Regulation of Protein Trafficking and Function by Palmitoylation

Mechanism and function of DHHC S-acyltransferases

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Abstract
Protein S-palmitoylation is a reversible post-translational modification of proteins with fatty acids. In the last 5 years, improved proteomic methods have increased the number of proteins identified as substrates for palmitoylation from tens to hundreds. Palmitoylation regulates protein membrane interactions, activity, trafficking and stability and can be constitutive or regulated by signalling inputs. A family of PATs (protein acyltransferases) is responsible for modifying proteins with palmitate or other long-chain fatty acids on the cytoplasmic face of cellular membranes. PATs share a signature DHHC (Asp-His-His-Cys) cysteine-rich domain that is the catalytic centre of the enzyme. The biomedical importance of members of this family is underscored by their association with intellectual disability, Huntington’s disease and cancer in humans, and raises the possibility of DHHC PATs as targets for therapeutic intervention. In the present paper, we discuss recent progress in understanding enzyme mechanism, regulation and substrate specificity.

DHHC proteins are PATs (protein acyltransferases)
2012 marks the tenth anniversary of the discovery that DHHC proteins are protein S-acyltransferases. The combination of yeast genetics and biochemistry yielded the molecular basis of the post-translational addition of palmitate and other long-chain fatty acids to proteins, a mechanism that eluded investigators for more than three decades [1,2]. Research in this field has expanded rapidly in the last 10 years, attracting investigators from diverse fields who are connecting biological functions to the DHHC proteins, some are anticipated, whereas others are not.

The DHHC cysteine-rich domain was first recognized in sequences encoding proteins of unknown function in the yeast genome as a variant of the C2H2 (Cys2His2) zinc finger motif [3]. Named NEW1, subsequent studies identified this domain in proteins encoded by the Drosophila and human genomes [4,5]. As additional sequences were identified, the NEW1 consensus motif was extended to include conserved hydrophobic residues at the C-terminus (Figure 1) and conservation of the DHHC motif in the NEW1 cysteine-rich domain led to use of the term DHHC proteins [5]. In mammalian genomes, the genes are annotated ZDHHC in reference to the zinc finger.

The connection of the DHHC domain to palmitoylation came from the discovery by Deschenes and co-workers of ERF2 (effect on Ras function), a yeast gene encoding a DHHC protein [6]. Seeking the gene products required for Ras palmitoylation, they developed a yeast strain harbouring a sensitized allele of Ras that required Ras palmitoylation for viability. Mutations in the gene named ERF2 caused wild-type Ras to be partially mislocalized on intracellular membranes and display reduced palmitoylation. The importance of the DHHC domain was evident from the loss of function associated with mutations in the DHHC domain. Two hypotheses for the role of Erf2

Key words: DHHC, fatty acylation, palmitoylation, protein acyltransferase, ZDHHC.
Abbreviations used: Akr, ankyrin repeat-containing protein; ER, endoplasmic reticulum; Erf, effect on Ras function; GFP, green fluorescent protein; HIP14, huntingtin-interacting protein 14; NMT, N-myristoyltransferase; PAT, protein acyltransferase; Pfa, protein fatty acyltransferase; SNAP-25, 25 kDa synaptosome-associated protein; SNARE, soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor; SH4, Src homology 4; TMD, transmembrane domain; Vac8, vacuole-related 8; Yck2, yeast casein kinase 2.

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in the palmitoylation of Ras were proposed. The first is that Erf2, a protein localized in the ER (endoplasmic reticulum), is a trafficking factor required to localize Ras at the plasma membrane where it can encounter its PAT. The second is that Erf2 is a component of the PAT for Ras. A subsequent study provided the biochemical evidence that the second hypothesis was correct [1]. Erf2, reconstituted with a second protein Erf4, was shown to have PAT activity for Ras in vitro that was dependent on an intact DHHC motif. In an independent investigation, Davis and coworkers established that a second DHHC protein in yeast, Akr (ankyrin repeat-containing protein) 1, is a PAT for Yck2 (yeast casein kinase 2) [2]. Akr1 was necessary for palmitoylation and plasma membrane localization of Yck2 in vivo; purified Akr1 acylated Yck2 in vitro. Together these studies lead to a hypothesis that proteins containing a DHHC domain are PATs. Studies describing PAT activity of DHHC proteins in mammals soon followed [7,8]. At present, there is a wealth of genetic and biochemical evidence establishing that PAT activity is a conserved function of DHHC proteins in eukaryotic organisms.

Structure and localization of DHHC PATs

DHHC proteins belong to multienzyme families that range in size from five members in Schizosaccharomyces pombe (http://www.pombase.org) to 23 members in humans [9]. The enzymes are polytopic integral membrane proteins; the signature feature is the DHHC domain (Figure 1). Membrane topology predictions indicate that the core structure of a DHHC protein is four TMDs (transmembrane domains), with the N- and C-termini in the cytoplasm. The DHHC domain is positioned in the cytoplasmic loop between the second and third TMDs and terminated in the third TMD. A smaller subset of the DHHC proteins has six predicted TMDs with an extended N-terminal region encoding ankyrin repeats. Akr1 is the founding member of this group and its predicted membrane topology has been confirmed experimentally [10]. There is little sequence homology outside the DHHC domain among the DHHC proteins. There is evidence from yeast two-hybrid analyses and other studies that the cytoplasmic N- and C-terminal domains mediate protein–protein interactions, representing one mechanism to bring a protein substrate into proximity of the catalytic DHHC domain. Examples include DHHC17/HIP14 (huntingtin-interacting protein 14) and its substrate huntingtin [7,11] and DHHC5/8 and the substrate GRIP1b (glutamate receptor-interacting protein 1b), which interacts with a PDZ ligand at the C-terminus of DHHC5 and DHHC8 [12].

DHHC proteins are localized in different subcellular compartments. Of the seven DHHC proteins in Saccharomyces cerevisiae, Erf2, Pfa (protein fatty acyltransferase) 4 and Swf1 (spore wall formation 1) are localized in the ER [6,13,14]. Akr1 and Akr2 are found in the Golgi [2,13]. Pfa3 is on the limiting membrane of the yeast vacuole and Pfa5 is at the plasma membrane [13,15]. Mammalian DHHC proteins appear to predominate on endomembranes, with a few localized at the plasma membrane [13]. It is noteworthy that many of the localization studies have been performed with proteins modified with an epitope or fluorescent protein tag. This is largely owing to the absence of good antibodies and low levels of expression. There are, however, a few exceptions. Localization of DHHC17/HIP14 at the Golgi apparatus and cytoplasmic vesicles was determined by immunogold labelling in electron micrographs [11]. Localization of endogenous DHHC3 [16] and DHHC7 [17] at the Golgi and DHHC2 at the plasma membrane and in dendritic
Enzyme mechanism of DHHC proteins

The initial studies of Erf2/Erf4 and Akr1 showed that the purified enzyme becomes palmitoylated in the presence of palmitoyl-CoA [1,2]. This process is referred to as autoacylation. Autoacylation and transfer of palmitate to a protein substrate are both blocked by mutation of the cysteine residue in the DHHC motif to a serine or alanine residue, suggesting that DHHC proteins use an acyl-enzyme intermediate to transfer palmitate to the protein substrate [1,2]. Mitchell et al. [21] proposed a two-step reaction mechanism based on kinetic analysis of the yeast Ras PAT, Erf2/Erf4. They demonstrated that the purified Erf2–Erf4 complex has palmitoyl-CoA hydrolease activity. Autoacylation is rapid and followed by a slower second step involving the transfer of palmitate to water. The addition of the Ras protein substrate increased Erf2–Erf4 consumption of palmitoyl-CoA, but slowed production of free palmitate, suggesting that the palmitate was now being transferred to Ras2 in the second reaction step.

We tested the acyl-enzyme intermediate hypothesis by performing a single turnover experiment with two mammalian PATs, DHHC2 and DHHC3 [22]. The enzymes were labelled in vitro with radioactive palmitate and then purified to remove any free palmitoyl-CoA. When protein substrate was added to the acylated enzyme in the presence of excess unlabelled palmitoyl-CoA, the radioactive palmitate was lost from the enzyme over time and accumulated on the protein substrate. A slower loss of palmitate was observed when incubated in the absence of protein substrate, probably owing to hydrolysis of the thioester-attached lipid. These data provide direct evidence of palmitate transfer from the enzyme to the protein substrate and are consistent with a ping–pong kinetic mechanism. This mechanism is further supported by the finding that the acyl-CoA specificity profile is the same for the autoacylation reaction and transfer to substrate.

An unresolved question is the identity of the cysteine residue that is palmitoylated in the autoacylation reaction. Although presumed to be the cysteine residue of the DHHC motif, this has not been directly demonstrated. The mutagenesis studies only show that autoacylation is dependent on the DHHC cysteine residue and it is possible that other residues are involved. In our single turnover experiments, we consistently observed that approximately 50% of the radioactivity remained associated with the enzyme, despite long incubation times [22]. It is unknown whether this represents inactive enzyme with lipid still attached or whether there are secondary acylation sites. The stoichiometry of autoacylation was measured at 0.5 palmitate per DHHC, similar to a value of 0.65 reported for the yeast DHHC protein Erf2 [21]. These data suggest that the stoichiometry of autoacylation in vitro is one acyl chain per DHHC. However, it has also been proposed that there is intramolecular transfer of the acyl group from the catalytic cysteine residues to distal cysteine residues [23,24]. An alternative possibility is that DHHC proteins behave as asymmetric dimers, with both subunits incorporating palmitate at a single site, but only one DHHC subunit transferring the palmitate to water or to a protein substrate. Consistent with this possibility, there is evidence that DHHC3 forms homomultimers and heteromultimers with DHHC7 [25]. Assessment of the quaternary structure of the purified active enzyme and mapping the sites of autoacylation with MS will be informative in addressing these possibilities.

Substrate specificity of DHHC proteins

Acyl-CoA specificity

In the course of investigating the kinetic mechanism of DHHC proteins we found a surprising difference in the acyl-CoA chain-length specificity of DHHC2 and DHHC3 [22]. Whereas DHHC2 efficiently translated acyl-chains of 14 carbon atoms and longer, DHHC3 activity was greatly reduced when acyl-CoAs with chain lengths longer than 16 carbon atoms were provided as substrates. Although palmitate is presumed to be the most abundant of the fatty acids attached to proteins, preferential modification of proteins with fatty acids other than palmitate has been detected [26–28], with functional consequences on lipid raft localization and signalling [29,30]. Our finding that DHHC proteins have different acyl-CoA substrate specificity may account for differential S-acylation of proteins observed in cells.

It will be of interest to determine the structural basis of the difference in acyl-CoA specificity between DHHC2 and DHHC3. The very strict acyl-CoA substrate specificity of the protein NMT (N-myristoyltransferase) is evident in the crystal structure and is achieved by the distance between an oxyanion hole binding the carbonyl of the fatty acid and the floor of the hydrophobic pocket. Furthermore, a hydrophobic groove induces bends in myristate that further restrict acyl-chain saturation and branching [31]. NMT is a soluble enzyme. More similar to DHHC proteins is the VLCFA (very-long-chain fatty acid) synthase complex. The Fen1p and Sur4p subunits of this complex are integral membrane proteins and display different acyl-CoA specificities [32]. The sizing mechanism results from the distance between a cytoplasmic active site and a lysine...
residue extending from a transmembrane helix within the lipid bilayer. By shifting the lysine residue up and down the turns of the helix, acyl-chain specificity was altered. Whereas DHHC3 does not contain lysine residues within its predicted TMDs, it does contain charged residues that may behave similarly in restricting acyl-chain length, a hypothesis that we are currently testing.

Protein substrate specificity

Prenylated and N-myristoylated proteins have well defined consensus sequences that are recognized by one of three prenyltransferases or one (or two, in mammals) NMTs. In contrast, there is no consensus sequence for palmitoylation. With the discovery of seven DHHC PATs in yeast and 23 in humans, one might anticipate that as substrates were identified for individual DHHC proteins, rules governing substrate specificity would emerge. As is often the case in biology, the situation is complex. Indeed the apparent functional redundancy of DHHC proteins emerged with the discovery of Erf2. Ras palmitoylation was only partially reduced when Erf2 was absent [6], suggesting that other proteins were compensating for the residual palmitoylation.

The question of substrate specificity of the DHHC proteins in yeast was addressed globally by a proteomic study from Davis and coworkers [33]. After defining the yeast palmitoylproteome through a combination of acyl-biotin exchange chromatography and MS, the profile of palmitoylated proteins was examined in yeast strains with deletions in DHHC proteins, individually or in combination. What emerged from this study was a finding that whereas palmitoylation of some proteins was dependent on a single DHHC PAT, others appeared to be modified by several enzymes. In mammalian cells, a screening strategy in which the 23 mouse DHHC proteins are individually co-expressed with a substrate of interest has typically revealed a subset of DHHC proteins that increase substrate palmitoylation [8,34]. In many cases, subsequent DHHC gene silencing experiments have confirmed the assignment of a cognate enzyme-substrate pair [34]. Characterization of DHHC-knockout mouse models has also been informative in assigning substrates [35–37].

Several recent studies have focused on elucidating the structural determinants of protein substrates that are recognized by their cognate DHHC proteins. We demonstrated that the vacuolar DHHC protein Pfa3 is a PAT for Vac8 (vacuole-related 8), an armadillo repeat protein that is localized at the yeast vacuole [15]. The first 18 amino acids of Vac8 comprise a so-called SH4 (Src homology 4) domain that is N-myristoylated and multiply S-palmitoylated. Nadolski and Linder [38] dissected the molecular determinants of Vac8 that enabled it to be recognized as a substrate in vitro by purified Pfa3. They showed that myristoylation was not absolutely required for, but significantly enhanced, palmitoylation of Vac8, consistent with in vivo data [39]. Pfa3 was capable of palmitoylating each of the three N-terminal cysteine residues in Vac8. Interestingly, a fusion protein of the Vac8 SH4 domain attached to GFP (green fluorescent protein) was sufficient for palmitoylation by Pfa3. However, the specificity of palmitoylation seen for the full-length protein was lost and the SH4 domain GFP protein was palmitoylated by all five of the yeast DHHC proteins tested. These data suggested that a region of the protein C-terminal to the SH4 domain is important for conferring specificity of palmitoylation. This region was identified as the eleventh armadillo repeat of Vac8 in substrate competition experiments.

The theme that there are multiple recognition elements in a substrate for recognition by a PAT was also observed for the Akr1 substrate Yck2 [40] and the synaptic SNARE (soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor) protein SNAP-25 (25 kDa synaptosome-associated protein) [41]. Davis and coworkers showed that Yck2 has a tripartite palmitoylation signal comprising a ten-residue-long C-terminal peptide that includes the palmitoylated dicysteine motif, the kinase catalytic domain and a glutamine-rich sequence of 176 amino acids that appears to be largely unstructured and links the C-terminal peptide with the kinase domain [40]. SNAP-25 is palmitoylated at a cysteine cluster within the unstructured interhelical domain that connects the two SNARE domains. Palmitoylation of SNAP-25 is mediated by several DHHC PATs, including DHHC3, 7 and 17 (HIP14). Interestingly, palmitoylation by DHHC17/HIP14 is dependent on a proline residue that is separated from the palmitoylated cysteine residues by 24 residues. This distance between the cysteine residues and the proline is also important for palmitoylation by DHHC17. In contrast with proteins that undergo dual lipidation with prenyl and palmitoyl or myristoyl and palmitoyl lipids, soluble proteins anchored to membranes solely by S-palmitoylation lack a mechanism to interact with membrane-bound S-acyltransferases. It has been proposed that the complex recognition sequences found in Yck2 and other soluble proteins enable DHHC PATs to actively recruit their substrates from the cytoplasm [40]. In contrast, substrates modified with a myristoyl or prenyl group have intrinsic membrane affinity and therefore, can more readily access PATs.

Concluding remarks

Schmidt and Schlesinger [42] at Washington University in St Louis brought palmitoylation into prominence in a 1979 Cell publication, reporting that viral glycoproteins are covalently modified with fatty acids. The concluding sentence was, “We have preliminary data that normal cells contain a subset of proteins with stably bound fatty acids. Thus, the post-translational modification described here for viral G protein is most probably a general one”. Numerous cellular proteins were shown to be palmitoylated subsequent to this work. Progress in understanding the mechanism and regulation of palmitoylation lagged behind studies of prenylation and N-myristoylation, however, the field has been energized in recent years. The discovery of the DHHC proteins is just one element of many that have accelerated progress in this
field. The development of chemical biology approaches and advances in MS and live cell imaging enable palmitoylation to be interrogated today with a level of detail and sophistication that will continue to provide insight into this fundamental biological process.

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