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REVIEW

The role of S-acylation in protein trafficking

Jose L. Daniotti[1,2] | Maria P. Pedro[1,2] | Javier Valdez Taubas[1,2]

[1]Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC), CONICET, Universidad Nacional de Córdoba, Córdoba, Argentina

[2]Departamento de Química Biológica Ranwel Caputto, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina

Correspondence
Jose L. Daniotti, Departamento de Química Biológica Ranwel Caputto, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, X5000HUA Córdoba, Argentina.
Email: daniotti@fcq.unc.edu.ar

Present address
Maria P. Pedro, Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

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Protein S-acylation, also known as palmitoylation, consists of the addition of a lipid molecule to one or more cysteine residues through a thioester bond. This modification, which is widespread in eukaryotes, is thought to affect over 12% of the human proteome. S-acylation allows the reversible association of peripheral proteins with membranes or, in the case of integral membrane proteins, modulates their behavior within the plane of the membrane. This review focuses on the consequences of protein S-acylation on intracellular trafficking and membrane association. We summarize relevant information that illustrates how lipid modification of proteins plays an important role in dictating precise intracellular movements within cells by regulating membrane-cytosol exchange, through membrane microdomain segregation, or by modifying the flux of the proteins by means of vesicular or diffusional transport systems. Finally, we highlight some of the key open questions and major challenges in the field.

KEYWORDS

acyl-protein thioesterase, DHHC, endomembrane trafficking, lipidation, palmitoylation, palmitoyltransferase, S-acylation

1 | INTRODUCTION

Many proteins have been shown to contain covalently bound lipids, and the lipidation of proteins in eukaryotic cells can be divided into two main categories: that occurring in the cytosol or on the cytoplasmic face of cellular membranes, and that taking place in the lumen of secretory organelles. Concerning the latter, the addition of glycosylphosphatidylinositol (GPI) in the endoplasmic reticulum is the best characterized. In this case, the proteins transit the exocytic path to the cell surface, where they remain anchored to the extracellular face of the plasma membrane. Another modification that occurs in secretory proteins (such as morphogens and cytokines) is the addition of cholesterol or fatty acids (N-acylation) through an ether or amide bond, respectively. Examples of these are the Hedgehog and Wingless proteins Wnt-1 and Wnt-3a. In addition, three main types of cytosolic lipid modifications take place: prenylation, the addition of an isoprenyl group such as 15-carbon farnesyl or 20-carbon geranylgeranyl to a cysteine through a thioether bond; N-myristoylation, the addition of a myristoyl group to a glycine residue through an amide bond; and S-acylation, the addition of long chain fatty acid, usually palmitate, to a cysteine through a thioester bond. There are some excellent general reviews on lipid modifications.[1–6]

This review will focus mainly on S-acylation of peripheral and integral membrane proteins, with particular emphasis on how this chemical modification regulates the membrane affinity and intracellular trafficking of a few selected proteins (Figure 1). We first

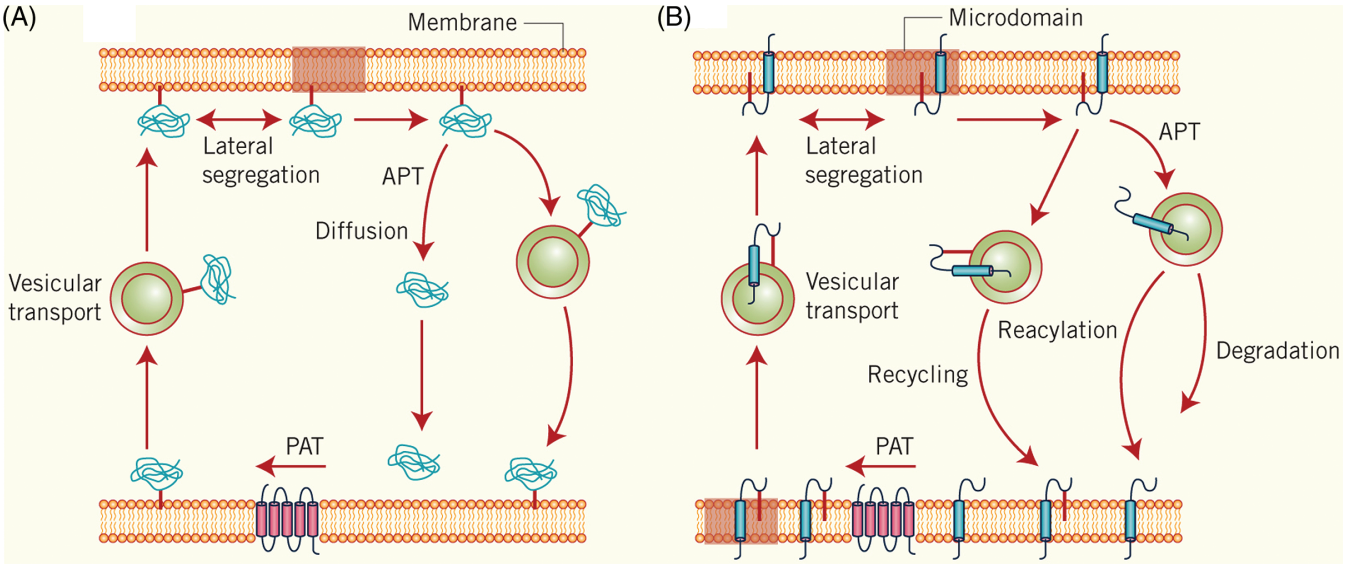

FIGURE 1 S-acylation cycle regulates the membrane association, segregation and intracellular transport of both peripheral and integral membrane proteins. Peripheral (A) and integral membrane (B) proteins may be palmitoylated by a PAT and depalmitoylated by an APT. This reversible S-acylation cycle, which can occur at multiple membranes including Golgi complex and plasma membrane regulates protein trafficking (vesicular and nonvesicular [diffusion] transport), recycling, targeting to specialized membrane microdomains (lateral segregation), biological function and degradation. Rods in red represent palmitates. Membrane microdomains are indicated in old rose

summarize below some general features of acylation and of the molecular machinery that carries it out.

2 | PROTEIN S-ACYLATION

To date, more than 2000 human proteins have been either shown or predicted to be palmitoylated, which represents approximately 12% of the proteome.[7] Even though there is no clear consensus on the amino acid sequence for this lipid modification, some features shared by different substrates can help to predict palmitoylation, and algorithms have been developed to identify potential palmitoylation sites.[8–10] In the case of single-spanning membrane proteins, palmitoylation may occur at cysteines located in the cytoplasmic domain or near the cytosolic border of the transmembrane domain. In contrast, for soluble proteins, S-acylatable cysteines are present close to regions with an affinity for membranes, such as a hydrophobic or basic stretch of amino acids, or at sites where other lipid modifications such as myristoylation or prenylation occur.[5]

S-acylation is mediated by protein acyltransferases (PATs) that belong to the DHHC (aspartate-histidine-histidine-cysteine) family, whereas acyl-protein thioesterases (APTs) are required for deacylation (Figure 1). PATs were first described in 2002 in two independent studies performed on *Saccharomyces cerevisiae*.[11,12] The number of PATs varies from 7 in yeast to 23 in humans, with a common feature of these enzymes being the presence of a cysteine-rich domain with a conserved DHHC motif, which is indispensable for enzyme activity.[13,14] The DHHC motif is located in one of the cytosolic loops of these 4 to 6-pass transmembrane proteins. Additionally, a palmitoyltransferase conserved C-terminus (PaCCT) domain is conserved in the majority of PATs from several eukaryotic organisms and is important for their function and localization.[15] These enzymes are mainly localized at the Golgi complex but have also been found in the endoplasmic reticulum, plasma membrane and endosomes.[13,16,17]

PAT enzymes are likely to display a certain degree of redundancy since they may have partially overlapping substrate specificities.[18,19] However, some particular substrates have been demonstrated to be dependent on an individual PAT for their efficient modification.[13] In yeast, Ras2 is mostly S-acylated by the yeast palmitoyltransferase Erf2-Erf4, although deletion of this enzyme does not completely block S-acylation.[11] On the other hand, deletion of Swf1, the PAT responsible for the palmitoylation of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment receptor) protein Tlg1, leads to a complete absence of Tlg1 palmitoylation, which is not rescued by overexpression of other yeast PATs.[20,21]

Some aspects of PATs biology that remain to be investigated in depth are: how PATs achieve their respective intracellular localizations, the relevance of posttranslational modifications on PAT activity and, very importantly, the dynamic exchange of PATs between membranes since these enzymes might be relevant for S-acylation of a specific substrate in different membrane environments. Currently, many studies show the important contribution of acylation to protein trafficking. However, PATs are generally regarded as "static actors," which in our opinion, may not represent the real situation. One of the few pieces of evidence that highlight the importance of these concepts came from the Chamberlain's laboratory. They showed that DHHC2, which mediates activity-dependent S-acylation of PSD95, is regulated by a dynamic cycling pathway that connects the plasma membrane with recycling endosomes, and they also identified domains that specify the membrane targeting of this protein.[22,23] Also, a previous report demonstrated that blocking synaptic activity, which leads to enhanced palmitoylation of PSD95, also modulates DHHC2 trafficking, promoting its accumulation to sites near the postsynaptic membrane.[24]

The enzymes that mediate deacylation have not been as extensively characterized as the PATs, with only two cytosolic APTs having been described to date: APT1 and APT2.[25–28] An additional protein, called APT1-like thioesterase, is active against the BK potassium channel,[29] although its role as a thioesterase is still debated.[30,31] More recently, ABHD17, a protein depalmitoylase, was also found to deacylate N-Ras and postsynaptic density-95 (PSD-95) protein.[32,33]

APT1 and APT2, which share a 64% homology at the amino acid level, mediate fatty acid turnover on many peripheral proteins and are thought to be selective because not all substrates are deacylated with the same efficiency.[26,29,30,34,35] APT1 and APT2 contain a catalytic triad made up of serine, histidine and aspartic acid, and also a glycine-X-serine-X-glycine motif, which is characteristic of the large family of α/β hydrolases and serine hydrolases.[36–39] The crystal structures of APT1 and APT2 have been determined, revealing an architecture containing a central structure made up of parallel β sheets, connected by loops and surrounded by α-helices.[38,40] Experimental evidence obtained in various laboratories (including ours) has indicated that both enzymes are mainly cytosolic with a highly hydrophilic character.[25,26,41,42] Recently, APT1 and APT2 were found to undergo palmitoylation on cysteine 2,[43–45] which was suggested to facilitate the steady-state membrane localization and function of these thioesterases. However, it should be mentioned that the acylatable cysteine in APT1 and APT2 is not conserved in all analyzed species,[46] with the exact role of this modification still being unclear.

During decades, radioactive labeling was used for detection of palmitoylation; this method is tedious, time-consuming and does not allow the identification of the S-acylated sites. Mass spectrometry approaches such as shotgun proteomic assays are challenging for S-acylated proteins because palmitoylated peptides are hydrophobic, water insoluble and have a high susceptibility to hydrolysis of the thioester-linked palmitate. To partially circumvent these limitations, new methodologies were developed such as the acyl-biotin exchange (ABE),[47,48] acyl-resin assisted capture (Acyl-RAC),[49] and metabolic labeling approaches involving alkyne-tagged analogous of palmitic acid which are later derivatized using click chemistry, allowing, affinity purification and analysis by shotgun proteomics.[50] Although these methods proved powerful for the identification of palmitoylated proteins, they do not provide information such as the identity of modified cysteine residues with multiple possible palmitoylation sites, and more importantly, to what extent are the proteins modified, as the unpalmitoylated proteins are not retained in these assays.

Another issue that remains a significant challenge in the field is the development of experimental strategies to study the regulation and dynamics of S-acylation in vivo. There is a need for methods to monitor the activity levels of PATs and APTs under physiological and pathological contexts. In this sense, a recent novel approach has been the development and application of a fluorescent probe for S-depalmitoylation activity, which reports on endogenous levels of depalmitoylation activity in live cells.[51] Particularly, this approach elegantly allowed the observation of the rapid growth factor-mediated inhibition of the depalmitoylation activity of APTs, revealing a novel regulatory mechanism of dynamic lipid signaling.

3 | ROLE OF S-ACYLATION ON THE TRAFFICKING OF PERIPHERAL MEMBRANE PROTEINS: THE PARADIGMATIC CASE OF THE SMALL GTPASE H-RAS

Ras family proteins are monomeric GTPases which couple extracellular signals to intracellular effector pathways, and have a role in the control of multiple biological functions including proliferation, differentiation, and survival, among many others.[52] Hyperactivating mutations in Ras are a hallmark of cancer,[53] with these gain-of-function mutations in RAS genes being found in approximately 20% to 35% of all human cancers, which has led to intensive research aimed at interfering with the Ras function.[54,55]

There are four Ras isoforms, namely H-Ras, N-Ras, K-Ras4A and K-Ras4B, and despite them sharing over a 90% homology, their functions are not redundant.[56–59] All of these isoforms have a CAAX sequence (in which C is a cysteine, A is an aliphatic amino acid and X is any amino acid) that directs the posttranslational modification of the C-terminus.[53,56,58,60] Ras proteins are first modified in the cytosol, where a farnesyltransferase enzyme transfers a 15-carbon isoprenyl group to cysteine 186 in the CAAX motif, thereby allowing the transient association of farnesylated Ras with the cytosolic surface of the endoplasmic reticulum (Figure 2). Then, the C-terminal AAX sequence is cleaved by the endopeptidase Rce1, and the farnesylated cysteine becomes methylated by the isoprenylcysteine carboxyl methyltransferase Icmt. Depending on the Ras isoform, a second signal for the membrane anchor is present in the vicinity of the farnesylated cysteine.[66] H-Ras is dually S-acylated at cysteines 181 and 184, whereas N-Ras is S-acylated at cysteine 181. In contrast, K-Ras4B is not further modified but contains a polybasic domain that binds to the phospholipid headgroups present in the internal layer of membranes. K-Ras4A is unique among the four Ras proteins in possessing (in addition to the farnesylated cysteine) an S-acylated cysteine and a polybasic region.[67–69]

After farnesylation, Ras moves to the Golgi complex, where S-acylation is believed to occur by the action of the DHHC9/GCP16 complex[70] and possibly by other PATs.[71] As mentioned above, in yeast this reaction is mediated by its orthologue Erf2/4.[11] Single or dual palmitoylation significantly increases the affinity of prenylated Ras to biological membranes, which is required for its correct intracellular transport. Farnesylated and palmitoylated N- and H-Ras move to the plasma membrane associated to the cytoplasmic leaflet of vesicular carriers (Figure 2). Then, once at the cell surface, Ras proteins can become depalmitoylated and released back to the cytosol where they travel by diffusion to the Golgi complex for another round of palmitoylation and exocytic delivery to the plasma membrane.[61,62] We have previously shown that H-Ras is endocytosed and delivered to the recycling endosome in a Rab5- and Rab11-dependent vesicular transport in CHO-K1 cells,[64] with this subcellular localization and route having also been described in other cell lines.[72–75] Additionally, diacylated GAP-43 was also found to localize to the recycling endosomes, arriving from the plasma membrane by vesicular transport.[76] For both H-Ras and GAP-43, double palmitoylation is necessary for them to be targeted to this location. More recently, an alternative exocytic route for N- and H-Ras has been described in COS-1 cells,

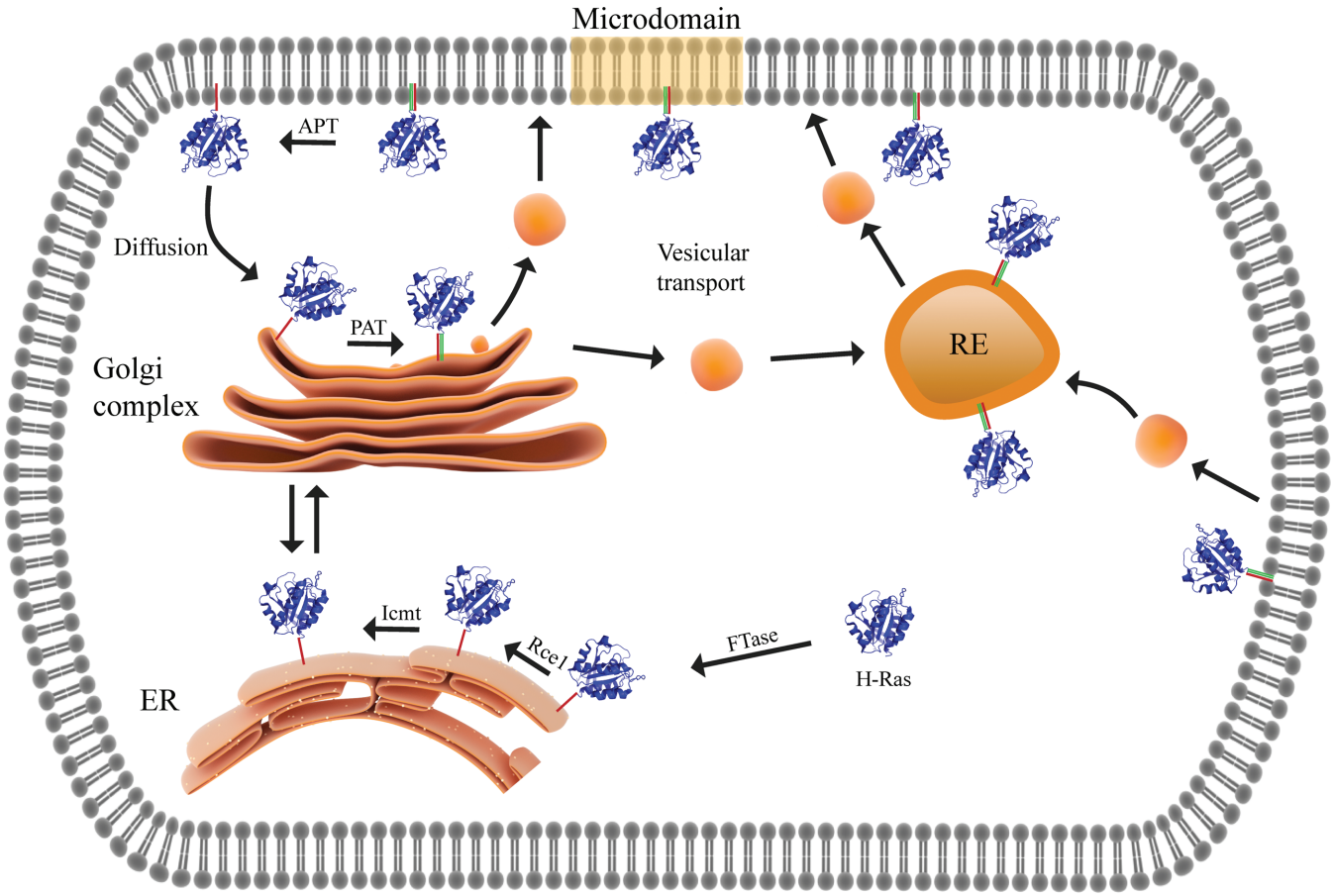

FIGURE 2 An overview of intracellular transport of diacylated H-Ras. After synthesis as a globular hydrophilic protein, H-Ras is first modified in the cytosol by a farnesyltransferase enzyme (FTase) which transfers a 15-carbon isoprenyl group to cysteine 186 in the CAAX motif, thereby allowing a transient association of farnesylated H-Ras with the cytosolic surface of the endoplasmic reticulum (ER). At this organelle, the C-terminal AAX sequence is cleaved by the endopeptidase Rce1, and the farnesylated cysteine becomes methylated by the isoprenylcysteine carboxyl methyltransferase Icmt. Then, H-Ras arrives at the Golgi complex, where a specific PAT S-acylates the cysteines 181 and 184. After single or dual acylation at the Golgi complex, H-Ras is included into the vesicular carriers directed to the plasma membrane.[61,62] An alternative exocytic route for H-Ras was described in COS-1 cells, in which recycling endosomes (RE) act as a way station along the post-Golgi pathway to the plasma membrane.[63] At the cell surface, dually acylated H-Ras is endocytosed, transported to RE and recycled back to the plasma membrane.[64,65] A fraction of H-Ras may eventually be deacylated by APTs, returning by nonvesicular transport (diffusion) to the Golgi complex, where it is then re-acylated.[61,62] H-Ras can eventually distribute in plasma membrane microdomains, which varies depending on the cell type, activation status and palmitoylation level. The structural representation of H-Ras was obtained from the RCSB Protein Data Bank (www.rcsb.org). Rods in red and green represent farnesyl and palmitate groups, respectively

in which the proteins go through recycling endosomes on their way to the plasma membrane from the trans-Golgi-network (TGN).[63]

H-Ras is potentially acylated on cysteines 181 and 184, and how these residues contribute individually to H-Ras trafficking is now being studied. Seminal work from Hancock's laboratory has demonstrated that mutation of acylation sites causes H-Ras to accumulate in different subcellular compartments.[77] Recently, our laboratory has shown that the S-acylated cysteines 181 and 184 contribute differentially to H-Ras membrane affinity and exhibit distinct APT sensitivities and deacylation kinetics.[65] In particular, it was found that mono-acylated H-Ras species are selectively incorporated into carrier vesicles at the level of the post-Golgi complex with different efficiencies. The mutant that is palmitoylated only in cysteine 184 is more efficiently incorporated into the Golgi to plasma membrane vesicular carriers. Notably, fluorescence photobleaching and photoactivation experiments revealed that both mono-acylated mutants exhibit deacylation-dependent and independent diffusion, with rapidly reversible membrane binding to different organelles. Consequently, the cysteines 181 and 184 each provide unique information on the spatial organization and trafficking of H-Ras. The interplay between PAT and APT activities, as well as protein stability and other posttranslational modifications, results in a heterogeneous distribution of acylated species, which may have physiological or even pathological implications due to the influence it may have on the connection of the small GTPase with downstream signaling molecules.

Although the relative activities of PATs and APTs are the primary factors regulating acylation stoichiometry, and hence the subcellular sorting of Ras isotypes, additional chemical modifications have important consequences on their acylation status, subcellular distribution, and lateral segregation on biomembranes. FKBP12, the best-characterized member of the FK506-binding protein (FKBP) family of prolyl isomerases, binds to diacylated H-Ras and promotes depalmitoylation in a proline 179-dependent fashion.[78] Moreover, it has been demonstrated that H- and N-Ras, but not K-Ras, are modified by

lysine 63-linked di-ubiquitin chains.[72,73] CAAX-mediated modification of H-Ras (farnesylation and palmitoylation) is necessary for its ubiquitination and association with endosome membranes, and more recently, Sasaki et al have demonstrated the monoubiquitination of K-Ras in lysine 147 resulted in enhanced GTP loading.[79]

The activation status of H-Ras also has profound consequences on its distribution in membrane microdomains and its deacylation kinetics. When H-Ras is in the GTP-bound state, it segregates to disordered lipid domains, whereas the inactive GDP-loaded H-Ras resides in liquid-ordered lipid raft domains.[80] Nevertheless, H-Ras distribution in plasma membrane microdomains varies depending on the cell type and is determined by the balance between palmitoylation and depalmitoylation.[81] Intriguingly, the oncogenic GTP-bound form of H-Ras is more accessible to APT(s) than the inactive one,[82] suggesting that depalmitoylation of H-Ras is mainly carried out in specialized membrane microdomains containing active H-Ras.

Caveolae are bulb-shaped plasma membrane microdomains enriched in cholesterol and glycosphingolipids. Investigations carried out by Parton's laboratory have indicated that caveolae regulate Ras nanoclustering and signal transduction by controlling plasma membrane organization.[83,84] Caveolin-1 deficiency affects cellular lipid composition and plasma membrane dynamics, which correlates with an increased K-Ras nanoclustering and signal transmission but a reduction in GTP-dependent lateral segregation of H-Ras, resulting in a compromised signal output from H-RasG12V nanoclusters.[83] Thus, these results illustrate the cross-talk between caveolae, lipid metabolism and key signal transduction pathways.

The regulation of many other peripheral proteins through acylation-deacylation cycles has been described in numerous original research articles and summarized in excellent reviews.[13,85–92] In brief, the dynamic acylation cycle acts as a molecular switch that regulates the spatial distribution of proteins at the micro- (ie, inter-organellar transport) and nano- (ie, segregation or clustering of proteins at membrane microdomains) scale dimensions.

4 | INFLUENCE OF S-ACYLATION IN THE TRAFFIC OF TRANSMEMBRANE PROTEINS

While the role of peripheral membrane protein palmitoylation is to regulate interactions between the modified proteins and membranes, palmitoylation of integral membrane proteins modulates their behavior within the plane of the membrane, due to the partition to different subdomains and/or the modulation of interactions with other proteins (Figure 1). In many cases, this will affect the traffic of the protein and therefore its final membrane localization, whereas in other cases, there are no gross changes in localization or the changes that may occur require more sophisticated tools for them to be detected (see below).

The role of lipid domains in membrane traffic has been extensively reviewed.[93,94] However, the nature of these domains and the affinity they have for palmitoylated proteins is still not entirely clear. There are examples of S-acylated proteins partitioning both in and out of traditional lipid rafts,[86,95,96] but the difficulties encountered in visualizing and characterizing these domains complicate our understanding of their relationship with palmitoylation. These, often contradictory, findings are not only a result of the technologies that have been used for raft study.[97] Cells may form ordered liquid membrane domains of various properties,[98] and the partition of a protein into these domains and the consequences it will have on the protein traffic will result from the emergent properties of complex systems and interactions. These include, in addition to the membrane composition and heterogeneity, the nature of the transmembrane domains (length, shape and composition),[99–101] the position of the acylatable cysteines, and importantly, the nature of the added lipid (length and saturation) since it is now becoming clear that some PATs will transfer lipids of different length to their substrates.[102,103] It is also possible that sorting events will be mediated by the partition of proteins into regions of different curvatures, and to discover how palmitoylation affects these events is an interesting line for future research. Concerning this, a study by van der Goot and coworkers showed that palmitoylation of calnexin (a type I membrane protein and a major endoplasmic reticulum chaperone involved in glycoprotein folding) leads to the preferential localization of calnexin to the perinuclear rough endoplasmic reticulum, at the expense of endoplasmic reticulum tubules.[104]

Overall, it is unlikely that general rules about the consequences of transmembrane protein palmitoylation will emerge, as these will be specific for each protein or family of proteins. Palmitoylation of transmembrane proteins can be classified according to the position of the palmitoylated cysteines with respect to the transmembrane domain. S-acylation of cysteines that are located in a cytosolic loop may have structural consequences on this protein region, leading to a variety of responses including modification of the interaction (either intra- or intermolecular), or partitioning to different lipid domains as reviewed in Reference 86. On the other hand, many single and multispanning membrane proteins have conserved cysteines either at the cytosolic border or within their transmembrane domain/s, which in many cases were found to be palmitoylated. The consequences of this latter type of palmitoylation are less obvious in terms of the changes they may produce in the structure of a protein, with the effects of this modification most likely being due to changes in the solubility of the transmembrane domain, as well as to the tilting of the transmembrane domain in the membrane (which will also affect solubility).[86,105] Finally, the interplay between palmitoylation and other posttranslational modifications, such as ubiquitination and phosphorylation, is also an important determinant of a protein's fate.

There are multiple examples of palmitoylated membrane proteins, and for many of these, palmitoylation affects their traffic and localization. Although a detailed review of this topic is beyond the scope of this article, there are some excellent reviews.[13,86,106] In the following paragraphs, we will cover a few examples obtained from our investigations and the literature, with emphasis on single-spanning membrane proteins, which are simpler to address. Particular focus will be placed on SNAREs (type II membrane proteins), since these are central to the trafficking machinery, with the SNAREs that are exclusively lipid-anchored to the membranes, such as SNAP25, being treated in the following section.

Palmitoylation of transmembrane SNAREs was first identified in yeast.[21,107] Interestingly, out of the 20 currently known yeast

SNAREs, only those that are localized in distal regions of the secretory pathway have conserved palmitoylated cysteines,[21,108] which are located at the cytosolic border of the transmembrane domains. Several mammalian SNAREs also have conserved cysteines in this region,[21] and some of these have been shown to be palmitoylated.[109,110] However, this distribution of cysteine-bearing SNAREs, which increases towards the late secretory pathway, is not observed in mammalian cells. This may be due to the properties of the yeast membranes, in particular, the yeast plasma membrane, as it is highly enriched in ergosterol and long chain lipids (reviewed in References 111–113).

The subcellular distribution of four acylated yeast SNAREs (Snc1, Syn8, Tlg1[21] and Sso1 [Valdez Taubas J, unpublished]) was analyzed in a yeast strain that has the cognate PAT deleted (*swf1Δ*). Although no gross changes in localization were observed for the majority of the SNAREs, when Tlg1 is not palmitoylated, it becomes ubiquitinated and degraded in the yeast vacuole. Also, nonacylatable versions of these proteins were observed in wild-type backgrounds with similar results.[21] The *swf1Δ* strain, however, has multiple phenotypes that cannot be ascribed to a lack of palmitoylation of any of its known substrates but are possibly a consequence of a general defect in membrane traffic, suggesting that this modification is important for the function of the SNAREs. The study of the influence of palmitoylation on the SNARE function in vivo is complicated by the fact that SNAREs are redundant,[114] and it is also difficult to test in vitro since this requires purification of palmitoylated SNAREs from sources other than bacteria. Nevertheless, as the reconstitution of SNARE-mediated fusion has been achieved in vitro,[115] palmitoylation is not essential for SNARE activity, although it is not known whether lipidation affects the efficiency of SNARE-mediated fusion.

The role of S-acylation in membrane traffic has been studied for two mammalian SNAREs. Palmitoylation did not influence the steady-state localization of syntaxin 8 in late endosomes, whereas syntaxin 7, which normally cycles between endosomes and the plasma membrane, was retained in the latter upon mutation of the palmitoylated cysteine.[109] The PAT/s responsible for mammalian transmembrane SNARE palmitoylation has not yet been identified, and therefore the global effect of a lack of SNARE palmitoylation has not yet been addressed.

Another important set of type II membrane proteins is Golgi complex glycosyltransferases (GTs), which catalyzes the stepwise addition of sugar residues to lipids and glycoproteins. Many GTs, particularly those involved in the synthesis of glycolipids, bear conserved cysteines that are modified by palmitoylation.[116] Again, the functional consequences of a loss of palmitoylation for these enzymes are not obvious, but the high conservation of the acylated cysteines indicates that this modification has indeed an important role. It is intriguing that GTs have not shown up in the many palmitoyl-proteomes already available,[7] with the exception of two mannosyltransferases identified in the yeast proteome,[108] which is probably due to the low levels of GT expression. Additionally, many GTs involved in the synthesis of glycolipids are both tissue-specific and regulated during development[117–119] and may have been absent from the samples altogether. As for SNAREs, it is unlikely that cysteine palmitoylation is required for the catalytic activity of type II GTs. Indeed, the catalytic domain is oriented towards the lumen of the Golgi complex. Moreover, there are multiple GTs that do not have conserved cysteines that can be palmitoylated. Therefore, it is more likely that palmitoylation is involved with the fine-tuning of the enzyme activity because of subtle changes in the segregation to membrane domains or in subtle associations that might not be picked-up in an in vitro reaction or by observing the localization of the overexpressed enzymes using conventional optic microscopy.

Synaptotagmin VII is a Ca^{2+} sensor that regulates lysosome exocytosis and plasma membrane repair,[120–122] which is a type I membrane protein that is palmitoylated in cysteine residues adjacent to the transmembrane domain. This palmitoylation is essential for the association of the protein with the tetraspanning CDC63, as it is required for localization of synaptotagmin in lysosomes. Mutation of the cysteines results in Golgi complex localization of synaptotagmin VII, and in the loss of its function.[123] Most members of the tetraspanin family are palmitoylated,[124] which is necessary for them to be able to organize signaling microdomains.[125]

A large number of G protein-coupled receptors are S-acylated at cysteines in their cytoplasmic tails following the last transmembrane domain (reviewed in Reference 126). This modification can have a variety of consequences which include defects in transport to the plasma membrane and targeting to lipid rafts (reviewed in Reference 13). S-acylation also controls the activity of a great number of ion channels and transporters.[13,127]

A recent example of a membrane protein whose localization is regulated by palmitoylation is the glucose transporter Glut4. This multispanning membrane protein plays a key role in the regulation of glucose homeostasis and is palmitoylated at a juxtamembrane cysteine (cysteine 223) adjacent to the transmembrane domain 6.[128] Glut4 normally localizes to the TGN and in small vesicles in tubular-vesicular structures, with the substitution of cysteine 223 abolishing Glut4 palmitoylation and insulin dependent Glut4 plasma membrane translocation. Glut4 palmitoylation is mediated by the PAT DHHC7, and the activity of this PAT is increased upon addition of insulin, representing one of the first examples of regulated PAT activity.[129]

From the examples described above and many others,[13] it can be inferred that S-acylation might affect the fate of a protein in multiple transport steps in the cell. For example, protein endocytosis of the anthrax receptor,[130] the transferrin receptor[131] and the DJ-1 protein[131,132] are affected by palmitoylation. Also, a massive endocytosis event (MEND) in cardiac cells can be triggered by palmitoylation,[133,134] and some proteins require S-acylation to leave the endoplasmic reticulum, as in the case of Chs3[135] and the Wnt receptor LRP6.[136] As already mentioned for Glut4, certain proteins require palmitoylation to leave the Golgi complex, and examples of endosomal/lysosomal traffic alterations due to palmitoylation have been described above, including Tlg1, Syn 7 and Synaptotagmin VII. Furthermore, the mannose 6-phosphate receptor and sortilin recycling are controlled by S-acylation.[137]

Overall, the importance of palmitoylation in membrane traffic is becoming increasingly recognized. However, while examples of the influence of S-acylation in the traffic of membrane proteins continue to be reported, the mechanisms by which this modification exerts its function in the different transport steps are still not well understood

and represent an interesting future line of research that might yield novel biological insights. Additionally, the apparent lack of consequences of palmitoylation for many proteins will likely be clarified as more sophisticated approaches are utilized for their study.[138] In both scenarios, detectable or nondetectable effects of palmitoylation, gene editing technologies such as CRISPR/Cas9 (clustered, regularly interspaced, short palindromic repeat-associated endonuclease Cas9) system will allow the analysis of cell lines devoid of specific PATs, and the detection of endogenous palmitoylated proteins fused to fluorescent tags.[139] Additionally, if palmitoylation causes the partition to specific membrane domains, these may be detected using super-resolution microscopy. However, if the domains remain too small and dynamic to be detected, it is possible to turn to yeast, in which membrane subdomains are sufficiently stable and big enough to be detected by optical microscopy both at the plasma membrane[140] and the vacuole membrane.[141]

5 | REGULATION OF INTRACELLULAR TRANSPORT PROCESSES BY PROTEIN S-ACYLATION

In addition to its specific role in the transport of multiple proteins, palmitoylation also plays a more general role in membrane transport by influencing the trafficking machinery of the cell. In the following paragraphs, we will cover a few examples of how protein S-acylation orchestrates intracellular transport and membrane fusion processes.

Phosphatidylinositol 4-phosphate (PI4P) has recently emerged as a TGN resident that establishes a signpost for the recruitment of trafficking proteins.[142,143] The major TGN clathrin adaptor, AP-1, binds PI4P and requires PI4P for TGN targeting.[144] Furthermore, the Golgi-localized γ-ear-containing Arf-binding proteins (GGAs), which mediate the trafficking between the TGN and endosomes, use PI4P to ensure organelle-specific targeting.[145] In addition, PI4P also participates in sphingolipid homeostasis by regulating the transport of glycolipid precursors at the TGN[146] as well as the delivery of lysosomal enzymes involved in sphingolipid degradation.[147]

Four distinct phosphatidylinositol 4-kinases have been identified in mammalian cells, which are able to catalyze the phosphorylation of phosphatidylinositol (PI) to synthesize PI4P. These 4-kinases localize to distinct membrane compartments and have specific roles in modulating inter-organelle lipid trafficking, phosphoinositide signaling and intracellular vesicle trafficking, as well as the Golgi function.[148,149] One of the phosphatidylinositol 4-kinase isoforms, PI4KIIα, has been shown to localize at endosomal compartments[148,150–154] but also at the Golgi complex.[144,145] This enzyme contains a conserved cysteine-rich motif (CCPCC) that is palmitoylated by 6 different PATs (ie, DHHC2, 3, 7, 14, 15 and 21).[155–157] Palmitoylation strongly enhances PI4KIIα activity, whereas unpalmitoylated PI4KIIα is unable to associate with the TGN or phosphorylate PI, resulting in a decrease in the Golgi pool of PI4P.[156] Consequently, the depletion of PI4P leads, among other effects, to the impaired recruitment of clathrin adaptor protein AP-1 to the TGN and inhibition of constitutive secretion from this site.[158] Additionally, PI4KIIα was shown to behave as both a cargo and enzymatic regulator of adaptor protein (AP)-3,[159,160] which participates in the targeting of membrane proteins from endosomes to lysosomes and synaptic vesicles. In the absence of AP-3, PI4KIIα redistributes to the Golgi complex area, suggesting that the steady-state localization of PI4KIIα reflects an equilibrium between the Golgi complex and the endosomal compartments.[153] According to these antecedents, this enzyme cycle might be highly dependent on the S-acylation status of PI4KIIα.

The c-Jun amino-terminal kinases (JNK) are crucial players in the stress response in neurons.[161] Among these, JNK3 isoform is S-acylated in two cysteines at the C-terminal.[162] Palmitoylated JNK3 is localized at the Golgi complex independently of its kinase activity,[163] with its palmitoylation impeding axon growth, a process that relies on active Golgi functions.[162] At this location, JNK3 interacts and retains the vesicular stomatitis virus G glycoprotein.[163] Moreover, Rab8 and Rab11, which facilitate anterograde transport along the secretory pathway, also selectively accumulate in the Golgi complex upon association with JNK3.[163] It was also reported that palmitoylated JNK3 reduces PI4P in the Golgi complex in both rat hippocampal neurons and COS7 cells, which is caused by the sequestration of the phosphatase SacI at the Golgi complex, where it binds to JNK3 and metabolizes PI4P to PI.[163] In this way, by regulating JNK3 subcellular localization, S-acylation controls the anterograde transport of several proteins.

Many components of the vesicle fusion machinery in neurons are S-acylated, including synaptosome-associated protein of 25 kDa (SNAP25), cysteine string protein (CSP), the vesicle-associated membrane protein, VAMP2, as well as the calcium sensor for fusion synaptotagmin 1 and multiple syntaxins.[13,164]

SNAP25 is a SNARE peripheral protein that is palmitoylated in four cysteines contained in a cysteine-rich domain protein and is involved in membrane fusion events at the plasma membrane and the endosomal system.[164,165] S-acylation controls membrane binding and the localization of SNAP25, thereby affecting its function.[22] It was also described that the localization in lipid rafts of SNAP25 and SNAP23 (a ubiquitously expressed homolog), regulates the SNARE function and therefore vesicle exocytosis, with this localization being associated with palmitoylation.[166,167]

The presynaptic protein CSP is a molecular chaperone that plays an essential role in regulated exocytosis pathways and is probably one of the most heavily palmitoylated proteins on a per mole basis.[164] The cysteine-rich domain of mammalian CSP contains 14 cysteines in a span of 24 amino acids, with the majority of these cysteines being palmitoylated.[164,168] S-acylation is essential for its association with membranes, subcellular trafficking, and activity.[164,168]

Taken together, these above examples illustrate the importance of S-acylation at several stages of intracellular trafficking. Moreover, they highlight the danger of using general palmitoylation/depalmitoylation inhibitors to infer the role of palmitoylation in the localization and transport of a particular protein, under the conditions in which the whole trafficking machinery is affected.

6 | CONCLUSIONS AND OPEN QUESTIONS

In summary, this review emphasizes the importance of S-acylation on protein-membrane association and intracellular trafficking. We expect

that emerging technologies will increase our ability to explore in a more systematic mode the functional consequences of fatty acid acylation of proteins, including S-acylation as well as the in vivo dynamics of these co- and posttranslational modifications. The development of specific activators and inhibitors for PATs and APTs is crucial for the field and will certainly open new research avenues. Particularly, it will allow exploring if the manipulation of protein palmitoylation represents an opportunity to fight diseases in which acylation or its misregulation is involved. In this sense, greater advances have been obtained for APTs than for PATs, with the recent discovery of the human APT1 and APT2 crystal structures, as well as isoform-selective inhibitors, which have provided new ways to probe the function of each enzyme.[40] More importantly, these pharmacological approaches represent a substantial advance for the development of rational therapies to control the oncogenic pathways driven by acylated proteins, as recently described for H- and N-Ras,[169,170] and also for the Scrib tumor suppressor in Snail-overexpressing epithelial cells,[171] in which a common feature of the inhibitors was to modulate the membrane binding and intracellular distribution of the target protein. Finally, an important aspect still to be elucidated in the field is the determination of the stoichiometry and dynamics of palmitoylation in proteins with multiple palmitoylation sites, with the interplay between PAT and APT activities being critical for this process. Undoubtedly, the physiological or pathological implications of the heterogeneous distribution of acylated species have been underestimated and poorly studied mainly due to methodological limitations, which will surely be overcome in the near future.

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Conflict of interests

The authors declare no conflict of interests.

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