thematic review

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Protein palmitoylation by a family of DHHC protein *S*-acyltransferases

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Abstract Protein palmitoylation refers to the posttranslational addition of a 16 carbon fatty acid to the side chain of cysteine, forming a thioester linkage. This acyl modification is readily reversible, providing a potential regulatory mechanism to mediate protein-membrane interactions and subcellular trafficking of proteins. The mechanism that underlies the transfer of palmitate or other long-chain fatty acids to protein was uncovered through genetic screens in yeast. Two related *S*-palmitoyltransferases were discovered. Erf2 palmitoylates yeast Ras proteins, whereas Akr1 modifies the yeast casein kinase, Yck2. Erf2 and Akr1 share a common sequence referred to as a DHHC (aspartate-histidine-histidine-cysteine) domain. Numerous genes encoding DHHC domain proteins are found in all eukaryotic genome databases. Mounting evidence is consistent with this signature motif playing a direct role in protein acyltransferase (PAT) reactions, although many questions remain. This review presents the genetic and biochemical evidence for the PAT activity of DHHC proteins and discusses the mechanism of protein-mediated palmitoylation.—Mitchell D. A., A. Vasudevan, M. E. Linder, and R. J. Deschenes. **Protein palmitoylation by a family of DHHC protein *S*-acyltransferases. *J. Lipid Res.* 2006.** 47: **1118–1127.**

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Protein palmitoylation was first documented >25 years ago, but only recently have the enzymes responsible for this modification been described (1, 2). Originally thought to simply anchor proteins in the membrane, palmitoylation is now known to occur on a wide variety of proteins, including peripherally associated and integral membrane proteins. Palmitoylation is also involved in protein trafficking, organelle inheritance, and vesicle fusion (3). Protein palmitoylation results in the modification of the side chain of cysteines, forming a thioester linkage. This has been referred to as *S*-palmitoylation and is reversible. The reversible nature of the thioester linkage lends itself to a variety of regulatory scenarios. When palmitoylation occurs at an N-terminal cysteine residue, an *S*-to-*N* transfer reaction occurs and results in the formation of a palmitoylamide (4). *N*-Palmitoylation is similar to *N*-myristoylation in that it is an irreversible modification.

The formation of a palmitoyl thioester linkage on peptides and proteins can occur spontaneously in vitro at neutral pH in the presence of palmitoyl-CoA, leading to the suggestion that *S*-palmitoylation occurs via a nonenzymatic mechanism in vivo (5–7). However, the discovery of two classes of protein-palmitoylating enzymes challenges this notion. Palmitoyltransferases carry out the thioesterification of the cysteine side chain with long-chain fatty acids, most often a palmitoyl moiety (1, 2, 8). Because other saturated and unsaturated fatty acyl groups also have been reported (9–11), we refer to these enzymes generically as thiol-directed protein acyltransferases (PATs). Examples include Erf2/Erf4, the yeast PAT that palmitoylates prenylated Ras, and Akr1, the PAT for yeast casein kinase I (12, 13). Erf2 and Akr1 share a domain referred to as the DHHC domain, a cysteine-rich domain with a conserved aspartate-histidine-histidine-cysteine signature motif. Evidence suggests that the DHHC domain is directly involved in the palmitoyl transfer reaction. The substrates for PATs are intracellular proteins. The second class of palmitoyltransferase acts on proteins that are modified in the lumen of the secretory pathway. Genetic evidence from *Drosophila melanogaster* suggests the existence of enzymes that modify secreted morphogens of the Wnt and Hedgehog families. Skinny hedgehog (also known as Rasp, Central missing, and Sightless) is required for the palmitoylation of Hedgehog (14) and Spitz (15) at N-terminal cysteines. Porcupine is required for the modification of Wnt proteins (16). Skinny hedgehog and Porcupine share limited

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sequence homology with *O*-acyltransferases (17) but lack the DHHC domain that is found in PATs. Based on the lack of sequence homology and the distinction between intracellular versus secreted substrates, it appears that at least two distinct classes of palmitoyltransferases have evolved. This review will focus on the family of DHHC domain-containing PATs.

Several members of the DHHC protein family have been demonstrated to possess PAT activity. Genes encoding DHHC proteins are found in all eukaryotes examined to date, with the number of examples ranging from 1 to 8 in unicellular fungi to >20 in metazoans. The large number of potential PATs in an organism raises important questions regarding substrate specificity and regulation. In the few examples in which they have been investigated, DHHC proteins were found to be expressed in diverse tissues and to reside in different subcellular locations (18–20). This raises the possibility that PATs play diverse roles in cells. This review will describe how DHHC proteins were discovered and the evidence that they represent the protein palmitoyltransferases responsible for modifying the diverse family of palmitoylated proteins found in eukaryotic cells.

IDENTIFICATION OF PATS IN *SACCHAROMYCES CEREVISIAE*

Protein palmitoyltransferases were first identified and isolated in *Saccharomyces cerevisiae*. The enzyme responsible for the palmitoylation of Ras was identified using a genetic screen based on palmitoylation-dependent Ras proteins (21). Yeast Ras proteins, like their mammalian counterparts, require membrane localization to function as signal transducers (22). Membrane localization in turn requires the posttranslational addition of a farnesyl moiety to the cysteine of a C-terminal CaaX box motif (23, 24). Farnesylation directs Ras to the cytosolic surface of the endoplasmic reticulum, where the CaaX box undergoes proteolytic cleavage to remove the aaX residues (25) and carboxylmethylation (26), resulting in a C-terminal farnesylated cysteinyl methylester. Although these modifications increase the affinity of Ras for membranes, alone they are not sufficient for the plasma membrane localization of Ras. This requires the addition of a second signal, in the form of a palmitoyl group covalently linked to the side chain of a second cysteine via a thioester linkage (27).

Attempts to biochemically isolate the enzyme responsible for the palmitoyltransferase reaction were unsuccessful, prompting many to consider that palmitoylation occurs via a nonenzymatic mechanism. The abundance and high reactivity of the palmitoyl-CoA moiety had made studying this reaction difficult and elucidating candidate enzymes nearly impossible because enzyme activity was unstable after solubilization (8). The identification of a palmitoylation-dependent Ras2 molecule provided the key to the discovery of genes required for Ras palmitoylation (28). This Ras2 allele was originally isolated based on its ability to support the viability of yeast without being prenylated. The protein contains a C-terminal extension of basic amino acid residues reminiscent of the mammalian K-Ras protein. The presence of the basic extension prevents prenylation, but palmitoylation is preserved at the cysteine palmitoylated in wild-type proteins. Presumably, it is the combination of the basic amino acids and the palmitoylation of the protein that allows it to substitute for the prenylation and palmitoylation found on the wild-type protein. This led to the notion that candidates for the palmitoyltransferase could be isolated through mutation of the yeast genome by screening for mutations that were lethal in the presence of the palmitoylation-dependent Ras2 protein. Using the palmitoylation-dependent Ras2 yeast strain, mutants were isolated that significantly reduced or blocked Ras2 palmitoylation, which resulted in lethality (21). These mutations were referred to as *ERF* (*Effectors of Ras Function*). Originally, three complementation groups were isolated, *ERF1*, *ERF2*, and *ERF4*. *ERF1* was allelic to the *RAS2* locus. *ERF2* was a previously uncharacterized gene in the yeast genome. In contrast, *ERF4* had been discovered in a screen for suppressors of hyperactive Ras (*SHR5*) and appeared to suppress the lethal effects of an activating *RAS2* allele by reducing the level of Ras2 palmitoylation, although a direct connection to palmitoyltransferase activity was not made at the time (29).

ERF2 encodes a 42 kDa protein with four predicted membrane-spanning domains. Deletion of the gene results in a decrease in Ras2 palmitoylation and a reduced presence on the plasma membrane. Localization studies place Erf2 on the endoplasmic reticulum membrane (21), which is consistent with the localization patterns of the other CaaX box-modifying proteins, Rce1 (30) and Ste14 (31). *ERF4/SHR5* encodes a 26 kDa protein, which copurifies and localizes with endoplasmic reticulum membranes despite lacking an apparent membrane association motif. Based on their ability to localize to the same membranous system, the observations that an *erf2 erf4* double mutant was no more severe than either of the single mutants, and that overexpression of *ERF2* suppressed some but not all alleles of *erf4*, suggested that the proteins were part of the same pathway and that they might physically interact. In fact, Erf2 and Erf4 copurify as a complex and interact in a yeast two-hybrid assay (32). Using palmitoyl-CoA as the palmitate donor, Ras undergoes Erf2/Erf4-dependent palmitoylation on the same cysteine residue that is modified in vivo. The enzyme activity can be isolated from yeast and also reconstituted in bacteria by coexpression of both *ERF2* and *ERF4*. Importantly, Ras PAT activity requires the heterodimeric complex of Erf2 and Erf4; neither Erf2 nor Erf4 alone is capable of carrying out the transfer reaction (12).

Erf2 is a polytopic four transmembrane-spanning protein (R.J.D., unpublished data). The defining characteristic of Erf2 is the DHHC domain located between transmembrane 2 (TM2) and TM3. A search of the yeast genome reveals seven other DHHC-containing proteins in addition to Erf2: Akr1, Akr2, Pfa3 (Protein fatty acyltransferase 3), Swf1, Pfa4, Pfa5, and a more distant member, Ynl155W. Three yeast DHHC proteins, Akr1 (13), Erf2

(12), and Pfa3 (33), have been demonstrated to catalyze *S*-palmitoylation. The DHHC motif is essential for catalytic activity in vitro and the function of these proteins in vivo. As discussed in more detail below, the enzymes become palmitoylated during the reaction, a process termed autoacylation. Mutation of the cysteine in the DHHC motif abolishes autoacylation and palmitoylation of the substrate, a property of all DHHC proteins studied to date.

Akr1 is an ankyrin repeat-containing protein that was originally isolated based on its role in cellular morphology, bud emergence, and mating pheromone receptor endocytosis (34–36). These phenotypes were also observed in mutants lacking type I casein kinase activity, suggesting involvement in the same processes (37). The yeast type I casein kinases Yck1, Yck2, and Yck3 all require membrane localization for function (37–39). Yck1 and Yck2 localize to the plasma membrane in a process dependent on the palmitoylation of a C-terminal cysteine-cysteine amino acid motif (13, 37, 40). Davis and coworkers (13) demonstrated that membrane localization of Yck2 was dependent on Akr1, which led them to postulate, and then demonstrate, that Akr1 is the palmitoyltransferase for Yck2. Akr1 and Erf2 are both polytopic integral membrane proteins containing related DHYC (aspartate-histidine-tyrosine-cysteine) and DHHC sequences. The topology of Akr1 has been mapped, and it was determined that the DHYC domain faces the cytosolic surface of the membrane (41). Like Erf2, Akr1 is palmitoylated when incubated with palmitoyl-CoA. Mutations in the Akr1 DHYC domain abolish Akr1 autopalmitoylation and the palmitoylation of Yck2, suggesting a direct participation of the DHYC domain in palmitoyl transfer (13). Recently, Yck3 was shown to be palmitoylated, but unlike Yck1 and Yck2, the palmitoylation of Yck3 is Akr1-independent, suggesting that it is palmitoylated by another DHHC protein (38).

Akr1-dependent palmitoylation is not restricted to the C terminus of the Yck proteins. In addition to Yck1 and Yck2, the sphingoid long-chain base kinase, Lcb4, has also been shown to be a substrate for Akr1 (42). Interestingly, the palmitoylcysteines of Lcb4 are internal to the protein and not at the C terminus, as they are in Yck1 and Yck2. The surprising sequence diversity of Akr1's substrates might be explained by the presence of the ankyrin repeat domain. These domains are involved in protein-protein interactions and could interact either directly or through another protein with substrates, thus providing recognition of the substrate independently of the sequence context surrounding the palmitoylated cysteine.

A PAT that recognizes an N-terminal palmitoylation motif has also been described in yeast (33, 43). Vac8 is a myristoylated and palmitoylated protein that localizes to the vacuolar membrane and is required for vacuolar fusion (44, 45). The association of Vac8 with vacuolar membranes requires myristoylation at its N-terminal glycine residue. Palmitoylation of up to three N-terminal cysteines is proposed to influence its function through localization of the protein to specific vacuolar membrane microdomains (3). It was suggested that the palmitoylation of Vac8 proceeds through a nonenzymatic mechanism, mediated by the N-terminal longin domain of Ykt6, a SNARE protein involved in several membrane-trafficking events (46). This conclusion was based on the observation that equimolar amounts of Ykt6 and Vac8 were required for Vac8 palmitoylation in vitro (47). More recently, however, the DHHC protein Pfa3 was described as a PAT for Vac8. Pfa3 was linked to Vac8 palmitoylation genetically based on its role in the vacuolar fusion process (33) and the fact that it is required for efficient targeting of Vac8 to the vacuole membrane (43). Under normal circumstances, cells lacking *PFA3* show normal vacuolar morphology. However, when the cells are stressed by the addition of a reducing agent, *pfa3Δ* cells have fragmented vacuoles. This phenotype is reminiscent of that of cells lacking *VAC8*, although not as severe, suggesting that these two proteins may function in the same process (33). In addition, Pfa3 resides on the vacuolar membrane, making it a prime candidate for the Vac8 PAT. In vivo, Vac8 palmitoylation is significantly reduced but not absent in cells lacking Pfa3 (33, 43). Ykt6 or other DHHC proteins may be responsible for this residual palmitoylation. In vitro, Pfa3 transfers palmitate to Vac8, demonstrating that Vac8 is indeed a substrate. The in vitro reaction is specific for Pfa3, in that Vac8 was not palmitoylated by Akr1, Erf2/Erf4, Pfa4, or Pfa5. Although the substrates of Pfa4 and Pfa5 are unknown, they appear to be active PATs based on their ability to autoacylate (33).

Finally, Swf1 is a DHHC protein that appears to be responsible for palmitoylating the yeast SNARES Snc1, Syn8, and Tlg1 at cysteine residues near the cytoplasmic side of their single transmembrane span (48). Deletion of the *SWF1* gene abolishes the palmitoylation of Snc1, Syn8, and Tlg1 in vivo, consistent with it being a PAT for transmembrane proteins palmitoylated at juxtamembranous cysteine residues. Tlg1 is an endosomal SNARE involved in the recycling of proteins from the endosome to the Golgi (49). Normally, palmitoylated Tlg1 resides at the endosome. However, if Tlg1 is not palmitoylated, it becomes a substrate for the ubiquitin ligase, Tul1. The ubiquitin on Tlg1 is a signal for entry into multivesicular bodies and results in its degradation in the vacuole. The study of Swf1 and Tlg1 suggests that palmitoylation can act as more than a lipid anchor or protein-trafficking signal. In this case, palmitoylation appears to act as a stability factor, protecting Tlg1 from the cellular quality control machinery (48). Before this study, it has been difficult to assign a functional role to palmitoylating a cysteine that is adjacent to a transmembrane span. The study by Valdez-Taubas and Pelham (48) clearly demonstrates that palmitoylation within this sequence context is not a spurious event attributable to the spontaneous acylation of a cysteine residue adjacent to the membrane.

To date, three (Erf2, Akr1, and Pfa3) of the eight yeast DHHC proteins have been shown to possess PAT activity, and substrates have been identified. Swf1 and its SNARE proteins almost certainly represent another authentic enzyme: substrate class pairing. It is expected that the other members of the DHHC family will likewise turn out to be PATs. Work is under way to test this idea. The

high level of sequence similarity within this domain has made it possible to identify and study DHHC proteins in higher eukaryotes.

SEQUENCE CONSERVATION AND PHYLOGENY OF THE DHHC DOMAIN

The DHHC-Cysteine Rich Domain (CRD) was first described in a novel human pancreatic cDNA library clone and a *Drosophila* open reading frame called DNZ1 (50, 51). The following consensus sequence was proposed:

$$Cx_2Cx_9HCx_2Cx_4DHHCx_5Cx_4Nx_3F$$

The DHHC domain is similar to C_2H_2 zinc finger motifs but sufficiently different to be categorized as a variant form of this motif (52). Although the sequence of this domain suggests that it is an excellent candidate for a zinc binding site, to date this has not been experimentally addressed. If the DHHC domain does bind zinc, it is likely to be in a more complex arrangement than the canonical four-ligand coordination found in other C_2H_2 zinc binding motifs ($Zn_1C_2H_2$) (52). The DHHC consensus sequence predicts a $Zn_nC_7H_3$ stoichiometry, which would either leave some cysteine or histidine side chains uncoordinated or involve bridging two zinc atoms with one side chain, as is seen in some zinc cluster proteins. This is clearly an issue that deserves to be addressed.

Searching translated genomic databases with a 51 amino acid region that encompasses the DHHC-CRD region revealed a large number of eukaryotic DHHC proteins, but no examples of prokaryotes or archea sequences have been found. This appears to coincide with the widespread occurrence of protein palmitoylation in eukaryotes and its apparent absence in prokaryotes. The sequence and predicted domain organization of a large number of eukaryotic DHHC proteins were compared with the hope of gleaning information about their function. The sequences included 8 DHHC proteins from *S. cerevisiae*, 9 in *Trypanosoma brucei*, 23 in *Drosophila melanogaster*, 16 in *Caenorhabditis elegans*, 18 in *Takifugo rubripes*, 31 in *Arabidopsis thaliana*, and 23 in *Homo sapiens* (GenBank). All DHHC proteins, with the exception of yeast Ynl155W, are polytopic integral membrane proteins with four or more transmembrane domains (**Fig. 1A**). The DHHC-CRD is generally located between TM2 and TM3, which is predicted to put it on the cytosolic face of the membrane along with the N terminus that precedes TM1 and the C terminus that lies after TM4. In the one case in which this topology has been tested experimentally, Akr1 was shown to have the predicted topology with the DHHC domain facing the cytosol (41).

A ClustalX alignment of the yeast and human DHHC proteins was performed (Fig. 1B). Although the original consensus sequence proposed for the DHHC domain appears to hold up well, additional residues are also highly conserved. For example, basic amino acids (lysine/arginine) often appear at positions 11 and 14 (numbering of the domain is as shown in Fig. 1), proline at 12 and 33, valine/isoleucine at 25, 35, and 39, asparagine at 37 and 43, glycine at 40, and tryptophan at 34. Accordingly, a new consensus sequence for the DHHC is proposed:

$$Cx_2Cx_3(R/K)PxRx_2HCx_2Cx_2Cx_4DHHCxW(V/I)xNC(I/V)Gx_2Nx_3F$$

In addition to the DHHC domain conservation, two other areas exhibiting significant homology are observed. A DPG (aspartate-proline-glycine) motif is typically found next to TM2 and a TTxE (threonine-threonine-x-glutamate) motif is found adjacent to TM4. The functional significance of these motifs has not been addressed, but it is interesting that the two conserved motifs (DPG and TTxE) and the DHHC domain are predicted to lie on the same side of the membrane.

Several exceptions to the DHHC consensus sequence are observed. For example, Akr1, Akr2, Pfa5, DHHC22, and Ynl155W are missing cysteines and histidine residues found in other DHHC proteins (Fig. 1B). Akr1 has been shown to possess PAT activity, whereas Pfa5, DHHC22, and Ynl155W have not been tested. By comparing the sequences of Erf2 and Akr1, it is possible to identify the residues that correlate with PAT activity and to rule out the direct involvement of residues found in Erf2 but missing in Akr1. This is likely to be related to the role of zinc in the structure or function of the DHHC domain, but this issue remains to be addressed.

The sequences of DHHC domains in human and yeast proteins were aligned using ClustalX, and a phylogram was generated (Fig. 1C). In most cases, the human DHHC proteins fall into groups that include a yeast protein. For example, the human DHHC proteins DHHC5, -8, -18, -14, -19, and -9 appear most related to the yeast Ras PAT Erf2. It is tempting to propose that this represents a related family with similar function, albeit with limited evidence. The yeast Ras PAT Erf2 and human DHHC9 fall into one such related family. DHHC9 was recently shown to be a subunit of a human Ras PAT (19). Another related group includes Akr1, Akr2, and the human DHHC proteins HIP14 (DHHC17) and HIP14L (DHHC13). This group constitutes a family of ankyrin repeat-containing DHHC proteins. HIP14 was shown to complement the temperature-sensitive growth phenotype and to rescue the defect in receptor endocytosis that results from deleting *AKR1* (53). Thus, in two cases, the function of Erf2-related and Akr1-related human DHHC proteins has been inferred from phenotypes in yeast. It remains to be seen whether this will be the case for other members of the family. For example, three human DHHC proteins (1, 4, 11) are grouped with Swf1, which was recently shown to be the PAT for the yeast SNARE protein Tlg1 (48). By extending the analysis, it might be possible to identify potential homologs among the families of DHHC-CRD proteins in any eukaryotic sequence database.

METAZOAN DHHC PATS

As discussed above, it is relatively straightforward to identify candidates for metazoan PATs using the DHHC

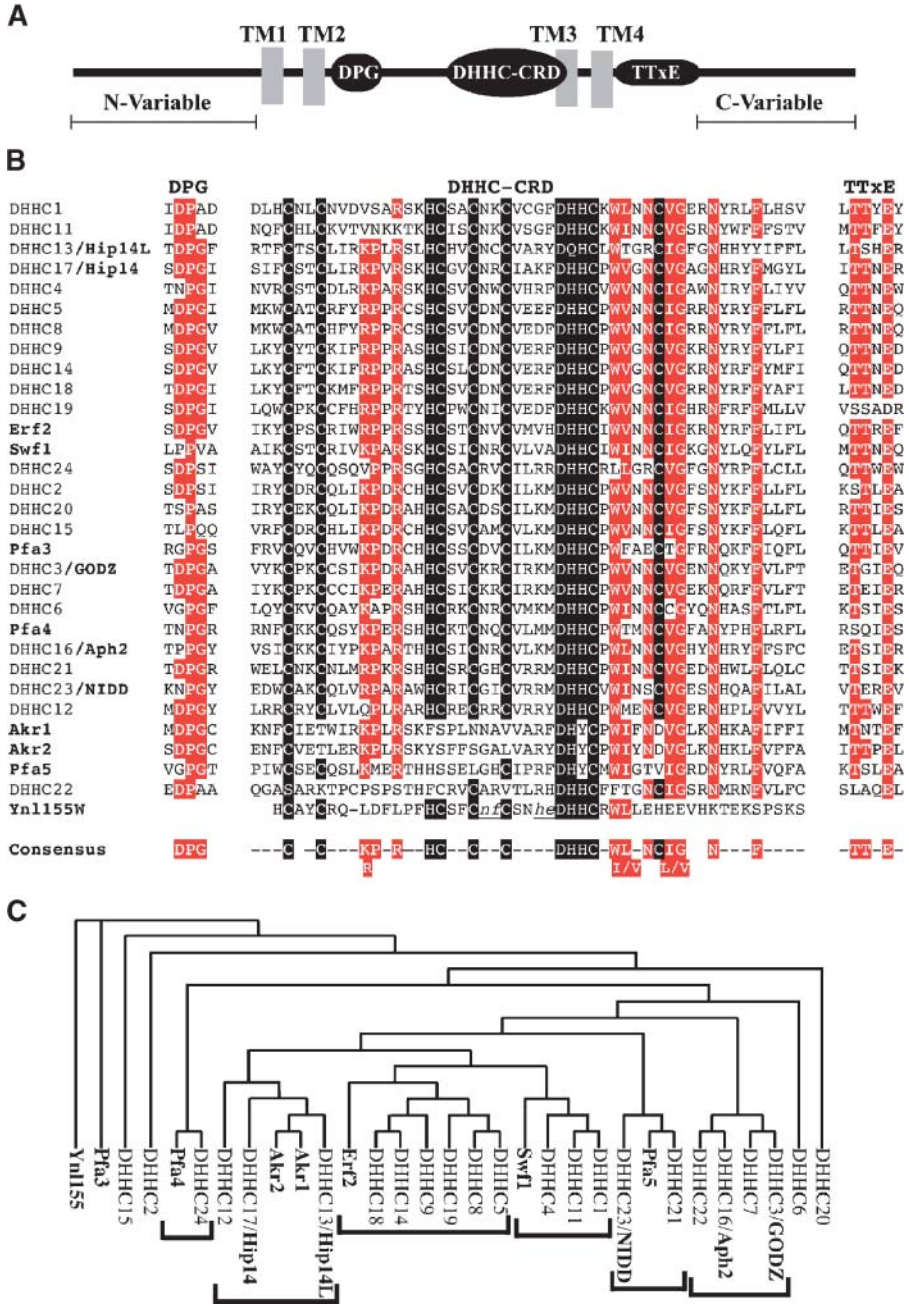

Fig. 1. Homology and phylogeny of DHHC (aspartate-histidine-histidine-cysteine) proteins. A: Schematic diagram of a DHHC domain protein. The N-terminal region, transmembrane domains (gray boxes; TM), DPG (aspartate-proline-glycine) motif, DHHC, TTxE (threonine-threonine-asparagine-glutamate) motif, and C-terminal regions are indicated. In most but not all cases, DHHC proteins are ~40 kDa in size. Exceptions involve differences in the N-variable and/or C-variable regions. B: The 51 amino acid core sequence derived from the DHHC of human and yeast Erf2 homologs was aligned using ClustalX (version 1.83). After the alignment, DPG and TTxE conserved regions were added to the alignment. Two classes of conserved residues are highlighted. The cysteine, histidine, and aspartate residues that make up the DHHC domain are shaded black. Additional conserved residues found in the DPG, DHHC, and TTxE motifs are shaded red. Two deletions were made in the Ynl155W sequence to obtain the best alignment with the DHHC domain. The letters *nf* represent the sequence NEDF (asparagine-glutamate-aspartate-phenylalanine), and the letters *he* represent the sequence HRLKE (histidine-arginine-leucine-lysine-glutamate). The sequence identification numbers for the human proteins are as follows: DHHC1 (NP_037436.1), DHHC2 (NP_057437.1), DHHC3 (NP_057682.1), DHHC4 (NP_060576.1), DHHC5 (NP_056272.2), DHHC6 (NP_071939.1), DHHC7 (NP_060210.1), DHHC8 (NP_037505.1), DHHC9 (NP_057116.2), DHHC11 (NP_079062.1), DHHC12 (NP_116188.2), DHHC13 (NP_061901.2), DHHC14 (NP_714968.1), DHHC15 (NP_659406.1), DHHC16 (NP_115703.2), DHHC17 (NP_056151.1), DHHC18 (NP_115659.1), DHHC19 (NP_653238.1), DHHC20 (NP_694983.2), DHHC21 (NP_848661.1), DHHC22 (NP_777636.1), DHHC23 (NP_775841.2), and DHHC24 (NP_997223.1) (74). C: Phylogenetic clustering of the human and *S. cerevisiae* DHHC proteins based on ClustalX alignment of the 51 amino acid DHHC core sequence using TreePuzzle (version 5.2). Using Neighbor (Phylip version 3.61), the outgroup was designated to be yeast Ynl155W, the least well conserved of the family, and the phylogram was generated using TreeView (version 1.6.6). Six potential subfamilies are indicated by brackets.

domain consensus sequence. However, characterizing the activity and identifying the substrates still present challenges. For example, the yeast Ras PAT Erf2 is closely related to DHHC9. However, expression of human DHHC9 in yeast fails to complement an *erf2Δ* strain, and attempts to demonstrate PAT activity from purified DHHC9 were unsuccessful. In yeast, Erf2 requires association with Erf4 for Ras PAT activity (12), prompting a search for a human homolog of yeast Erf4. GCP16, a Golgi-associated protein (54), exhibits weak sequence similarity with Erf4. Expression of GCP16 along with DHHC9 results in the formation of a complex that has PAT activity toward H-Ras and N-Ras substrates (19). The reaction is specific for prenylated Ras proteins in that minimal palmitoylation was observed for $G_{\alpha i1}$ or for GAP-43, two proteins with N-terminal palmitoylation motifs. This raises the possibility that other DHHC proteins in the Erf2 cluster are also palmitoyltransferases and may have specificity for substrates related to prenylated Ras or Rho proteins.

The Ras PAT activity of DHHC9/GCP16 appears to be restricted to a subset of tissues. DHHC9 transcripts were detected in kidney, skeletal muscle, brain, lung, liver, placenta, heart, colon, and small intestine. Intriguingly, GCP16 transcripts were observed in the same tissues except for colon and were present in peripheral blood leukocytes and spleen (19). This observation raises several questions regarding the potential subunit composition of palmitoyltransferases. For example, does the colon have DHHC9-specific palmitoyltransferase activity? If so, is there another Erf4-like molecule that is required for this activity? Likewise, can GCP16 associate with other catalytic subunits in the spleen and peripheral blood leukocytes to form an active palmitoyltransferase? Finally, what genetic *cis*-acting elements underlie the tissue-specific regulation of these genes? The answers to these questions await further study.

Palmitoylation plays a vital role in the nervous system, where substrates are abundant (3, 55). These include synaptotagmins, the exocytic SNARE SNAP-25, GAD65, GAP-43, and the neuronal scaffold PSD-95 as well as numerous ion channels and receptors. Axonal pathfinding, neuronal protein trafficking, and clustering of receptors and their associated proteins at synapses are targets for regulation by palmitoylation. As an example, glutamate receptor activity appears to regulate palmitate cycling on PSD-95 at the synapse (56). Treatment of neurons with the palmitoylation inhibitor 2-bromopalmitate results in the dispersal of PSD-95 and AMPA-type glutamate receptors from synaptic clusters and reduces the amplitude and frequency of AMPA-mediated miniature excitatory postsynaptic currents. Glutamate-induced internalization of AMPA receptors requires palmitoylation of PSD-95. Internalization was enhanced with the overexpression of palmitoylated PSD-95 but abolished when cells expressed PSD-95-prenyl, which is stably associated with membranes. Thus, palmitate turnover on PSD-95 regulates the cell surface expression of AMPA receptors in response to glutamate, which in turn may regulate synaptic strength. Hence, identification of the enzymes that mediate palmitate cycling on PSD-95 and other neuronal proteins is an important step for a mechanistic understanding of these processes. To date, at least five DHHC proteins are candidates for PSD-95 palmitoyltransferases. HIP14 (DHHC17) is a neuronal protein that was first identified by its interaction with huntingtin (htt) and has the greatest similarity with the Akr1 branch of the DHHC proteins (53). HIP14 resides on the Golgi and can be observed on cytoplasmic vesicles. Based on its DHHC motif, Huang et al. (57) tested HIP14 for palmitoyltransferase activity toward htt and other neuronal substrates in vitro. HIP14 had PAT activity for the N-terminal fragment of htt(1–548), SNAP-25, PSD-95, GAD65, and synaptotagmin I but not for synaptotagmin VII and paralemmin. Consistent with the biochemical experiments, perinuclear accumulation of PSD-95 and SNAP-25, but not paralemmin and synaptotagmin VII, increased when they were cotransfected with HIP14 in neurons. In neurons treated with HIP14 short, interfering RNA, synaptic clustering of endogenous PSD-95 was reduced by 50%. These data support a role for HIP14 as a regulator of neuronal protein trafficking mediated by its PAT activity.

Other candidates have emerged for the PSD-95 PAT. Fukata et al. (58) cloned and expressed 23 murine DHHC proteins and screened them for the ability to increase radioactive palmitate incorporation into PSD-95 in cells. DHHC15 and, to a lesser extent, DHHC2, DHHC3, and DHHC7 were the most effective. The authors refer to this group of DHHC proteins as the P-PATs for PSD-95-palmitoylating enzymes. Analysis of other substrates using this assay suggested DHHC substrate selectivity. SNAP-25 and G_{α} proteins were more efficiently palmitoylated when DHHC3 and DHHC7 were expressed, whereas GAP-43 incorporated more palmitate when DHHC7 and DHHC15 were expressed. Surprisingly, HIP14 (DHHC17) did not significantly increase PSD-95 palmitoylation in this assay. HIP14 (DHHC17) was active because its expression resulted in increased palmitoylation of the nonreceptor tyrosine kinase, Lck.

PAT activity of DHHC15 for PSD-95 was reconstituted in vitro using purified components. Substrate specificity was preserved in vitro. The P-PAT DHHC2 palmitoylates PSD-95, whereas DHHC18 does not. To address whether DHHC15 was linked to PSD-95 palmitoylation in vivo, the authors expressed wild-type and a catalytically inactive form of DHHC15 (C159S in the DHHC motif) in neurons and assessed their effects on PSD-95 palmitoylation. Catalytically inactive DHHC15 appeared to act as a dominant negative mutant. Whereas wild-type DHHC15 enhanced radiolabeling of endogenous PSD-95 by 4-fold, expression of the catalytically inactive mutant suppressed it by 60%. Expression of DHHC15 (C159S) reduced PSD-95 synaptic clustering as well as the clustering of cell-surface AMPA receptor GluR2 subunits, which is dependent upon PSD-95 palmitoylation. There was also a corresponding reduction in AMPA receptor-mediated miniature excitatory postsynaptic currents (58). Together, these data support DHHC15 as a regulator of PSD-95 palmitoylation in vivo.

Both of these studies (57, 58) provide a comprehensive analysis of the respective PSD-95 PATs, and the simplest conclusion is that there are multiple enzymes that regulate the activity of PSD-95 in vivo. However, if DHHC15 and HIP14 are functionally redundant, the specificity and magnitude of the effects on PSD-95 localization seen with HIP14 (DHHC17) short, interfering RNA or with the dominant interfering mutant of DHHC15 are surprising (59). Further exploration of these effects in HIP14 or DHHC15 knockout animals could help reconcile these findings. It will also be important to determine the mechanism by which the catalytically inactive DHHC15 interferes with PSD-95 palmitoylation and function and whether it can be extended to other DHHC proteins. Discrepancies with respect to the substrate specificity of HIP14 (DHHC17) are also apparent in these studies, which can be addressed with a comprehensive kinetic analysis.

HIP14 is ubiquitously expressed, and its functions may extend beyond the nervous system. HIP14 was discovered independently in a screen for proteins that could activate the Jun kinase (JNK) pathway and named AKRL1 (Akr1-like) (60). The JNK pathway is activated in response to inflammatory cytokines and environmental stresses. The mechanism of the HIP14/AKRL1 activation of JNK is unknown, but it appears to be upstream of MKK4 and MKK7, the kinases that directly phosphorylate JNK. HIP14 has also been linked to abnormal cellular proliferation (61). Overexpression of HIP14 transformed fibroblasts and caused tumors in nude mice. Interestingly, HIP14 palmitoyltransferase activity was required for its oncogenic activity. Mutation of the cysteine in the DHHC motif abolished PAT activity in vitro and its oncogenic potential in vivo. In this study, HIP14 was shown to have PAT activity for farnesylated peptides derived from the C terminus of Ras proteins, suggesting that HIP14's oncogenic properties are mediated through Ras proteins. This study also adds to the complexity of HIP14's substrate specificity. Myristoylated peptides with a palmitoylation motif found in $G_{\alpha i}$ and nonreceptor tyrosine kinases were not palmitoylated by cell extracts derived from HIP14-expressing cells, consistent with the results of Huang et al. (57) but in contrast to those of Fukata et al. (58). It is clear that the substrate specificity of HIP14 warrants further investigation. HIP14 may have broad substrate specificity that manifests differently in proliferating versus nonproliferating cells.

GODZ (DHHC3) represents another example of a DHHC protein being involved in neuronal regulation. Identified in yeast two-hybrid screens as an interactor with the GluRα1 glutamate receptor subunit and with the γ2 subunit of the $GABA_A$ receptor, GODZ is a Golgi-specific DHHC protein with four putative transmembrane domains (18, 62). It is expressed in a wide array of tissues, including heart, lung, kidney, spleen, liver, muscle, testis, and brain. GODZ enhanced palmitoylation of the γ2 subunit of the $GABA_A$ receptor in heterologous cells, suggesting that the physical interaction also represents an enzyme-substrate relationship (62). Hayashi, Rumbaugh, and Huganir (63) investigated GODZ's role in the palmitoylation of AMPA-type glutamate receptors. The receptor subunits GluR1–GluR4 are palmitoylated at two cysteine residues, one at the cytoplasmic end of transmembrane domain 2 (TMD2) and a second at the end of TMD4 near the C terminus. GODZ is likely to be the PAT for palmitoylation at the TMD2 site. When expressed in nonneuronal cells, GluR2 is only palmitoylated at the C-terminal site. Coexpression of GODZ with GluR2 results in palmitoylation at the TMD2 cysteine. Palmitoylation of the TMD2 site is associated with retention of the receptor in the Golgi apparatus, resulting in decreased steady-state levels of the receptor at the plasma membrane in heterologous cells and in neurons. These properties are dependent upon the DHHC cysteine in GODZ, demonstrating a requirement for GODZ's PAT activity. Palmitoylation at the C-terminal cysteine does not affect the steady-state distribution of receptors, but it is necessary for acute receptor internalization in response to agonist. The PAT for the C-terminal cysteine is unknown. Like HIP14, GODZ may have broad substrate specificity. As noted above, GODZ is one of the P-PATs identified in the study by Fukata et al. (58), and its expression resulted in increased palmitoylation of SNAP-25 and G_α proteins but not H-Ras. GODZ has not been characterized biochemically, and it will be important to determine whether the candidate substrates identified in the heterologous expression assays are direct targets of GODZ activity.

Neuronal nitric oxide synthase (nNOS) is an inducible, PDZ domain-containing protein that interacts with NIDD [nNOS-interacting DHHC domain-containing (DHHC23)], a DHHC protein expressed in the postsynaptic density fraction of neurons (20). Like other members of the PAT family, NIDD is a polytopic, integral membrane protein with a conserved DHHC domain. In addition, NIDD contains a PDZ domain binding motif, EDIV, that binds to the PDZ domain of nNOS. This interaction appears to play an important role in the subcellular targeting of nNOS, with overexpression of NIDD increasing the membrane association of nNOS and an increase in nitric oxide levels. Although it is tempting to propose a role for palmitoylation, this remains to be addressed. The identity of the protein or proteins palmitoylated by NIDD is unknown. In contrast to endothelial NOS (64), nNOS has not been shown to be a palmitoylated protein.

The functional importance of DHHC proteins extends to plants as well as animals. Recently, the gene for TIP1 (Tip Growth Defect 1) was cloned in *A. thaliana* (65). Phenotypically, tip1-1 and tip1-2 mutants elicit wider, shorter, and irregularly initiated root hairs compared with the wild-type gene. TIP1 is also involved in the germination and growth of the pollen tube and plays a role in regulating organ size. The deduced amino acid sequence revealed six ankyrin repeats and a DHHC motif, similar to the *S. cerevisiae* Akr1 and human HIP14 proteins, suggesting that TIP1 is a PAT. This is further substantiated by the observation that treatment of wild-type plants with the palmitoylation inhibitor 2-bromopalmitate produces plants that phenocopy the

tip1 defect. Like human HIP14, overexpression of the TIP1 gene product in *akr1Δ* yeast cells rescues phenotypes associated with the loss of *AKR1* function. In this study, the slow growth, morphological defects, and mislocalization of the Akr1p substrate Yck2p in *akr1Δ* cells were corrected when TIP1 was expressed, suggesting that it can palmitoylate Yck2 in yeast. TIP1 expressed in yeast is palmitoylated (65), similar to Akr1 (13). Palmitate labeling was abolished when cysteine 401, the cysteine residue of the DHHC motif, was mutated. TIP1 represents a new member of the ankyrin repeat-containing DHHC proteins. The identity of TIP1's substrates will be informative because it will help address the question of whether the evolutionary relationship between Akr1, HIP14, and TIP1 is conserved with respect to substrate specificity.

THE DHHC DOMAIN AND PROTEIN PALMITOYLATION

It is evident from the biochemical and genetic data described above that DHHC proteins mediate the palmitoylation of protein substrates. However, many questions remain. For example, what is the mechanism of palmitate transfer, and what role does the DHHC domain play in the reaction? Is the DHHC domain a zinc binding motif, and if so, how many zinc ions are bound and do they play a structural or catalytic role? How do PATs recognize their substrates, palmitoyl-CoA and the diverse array of palmitoylated proteins found in eukaryotic cells? These questions are just now beginning to be addressed.

All DHHC proteins examined to date are palmitoylated when incubated with palmitoyl-CoA (12, 13, 19, 33, 57, 58). The functional significance of autopalmitoylation is not clear at this time, but one possibility is that it serves as a covalent enzyme intermediate required for the PAT reaction. The formation of palmitoyl-Erf2 is sensitive to hydroxylamine, consistent with a labile thioester linkage to cysteine (12). This is further supported by the abolition of autopalmitoylation when the cysteine residue of the DHHC motif is mutated (12, 13). The DHHC cysteine is a strong candidate for the site of modification, but it is also possible that the DHHC is required for palmitoylation at another cysteine residue within the domain. Autopalmitoylation occurs very rapidly and precedes the transfer of palmitate to substrate (19, 33). Together, these data suggest, but by no means prove, that autopalmitoylation represents an acyl-enzyme intermediate with subsequent transfer of the palmitoyl moiety to the substrate. The first histidine residue within the DHHC motif is also important for the palmitoylation of the substrate (12). In Erf2, mutation of histidine 201 to alanine does not affect the formation of the palmitoyl-Erf2 intermediate, but it does result in a pronounced reduction in palmitate transferred to a prenylated Ras substrate. This could reflect a direct role for histidine 201 in palmitate transfer or reduced binding of the Ras substrate to the mutant enzyme. In Akr1, mutation of the aspartate-histidine of the DHYC motif to alanine-alanine abolishes the palmitoylation of both the enzyme and the substrate (13). The individual roles of the aspartate and histidine residues were not analyzed. The model above proposes that the DHHC protein palmitoyl adduct is an intermediate in the transferase mechanism. However, it may not represent an intermediate but instead may serve to allosterically regulate the enzyme, perhaps making it competent to bind its lipid and protein substrates.

The lipid substrate specificity of DHHC proteins in vitro is consistent with the heterogeneity found in fatty acylated proteins isolated from tissues and cells (9–11). DHHC proteins use acyl-CoA as the lipid substrate; palmitate alone is not reactive with the enzyme (57). The acyl-CoA specificity was examined for Erf2/Erf4 by monitoring the ability of unlabeled acyl-CoAs to compete with radiolabeled palmitoyl-CoA assays of Ras PAT activity (12). Long-chain fatty acyl-CoAs were the most potent inhibitors (C16:0, C16:1, 18:0, 18:1), with the enzyme being indifferent to acyl chain saturation. Myristoyl-CoA (C14:0) and lauryl-CoA (12:0) could compete to a lesser extent; decanoyl-CoA (C10:0) was ineffective. The lack of acyl-CoA substrate selectivity is in stark contrast to that of *N*-myristoyltransferase (NMT), which is specific for myristoyl-CoA (66). The structure of NMT has features that act as rulers for the acyl-CoA chain length, including a deep narrow pocket located within the protein core that accommodates carbons 7–14 of the myristoyl chain (66). NMT is a cytoplasmic protein that sequesters its acyl-CoA substrate. DHHC proteins are integral to the membrane, and conserved residues included in the DHHC consensus sequence are predicted to include a transmembrane span. Thus, the lipid binding site in DHHC proteins may be at the membrane interface or embedded in the lipid bilayer. The preference for longer chain acyl-CoAs may be a function of their ability to partition into membranes more readily.

Determining the protein composition of an active PAT is another important issue to resolve. Whereas Akr1, HIP14, DHHC15, and Pfa3 are active in the absence of a binding partner (13, 33, 57, 58), the activity of yeast Erf2 and mammalian DHHC9 Ras PATs requires an association with Erf4 and GCP16, respectively (12, 19). Both are small proteins with limited sequence homology and no apparent conserved motifs or domains that would place them in a larger protein family. The function of the Erf4 and GCP16 subunits is unknown. One role may be to stabilize the Erf2 subunit. In yeast, Erf2 that is complexed with Erf4 has a half-life of ~100 min. However, in strains lacking Erf4, the Erf2 half-life decreases to ~9 min (R.J.D., unpublished data). Similarly, DHHC9 is unstable when it is expressed and purified in the absence of GCP16 (19). Another possibility is that Erf4 and GCP16 play a more direct role in catalysis. As described above, Erf2 and DHHC9 are phylogenetically clustered with other DHHC proteins (DHHC8, -9, -14, -18, and -19). It will be important to determine whether other members of this family also complex with GCP16 or other proteins for PAT activity and/or stability. None have been purified and assayed to date.

CONCLUDING REMARKS

The field of protein lipidation has made substantial progress since the discovery of protein prenyltransferases in the late 1980s and early 1990s (67). Farnesylation and geranylgeranylation are now well-recognized posttranslational modifications (68). As discussed in earlier contributions to this series, CaaX box-directed farnesyl and geranylgeranyl transferases have also become important targets for anticancer and antiparasitic chemotherapeutic design (69, 70). Work in the ensuing years also identified the genes of enzymes responsible for the postprenylation modifications, including aaX proteases and a carboxyl methyltransferase (30, 71, 72). The subsequent posttranslational processing of prenylated proteins and the importance of these modifications in disease pathogenesis were also covered in an earlier review in this series (73). Although the study of protein prenylation proceeded briskly, protein palmitoylation lagged, largely because the enzyme or enzymes that carry out this ubiquitous modification were not known. This changed with the identification of two palmitoyltransferases using genetic screens in yeast (13, 21). It should be noted that yeast genetics has played a seminal role in the discovery of prenylation enzymes, aaX proteases, and the carboxyl methyltransferase. With the discovery of a palmitoyltransferase for Ras and a separate palmitoyltransferase for Yck2, it has become apparent that DHHC proteins are the palmitoyltransferases that had been sought for many years (12, 13). Clearly, many questions remain and much work is still needed, but the identification of DHHC PATs has provided an important new tool with which to study protein palmitoylation.

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