Model organisms lead the way to protein palmitoyltransferases

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Summary

The acylation of proteins with palmitate and related fatty acids has been known for over 30 years, but the molecular machinery that carries out palmitoylation has only recently emerged from studies in the model organisms *Saccharomyces cerevisiae* and *Drosophila.* Two classes of protein acyltransferases (PATs) have been proposed. In yeast, members of a family of integral membrane proteins harboring a cysteine-rich domain (CRD) containing a conserved DHHC (Asp-His-His-Cys) motif are PATs for cytoplasmic signaling molecules. The DHHC-CRD protein Erf2p, together with an associated subunit Erf4p, palmitoylates yeast Ras proteins, and Akr1p catalyzes the palmitoylation of the yeast casein kinase Yck2p. The existence of a second class of PATs that modify secreted signaling proteins has been suggested from work in *Drosophila*. Rasp is required in vivo for the production of functional Hedgehog and shares sequence identity with membrane-bound *O*-acyltransferases, which suggests that it catalyzes the palmitoylation of Hedgehog. With the identification of PATs in model genetic organisms, the field is now poised to uncover their mammalian counterparts and to understand the enzymology of protein palmitoylation.

Key words: Palmitate, Protein acyltransferase, PATs, Signaling

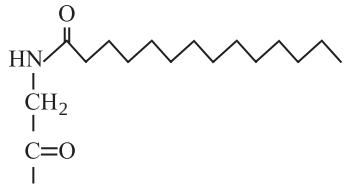
Introduction

The number and diversity of proteins known to be covalently modified by fatty acids has grown steadily since protein palmitoylation was first described over 30 years ago (Linder and Deschenes, 2003). The two most common forms of protein fatty acylation in eukaryotic cells are *S*-palmitoylation [the addition of palmitate (C16:0) or other long-chain fatty acids to proteins at cysteine] and N-myristoylation [the addition of myristate (C14:0) to proteins at the N-terminal glycine residue] (Fig. 1). Of the two, palmitoylation more effectively increases the hydrophobicity of proteins or protein domains and thus contributes to membrane association, subcellular trafficking of proteins between membrane organelles and their trafficking within membrane microdomains. In some cases, palmitoylation contributes directly in protein-protein interactions (Bijlmakers and Marsh, 2003; Dunphy and Linder, 1998; El-Husseini Ael and Bredt, 2002; Milligan et al., 1995; Resh, 1999; Silvius, 2002). Whereas the enzymology of *N*-myristoylation is well characterized, uncovering the molecular mechanisms underlying protein palmitoylation has proven to be very difficult. The recent discovery of amide-linked palmitate at the N-terminal cysteine residue of Hedgehog (Hh) proteins raises the possibility of multiple mechanisms of palmitate attachment (Fig. 1). Although biochemical strategies to purify PAT enzymes have been largely unsuccessful, genetic strategies in yeast and flies have recently led to the identification of two classes of PAT enzyme. Here, we focus on the enzymology of these newly discovered palmitoyltransferases.

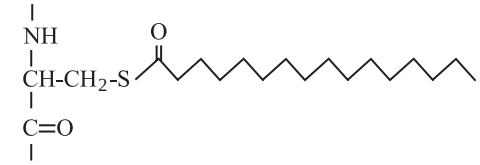
Palmitoyltransferases that modify intracellular proteins

Plasma membrane localization of the yeast Ras homologs Ras1p and Ras2p requires a series of post-translational modifications beginning with farnesylation of the cysteine residue of the C*aaX* box (Cys-aliphatic-aliphatic-X, where X is the C-terminal residue), proteolytic cleavage of the *-aaX* residues, and methylation of the resulting carboxyl group. A second cysteine immediately upstream of the farnesylated cysteine then undergoes *S*-palmitoylation (Bhattacharya et al., 1995; Deschenes and Broach, 1987; Fujiyama and Tamanoi, 1986) (Fig. 2). Preventing palmitoylation by mutating the modified cysteine residue to alanine or serine has a relatively minor effect on yeast viability as long as Ras can be farnesylated. However, Mitchell et al. created a mutant Ras protein in which the C*aaX* box was replaced by a stretch of basic residues (Mitchell et al., 1994). The mutant protein was palmitoylated but not farnesylated, and the viability of cells expressing the mutant Ras depends on its palmitoylation. This made possible a genetic screen for the Ras PAT (Bartels et al., 1999) and yielded two genes: *ERF2* and *ERF4/SHR5*. Deletion of *ERF2* and/or *ERF4/SHR5* causes a decrease in Ras palmitoylation and partial mislocalization of Ras2p from the plasma membrane (PM) to endomembrane compartments (Bartels et al., 1999; Jung et al., 1995). *ERF2* encodes a 42 kDa protein that has four predicted membrane-spanning domains and a cysteine-rich domain (CRD) containing a conserved DHHC motif. The DHHC-CRD has also been referred to as the NEW1 domain and the zf-DHHC motif (Mesilaty-Gross et al., 1999; Putilina et al., 1999). Orthologs

***N*-myristoylglycine**



***S*-palmitoylcysteine**



***N*-palmitoylcysteine**

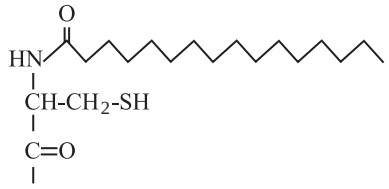


Fig. 1. Structures of *N*-myristoylglycine, *S*-palmitoylcysteine and *N*-palmitoylcysteine.

of *ERF2* can be found in the genomes of every eukaryote examined to date. Erf4p is a novel 26 kDa protein that has no recognizable motifs. The isolated Erf2p-Erf4p complex palmitoylates yeast Ras2p using palmitoyl-CoA as palmitate donor (Lobo et al., 2002). Mutations in the conserved DHHC motif (DAHC or DHHS) abolish PAT activity. The enzyme exhibits protein and lipid substrate selectivity. It works best with yeast Ras2p and less well with mammalian myristoylated $G_{i\alpha}$ subunits or mammalian H-Ras. In addition, long-chain acyl-CoA substrates (C16 and C18) are preferred over shorter acyl chains (<C14).

The discovery of Erf2p-Erf4p filled in the gap in the Ras post-translational processing pathway (see Fig. 2). Since all of the post-prenylation processing events occur on the endoplasmic reticulum (ER) membrane, the enzymes might form a complex to facilitate efficient processing. In the case of yeast Ras, palmitoylation is required for transit from the ER to the PM. It has recently been shown that ER-to-PM translocation of Ras does not require the Golgi or the classical secretory pathway (Dong et al., 2003), which raises the possibility that palmitoylation is a key determinant for the trafficking of Ras proteins between membrane compartments in yeast. However, this may not be a general feature of all palmitoylated Ras proteins. Mammalian K-Ras, which is not palmitoylated, uses a non-classical pathway for ER-to-PM translocation, whereas PM localization of palmitoylated H-Ras does require a functional classical secretory pathway. Work is still needed to define the intracellular trafficking pathways for lipid-modified proteins.

A related yeast protein, Akr1p, is also a PAT. Akr1p differs from Erf2p in that the DHHC motif is replaced by DHYC and Akr1p also has ankyrin repeats not present in Erf2p. The highly conserved Cys residues of the Erf2 CRD proteins are either missing or have altered spacing. Akr1p was initially identified as a component of the pheromone response pathway (Givan and Sprague, 1997; Kao et al., 1996; Pryciak and Hartwell, 1996). *AKR1* mutants exhibit a reduced rate of pheromone receptor internalization (Givan and Sprague, 1997), a phenotype shared with mutants that have defective casein kinase genes (*YCK1* and *YCK2*) (Feng and Davis, 2000). This led investigators to examine Yck2p trafficking in an *akr1* mutant strain. Deletion of *AKR1* prevents PM localization of Yck2p, producing diffuse cytosolic staining. The molecular basis for this was made clear by the demonstration that Akr1p is a palmitoyltransferase for Yck2p (Roth et al., 2002). Yck1p and Yck2p are palmitoylated on a dicysteine motif at the C-terminus and palmitoylation appears to be the only lipid modification. Akr1p and Erf2p-Erf4p PATs therefore recognize distinct substrates. The Erf2p-

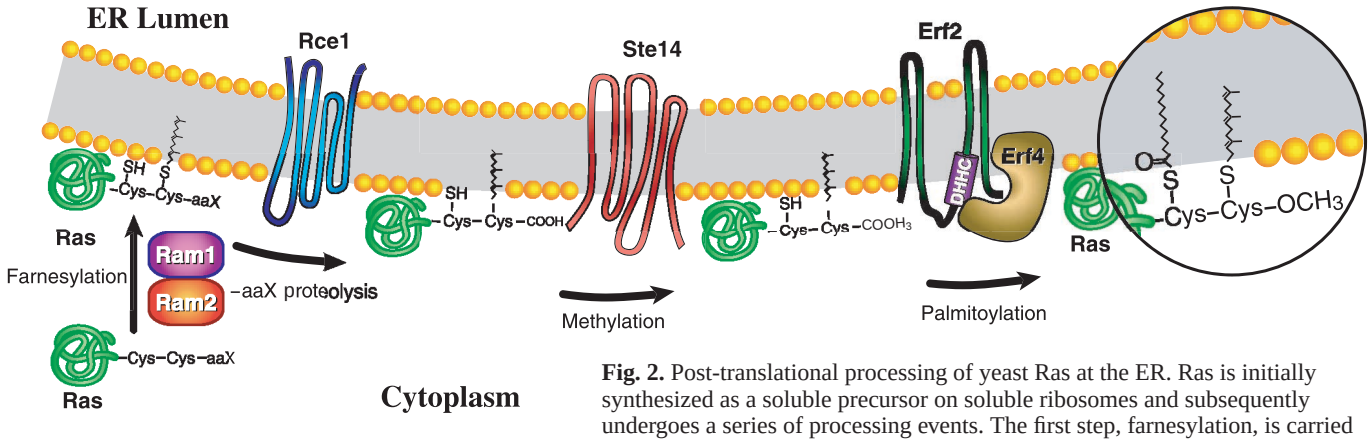


Fig. 2. Post-translational processing of yeast Ras at the ER. Ras is initially synthesized as a soluble precursor on soluble ribosomes and subsequently undergoes a series of processing events. The first step, farnesylation, is carried out by Ram1p/Ram2p, which modifies Ras and escorts it to the ER membrane. Farnesylated Ras is then proteolyzed by the C*aaX* protease Rce1, which removes the C-terminal *-aaX* sequence. The Ste14p carboxylmethyltransferase then methylates the farnesylated cysteine. Finally, the Erf2p/Erf4p PAT palmitoylates Ras at a cysteine residue adjacent to the farnesylated cysteine. The fully processed Ras protein is competent for transport to the plasma membrane. The topology model of Ste14p is from Romano and Michaelis (Romano and Michaelis, 2001), whereas the membrane topology of Rce1p, Erf2p and Erf4p is speculative and inferred from hydropathy plots. The DHHC-CRD domain of Erf2 is indicated by a purple cylinder.

Erf4p complex palmitoylates a cysteine residue adjacent to a farnesylated cysteine, whereas Akr1p palmitoylates cysteine residues in the absence of other modifications.

There are ~23 DHHC-CRD genes in the mouse and human genome databases. Although it is too early to assume that they are palmitoyltransferases, there are indications that they might be. Perhaps the best candidate is the gene encoding HIP14 (gi # 29244581), which shares significant sequence identity with yeast Akr1p. HIP14 rescues the temperature-sensitive and endocytosis-deficiency phenotypes of the yeast lacking *AKR1*. It was initially identified as a protein that interacts with huntingtin (Htt) in a yeast two-hybrid assay (Singaraja et al., 2002). The interaction between HIP14 and Htt inversely correlates with the poly(Q) track length in Htt, which suggests that a decreased interaction between HIP14 and Htt could be involved in the neuronal dysfunction in Huntington's disease. The highest expression level of HIP14 is in the brain, where it localizes to the Golgi in spiny neurons (Singaraja et al., 2002). The role of the DHHC-CRD in HIP14 function has not been assessed in mammalian cells or in yeast. It will be interesting to determine whether palmitoylation plays a role in the etiology of Huntington's disease.

GODZ (gi#7706133) is another example of a mammalian DHHC-CRD protein that shares sequence identity with Erf2p It was isolated in a screen for proteins that interact with the C-terminal tail of the GluRα1 glutamate receptor (Uemura et al., 2002). GODZ colocalizes with the trans-Golgi network, and overexpression of GODZ in COS7 cells interferes with subcellular trafficking of GluRα1. Although some glutamate receptors are palmitoylated, GluRα1 is not modified. However, several proteins that associate with glutamate receptors are palmitoylated (DeSouza et al., 2002; El-Husseini Ael and Bredt, 2002). Whether GODZ is a PAT and, if so, whether it plays a role in the palmitoylation and the regulation of glutamate receptor trafficking remain to be examined.

Abl-philin (Aph2) is an Abl-associated protein that colocalizes with an ER pool of this kinase. Aph2 has a DHHC-CRD motif and predicted topology very similar to that of Erf2p (Li et al., 2002). The functional significance of the Aph2-Abl interaction is not clear, but it is tempting to consider in the context of recent findings (Hantschel et al., 2003; Harrison, 2003). Differential splicing yields two isoforms of Abl. The Abl1b isoform is myristoylated at the N-terminus and the myristoyl group is essential for forming a clamp that regulates kinase activity. The myristoylation site is missing in Abl1a, but an N-terminal cap still plays a role regulating the kinase activity. It has been suggested that the hydrophobic residues play the role of the myristoyl moiety in Abl1b, but this has not been demonstrated. An alternative possibility, suggested by the interaction with Aph2, is that Abl is palmitoylated and palmitoylation plays role in kinase autoregulation. There are three cysteine residues in the N-terminal region of Abl1a; it is therefore a potential substrate for palmitoylation.

Finally, a gene encoding a mammalian DHHC-CRD protein has been reported at a locus associated with susceptibility to schizophrenia in humans (Liu et al., 2002). Relevant genes in this region include *KIAA1292* (gi#17484904; ZDHHC8), which encodes a DHHC-CRD protein that shares sequence similarity with yeast Erf2p. Whether ZDHHC8 is causally associated with schizophrenia remains to be determined.

Palmitoyltransferases that modify secreted proteins

Hh and Wnt represent families of secreted signaling molecules that are important for the development of metazoan organisms (Cadigan and Nusse, 1997; Hammerschmidt et al., 1997). Members of both families are substrates for palmitoylation. Hh is synthesized with a signal sequence that directs translocation into the lumen of the ER (Fig. 3A). Cleavage of the signal sequence yields a cysteine residue at the N-terminus. Hh then undergoes a complex series of post-translational processing steps beginning with a self-directed internal cleavage that generates the N-terminal signaling domain (Porter et al., 1996). The signaling domain is modified at its C-terminus with cholesterol (Porter et al., 1996) and at its N-terminus with palmitate (Pepinsky et al., 1998). Palmitate is linked to cysteine through a stable amide bond (Fig. 1, *N*-palmitoylcysteine). Mutation of the cysteine residue to serine abolishes palmitoylation, which suggests that acylation occurs through a thioester intermediate (Fig. 1, *S*-palmitoylcysteine), followed by a rearrangement that results in attachment of palmitate to the α-amino group of the N-terminal cysteine (Fig. 1, *N*-palmitoylcysteine; Fig. 3A).

Genetic screens performed in *Drosophila* to identify additional components in the Hh signaling pathway revealed an excellent candidate for the Hh palmitoyltransferase (Chamoun et al., 2001; Lee and Treisman, 2001; Micchelli et al., 2002). Three groups independently identified *skinny hedgehog(ski)/sightless(sit)/rasp* as a gene required for activity of Hh proteins. We refer to the gene as *rasp* hereafter (the *Drosophila* nomenclature used is from Flybase, http://flybase.bio.indiana.edu/). The *rasp* gene product is required in Hh-producing cells but does not affect the synthesis, accumulation, secretion or cholesterol modification of Hh protein. A clue to the function of Rasp is its short but significant sequence similarity to a superfamily of membrane-bound *O*-acyltransferases (MBOAT) proteins (Hofmann, 2000). These proteins have multiple membrane-spanning regions (typically 8-10) and share sequence similarity in a region that includes a highly conserved histidine residue that is a presumptive active site. All of the biochemically characterized members of this family are enzymes that transfer fatty acids (or other organic acids) onto hydroxyl groups of membrane-bound, non-proteinaceous targets. In *rasp*, mutation of the two most highly conserved residues (including the presumptive active site histidine) in the MBOAT homology sequence results in significant loss of Hh signaling activity (Chamoun et al., 2001). Furthermore, Hh proteins isolated from *rasp* mutant cells lack the palmitate modification. Together, these findings support the hypothesis that *rasp* encodes an enzyme that transfers palmitate to the N-terminal cysteine (Chamoun et al., 2001). This remains to be demonstrated biochemically. Given the requirement for cysteine (and not serine) for palmitoylation, Rasp might represent the first *S*-acyltransferase in the MBOAT family.

The latest additions to the list of palmitoylated proteins are members of the Wnt family (Willert et al., 2003). Wnt proteins are cysteine-rich, secreted glycoproteins. They have been difficult to purify in active form to permit biochemical characterization. However, Willert et al. recently succeeded in isolating active Wnt3a and other isoforms from conditioned medium derived from cultured cells stably transfected with the corresponding cDNA (Willert et al., 2003). The hydrophobic character of the purified growth factor was not consistent with

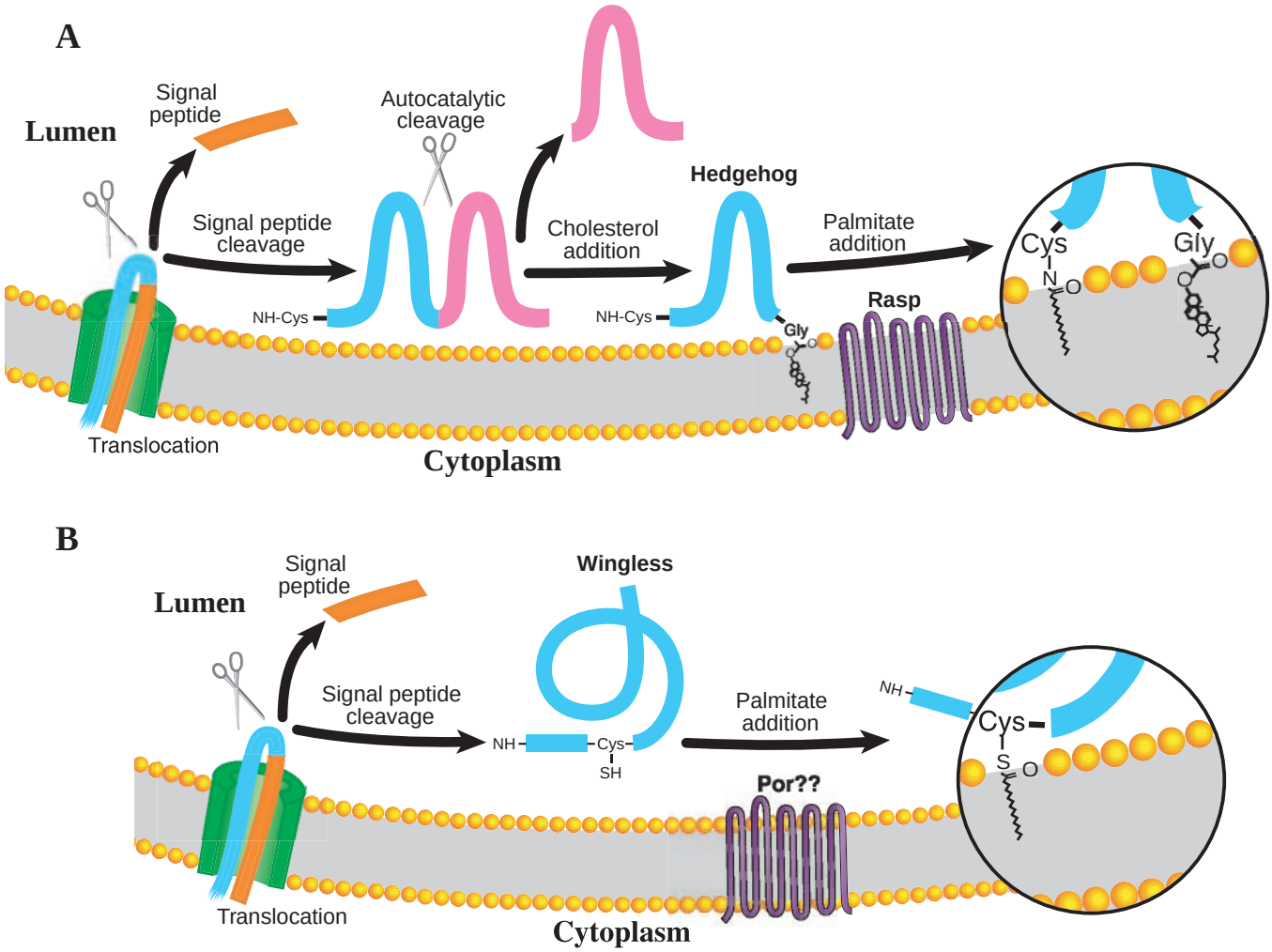


Fig. 3. Post-translational processing of secreted palmitoylated proteins. (A) Hedgehog is synthesized on membrane-bound ribosomes and translocated into the lumen of the ER by a signal-peptide-mediated process (orange). The hedgehog precursor undergoes proteolytic processing events to remove the signal peptide and a second autocatalytic cleavage generating the N-terminal signaling domain (blue) and a C-terminal autoprocessing domain (pink). Following cleavage, cholesterol is added at the C-terminus of the signaling domain. The N-terminus is palmitoylated, presumably by the Rasp PAT. Modification with cholesterol is coupled with autocatalytic cleavage, but is independent of palmitate addition. The order of addition of the two lipids is unknown. Although shown as occurring in the ER, the intracellular compartment where autocatalytic cleavage and lipidation of Hedgehog occurs is unknown. In the predicted structure of the unmodified signaling domain of Hedgehog, both lipid modifications emerge from the same face of the molecule, presumably tethering the protein to the membrane (Ho and Scott, 2002). The lipid modifications might facilitate targeting of Hedgehog to lipid rafts on the cell surface (Rietveld et al., 1999). However, Hedgehog is secreted in a hexameric form (Zeng et al., 2001), raising the possibility that the lipid modifications might form a hydrophobic core (Ho and Scott, 2002). (B) The Wingless precursor also enters the ER lumen via a signal-peptide-mediated translocation followed by signal peptide cleavage (orange). In the model described in the text, Wingless is palmitoylated by Por (purple) on a conserved cysteine residue prior to glycosylation. Rasp and Por are integral membrane proteins, but their topology is not known.

its amino acid composition or sequence, which suggested that a lipid modification is present. They further demonstrated that Wnt is in fact palmitoylated by incorporating radioactive palmitate into the protein and showed the susceptibility of the hydrophobic modification to cleavage by an acylprotein thioesterase (APT1) (Duncan and Gilman, 1998; Willert et al., 2003). Proteolytical cleavage of mammalian Wnt3a (or *Drosophila* Wnt8) yields a cysteine-containing peptide that has a mass increase of 238 Daltons (compared with its predicted mass), which is the size anticipated for thioester-linked palmitate (Fig. 3B). The palmitoylation site maps to C77 in Wnt3a and C51 in Wnt8. Sequence alignment of Wnt family members reveals that this is the first cysteine residue in the sequence that is conserved in all family members. Thus, palmitoylation is likely to be found on all Wnt signaling proteins. Several lines of evidence support the functional importance of Wnt palmitoylation. First, loss-of-function mutations in *Drosophila wingless* (*wg*) (Couso and Arias, 1994) and *Caenorhabditis elegans Egl-20* (Maloof et al., 1999) map to the conserved palmitoylated cysteine. Second, enzymatic depalmitoylation of purified Wnt3a protein results in the loss of its ability to stabilize β-catenin in mouse L cells, which is a measure of Wnt signaling activity (Willert et al., 2003).

Hofmann suggested a connection between MBOAT proteins and Wnt signaling when the protein Porcupine was identified as a member of the MBOAT family (Hofmann, 2000). The founding member of an evolutionarily conserved gene family, *porcupine* (*por*) is a segment polarity gene in *Drosophila* that is required for processing and secretion of Wg (Kadowaki et al., 1996). In *por* mutants, Wg is confined to cells where it is synthesized. In tissue culture systems, *N*-glycosylation of Wg is enhanced by coexpression of Por. Por binds the N-terminal 24-residue domain (residues 83-106) of Wg (Tanaka et al., 2002), the region that includes the putative palmitoylation site. Because Por is an MBOAT protein, it is tempting to speculate that it is the PAT for Wnt proteins (Fig. 3B). In vitro assays for PAT activity and in vivo analysis of Wg palmitoylation in *por* mutants will be required to establish this conjecture as fact.

Palmitoylation of Ras is an important signal for its trafficking in yeast and mammalian cells. If Por is an acyltransferase, then defective secretion of Wg in *por* mutants could be due to the loss of this trafficking signal. Another facet of Por function is its stimulation of Wg *N*-glycosylation. How would palmitoylation facilitate glycosylation? The oligosaccharyl transferase (OST) complex is localized to the ER membrane close to the translocon. Most proteins are cotranslationally *N*-glycosylated. However, Tanaka et al. demonstrated that *N*-glycosylation of Wg occurs post-translationally (Tanaka et al., 2002). They suggested that acylation might tether Wnt to the membrane to facilitate its interaction with the OST complex. *N*-glycosylation is an important parameter of quality control and protein folding in the ER. Generating functional Wnt proteins that can exit the ER might require coupling of these two post-translational modifications.

Conclusions and perspective

Characterization of membrane-bound enzymes has been a challenge for generations of biochemists. In some cases, genetic approaches can provide the key to success. For example, genetic screens in yeast have revealed the genes for all of the enzymes required for the post-translational processing of Ras proteins. *ERF2* and *ERF4* fill in the final step of Ras processing at the ER membrane. However, in cells that lack *ERF2* and *ERF4*, some residual palmitoylation of Ras is observed, which suggests that another Ras PAT is lurking in the wings. With six additional DHHC-CRD proteins in yeast, there are candidates. Akr1p has already been shown to be a PAT for a different class of palmitoylated proteins, the membrane-associated casein kinases. Yeast should continue to be a fruitful ground for identifying other enzyme-substrate pairs.

The large family of mammalian DHHC-CRD proteins is unexplored territory at this point. Do all of the DHHC-CRD proteins act as PATs? A challenge to be met is to find out whether partner proteins are required for activity. In yeast, Erf2p requires Erf4p for activity, whereas Akr1p appears to act alone. Given the dearth of Erf4p orthologs evident by sequence alignment, strategies will need to be developed to identify proteins that play a similar role.

Biochemists have yet to tackle the Rasp and Por proteins. Assays to measure PAT activity in vitro will be required to establish whether these proteins are bona fide enzymes. Several of the MBOAT proteins have been characterized as enzymes and can be used as models to establish assay systems. Functional mammalian orthologs of Por have been identified, permitting a rapid screen of these proteins for Wnt PAT activity. Sequence alignments reveal several candidates in mammalian genomes for Rasp orthologs. After many quiet years, the enzymology of protein palmitoylation is ready for full exploration.

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