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Review

Protein palmitoylation and subcellular trafficking

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ABSTRACT

Protein S-palmitoylation, the covalent lipid modification of the side chain of Cys residues with the 16-carbon fatty acid palmitate, is the most common acylation of proteins in eukaryotic cells. This post-translational modification provides an important mechanism for regulating protein subcellular localization, stability, trafficking, translocation to lipid rafts, aggregation, interaction with effectors and other aspects of protein function. In addition, N-terminal myristoylation and C-terminal prenylation, two well-studied post-translational modifications, frequently precede protein S-palmitoylation at a nearby spot of the polypeptide chain. Whereas N-myristoylation and prenylation are considered essentially irreversible attachments, S-palmitoylation is a tightly regulated, reversible modification. In addition, the unique reversibility of protein palmitoylation also allows proteins to rapidly shuttle between intracellular membrane compartments in a process controlled, in some cases, by the DHHC family of palmitoyl transferases. Recent cotransfection experiments using the DHHC family of protein palmitoyl transferases as well as RNA interference results have revealed that these enzymes, frequently localized to the Golgi apparatus, tightly control subcellular trafficking of acylated proteins. In this article we will give an overview of how protein palmitoylation regulates protein trafficking and subcellular localization.

1. Protein modification by lipids

It was 60 years ago when Folch and Lees [1] described the covalent modification of proteins with lipids for the first time in brain tissue. The covalent attachment of lipids to proteins plays different and important roles in their localization and function. It can be easily understood that lipidic modifications modulate the association of the modified proteins with cellular membranes. However, the effect of the lipid attachment goes further away, since it mediates membrane association of soluble proteins, protein–protein interactions, protein trafficking, subcellular targeting, partitioning of proteins into specific membrane domains, changes on structure and regulation of protein stability [2–6].

Proteins are covalently modified with a great variety of lipids: octanoic acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, a farnesyl or geranylgeranyl group, cholesterol, etc. (Box 1). Most of these modifications take place in the cytoplasm or in the cytoplasmic face of membranes. This review will focus on protein thioacylation, also called S-acylation (from now on palmitoylation), a reversible post-translational attachment of a palmitic acid (C16:0) group onto cysteine residues via a thioester linkage. Protein palmitoylation is a modification dynamically regulated by enzymes, that is, the lifetime of the modification is shorter than the lifetime of the protein it is modifying.

The amount of S-palmitoylated proteins is large and diverse; some of them are synthesized on soluble ribosomes but others are transmembrane-spanning proteins. Some of these proteins are modified sequentially with different lipids, but others are exclusively S-palmitoylated. In the past few years, the development of non-radioactive labeling techniques has allowed researchers to analyze the yeast [7], *Drosophila* [8], mouse [9], dendritic cell line [9] and Jurkat T-cell [10] palmitoylome. This large output of novel data regarding palmitoylated proteins has revealed previously unrecognized protein palmitoylation sites. In addition, using available databases together with reported palmitoylation sites in the literature prediction algorithms for palmitoylated proteins has also been developed [11,12].

Despite protein palmitoylation first being discovered several decades ago, the identification of palmitoylating enzymes has been difficult, mostly due to the fact that the cellular enzymes responsible for catalyzing the palmitate attachment to other protein substrates are membrane-bound and difficult to purify. The seminal work of Linder and Deschenes using the yeast *Saccharomyces cerevisiae* as model organism revealed that the Erf2–Erf4 (*effect on Ras function*) complex is responsible for yeast Ras2p palmitoylation [13–15]. Further analysis revealed that Erf2, a membrane integral protein, contains a conserved aspartate–histidine–histidine–cysteine (DHHC) cysteine-rich domain of approximately 55 amino acids, somehow resembling a C_2H_2 zinc finger motif, considered the active site [15]. Whereas there are seven DHHC proteins expressed in yeast, approximately 24 DHHC genes are present in mammalian cells (25

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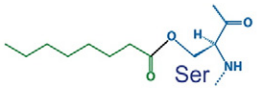
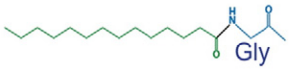
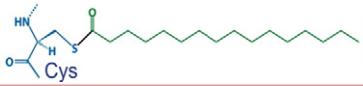
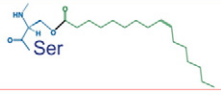
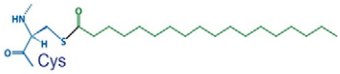
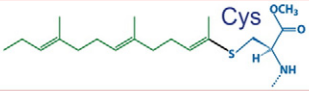
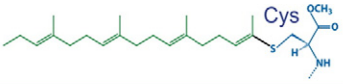
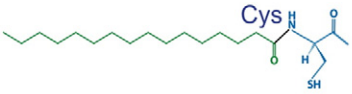
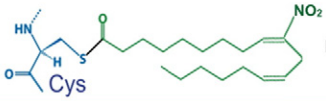
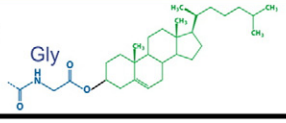
Box 1

Protein lipidation in mammalian cells. The attached lipid is shown in green whereas the protein moiety is shown in blue. The covalent bond that links the lipid to the protein is depicted in black. When known, the enzyme that catalyzes the lipidation process is shown. A representative lipidated protein example is also shown [35,198–202].

Attached lipid	Amino acid that becomes modified	Molecular structure	Enzyme that catalyzes the attachment	Representative protein	Reference
Octanoic acid	Serine		Ghrelin O-acyl Transferase (GOAT)	Ghrelin	[28]
Myristic acid	N-terminal Glycine		N-Myristoyl Transferase	Recoverin	[198]
Palmitic acid	Cysteine		Palmitoyl Transferases	PSD-95	[199]
Palmitoleic acid	Serine		Porcupine	Wnt3a	[200]
Stearic acid	Cysteine		Unknown	GAP-43	[35]
Farnesyl group	Cysteine		Farnesyl Transferase	H-Ras	[63]
Geranylgeranyl group	Cysteine		Geranylgeranyl Transferase I	RhoA	[136]
Palmitic acid	N-terminal Cysteine		Hedgehog acyl Transferase (Hhat)	Hedgehog	[201]
10-nitro-9,12-*cis*-octadecadienoic acid	Cysteine		Non-enzymatic	Keap1	[37]
Cholesterol	Carboxylate group of a carboxy-terminal Glycine		Autocatalytic	Hedgehog	[202]

in humans [16]), all of them multispanning transmembrane enzymes [17,18]. The palmitoyl-transferase activity of many of these DHHC motif-containing proteins has been shown in coexpression experiments using a growing number of protein substrates (PSD95, SNAP25, eNOS, Fyn, GAP43, R-Ras, etc.) [18]. In addition depletion of the protein levels of a certain DHHC palmitoyl transferase isoform has also been shown to decrease the amount of palmitate that becomes incorporated in the substrate protein [19,20]. When the subcellular localization of GFP-tagged human DHHC isoforms was analyzed in transfected HEK293 cells multiple staining patterns could be observed. Certain isoforms, such as DHHC1 and DHHC6 localized to the ER, DHHC2 and DHHC9 localized to the ER/Golgi and isoforms DHHC7 and DHHC8 localized to the Golgi. Remarkably, isoforms DHHC5, DHHC20 and DHHC21 displayed a distinctive plasma membrane localization [17]. As we will see below, the palmitoyl-transferases of the DHHC family regulate the subcellular localization of multiple protein substrates and also affect their proper intracellular sorting. Certainly, protein palmitoylation has been shown to act as a highly versatile sorting signal, regulating protein trafficking to many distinct intracellular compartments. In this regard, palmitoylation is known to regulate either retention or anterograde trafficking of proteins at the ER–Golgi as well as protein cycling within the endosomal/lysosomal system [5,21]. Before focusing on protein palmitoylation we will comment on other types of protein acylation. Selected examples of each of them and the proteins that catalyze these modifications are shown in Box 1.

Remarkably, not only protein acylation is an enzymatic process, but also cellular protein deacylation, a process catalyzed by enzymes that remove the acyl moiety. Although some deacylases might remain to be discovered, the catalytic activity of protein thioesterases APT1 (Acyl Protein Thioesterase 1) and also PPT1 (Protein-Palmitoyl Thioesterase) are well characterized [22]. For instance, APT1 overexpression is responsible for SNAP-23, GαQ and eNOS depalmitoylation [23–25]. On the other hand, PPT1 is very likely involved in the degradation of S-acylated proteins [26].

Ghrelin, a circulating peptide hormone that stimulates food intake and adiposity in humans, is synthesized as a precursor protein that renders the 28-amino acid mature octanoylated ghrelin after

intracellular processing [27]. Ghrelin, like many other peptide hormones, is generated from a precursor protein. Prepro-ghrelin contains 117 amino acids [28]. After removing the signal sequence by cleavage after amino acid-23, the pro-ghrelin peptide sequence (94 amino acids) becomes the substrate of a prohormone convertase PC1/3 which then cleaves pro-ghrelin after Arg28, generating the mature 28-amino acid ghrelin peptide. During these post-translational modifications ghrelin becomes octanoylated, thus forming an ester bond with the side chain of Ser3. The nonacylated form of ghrelin also exists at significant levels in both stomach and blood, although it does not replace radiolabeled acylated ghrelin at its binding sites in hypothalamus and pituitary and shows no growth hormone-releasing and other endocrine activities in rats [28]. This covalent attachment of octanoic acid to Ser3 of ghrelin is catalyzed by a Membrane Bound O-Acyl Transferase (MBOAT) [29,30]. Members of this MBOAT family are also multispanning transmembrane enzymes that usually catalyze the addition of a fatty acid to a hydroxy group. Two additional members of this family are Hedgehog acyltransferase (Hhat) which facilitates the N-terminal palmitoylation of Hedgehog and Porcupine (Porc) which catalyzes the acylation of Ser209 of Wnt with palmitoleic acid [31] (Box 1). In fact, all members of the Hedgehog family of morphogens undergo a unique series of post-translational processing reactions involving palmitoylation and cholesterol attachment. [32]. Palmitoylation of Hedgehog is essential for proper signaling and mutation of the N-terminal Cys to Ser diminishes Sonic hedgehog patterning activity in the mouse limb and neural tube [33,34]. In *Drosophila*, the absence of palmitoylation of Hedgehog essentially eliminates signaling activity [33].

Interestingly, there can be certain heterogeneity in the fatty acids that are naturally attached to a specific protein in vivo. Using mass spectrometry to identify the acyl groups attached to the N-terminus of GAP43 (neuromodulin) purified from transfected mammalian cells, Resh and coworkers found not only palmitate (C16:0), but also stearate (C18:0) [35]. The finding that GAP43 can be heterogeneously fatty acylated might indicate that either palmitoyl transferases use several acyl-CoA species as substrates or that other, yet unrecognized, cellular palmitoyl transferases exist that can transfer longer fatty acids such as stearate to protein substrates.

In addition, covalent post-translational protein adduction by electrophilic fatty acid derivatives is known to alter the structure, trafficking and catalytic activity of many unrelated proteins such as insulin, cathepsin B, glyceraldehyde-3-phosphate dehydrogenase and Keap1 [36,37]. For instance, alkylation of Keap1 cysteines with nitrated derivatives of linoleic acid (such as 10-nitro-9,12-cis-octadecadienoic acid) (Box 1) when part of the Keap1/Nrf2 heterodimer induces a conformational change in Keap1 hence liberating Nrf2 to translocate to the nucleus, bind to the *cis*-acting DNA regulatory antioxidant response element and thereby transactivating Nrf2-dependent gene transcription [37].

Perhaps the best understood protein acylation process is N-myristoylation, a cotranslational modification which involves the covalent addition of the C14:0 saturated fatty acid myristate via an amide bond to the N-terminal glycine residue after the initiating methionine is cleaved by an aminopeptidase [38]. This protein modification is catalyzed by a well-characterized enzyme called N-Myristoyl Transferase (NMT) that uses myristoyl-CoA and the peptide N-terminus as cosubstrates after recognition of a GXXX(S/T/C) N-terminal consensus sequence [39,40]. It has been estimated that approximately 0.5% of eukaryotic proteins are myristoylated [41]. In many cases, protein N-myristoylation is required but not sufficient for stable and permanent membrane anchoring [42–44] and often occurs together with S-acylation of proximal cysteine residues or a polybasic amino acid domain next to the protein N-terminus. N-myristoylation can also occur post-translationally when an internal glycine becomes exposed by caspase-mediated proteolytic cleavage and the resulting proteolytic product becomes a NMT substrate [45]. When N-myristoylation and palmitoylation concur at the N-terminal end of a protein the former covalent modification is considered irreversible whereas the latter is reversible and modulated hence regulating protein localization and function. Remarkably, palmitoylation of a previously myristoylated de novo-designed protein that lacked hydrophobic or basic amino acids required the palmitoylatable Cys residue to be positioned no further away than position 15 [44]. A few selected examples of myristoylated plus palmitoylated human proteins are shown in Box 2. Several palmitoyl transferases of the DHHC family are known to increase palmitate transfer to previously myristoylated protein substrates [19]: that is the case for eNOS [46], Giα2 [47], Fyn [48] and Lck kinase [49].

Recent reports seem to suggest that the Golgi apparatus might function as a hub for palmitoylation of peripheral proteins [50], and in fact, this might be well the case for certain protein substrates of the DHHC family of palmitoyl transferases. Dispersal of the cis and medial Golgi using brefeldin A treatment, which inactivates Arf1, is known to lead to the dissociation of COP I and other peripheral proteins from Golgi membranes, resulting in Golgi enzymes redistributing to the endoplasmic reticulum as the Golgi structure disassembles [51]. Although brefeldin A may display non-specific effects in addition to these aforementioned effects on Golgi traffic, sensitivity to brefeldin A has involved the Golgi apparatus in the palmitoylation of tetraspanin proteins CD151 and CD9 [52] as well iNOS [53]. Conversely, palmitoylation of other proteins, such as Lck kinase or H-Ras is unaffected by brefeldin A treatment, even if their transport toward the plasma membrane is partially inhibited [54–56]. In addition, p63 an ER resident protein that is normally palmitoylated in low levels was found to increase palmitoylation significantly in cells treated with brefeldin A, which suggests that a Golgi-associated palmitoyl transferase might redistribute toward the ER [57]. Subsequent studies have identified the p63 palmitoyl transferase as DHHC2 [58].

Prenylation, a post-translational modification which is thought to take place in the cytoplasm, involves the formation of a covalent thioether bond between a cysteine residue of the target protein and the 15 or 20-carbon isoprenoids farnesylpyrophosphate (FPP) or geranylgeranylpyrophosphate (GGPP), respectively [59] (Box 1). The isoprenylated cysteine is part of a C-terminal CaaX box (where "C" is cysteine, "a" is usually an aliphatic residue and "X" is usually Ser, Met,

Box 2

Selected examples of human proteins that become N-terminally myristoylated and palmitoylated. The myristoylated Gly residue is shaded in green whereas the palmitoylated Cys residues are shaded in yellow. Positively and negatively charged residues are shaded blue and red respectively. 25 amino acids are shown in every case.

Protein	N-terminal sequence
Tyrosine kinases of the Src family	
Yes (Yamaguchi sarcoma homolog, p61)	MGCIKSKENKSPAIKYRPENTPEPVSTSVS...
Fyn (p59)	MGCVQCKDKEATKLTEERDGSLNQSSGYRY...
Lyn (Lymphocytes)	MGCIKSKGKDSLSDDGVDLKTQPVPESQLL...
Lck (T-cell specific kinase, p56)	MGCGCSSHPEDDWMENIDVCENCHYPIVPL...
Hck (Hematopoietic cells kinase, p59)	MGCMKSKFLQVGGNTFSKTETSASPHCPVY...
Fgr (Gardner-Rasheed feline sarcoma viral)	MGCVFCKKLEPVATAKEDAGLEGDFRSYGA...
Alpha subunits of G proteins	
Gα O1	MGCTLSAEERAALERSKAIEKNLKEDGISA...
Gα Z	MGCRQSSEEKEAARRSRRIDRHLRSESQRQ...
Gα i1	MGCTLSAEDKAAVERSKMIDRNLREDGEKA...
Gα olfactory	MGLCYSLRPLLFGGPGDDPCAASEPPVEDA...
Endothelial Nitric Oxide Synthase (eNOS)	MGNLKSVAQEPGPPCGLGLGLGLGLCGKQG...
A-kinase anchor protein 7	MGQLCCFPFSRDEGKISELESSSSAVLQRY...

Cys, Ala, Gln or Leu, and the identity of this final amino acid determines if the protein becomes farnesylated or geranylgeranylated) [60]. After prenylation, processing of the CaaX box involves the proteolytic cleavage of the final three amino acids followed by the methylation of the free carboxyl group of the isoprenylated cysteine [61]. In the case of isoprenylated proteins, this acylation is assumed to promote the interaction of the modified protein with cellular membranes so that a subsequent palmitoylation can occur in cysteine residues adjacent to the farnesylated or geranylgeranylated cysteine of the CaaX box. In all cases tested, site-directed mutagenesis of the Cys residue from the CaaX box, hence abrogating farnesylation or geranylgeranylation results in the absence of palmitoylation [62,63]. Eight members of the Ras family of small GTPases (Box 3) and seven members of the RhoB family of small GTPases (Box 4) display palmitoylatable cysteine residues.

Among the Ras family of small GTPases, palmitoylation of H-Ras at Cys181 and Cys184 and N-Ras at Cys181 was described as early as 1989 [63]. Subsequently, other members of this family displaying Cys residues proximal to the prenylation site were also analyzed in terms of palmitate incorporation, and soon K-Ras4A [64], all three Rap2A, Rap2B and Rap2C [9,10,65] and R-Ras [66,67] were found to incorporate this fatty acid.

Among the RhoB family of small GTPases, palmitoylation has also been shown to occur in cysteine residues that are adjacent to the isoprenylated Cys of the CaaX box. Using radiolabeled palmitate both cysteine residues at 189 and 192 upstream of the CaaX box in RhoB have been shown to be sites for palmitoylation [68]. Likewise, TC10 palmitoylation has been shown using 2Br-palmitate as a palmitoylation inhibitor, since this compound induced significant localization changes on a GFP-tagged chimera transfected in mammalian cells [69]. The possibility of Rac1 becoming palmitoylated in vivo is currently under debate with results from various groups being contradictory (see below).

There are excellent recent reviews dealing with palmitoylation and sorting of H-Ras, N-Ras, SNAP25, G-Protein Coupled Receptors as well as transmembrane proteins such as the co-receptors CD4 and CD8 and the adaptor LAT [70–75]. In addition to all these proteins, we will also describe herein other selected examples of proteins in which palmitoylation regulate intracellular sorting, localization and function as well.

2. Subcellular trafficking of myristoylated plus palmitoylated proteins

Although protein myristoylation increases the total hydrophobicity of the modified protein, this modification, by itself, is not sufficient to promote membrane association [44,76,77]. In fact, certain myristoylated proteins, such as some Guanylate Cyclase Activating Proteins are soluble proteins that hide the myristoyl moiety within the hydrophobic protein core [78]. In contrast, two closely positioned lipid modifications, such as myristoylation and palmitoylation, promote stable membrane attachment [43]. The N-terminal palmitoylation of previously myristoylated proteins promotes membrane association and, very frequently, protein translocation to rafts/caveolae or intracellular liquid-ordered domains [76,77,79,80]. In dually acylated proteins, myristoylation is always a prerequisite for palmitoylation to occur, since mutation of the N-myristoylation site through the elimination of Gly2 prevents subsequent palmitoylation and typically results in protein translocation to the cytoplasm and a complete absence of membrane association [44,76,77,81,82].

Most of the members of the Src family of protein tyrosine kinases (although not Src itself) and the Giα subfamily of alpha subunits of G proteins are cotranslationally N-myristoylated and subsequently post-translationally palmitoylated. This dual acylation is responsible for the correct subcellular trafficking and the precise coupling of extracellular stimuli and intracellular signaling [4,83,84]. A few selected examples of dually acylated proteins will be described in more detail below.

2.1. Tyrosine kinases of the Src family

With the exception of Src and Blk, all members of the Src family of tyrosine kinases are cotranslationally myristoylated at Gly2 and then post-translationally palmitoylated at Cys3 (Box 2). In addition, Lck can also become palmitoylated at Cys5 and Fyn and Fgr at Cys6 [85,86]. In all cases, these acylations occur in the N-terminal SH4 domain, and influence the interaction of the acylated proteins with cellular

Box 3

Selected examples of human small GTPases of the Ras family with palmitoylatable Cys residues in their carboxy-terminus. The CaaX box is shaded in green whereas the palmitoylated Cys residues are shaded in yellow. Positively and negatively charged residues are shaded blue and red respectively. 25 amino acids are shown in every case.

GTPase	Carboxy-terminal sequence	Farnesylated or Geranylgeranylated
H-Ras	...QHKLRKLNPPDESGPGCMSCKCVLS	Farnesyl
N-Ras	...QYRMKKLNSSDDGTQGCMGLPCVVM	Farnesyl
K-Ras4A	...QYRLKKISKEEKTPGCVKIKKCIIM	Farnesyl
Rap2A	...EIVRQMNYAAQPDKDDPCCSACNIQ	Farnesyl
Rap2B	...EIVRQMNYAAQPNGDEGCCSACVIL	Geranylgeranyl
Rap2C	...EIVRQMNYSSLPEKQDQCCTTCVVQ	Farnesyl
R-Ras	...QEQELPPSPPSAPRKKGGGCPCVLL	Geranylgeranyl
R-Ras2/TC21	...EQECPPSPEPTRKEKDKKGCHCVIF	Geranylgeranyl

Box 4

Selected examples of human small GTPases of the RhoB family with palmitoylatable Cys residues in their carboxy-terminus. The CaaX box is shaded in green whereas the palmitoylated Cys residues are shaded in yellow. Positively and negatively charged residues are shaded blue and red respectively. Please note that TCL has two Cys residues upstream the CaaX box in which incorporation of palmitate has not yet been described. The CaaX box of Wrch-1 is non-functional (see text for details) whereas Wrch-2 lacks a CaaX box. 25 amino acids are shown in every case.

GTPase	Carboxy-terminal sequence	Farnesylated or Geranylgeranylated
RhoB	...ETATRAALQKRYGSQNGCINCCKVL	Farnesyl or Geranylgeranyl
TC10/RhoQ	...AILTPKKHTVKKRIGSRCINCCLIT	Farnesyl or Geranylgeranyl
TCL	...AILTIFHPKKKKKRCSEGHSCCSII	Farnesyl
Rac1	...VFDEAIRAVLCPPPVKKRKRKCLLL	Geranylgeranyl
Rac2	...VFDEAIRAVLCPQPTRQQKRACSLL	Geranylgeranyl
Rac3	...VFDEAIRAVLCPPPVKKPGKKCTVF	Geranylgeranyl
Wrch-1/RhoU	...KSKSRTPDKMKNLSKSWWKKYCCFV	----------
Wrch-2/Chp	...LEKKLNAKGVRTLSRCRWKKFFCFV	----------

membranes [76,77]. It is generally accepted that Src family kinases are predominantly located at the cytoplasmic face of the plasma membrane through myristoylation and subsequent palmitoylation, although appreciable fractions are also found in the Golgi complex, phagosomes, endosomes and secretory granules [76,87]. Site directed mutagenesis studies have revealed that myristoylation is always a prerequisite for subsequent palmitoylation and by itself mediates nuclear exclusion. In addition, the non-palmitoylated myristoylated kinases are unable to associate productively with the plasma membrane and cannot mediate signaling [76,88–91].

It has been proposed that newly synthesized myristoylated kinases such as Lyn and Yes initially enter the Golgi system where they become singly palmitoylated at Cys3 hence providing access to the membrane secretory transport pathway en route to the plasma membrane [85,87]. In addition, Rab11 might be involved in the exocytic transport of Lyn and Yes. However, a non-vesicular traffic has been proposed in the case of the direct plasma membrane-targeting pathway for myristoylated and dually palmitoylated Fyn [89].

Cotransfection experiments have shown that Lck palmitoylation is considerably augmented in the presence of DHHC3, DHHC17, DHHC18 and DHHC21 [19,47,49] whereas Fyn palmitoylation increases when cotransfected with DHHC2, DHHC3, DHHC7, DHHC10, DHHC15, DHHC20 and DHHC21 [19,48] .

2.2. eNOS

The endothelial isoform of Nitric Oxide Synthases (eNOS) is responsible for nitric oxide synthesis in the cardiovascular system. Nitric oxide serves as an endogenous vasodilator, antioxidant, and platelet inhibitor and regulates the vascular endothelium by sustaining its anti-thrombogenic and anti-coagulant properties. In quiescent endothelial cells, eNOS is specifically targeted to the Golgi apparatus and also toward small invaginations of the plasma membrane called caveolae. These invaginated membrane microdomains are defined by the presence of the tripalmitoylated scaffolding protein caveolin [92,93]. Caveolae not only sequester eNOS, but also diverse receptors and signaling proteins from a variety of signal transduction pathways, including G-protein coupled receptors, G-proteins, growth factor receptors and calcium channels. In endothelial cells, eNOS is activated in response to a variety of stimuli, including acetylcholine, thrombin, histamine, bradykinin, substance P, ATP, endothelin-1, angiotensin II and vascular endothelial growth factor. In fact, eNOS interacts directly with bradykinin B2 receptor, angiotensin II AT1 receptor and endothelin-1 receptor [94]. On the other hand, caveolin-1 is an eNOS negative effector since it binds directly to eNOS and inhibits its activity [95–97].

eNOS is N-terminally myristoylated and subsequently palmitoylated on cysteines 15 and 26 [98,99]. The presence of multiple hydrophobic amino acids in the proximity of those Cys residues (a $(Gly\text{-}Leu)_5$ repeat) is probably responsible for the linkage of the palmitate moiety in such distant positions (Box 2). In fact, mutagenesis of the five Leu residues which flank the palmitoylated cysteines prevents palmitoylation [82]. Both the myristoylation and palmitoylation sites of eNOS lie outside the catalytic core of the enzyme, and purified recombinant eNOS lacking the N-terminal 52 amino acids shows identical activity as its wild-type counterpart [100]. When transfected HEK293 cells express identical amounts of wild-type eNOS or the palmitoylation-defective Cys15/26Ser mutant, their catalytic activity is undistinguishable [101]. However, cell treatment with ionomycin results in a three-fold increase in nitric oxide synthesis in cells expressing wild-type eNOS when compared with its palmitoylation-defective counterpart. Hence, palmitoylation is responsible for the appropriate subcellular localization of eNOS and the stimulation-dependent release of nitric oxide [101]. In fact, the Cys15/26Ser mutant of eNOS, although membrane associated to similar extent as its wild-type counterpart [82,101], fails to selectively target eNOS toward the plasma membrane caveolae [102,103]. Consequently, the role of palmitoylation is to target eNOS into caveolae, hence restricting nitric oxide signaling to specific targets within a limited microenvironment at the cell surface and modulating signal transduction through caveolae [103]. Likewise, studies performed with synthetic peptides corresponding to the N-terminus of eNOS have revealed that the myristoylated plus doubly palmitoylated peptide associated preferentially with membranes enriched in cholesterol and sphingomyelin, two lipid species highly enriched in caveolae/rafts [104].

Elegant studies performed in endothelial cells with GFP-tagged eNOS mutant proteins have shown that the Cys15/26Ser palmitoylation-defective mutant of eNOS is excluded from the plasma membrane and was concentrated in a diffuse perinuclear pattern [92]. In addition, fluorescence recovery after photobleaching of the palmitoylation mutant is two times faster than that of wild-type eNOS-GFP, indicating that palmitoylation can influence the rate of trafficking. In fact, palmitoylation of eNOS renders the protein less mobile in lipid bilayers, thus supporting the idea that this post-translational modification is a "kinetic trapping mechanism" that mediates eNOS interactions in Golgi and plasmalemmal microdomains [92]. Finally, overexpression of Acyl-Protein Thioesterase-1 (APT-1) accelerates the depalmitoylation of eNOS in COS7 cells cotransfected with eNOS and APT1, and remarkably, the APT1-catalyzed depalmitoylation of eNOS is potentiated by Ca^{2+}-calmodulin, a key allosteric activator of eNOS [23].

3. Subcellular trafficking of isoprenylated plus palmitoylated proteins

For small GTPases of both the Ras (Box 3) and RhoB (Box 4) families palmitoylation occurs in the hypervariable domain, which is located close to the C-terminal end of the protein. The hypervariable region of Ras and Rho GTPases contains all of the targeting information necessary to regulate localization on cellular membranes [69] and the precise subcellular localization of these GTPases contributes to their functions [105]. Palmitoylation frequently occurs on Cys residues that are in the proximity of the isoprenylated Cys residue of the CaaX box, independently of this being farnesylated or geranylgeranylated. Exceptions to this rule are Wrch-1 and Wrch-2 that are not isoprenylated but palmitoylated at Cys residues positioned at the carboxy terminus.

3.1. Small GTPases of the Ras family

After farnesylation of the cysteine in the CaaX-box, the various Ras isoforms are targeted to the cytosolic surface of the endoplasmic reticulum where the CaaX processing occurs [106,107]. Whereas the prenyltransferases are soluble heterodimers [108], the activities of the enzymes that act subsequent to prenyltransferase, including the prenyl-CaaX protease [109], prenylcysteine-directed carboxylmethyltransferase [110] and palmitoyl transferases [19,20] are associated with membranes. Whereas the first 185 amino acids of all Ras proteins exhibit a high degree of homology between isoforms and contain the nucleotide and effector interacting domains required for signaling, acylation occurs within the hypervariable domain positioned at the carboxy-terminus (Box 3). Mammalian H-Ras isoforms become isoprenylated at Cys186 and doubly palmitoylated at Cys residues 181 and 184. It is generally accepted that palmitoylation takes place in the ER/Golgi region, with this acylation being required for intracellular trafficking. The prenylated, carboxyl-methylated, but unpalmitoylated form of H-Ras can undergo rapid, non-vesicular exchange with other cellular membranes or can undergo palmitoylation and vesicle-mediated trafficking to the plasma membrane (Fig. 1). When modified by both farnesyl and palmitate, H-Ras is ~95% membrane bound and fully active whereas with farnesyl as the only lipid ~10% of H-Ras is attached to membranes and biological activity is severely compromised [111]. Indeed, the extended conformation of

the palmitoyl moieties for membrane-bound H-Ras-GDP results in deeper insertion into the bilayer than in the GTP-loaded conformation [112].

The regulation of H-Ras through acylation–deacylation cycles has been recently summarized in numerous excellent review articles [74,75,113,114], so only a summary of the current knowledge will be included below. The abundant experimental evidence (detergent resistance experiments, antibody patching, FRAP, electron microscopy, etc.) suggests that doubly palmitoylated H-Ras is raft-associated [115,116] (Fig. 1). Ras isoform sublocalization on the plasma membrane depended heavily on electron microscopic analysis performed by the Hancock and Parton groups. Remarkably, only the inactive, GDP-loaded H-Ras is found raft-resident, whereas activation through GTP binding leads to its translocation to non-raft domains where it activates its downstream kinase Raf [74,114]. Insolubility in Triton X-100, a widely used assay for lipid raft association, was used to determine that approximately 13% of H-RasGly12Val (activated GTP-bound) was insoluble in low concentrations of this detergent, compared with 34% of its wild-type counterpart [115]. Thus, H-Ras palmitoylation enables access to cholesterol-sensitive nanodomains or clusters. Interestingly, the positioning of the palmitate moiety relative to the farnesylated C-terminal cysteine is important for both trafficking and eventual subdomain translocation within the plasma membrane. H-Ras palmitoylated on Cys181, a position shared by N-Ras and H-Ras, traffics to cell surface cholesterol-dependent nanoclusters, whereas mutant H-Ras monopalmitoylated on Cys184 remains confined in the Golgi area [117]. It has been known for a while that palmitoylation is labile, and that H- and N-Ras activation radically decreases the half-life of the attachment of their palmitoyl groups from hours to minutes [118,119].

As in the case of H-Ras, N-Ras palmitoylation is indispensable for its cellular activity. For instance, Cys181 palmitoylation is essential for leukemogenesis by oncogenic N-Ras [120]. Intriguingly, a dependence of the balance between cholesterol-dependent and independent nanoclusters on the GDP/GTP loading state has also been observed for N-Ras, although in this case active GTP-bound N-Ras preferentially localizes to cholesterol-sensitive clusters [117]. Alternatively, other researchers using time-lapse tapping-mode atomic force microscopy, have concluded that partitioning of N-Ras occurs preferentially into the liquid-disordered/liquid-ordered phase boundary region and neither the GDP-bound nor the activated GTP-bound N-Ras could be detected in the bulk raft-like lo domains [121]. The most likely scenario suggests that when N-Ras is farnesylated and palmitoylated at Cys181 strong intermolecular interactions foster N-Ras self-association and formation

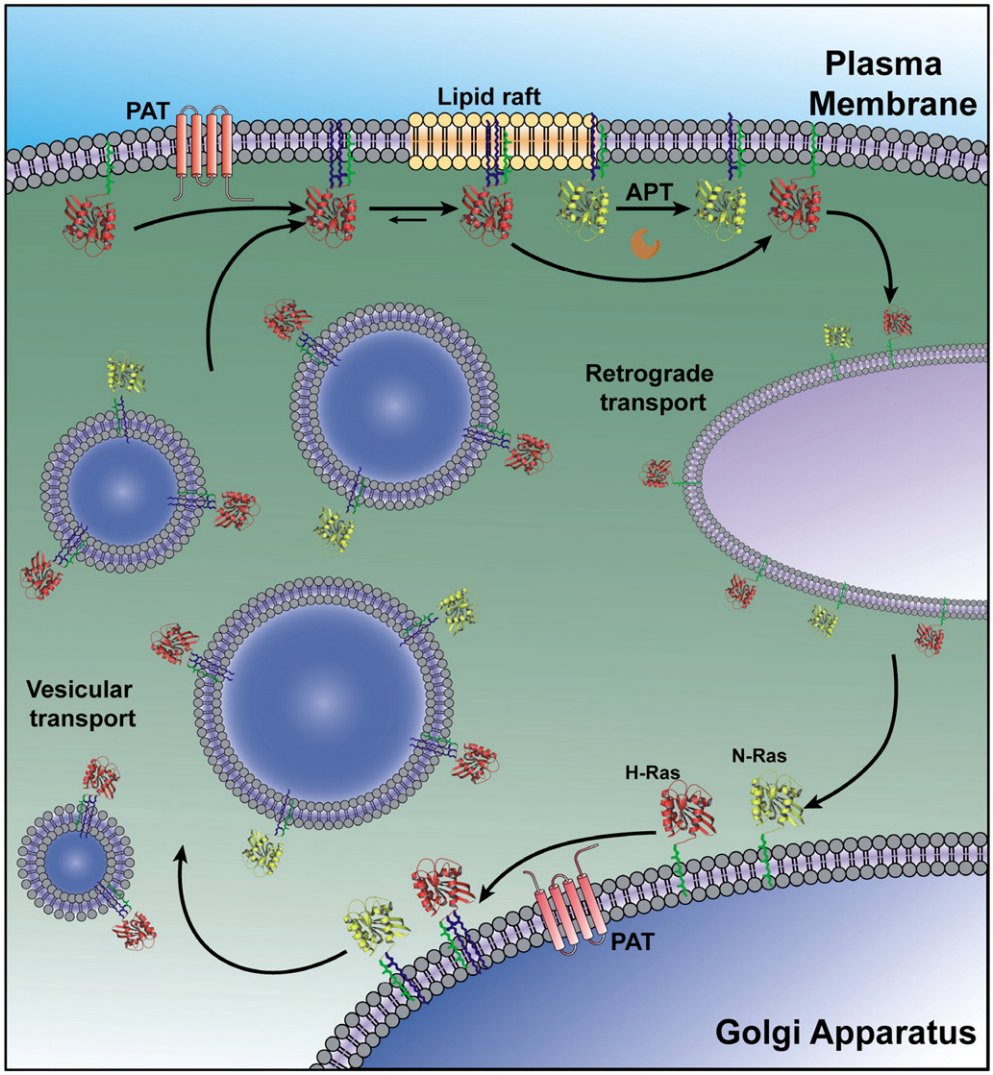


Fig. 1. Intracellular palmitoylation–depalmitoylation cycle of H-Ras and N-Ras. Both H-Ras (red) and N-Ras (purple) can become palmitoylated in the Golgi apparatus by a transmembrane Palmitoyl-Acyl-Transferase (PAT). Palmitoylated H-Ras and N-Ras then traffic toward the plasma membrane using vesicular transport. Palmitoylated H-Ras can translocate to lipid rafts whereas palmitoylated N-Ras typically becomes enriched in lipid raft boundaries. The action of an Acyl Protein Thioesterase (APT) depalmitoylates H-Ras and N-Ras which can traffic in a retrograde manner toward endomembranes and the Golgi apparatus where they become repalmitoylated (please see text for details).

of nanoclusters at the domain boundaries, which might serve as a reaction platform for GTP activation and for recruitment of further membranous and cytosolic regulators as well as downstream effectors, such as Gap and Raf [121]. On the other hand, inactive GDP-loaded farnesylated N-Ras is proposed to partition into the fluid-like phase of the membrane and subsequently diffuses to the ld/lo phase boundaries [74,121].

The general model for H-Ras and N-Ras trafficking en route for the plasma membrane and recycling toward perinuclear locations has been elegantly analyzed by Bastiaens and coworkers [16,114,122]. Farnesylated non-palmitoylated H-Ras and N-Ras are distributed homogenously among membrane compartments in the cell. The palmitoyl transferase activity, located to the Golgi apparatus [122] then enables this membrane compartment to trap newly palmitoylated H-Ras and N-Ras. This Golgi trapping occurs because palmitoylation enhances the stability of the interaction of H-Ras and N-Ras with the bilayer, thereby slowing its diffusion [123]. However, H- and N-Ras do not accumulate at the Golgi, but start their transit toward the plasma membrane via the secretory vesicular pathway [16,124]. Once H- and N-Ras are away from the palmitoyl transferase activity of the Golgi, the palmitoyl moiety is removed by the ubiquitous thioesterase activity (Fig. 1). This depalmitoylated, farnesylated H-Ras (or N-Ras) redistributes over all endomembranes, and this retrograde transport enhances the chance of re-encounter with a DHHC palmitoyl transferase and becoming trapped in the Golgi [114]. The repeated cycles of de/repalmitoylation together with the Golgi trapping by palmitoylation and directionality of the vesicular secretory pathway thus comprise a spatially organizing system that counters the entropy-driven re-equilibration of lipidated Ras within cellular membranes. Truly, Ras depalmitoylation is important for correct localization, because when non-hydrolysable acyl groups are attached to H-Ras, it partitions non-specifically into the entire endomembrane system [16,122]. However, the plasma membrane cannot be evoked as the exclusive platform from which H-Ras regulates signaling. In fact, oncogenic H-Ras and N-Ras can engage Raf-1 on the Golgi membrane and, furthermore, Golgi-resident H-Ras and ER-resident unpalmitoylated H-Ras are activated in response to mitogens [125].

Finally, although both purified recombinant H-Ras and N-Ras are substrates for purified recombinant DHHC9 in vitro [126] short hairpin RNA-mediated knockdown of DHHC9 showed no detectable effect on Ras palmitoylation [16]. In fact, both H-Ras and N-Ras are ubiquitously expressed, whereas DHHC9 is not expressed in the thymus, skeletal muscle, spleen and leukocytes [17]. In addition, H-Ras palmitoylation is significantly increased when cotransfected with DHHC18 [49], a palmitoyl transferase that clearly exhibited a perinuclear staining pattern, most likely corresponding to the Golgi apparatus [17].

K-Ras occurs in two alternatively spliced forms: Ki(A)-Ras (or K-Ras4A) and Ki(B)-Ras (or K-Ras4B), deriving from *Kras-2* gene expression [127]. Using, radiolabeled palmitic acid, the palmitoylation of K-Ras4A was unambiguously shown [64]. The trafficking of alternatively spliced K-Ras4A has not been studied in detail, although as it is palmitoylated on Cys180 and has no polybasic domain (Box 3) it would be expected to follow a vesicular transport analogous to that of H-Ras and N-Ras.

The Ras superfamily of GTPases also includes Rap2A, Rap2B and Rap2C (Box 3), all three with two palmitoylatable Cys residues in tandem (Cys176 and Cys177) and in the proximity to the CaaX box. As expected, during its maturation, the Rap2 proteins become modified by the attachment of both palmitate and polyisoprenoid groups [65]. In human platelets palmitoylation of Rap2b, but not of Rap1b (which lacks palmitoylatable Cys residues), results in its translocation to lipid rafts. Furthermore, raft disruption by cholesterol depletion with methyl-β-cyclodextrin strongly impaired Rap2b activation [62]. Since Rap2 activity can be monitored through the activation of the Traf2- and Nck-interacting kinase (TNIK) the role of Cys176/Cys177 palmitoylation in activity was compared among Rap2A (farnesylated), Rap2B (geranylgeranylated) and Rap2C (farnesylated). In all three cases, palmitoylation enabled endosome localization and subsequent TNIK activation. Interestingly, non-palmitoylated Rap2A and Rap2C failed to activate TNIK whereas non-palmitoylated Rap2B, whose interaction with cellular membranes was stronger, activated TNIK effectively [128]. Taken together with the different subcellular fractionation data displayed by non-palmitoylated Ras2A, Ras2B and Ras2C isoforms, these results imply that endosome targeting and membrane-association are necessary and sufficient for TNIK activation.

R-Ras, which shows 55% identity with H-Ras, regulates cell adhesion, spreading and phagocytosis by activating integrins, and also incorporates radiolabeled palmitic acid [66]. As shown by site directed mutagenesis studies, the site of palmitoylation in R-Ras is Cys213 (Box 3) in the proximity of the geranylgeranylated Cys residue [67], hence indicating that, as expected, the hypervariable region of R-Ras is crucial for membrane targeting and transport. Moreover, the Cys213Ala mutant of R-Ras showed an increased accumulation in perinuclear Golgi areas and consequently a decreased appearance on the plasma membrane when compared with its wild-type counterpart. Remarkably, although intracellular trafficking of the palmitoylation-defective mutant of R-Ras is clearly altered, it can still reach focal adhesions [67]. The palmitoyl transferase DHHC19 can use R-Ras as a substrate, but not H-Ras, N-Ras, KRas4A, RhoB or Rap2 [129]. Cotransfection of DHHC19 with R-Ras increased the amount of palmitate that became incorporated in the small GTPase resulting in a stronger general membrane association and an increase in the association of R-Ras with lipid raft/caveolae. Interestingly, the carboxy-terminus of mammalian DHHC19 possesses a CaaX that can be farnesylated in vitro with purified farnesyltransferase heterodimer [129].

3.2. Small GTPases of the Rho family

Rho proteins are Ras-related GTPases that regulate a variety of cellular processes. Cellular RhoB localized in a pattern similar to that of H-Ras, with prominent fluorescence in the plasma membrane and in a discrete perinuclear structure, essentially the Golgi apparatus [69,130]. In addition, RhoB is also associated with early endosomes and a pre-lysosomal compartment [69,131]. Isoprenylation alone is not sufficient to determine the subcellular location of Rho GTPases and, as in the case of the Ras protein family, a second C-terminal signal is required. Inspection of the amino acid sequence of RhoB reveals the presence of two palmitoylatable Cys residues (Cys189 and Cys192) in the proximity of the isoprenylated Cys residue (Cys193) of the CaaX box (Box 4). Radiolabel incorporation studies soon revealed that cellular RhoB is palmitoylated [69] and site directed mutagenesis experiments subsequently uncovered that both Cys189 and Cys192 incorporated a palmitate acyl chain [69,132]. As expected, a Cys193Ser mutant of RhoB failed not only to incorporate the geranylgeranyl moiety, but also palmitic acid in the neighboring Cys residues [132]. Remarkably, palmitoylation of Cys192, rather than Cys189, together with Cys193 geranylgeranylation are required for RhoB tumor-suppressive and pro-apoptotic activities [132]. In fact, the carboxy-terminal eight-amino acid motif present in RhoB (CINCCKVL) contains a targeting sequence for sorting into multivesicular bodies and rapid lysosomal degradation [133]. Moreover, this RhoB sequence, containing the isoprenylation and palmitoylation sites (CINCCKVL) is sufficient to promote a RhoB-like localization and degradation rate when fused to the C-terminal end of several unrelated proteins. Similarly to the case of H-Ras previously discussed, both palmitoylation sites contribute to the localization and degradation of RhoB, although to different extents. Mutation of the first palmitoylation site (Cys189Ser) induced a diffuse cytosolic distribution with a perinuclear accumulation and partially impaired lysosomal degradation, whereas mutation of the second palmitoylation site (Cys192Ser) completely stabilized RhoB, to the same extent as the double Cys189,192Ser mutation [133]. This endolysosomal sorting governed by the RhoB carboxy-terminus is exquisitely determined not

only by the three lipid moieties, but also by its amino acid sequence, since other isoprenylated and bipalmitoylated small GTPases, such as H-Ras, Rap2A, Rap2B and TC10 were not accumulated into multivesicular bodies and were stable [134]. Finally, coimmunoprecipitation studies have also shown that RhoB palmitoylation interferes with its binding to RhoGDIα [69].

Intriguingly, the carboxy-terminus of TC10 is very similar to that of RhoB (Box 4), and, although the position of a Thr residue at the final position of the CaaX box of TC10 governs its farnesylation, in the presence of farnesyl-transferase inhibitors it can be partially geranylgeranylated as well [135]. This is reminiscent of the behavior shown by RhoB, which can be farnesylated or geranylgeranylated [135,136]. In transfected cells, TC10 can be observed mostly in plasma membrane and internal membranes [69]. Palmitoylation of TC10 has also been demonstrated using radioactive labeling, a biotin switch assay and further confirmed upon treatment of the GFP-TC10 transfected COS1 cells with the palmitoylation inhibitor 2Br-palmitate [69,136]. Farnesylation occurs at Cys210 whereas the palmitoylatable residues are Cys206 and Cys209. TC10, which has both a palmitoylation site and a polybasic region, is targeted to the plasma membrane and endosomes [69,136]. In adipocytes, the newly synthesized TC10 protein undergoes farnesylation and subsequently associates with the ER for proteolytic trimming and carboxyl-methylation [137]. The majority of the modified TC10 protein enters the Golgi system, where palmitoylation is supposed to occur, and then follows the membrane secretory transport pathway en route to lipid rafts/caveolae of the plasma membrane. Subcellular targeting and membrane localization of the TC10 Cys206Ser mutant were indistinguishable from those of wild-type TC10. In contrast, both the single Cys209Ser and the double Cys206/209Ser TC10 mutants displayed plasma membrane localization, but they were excluded from both the secretory membrane system and the lipid raft compartments [137]. Nevertheless, it has been reported that, at least in adipocytes, the exocytotic trafficking of TC10 occurs through both classical and nonclassical secretory transport pathways [137]. Support for this hypothesis was obtained when inhibition of Golgi membrane transport with brefeldin-A treatment did not prevent the plasma membrane localization of TC10. Consequently, a second potentially soluble, parallel pathway can escape palmitoylation and direct TC10 to the non-lipid raft plasma membrane regions. It has been proposed that TC10 may be continuously exchanging between these two pathways through a regulatory balance of palmitoylation–depalmitoylation reactions [137]. In addition, as in the case of RhoB, TC10 palmitoylation prevents its recognition by RhoGDIα [69].

TCL also possesses two carboxyl-terminal putative palmitoylation sites similar to TC10 that may serve as additional signals to control proper subcellular localization (Box 4). Despite the prediction of palmitoylation in these Cys residues [138], TCL palmitoylation has remained elusive. In mammalian cells, TCL, as was the case for TC10, becomes farnesylated and localizes at the plasma membrane and in early endocytic compartments [136,139]. Ectopically expressed TCL also exhibited perinuclear endosomal localization and induced filopodia formation. Interestingly, 2Br-palmitate treatment of mammalian cells transfected with GFP-tagged TCL had no effect on the subcellular localization of the chimera [136], and likewise, the biotin switch assay for palmitoylated proteins also tested negative [136]. It is then conceivable that TCL might become only very marginally palmitoylated in vivo, as previously reported for R-Ras, in which palmitate incorporation is far less evident than in the case of H-Ras when compared under identical labeling conditions [67].

There are three Rac proteins in humans, Rac1, 2 and 3, and all three contain a functional CaaX box with a Cys residue that becomes geranylgeranylated. Rac1, which has a strong polybasic region, is targeted like K-Ras4B primarily to the plasma membrane although 2Br-palmitate treatment of cells transfected with a GFP-Rac1 chimera did not result in significant changes in the subcellular localization [69] and a biotin switch assay also seems to indicate that Cys178 of Rac1 is not palmitoylated in vivo [136]. This observation contrasts with the identification of Rac1 as a palmitoylated protein in a general screen for palmitoylated proteins rat brain [140] and with the reduced colony formation capacity of a Rac1 Cys178Ser mutant [136].

Finally, palmitoylation of a carboxy-terminal Cys has also been reported for Wrch-1 and Wrch-2, two additional small GTPases of the RhoB family. The carboxy-terminus of Wrch-1 displays a KKYCCFV amino acid sequence although its CaaX box seems to be non-functional (Box 4). Unusually, Wrch-1 is not isoprenylated but is instead modified by palmitoylation [141] being this acylation required for both its subcellular localization and biological activities [142]. Significantly, the carboxy-terminal Cys256, but not Cys255, becomes palmitoylated and is required for Wrch-1 transformation [141]. In addition, lacking any isoprenyl group, Wrch-1 does not bind RhoGDI [143]. In the case of Wrch-2, palmitate incorporation was absent in a Cys234Ser mutant protein, but, remarkably, the Cys227Ser mutant had similar amounts of labeling as wild type Wrch-2, suggesting that this small GTPase contains a single palmitoylation site in the carboxyl terminus at residue Cys234 [144].

4. Subcellular trafficking of palmitoylated proteins

Unlike N-myristoylation and isoprenylation, S-acylation has no clear sequence requirement other than a cysteine residue. Site directed mutagenesis of Cys residues in the proximity of myristoylated N-terminal Gly residues and in the proximity of isoprenylated C-terminal Cys residues has been extensively used for the identification of palmitoylation sites. Palmitoylation sites are also found in the proximity of transmembrane helices. That is the case of the carboxy-terminus intracellular loop of many G-protein coupled receptors [72,145], transferrin receptor [146], certain viral glycoproteins that traverse the bilayers [147], the transmembrane linker for activation of T cells [148], BACE [149] and also Surfactant Protein C [150]. In the case of proteins with a single transmembrane helix palmitoylation occurs on Cys residues facing the cytoplasm, both in case of type I (CD4, CD8, BACE) or type II (Transferrin Receptor, Surfactant Protein C) topology (Box 5). Protein palmitoylation in the lumen of the endoplasmic reticulum is characteristic of secreted proteins that follow the anterograde sorting route such as Hedgehog.

Protein palmitoylation at the N-terminus in the absence of myristoylation (Box 5) has been described in the case of PSD-95 and PSD-93 [151,152], iNOS [53,153] and GAP43/neuromodulin [154]. It is interesting to remark that some of these non-myristoylated, N-terminally palmitoylated proteins have a palmitoylatable Cys residue at position 3, but never at position 2. Both PSD-95 and GAP43 are substrates for DHHC7 and DHHC15 [19], hence indicating that these palmitoyl transferases might transfer the palmitoyl moiety selectively to position 3.

Synaptic vesicles, which are specialized secretory organelles that store and secrete neurotransmitters during synaptic transmission in neurons, are enriched in numerous membrane-associated proteins, very frequently palmitoylated. That is the case of synaptobrevin 2, synaptotagmin, SNAP23, SNAP25, AQP4 and GAD65 [155,156]. SNAP25 is a neuronal SNARE protein that has a Cys string motif which is palmitoylated in 4 Cys among residues 83–95 within its membrane-targeting sequence. 25 amino acids downstream of the cysteine motif there is a five amino acid motif (QPARV) necessary for efficient palmitoylation and membrane targeting [157]. Recent studies have revealed that coexpression of SNAP25 with DHHC3, DHHC7 and DHHC17 is sufficient to promote membrane association in HEK293 cells [158]. Recent review articles have analyzed in detail the palmitoylation of SNAP25 [70] as well as the co-receptors CD4 and CD8 [71].

One of the first proteins with transmembrane domains to be recognized as palmitoylated was the transferrin receptor [146]. In this case, Cys62 and Cys67, both adjacent to the predicted single

Box 5

Selected examples of human palmitoylated proteins in the absence of myristoylation or isoprenylation. The predicted transmembrane helices are shaded in gray whereas the palmitoylated Cys residues are shaded in yellow. Positively and negatively charged residues are shaded blue and red respectively. 25 amino acids are shown in every case. In all cases, proteins with a single transmembrane stretch display the palmitoylatable Cys residues facing the cytoplasm.

Protein	Amino acid sequence
PSD-95	MDCLCIVTTKKYRYQDEDTPPLEHS...
PSD-93/Chapsyn-110	MFFACYCALRTNVKKYRYQDEDAPH...
iNOS	MACPWKFLFKTKFHQYAMNGEKDIN...
GAP43	MLCCMRRTKQVEKNDDDQKIEQDGI...
Gα Q	MTLESIMACCLSEEAKEARRINDEI...
Gα 11	MTLESMMACCLSDEVKESKRINAEI...
Gα 14	MAGCCCLSAEEKESQRISAEIERQL...
Gα 16	MARSLTWRCCPWCLTEDEKAAARVD...
SNAP23	...KCCGLCVCPCNRTKNFESGKAYKTT...
SNAP25a	...KCCGLFICPCNKLKSSDAYKKAWGN...
SNAP25b	...KFCGLCVCPCNKLKSSDAYKKAWGN...
Transferrin receptor	...KPKRCSGSICYGTIAVIVFFLIGFM...
Synaptotagmin VII	...VSAIITVSLSVTIVLCGLCHWCQRK...
Surfactant Protein C	...RGRFGIPCCPVHLKRLLIVVVVVVL...
CD4	...VLGGVAGLLLFIGLGIFFCVRCRHR...
CD8 α	...LAGTCGVLLLSLVITLYCNHRNRRR...
CD8 β	...VAGVLVLLVSLGVAIHLCCRRRRAR...
BACE	...VMAAICALFMLPLCLMVCQWRCLRC...
LAT (linker for activation of T-cells)	...ILVPCVLGLLLLPILAMLMALCVHC...
PAG1	...TLWGSLAAVAIFFVITFLIFLCSSC...

transmembrane helix incorporate the fatty acid, a modification reported to regulate the rate of endocytosis of the receptor [159].

Synaptotagmins comprise a family of type I membrane proteins characterized by a short N-terminal region, a single transmembrane domain, a spacer region and two highly conserved cytosolic C2 domains. One of its members, Syt VII is a Ca^{2+} sensor that regulates lysosome exocytosis and plasma membrane repair. Syt VII displays three cysteines within and adjacent to the transmembrane domain, Cys35, Cys38 and Cys41, known to become palmitoylated [160] (Box 5). Mutation of Syt VII palmitoylation sites blocks its trafficking to lysosomes [161] hence demonstrating a palmitoylation-dependent targeting to this organelle.

4.1. Surfactant Protein C

Surfactant Protein C (SP-C), a small, hydrophobic, 3.9 kDa polypeptide composed of just 35 amino acids is one of the four specific proteins isolated from pulmonary surfactant [150,162,163]. It is synthesized in the ribosome as a precursor protein of 21 kDa (proSP-C) that is proteolytically processed along the secretory pathway at both the C-terminal and N-terminal ends [164,165] rendering mature SP-C, which ultimately becomes secreted to the alveolar spaces as part of the surfactant complex. In addition, both the amino acid sequence and the palmitoylated Cys residues of SP-C, are highly conserved among species [166]. This palmitoylated integral membrane protein (Box 5) adopts a type II orientation (N cytosol/C lumen) and a 24° average angle of orientation with respect to the phospholipid bilayer [167].

SP-C is present in lipid–protein surfactant complexes assembled in alveolar pneumocytes in the form of tightly packed membranes, which are stored in specialized organelles called lamellar bodies that subsequently become secreted. SP-C isolated from mammalian broncho-alveolar lavage is dipalmitoylated on cysteines 5 and 6 [166]. The palmitoyl chains are attached to cysteines 28 and 29 of proSP-C (the equivalent positions to Cys5 and Cys6 in the mature protein) before its processing to SP-C, since the precursor polypeptides also incorporate radioactive palmitate [168,169]. Palmitoylation of proSP-C probably occurs immediately after insertion of its only transmembrane helix in the ER membrane and depends largely on the conformation of its cytosolic domain. Delivery of proSP-C to distal processing organelles is dependent upon this N-terminal cytoplasmic SP-C propeptide, which contains a conserved PPDY motif capable of interaction with several WW domains found in the Nedd4 family of E3 ligases [170]. SP-C palmitoylation early in the sorting process, probably in the ER–Golgi intermediate compartment (ERGIC) or cis-Golgi, is further verified by the observation that it is not affected by the Golgi disturbing agent brefeldin A [169,171,172]. Interestingly, proSP-C contains two positively charged residues (Lys34 and Arg35) in the proximity of the post-translationally palmitoylated Cys residues (Cys28 and Cys29). Substitution of these residues with uncharged Gln residues results in a complete inversion of the topology of proSP-C and the palmitoylatable Cys residues become translocated to the lumen of the endoplasmic reticulum and palmitoylation is lost [173]. Thus, palmitoylation of proSP-C is dependent on those N-terminal positively charged residues [171]. In addition, fluorescence microscopy revealed that this mutant SP-C protein appeared retained in the ER, suggesting that the Lys and Arg residues influence transport of proSP-C to compartments distal to the ER.

The successful recombinant expression and purification of mature SP-C has allowed the analysis in detail of the significance of the acyl moiety [174]. The behavior of native palmitoylated SP-C and recombinant unpalmitoylated versions of SP-C produced in bacteria were compared to understand the importance of the palmitic chains to optimize interfacial performance of cholesterol-containing surfactant films [129,175]. Palmitoylation of SP-C was determined not to be essential for the protein to promote rapid interfacial adsorption of phospholipids to equilibrium surface tensions (22 mN/m), in the presence or absence of cholesterol, but it was critical for cholesterol-containing films to reach surface tensions $\leq$1 mN/m at the highest compression rates assessed in a captive bubble surfactometer, in the presence of SP-B. Nevertheless, although SP-C palmitoylation is necessary for its proper function in pulmonary surfactant, palmitoylation itself does not seem to guide its subcellular traffic. The sorting activity of the palmitoylated cysteines was tested by mutating both Cys residues to Ser or Ala in the context of the SP-C proprotein. The successful sorting followed by extracellular secretion of the mutant proteins was confirmed using chromogranin staining (a marker for dense core granules) and was found indistinguishable from that of the wild-type counterpart [176].

4.2. Alpha subunits of G proteins

Heterotrimeric G proteins, composed of α, β and γ subunits, relay signals between cell surface receptors and membrane-bound effectors in numerous signaling cascades. Activation of a heptahelical transmembrane G protein-coupled receptor by an extracellular agonist, such as hormones, light or odorants, activates the G protein by catalyzing the release of GDP from Gα. In order to ensure specificity as well as effective concentrations and speed of interactions, these signaling components are usually translocated to the cytoplasmic side of the plasma

membrane. Whereas the Gγ subunit is irreversibly isoprenylated, the N-terminus of the Gα subunits of heterotrimeric G proteins can be myristoylated, palmitoylated or myristoylated plus palmitoylated [177–179]. In fact, the N-termini of all Gα are regions of great diversity, not only in terms of types and combinations of lipid modifications, but also in terms of amino acid sequence and length (ranging from 29 amino acids in Gαt to 55 amino acids in Gα12 before the beginning of the highly conserved β-sheet 1) [179]. Myristoylation in the absence of palmitoylation occurs in the Gα subunits of Gαt1, Gαt2 and Gαgust whereas palmitoylation is found in the four members of the GαQ family (GαQ, Gα11, Gα14 and Gα16/Gα15) as well as in Gα12, Gα13, GαS and Gα olfactory [177,180,181]. Myristoylation plus palmitoylation occurs in Gαi1 Gαi2 Gαi3, Gα0 and GαZ, and in every case, site directed mutagenesis of Gly2, which abolishes myristoylation, leads to the absence of N-terminal palmitoylation and to the translocation to the cell cytoplasm [81,182,183]. Translocation of Gα subunits to the plasma membrane has been explained by the "two-signal model" which suggests that peripheral membrane proteins such as Gα subunits require more than one signal to firmly attach them to the plasma membrane [180]. In addition to these lipid modifications, other mechanisms contribute to plasma membrane targeting of the heterotrimeric G proteins, such as the direct interaction of Gα with Gβγ [184] or the clusters of basic amino acids present at the N-terminus [181,185]. In fact, when site directed mutagenesis of residues that interact directly with the Gβγ heterodimer was performed in GαS or GαQ, efficient palmitoylation of the Gα subunits was clearly reduced, hence indicating that formation of the heterotrimer and Gα palmitoylation are concerted processes [186]. Thus, the plasma membrane targeting of Gα subunits requires both interaction with the Gβγ complex and subsequent palmitoylation of Gα. Nowadays, it is accepted that the spatiotemporal dynamics of Gα subunits involve continuous shuttling between the plasma membrane and intracellular membranes, a process that requires efficient palmitoylation [187].

Elegant studies have been recently performed in order to determine the functional significance of palmitoylation at N-terminal Cys residues of GαQ (Box 5) in terms of subcellular localization and coupling to G-protein coupled receptors and signaling. Taking advantage of photoconvertible fluorescent proteins, the anterograde and retrograde traffic between plasma membrane and endomembranes of GαQ transfected in HeLa cells has been studied [47]. Indeed, palmitoylation of GαQ is essential for plasma membrane targeting, as shown when transfected cells were incubated with the palmitoylation inhibitor 2Br-palmitate [47] or when site directed mutagenesis eliminated the palmitoylatable Cys residues present at the N-terminus [188] since under these circumstances, GαQ relocalized from the plasma membrane toward endomembranes and the cytoplasm. A general screening using 23 DHHC palmitoyl transferases cotransfected with GαQ showed that DHHC3 and DHHC7 coexpression increased GαQ palmitoylation significantly [47]. As expected siRNA-mediated silencing of DHHC3, DHHC7 or both significantly diminished GαQ palmitoylation, induced its translocation from the plasma membrane toward the cytoplasm and abrogated its signaling mediated by the α_{1A}-AR. Colocalization studies with GM130 revealed that DHHC3 is specifically detected at the Golgi apparatus where it partially overlaps with GαQ. Hence it has been proposed that GαQ shuttles between the plasma membrane and the Golgi apparatus through de/repalmitoylation cycles and this dynamic movement allows cells to adjust to extracellular stimulation [47].

4.3. Inducible Nitric Oxide Synthase (iNOS)

iNOS is a transcriptionally-regulated enzyme expressed in several cell types, such as macrophages, mesangial cells, muscular myotubes, hepatocytes and even endothelial cells, in most cases in response to an exogenous stimuli, fundamentally proinflammatory cytokines or bacterial lipopolysaccharide. Remarkably, •NO synthesis by iNOS is tightly regulated, occurring in a vectorial fashion at certain discrete subcellular sites [189]. Although early reports remarked that iNOS suffered a post-translational modification at its N-terminus, the identity of this modification remained elusive [190]. Using radiolabelled palmitic acid, we showed that both transcriptionally induced iNOS in muscular myotubes as well as transfected iNOS becomes palmitoylated at Cys3 [53,153]. In fact, iNOS palmitoylation is completely necessary for its intracellular trafficking toward subcellular domains where nitric oxide release is required. The correct targeting of palmitoylated iNOS allows its proper localization within the cells, therefore preventing the toxic effects of an uncontrolled release of nitric oxide. Using a Transwell cell culture system that allowed the measurement of the amount of released •NO at both apical and basolateral chambers, we showed that iNOS targets the apical membrane selectively, without •NO being released to the basolateral compartment [189,191].

The mutant iNOS Cys3Ser did not incorporate palmitic acid, forms irreversibly aggregates in the Golgi and is unable to progress along the secretory pathways, becoming completely inactive. This is especially important in polarized cell types in which a pro-inflammatory stimulus results in iNOS expression, since the carboxy-terminal tail of iNOS is known to subsequently attach to PDZ domains of certain transporter proteins such as EBP50 or CAP70 becoming selectively delivered to the apical surface [189,191]. In fact, N-terminal palmitoylation of iNOS at Cys3 together with the binding of its carboxy-terminus to these PDZ domain-containing proteins are equally indispensable requirements for the proper apical release of nitric oxide [53,153,191]. Support for the important role of iNOS palmitoylation is strengthened by the observation that treatment of COS7 cells transfected with iNOS or muscular myotubes treated with pro-inflammatory cytokines with both 2Br-palmitate or 8Br-palmitate severely affects nitric oxide synthesis. Site directed mutagenesis studies of the residues surrounding Cys3 followed by measurement of [^{3}H]-palmitate incorporation concluded that the hydrophobic amino acid Pro4 as well as the basic residues Lys6 and Lys10 is indispensable for the incorporation of the fatty acid. Unlike eNOS, in which palmitoylation is necessary for caveolar targeting, interaction with caveolin and protein inactivation, iNOS palmitoylation does not result in caveolar localization, but rather is necessary for proper sorting along the secretory route and its exit from the endoplasmic reticulum. Nevertheless, iNOS can become associated with caveolin-1 and marginally with caveolin-2 and -3 within muscle cells and this interaction abrogates nitric oxide synthesis [192], although in mature myotubes the same cytokine stimuli that induce iNOS expression lead to the downregulation of all three caveolin isoforms, hence allowing iNOS to be completely active [192,193]. Interestingly, the transit of iNOS along the secretory pathway is exquisitely regulated through palmitoylation at Cys3, and this post-translational modification cannot be replaced by a surrogate myristoylation at glycine 2 nor by an additional palmitoylatable cysteine at position 2 [53].

4.4. AMPA receptor subunits

Several subunits of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate) receptor, an ionotropic glutamate, ligand-gated cation channel that mediates the fast component of excitatory postsynaptic currents in the central nervous system are also palmitoylated [194]. All AMPA receptors consist of four types of subunits, GluR1–4 [195], and can be palmitoylated at the side chain of two Cys residues: one in the second transmembrane domain (TM2), the other in the C-terminal intracellular region, hence regulating receptor trafficking. TM2 palmitoylation has a profound effect on the delivery of the receptor from the Golgi to the cell surface, leading to an accumulation of the receptor in the Golgi and a reduction of receptor surface expression. Nevertheless, C-terminal palmitoylation did not affect the trafficking toward the cell surface but instead decreased the interaction of the AMPA receptor with the 4.1N protein, mediating agonist-induced AMPA receptor internalization [196]. Therefore, regulated palmitoylation

of AMPA receptor subunits on distinct cysteines modulates receptor trafficking and therein regulates the levels of receptor surface expression and may be important for synaptic plasticity [197]. Over-expression experiments indicate that DHHC3/GODZ increase palmitoylation of the AMPA receptor subunits GluR1 and GluR2 [197].

5. Current challenges and future perspectives

Although in the past few years the identification of several palmitoyl transferases as well as their protein substrates has brought major advances to the protein palmitoylation field many central questions remain. Considering that there are hundreds or thousands of cellular proteins that become palmitoylated and only several dozen of palmitoyl transferases it remains to be established how the substrate recognition takes place. It is not well determined if palmitoyl transferases specifically recognize an amino acid sequence, a subcellular localization of the substrate or a previous acylation (i.e. myristoylation or prenylation) in the proximity of a palmitoylatable Cys residue. Likewise, although the palmitoylation–depalmitoylation cycle of proteins such as H-Ras, N-Ras or PSD-95 are beginning to be unraveled it is not well known if these cycles also apply to other palmitoylated protein substrates. Finally, the inter-relationship between protein palmitoyl transferases and thioesterases, their precise subcellular localization as well as their activity in terms of time and space are questions that, no-doubt, will be addressed in coming years.

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