC proteins preferentially modify soluble proteins, while others appear to require an additional modification, such as myristoylation or prenylation [2, 41]. Interestingly, Swf1 showed preference towards transmembrane proteins and cysteines that are in close proximity to TMDs [2]. In humans, it was found that certain substrates can be modified by a restricted set of DHHC proteins, for example the SNAP receptor (SNARE) protein SNAP25 can be modified on its 4 cysteines by DHHC3, DHHC7 and DHHC17 [42]. In contrast the transmembrane ER chaperone calnexin is exclusively palmitoylated on its
juxtamembranous double cysteine motif by DHHC6 [27], while integrin α6β4 is specifically modified by DHHC3 [8].

So far little information is available regarding the regulation of DHHC proteins, both in terms of expression and activity. It has been shown, in yeast and in mammals, that palmitoylation of Ras requires the formation of a complex between the palmitoyltransferase Erf2 (DHHC9 in mammals) and the interacting protein Erf4 (GCP16 in mammals) [43, 44]). Interaction of DHHC proteins with co-factors might be more widespread. Also they might undergo specific post-translational modifications in response to specific stimuli or stress conditions. In this context, it is worth mentioning that a large-scale proteomic analysis of palmitoylated proteins suggested that DHHC5, DHHC6 and DHHC8 are themselves palmitoylated [4] but these hits were not further validated, nor were the consequences of palmitoylation addressed.

In the cytosol, S-palmitoylation is reversed by acyl protein thioesterases (APTs) of which three have so far been identified, APT1, APT2 and APT1-like [45, 46]. Only a handful of studies have addressed their implication in the palmitoylation-depalmitoylation cycles of proteins. APT1 was shown to depalmitoylate Gα proteins [47, 48], APT2 to depalmitoylate growth-associated protein-43 [46, 49], both were found to depalmitoylate Ras [49] and APT1 and APT1-like were found to depalmitoylate the calcium-activated large potassium channel (BK) [50].

Altering the activity or expression levels of APTs clearly constitutes an effective method of regulating protein palmitoylation levels. For example, based on a functional screen to identify microRNAs involved in dendritic spine morphogenesis, microRNA-138 was found to be highly enriched and to regulate expression of APT1 in dendrites, thereby controlling palmitoylation of Gα13 [48].
Effects of palmitoylation on the conformation of transmembrane proteins

S-palmitoylation frequently occurs on cysteins adjacent to TMDs. Since this clearly does not have a membrane anchoring function, it was proposed early on that palmitoylation of juxtamembranous cysteines could induce tilting of TMDs [51]. Joseph and Nagaraj showed that palmitoylation of a hydrophobic synthetic peptide reconstituted into liposomes affected the orientation of the TMD. This concept has remained attractive and has been utilized to explain the effect of palmitoylation on the behavior of several membrane proteins [52-55]. The effect of palmitoylation on the conformation of a transmembrane protein was however not explicitly addressed. We have approached the issue when studying the palmitoylation of lipoprotein receptor-related protein 6 (LRP6) [30]. LRP6 has a well-established role in canonical Wnt signaling, a key pathway in early embryonic development as well as cancer. Upon binding of Wnt proteins to their receptors of the Frizzled family, a ternary Wnt-Frizzled-LRP6 is formed and transmits the signal across the membrane. LRP6 is a type I membrane protein with two juxtamembrane palmitoylation sites [30]. Upon mutation of the palmitoylation sites, LRP6 is recognized by the ER quality control and retained. LRP6 has a 23 residue long TMD, i.e. 2 residues longer than the vast majority of single pass membrane proteins. Since the ER membrane has been proposed to be thinner than the plasma membrane, a long TMD might lead to a hydrophobic mismatch. Tilting of the TMD through palmitoylation, as proposed by Joseph and Nagaraj [51], would reduce the effective hydrophobic length. To test this possibility, we shortened of the TMD of LRP6. In the absence of palmitoylation, shortening relieved the ER retention, whereas in the presence of palmitoylation
shortening led to ER retention, consistent with an effect of palmitoylation of the
conformation of the TMD [30].

Interestingly, ER retention of palmitoylation-deficient LRP6 was due to
ubiquitination of a lysine residue in the close proximity of the TMD [30]. Mutation of
this lysine relieved ER retention. The protein was then properly targeted to the plasma
membrane and competent for Wnt signaling. Intriguingly however, mutation of the
juxtamembranous lysine on the WT background, while not affecting plasma
membrane targeting, drastically reduced Wnt signaling [30]. Thus both in the ER and
at the plasma membrane there appears to be a cross-talk between palmitoylation and
ubiquitination of LRP6 (see below).

Association with membrane domains

A major focus of studies on the palmitoylation of membrane proteins has been the
role of this modification in promoting their association with lipid rafts—cholesterol
and sphingolipid rich membrane domains [56]. Palmitoylation of many proteins will
indeed promote their association with lipid rafts (for review see [57]). A recent
example is the death receptor 4 that undergoes DHHC3-mediated palmitoylation [58]
on 3 cytosolic cysteines, thus allowing its proper targeting to the plasma membrane
and its association with lipid rafts, which was found to be required for ligand-induced
cell death [59]. Palmitoylation-dependent raft association has also been shown
recently for β-secretase BACE1 [23], cannabinoid receptor [19], influenza virus M2
protein [60] and Close Homolog of Adhesion Molecule L1 (CHL1) [50]. Exceptions
do exist however such as the G protein of vesicular stomatitis virus and the transferrin
receptor which are both palmitoylated on three sites yet localize to non-raft domains
(for review see [57]), or the anthrax toxin receptor Tumor endothelial marker 8
(TEM8), for which palmitoylation was actually found to negatively regulate raft association [32].

The ability of palmitoylation to target proteins to specific membrane domains might however not be restricted to lipid rafts. It was indeed found that palmitoylation of the chaperone calnexin and the thioredoxin-related transmembrane protein TMX, two transmembrane proteins of the ER, is required for their targeting to specific ER domains, the ER-mitochondrial interaction sites [26]. Palmitoylation of calnexin also appears to be necessary for its localization to the nuclear envelope [27]. Palmitoylation of the ER E3 ligase gp78, involved in particular in the down regulation of HMGCoA reductase, was recently found to regulate the localization of the protein to the peripheral ER [25]. Although detergent-resistant ER domain have been observed [61], the very low cholesterol content of this compartment suggests that these domains must have different assembly mechanisms, and thus do not fulfill the definition of lipid rafts.

By controlling the association of membrane proteins with specific membrane domains/compartments, palmitoylation can bring together, or alternatively segregate, proteins that have the ability to interact under specific circumstances. For example, Neurotensin receptor-1, a G-protein coupled receptor (GPCR) implicated in breast cancer progression is palmitoylated, allowing it to interact with cholesterol-rich domains where its downstream Gα protein resides [62]. Thus in the absence of palmitoylation signaling is reduced. The opposite situation was observed for the anthrax toxin receptor TEM8, where palmitoylation negatively regulates raft-association, thus sequestering the receptor away from its E3 ubiquitin ligase Cbl [32]. In the absence of palmitoylation, TEM8 is prematurely ubiquitinated at the cell surface and targeted to lysosomes.
How palmitoylation affect rafts association of proteins is unclear. It could be due to the affinity of palmitate for specific lipids or lipid domains. This is suggested by studies of different isoforms of Ras that are or not palmitoylated [63]. Palmitoylation was shown to promote association with cholesterol-rich nanoclusters, while non-palmitoylated Ras was excluded from these domains [64]. In the case of transmembrane proteins, modified raft-association could however also be due to the effect of palmitoylation on the conformation of TMDs. Indeed, since cholesterol rich domains are though to be thicker than other parts of the membrane due to the presence of cholesterol, modification of TMD tilting through palmitoylation might affect the effective length of the hydrophobic segments leading to protein partitioning through membrane thickness.

**Effect on protein-protein association**

Palmitoylation of membrane proteins also appears to affect their ability to associate with other, soluble or transmembrane, proteins. One striking example is provided by the so-called tetraspanin webs, large protein complexes that include multiple palmitoylated tetraspanin proteins [12, 65-67] as well as palmitoylated integrin subunits such as α3, α6 and β4 [8, 68, 69]. Palmitoylation may occur on multiple sites as shown for KAI1, a member of tetraspanin family that inhibits migration and invasion of metastatic prostate cancer cells, and is palmitoylated on 5 cysteines [65]. Palmitoylation regulates interactions between tetraspanins and between tetraspanins and integrins, and this can occur in rather sophisticated ways as recently shown for CD151 for which palmitoylation differentially affects its interaction with α3β1 vs. α6β4 integrins [12].
We have recently found that palmitoylation of the ER chaperone calnexin is required for the formation of a super-complex that includes the ER translocon, the ribosome, the oligosaccharyltransferase and calnexin, and is stabilized by polymerized actin [27]. This complex allows newly synthesized proteins to be sequentially handled by the enzymes and chaperones that allow it to reach its native state.

Palmitoylation can however also negatively affect protein multimerization as was shown for the neural cell adhesion molecule (NCAM) 140 that is essential for axonal growth. DHHC7-dependent palmitoylation of NCAM140 [70] leads to raft association, but negatively regulates the hemophilic dimerization of extracellular NCAM domains in cis [71]. Interestingly, neuritogenesis requires interplay between NCAMs and Fibroblast Growth Factor receptor signaling, and FGF leads to the activation of DHHC7 that in turn enhances NCAM palmitoylation [70] further promoting axonal growth [72].

Palmitoylation may also control the interaction of membrane proteins with cytoplasmic proteins as shown for the Cation-dependent Mannose-6-phosphate receptor (CD-M6PR), a type I transmembrane protein, responsible for sorting of newly synthesized acid-hydrolases from the trans-Golgi network to endosomes. Once the M6PR has delivered its hydrolases to late endosomes, it is recycled back to the Golgi for re-utilization. This step depends on palmitoylation of M6PR [73] by DHHC15 [74]. More specifically it was found that palmitoylation allows the interaction of the cytosolic tail of M6PR with the retromer complex [74].

Palmitoylation is also important for the function of a wide array of GPCRs, one of the largest classes of transmembrane proteins, involved in variety of signaling pathways. A well-characterized example is the β-2-adrenergic receptor (β-2AR) which triggers activation of an adenylate cyclase, production of cyclic adenosine monophosphate
(cAMP) and activation of protein kinase A (PKA). In the late 1980s it was reported that β-2AR is palmitoylated and that this is important to prevent uncontrolled receptor desensitization and thereby loss of signaling [75]. Recently it was demonstrated that palmitoylation enables β-2AR to interact with cAMP degradation enzyme phosphodiesterase 4D as well as with the adaptor protein β-arrestin 2, which is required for agonist-induced receptor internalization [18]. Thus by interfering with desensitization, the lack of β-2AR palmitoylation leads to enhanced receptor-induced PKA activities in the cytoplasm. Consistent with these conclusions, palmitoylation-deficient β-2AR was found to be phosphorylated even in the absence of stimulus [76, 77]. Interestingly, stimulation with the agonist has also been proposed to induce depalmitoylation of the receptor [78]. A full understanding of how cycles of palmitoylation-depalmitoylation regulated β-2AR signaling will require further inquiry.

**Interplay between palmitoylation and other post-translational modifications**

As just illustrated by β-2AR, there are interplays between palmitoylation and other post-translational modifications on the same protein, generally in close proximity of the palmitoylation sites. As mentioned earlier, we found that palmitoylation deficient LRP6 undergoes ubiquitination in the ER of juxtamembranous lysine residues [30]. Similarly we found that palmitoylation deficient TEM8 undergoes ubiquitination at the cell surface, again in the vicinity of the palmitoylation sites, resulting in destabilization of TEM8 and its premature degradation [32]. Premature degradation of palmitoylation deficient mutants has been observed for a variety of proteins both in tissue cultures cells such as for CCR5 [79, 80] but also in knock-in mice for rhodopsin [13]. Interestingly, rhodopsin destabilization was not apparent in tissue
culture studies but clearly evident in mice. Also in yeast, palmitoylation of the SNARE protein (see below) Tlg1 was found to prevent ubiquitination and degradation [52]. Although palmitoylation is generally found to stabilize proteins, the reverse has also been observed as for the E3 ubiquitin ER ligase gp78 [25].

Interplay also occurs between palmitoylation and post-translational modifications other than ubiquitination. This is the case for the large conductance calcium-activated potassium (BK) channels, and especially those containing the stress-regulated exon (STREX) splice variant [16, 81, 82]. BKs are six-spanning membrane proteins with a long cytosolic tail, corresponding to the site of insertion of the STREX domain. Palmitoylation was found to occur on three sites in the cytoplasmic loop separating TMD1 and 2 [50] and on 2 sites in the STREX domain [82]. Modification of the cytoplasmic loop is mediated by DHHC22 and DHHC23 and reversed by APT1 and APT1L, and the palmitoylation-depalmitoylation cycle is required to tune expression of BK channels, but does not affect activity [50, 83]. Modification of the STREX domain is mediated by DHHC3, DHHC5, DHHC7, DHHC9 and DHHC17 and also reversed by APT1 and APT1L [50, 82, 83]. Palmitoylation brings the STREX domain to the membrane, this alters the conformation of the channel, renders it insensitive to phosphorylation by PKC and thus to its inhibitory effect [83]. Depalmitoylation or phosphorylation by PKA leads to disassembly of the STREX domain from the membrane, rendering it sensitive to PKC inhibition [83].

Although it is not relevant to transmembrane proteins, it is worth mentioning that palmitoylation of soluble proteins may require a prior, irreversible, lipid modification such as prenylation. This is the case for Ras, which undergoes farnesylation shortly after synthesis, allowing it to weakly interact with the ER and the Golgi [63]. Subsequent palmitoylation of specific Ras isoforms on the Golgi leads to stable
membrane association and vesicular traffic-mediated transport to the plasma membrane [84], whereas depalmitoylation leads to non-vesicular transport of Ras back to the Golgi [85].

Concluding remarks

S-Palmitoylation has been known for many decades. With the identification of both the enzymes that mediated the modification and those that reverse it, as well as the advent of new technologies to determine cellular palmitomes, the field has however entered a new era and the pace of discoveries has hugely accelerated. Some mechanistic consequences have already emerged, as reviewed here, but many open questions remain, such as the importance of palmitoylation-depalmitoylation cycles and the global consequences of palmitoylation at the cellular and organism levels.

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FIGURE LEGEND
Figure 1: Consequences of the palmitoylation of membrane proteins.

Palmitoylation of proteins in the proximity of their transmembrane domain has been proposed to 1) regulate in a positive, but also possibly negative, manner their association with lipid rafts, 2) to affect the conformation of their transmembrane domain such as tilting it, 3) to prevent ubiquitination of lysines in proximity of the palmitoylation site, 4) to promote the formation of protein complexes. These various effects are not mutually exclusive and might actually explain one another, for example tilting of the transmembrane domain could promote its association with a specific domain which could sequester it away from its E3 ubiquitin ligase or/and promotes its association with proteins present in this domain.
REFERENCES


1. Initiation of conformational changes

2. Regulation of membrane domain association

3. Regulation of protein complex formation

4. Interplay with other post-translational modifications

- Ex.: LRP6, TEM8, BK channels
- Ex.: Neurotensin receptor, gp78, TMX
- Ex.: tetraspanins, integrins, calnexin