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On the mechanism of protein palmitoylation

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Protein palmitoylation or, more specifically, *S*-acylation is a reversible post-translational lipid modification. Despite the identification of several proteins that are altered in this way, our understanding of the enzymology of this process has been hampered by the lack of well-characterized acyltransferases. We now know of three proteins in *Saccharomyces cerevisiae* that promote palmitoylation: effector of Ras function (Erf2), ankyrin-repeat-containing protein (Akr1) and the SNARE protein Ykt6. Erf2 and Akr1 are integral membrane proteins that contain a cysteine-rich domain and an Asp-His-His-Cys motif, both of which catalyse acylation at the carboxyl terminus of their target proteins. Recently, we discovered that Ykt6 mediates the amino-terminal acylation of the fusion protein Vac8. Even though these three proteins differ in sequence, topology, size and substrate specificity, they might function in a similar manner. In this review, we discuss these observations in the context of a potential general mechanism of acylation.

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Introduction

Lipid modifications are required for the membrane targeting of proteins and for their enrichment in microdomains on organelles (Resh, 1999; Berthiaume, 2002; Hancock, 2003). Protein palmitoylation is the addition of fatty acids (mainly palmitic acid) through an N-amide bond or a thioester (*S*-acylation). *S*-acylation is unique in that it is the only reversible lipid modification (Linder & Deschenes, 2003; Smothrys & Linder, 2004). In this review, we use the term palmitoylation exclusively to describe *S*-acylation.

Similar to other lipid modifications, protein palmitoylation is thought to be an enzymatic reaction, which is mediated by a protein that is known as a palmitoyltransferase (PAT) or an acyltransferase. However, the existence of such proteins has long been questioned for several reasons: first, although biochemical studies were successful in enriching PAT activity, they either failed to identify the respective protein (Kasinathan *et al*, 1990; Berthiaume & Resh, 1995; Das *et al*, 1997) or identified false positives (Liu *et al*, 1996); second, palmitoylated proteins have no clear consensus sequence for the modification—the common denominator for most palmitoylated proteins is a membrane-targeting sequence in the vicinity of the target cysteines that consists of positive charges, adjacent lipid anchors or transmembrane domains (Bijlmakers & Marsh, 2003); third, proteins containing a target cysteine can be autoacylated *in vitro* in the presence of palmitoyl-CoA (Pal-CoA; Duncan & Gilman, 1996; Veit *et al*, 1998; Veit, 2000; Bizzozero *et al*, 2001). The spontaneous nature of this modification raised doubts as to whether an enzyme was required by cells. However, during the past two years, three putative acyltransferases have been identified in the yeast *Saccharomyces cerevisiae*: two proteins with a conserved Asp-His-His-Cys (DHHC) motif—effector of Ras protein (Erf2) and ankyrin-repeat-containing protein (Akr1)—and the SNARE protein Ykt6 (Table 1; Lobo *et al*, 2002; Roth *et al*, 2002; Dietrich *et al*, 2004). In this review, we compare the recent findings on these acyltransferases and discuss a potential general mechanism of acylation.

Palmitoyltransferases in yeast

The DHHC proteins. The identification of Erf2 and Akr1 as acyltransferases brought into focus a family of proteins with a DHHC consensus sequence and a cysteine-rich domain (CRD; Lobo *et al*, 2002; Roth *et al*, 2002). Both Erf2 and Akr1 are polytopic membrane proteins with four predicted transmembrane domains. The traits that identify these proteins as acyltransferases are summarized below.

In mammalian cells, two members of the RAS superfamily, N-RAS and H-RAS, are farnesylated and require palmitoylation for their localization and function at the plasma membrane. Yeast has two redundant Ras homologues: Ras-like protein 1 (Ras1) and Ras2. Deschenes and colleagues identified the proteins Erf2 and Erf4 as necessary for the targeting of Ras2 to membranes (Bartels *et al*, 1999). Both proteins seemed to be involved in Ras2 palmitoylation, as this modification was suppressed in *erf2*- and *erf4*-deletion mutants (Bartels *et al*, 1999). Erf2 is a transmembrane protein that recruits Erf4 to the endoplasmic reticulum (Zhao *et al*, 2002). Subsequent purification of the Erf2/4 complex from yeast and *Escherichia coli* showed that it has acyltransferase activity for farnesylated yeast Ras2 (Lobo *et al*, 2002). Erf2 contains a DHHC box, and mutations in this sequence inhibit the *in vitro* palmitoylation activity of Erf2/4 and interfere with Ras2 palmitoylation *in vivo*. These findings indicate that Erf2 might act as the acyltransferase. Erf2 mediates palmitoylation in a substrate-specific manner: mammalian H-RAS and the amino (N)-terminal Src-homology 4 (SH4) domain of $G\alpha_i$ are not acylated by Erf2 *in vitro*. Less specificity is observed for the lipid moiety, with palmitate (C_{16}) and oleate (C_{18}) being preferred over myristate (C_{14}) and shorter length CoA derivatives (Lobo *et al*, 2002). The specificity of Erf2 for Ras2 is, at least in part, determined by lysine residues that are upstream of the target cysteine (Dong *et al*, 2003).

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Table 1 | Overview of characterized PATs and their substrates

Palmitoyl transfer protein				Substrate		
Name	**Localization**	**Anchored by**	**Domain**	**Name**	**Target cysteines**	**Reference**
Akr1	Golgi	4–5 transmembrane domains	DHHC–CRD	Yck2	—CC	Roth *et al*, 2002
Erf2	Endoplasmic reticulum	4–5 transmembrane domains	DHHC–CRD	Ras2	—C…CIIS	Lobo *et al*, 2002
Ykt6	Ubiquitous	Farnesylated Palmitoylated	Longin domain	Vac8	MGSCCSC—	Dietrich *et al*, 2004

Akr1, ankyrin repeat-containing protein 1; CRD, cysteine-rich domain; DHHC, an Asp-His-His-Cys motif that is found in many putative acyltransferases; Erf2, eukaryotic peptide chain-release factor GTP-binding subunit 2; Ras2, Ras-like protein 2; Vac8, vacuolar protein 8; Yck2, yeast casein kinase 2.

The second DHHC protein, Akr1, was initially identified as being required for plasma-membrane targeting of two casein kinase I homologues: Yck1 and Yck2 (Feng & Davis, 2000). Both of these proteins have a carboxy (C)-terminal CC-sequence and were initially thought to be prenylated (Feng & Davis, 2000). However, Davis and co-workers showed that the Yck2 C-terminus is, in fact, palmitoylated in an Akr1-dependent manner (Roth *et al*, 2002). Akr1 was shown to palmitoylate Yck2 at the Golgi, which then moved to the plasma membrane by vesicular transport (Babu *et al*, 2004). Purified Akr1 from yeast promoted the palmitoylation of both Yck2 and itself; the self-acylation was independent of substrate palmitoylation as it remained unaltered irrespective of whether Yck2 was added to the reaction, and both activities were lost on mutating the DHHC box of Akr1. Interestingly, *in vitro* acylation was stimulated by ATP, although the reason for this is unclear. Davis and colleagues noted that Akr1 has no clear consensus sequence for ATP binding and other nucleotide analogues were not tested in the reaction. It therefore remains to be seen whether ATP is a specific effector in the Akr1-mediated reaction.

These examples could mark the DHHC box as a motif of palmitoyltransferases. Indeed, further DHHC proteins have recently been identified and implicated in protein palmitoylation. In neuronal cells, the Golgi-specific DHHC zinc-finger protein (GODZ) is required for the palmitoylation of the γ2-subunit of the $GABA_A$ receptor (Keller *et al*, 2004). In addition to Erf2 and Akr1, five DHHC proteins have been identified through sequence homology in yeast (Linder & Deschenes, 2003). One of these, the hypothetical zinc-finger membrane protein Ynl326c, has been localized to the vacuoles (Huh *et al*, 2003) to which palmitoylation activity has also previously been mapped (Veit *et al*, 2003). However, identifying the precise role of each of these DHHC proteins and their substrate specificities will require further studies.

The longin Ykt6. It has been known for some time that Pal-CoA stimulates yeast vacuole fusion (Haas & Wickner, 1996), which indicates a requirement for protein palmitoylation. The fusion factor Vac8 was subsequently identified as a target of palmitoylation on the yeast vacuole (Veit *et al*, 2001; Wang *et al*, 2001). Vac8 contains an N-terminal SH4 domain that is myristoylated at a glycine residue and is palmitoylated at up to three cysteine residues (Fleckenstein *et al*, 1998; Pan & Goldfarb, 1998; Wang *et al*, 1998). Palmitoylation of Vac8 occurs during an early stage of *in vitro* vacuole fusion and is required for completion of the process (Veit *et al*, 2001; Wang *et al*, 2001). We showed recently that Ykt6 is required for the acylation of Vac8 (Dietrich *et al*, 2004). Ykt6 is a highly conserved SNARE (Tochio *et al*, 2001) that consists of three domains: an N-terminal longin domain, which is a tightly folded domain that is found in a subset of v/R-SNAREs; the coiled-coil/SNARE domain; and a farnesylation consensus sequence at the C-terminus (Filippini *et al*, 2001). Sequence comparison indicates that the longin domain has some similarity to the human β-ketoacyl synthase, which is a subunit of the fatty-acid synthase that conjugates acetyl-CoA and malonyl-CoA. Consistent with this finding, the Ykt6 longin domain is able to bind Pal-CoA, CoA or palmitate. Antibodies to the longin domain inhibit both fusion and palmitoylation (Dietrich *et al*, 2004); we therefore reasoned that Ykt6 could function in the palmitoylation reaction. This theory was verified by palmitoylating recombinant Vac8 *in vitro* in the presence of recombinant Ykt6 or its longin domain, all of which were purified from *E. coli.* Surprisingly, palmitoylation required a roughly equimolar ratio of Vac8 and Ykt6. This finding is not consistent with a typical catalytic mechanism, in which the enzyme can be reused in several reactions. Interestingly, Akr1- and Erf2-mediated protein acylation were also performed at equimolar concentrations of substrate and enzyme (Lobo *et al*, 2002; Roth *et al*, 2002). The following section offers a possible explanation for these observations in the context of mechanistic studies on protein palmitoylation.

An integrated model for protein palmitoylation

It has been proposed that acyltransferases might act as enzymes that recognize specific sequences on proteins and transfer lipids to the target cysteine. This reaction is thought to occur through a thioester intermediate, which is similar to known acyltransferases in lipid metabolism (Fig 1B). However, research on protein palmitoylation has been plagued by an inherent property of the target proteins: under appropriate conditions, they can be autopalmitoylated in the absence of an apparent enzyme (Duncan & Gilman, 1996; Veit *et al*, 1998; Dunphy *et al*, 2000; Veit, 2000; also reviewed by Bijlmakers & Marsh, 2002; Smotrys & Linder, 2004). Autoacylation of purified proteins occurs at the same specific sites as in the presumably enzyme-mediated reaction *in vivo*, albeit at a slower rate (Leventis *et al*, 1997; Bano *et al*, 1998; Veit *et al*, 1998; Dunphy *et al*, 2000). This has led to the speculation that protein palmitoylation is not necessarily enzyme mediated (Bano *et al*, 1998; Bizzozero *et al*, 2001). Consistent with this theory, Bizzozero and colleagues noted that, under appropriate conditions, the activation energy that is required for transferring palmitate from Pal-CoA to a peptide is only one-fifth of the energy that is required for enzyme-catalysed acyl-transfer reactions (Bharadwaj & Bizzozero, 1995). Therefore, palmitoylation can occur spontaneously. The crucial aspect of this transfer reaction is the formation of a reduced deprotonated cysteine (a thiolate) as the target for palmitate (Bizzozero *et al*, 2001). The thiolate anion can then act as a nucleophile on the thioester bond in Pal-CoA to catalyse the generation of the palmitoylated protein (Fig 1A).

If autoacylation is indeed the basis for *S*-acylation, this raises the question of how the spontaneous transfer of palmitate to target

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proteins is regulated in cells. Control might occur at two levels: the formation of the thiolate and the Pal-CoA availability (Fig 2).

Focusing on the first level, what is known about the formation of thiolates? The sulphydryl group of a cysteine has an ionization constant pK_A of 8.5, which makes the formation of a thiolate under the cytosolic conditions of the cell (pH 7.2–7.4) unlikely. However, in the context of a peptide or a protein, proximal polar or charged side chains can notably modulate the pK_a of a cysteine and, therefore, its potential to form a thiolate. Indeed, the pK_a of a cysteine can be reduced by as much as six orders of magnitude to a value of 3 (Mossner *et al*, 2000), which makes the formation of thiolates highly likely. The dependence of the pK_a on the position of a cysteine in a protein could also explain the peculiar observation that autoacylation targets the same cysteines that are acylated *in vivo* (O'Brien *et al*, 1987; Quesnel & Silvius, 1994; Bharadwaj & Bizzozero, 1995; Schroeder *et al*, 1996, 1997; Bano *et al*, 1998).

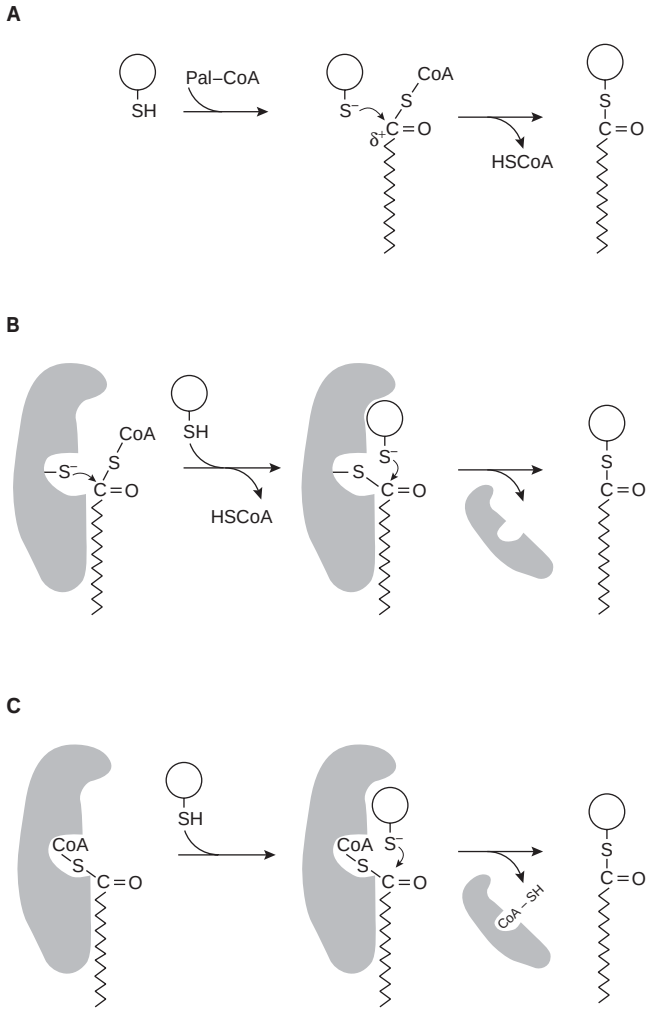

Fig 1 | Potential mechanisms of palmitoylation. (**A**) Basic mechanism. The reduced sulphydryl group on the target protein is deprotonated to form a thiolate. The thioester bond between the protein and palmitate is formed as a consequence of a nucleophilic attack of the thiolate on the α-carbon of palmitoyl-CoA (Pal-CoA). (**B**) Formation of a thioester intermediate. The palmitoyltransferase forms a thioester intermediate with palmitate, then binds to a target protein and catalyses the transfer of palmitate. For simplicity, the targeting of the substrate protein to the membrane is not shown. (**C**) Transfer protein-assisted palmitoylation. The Pal transfer protein binds to CoA or Pal-CoA through a binding pocket and presents Pal-CoA to a target protein, which might then bind to the transfer protein. Nucleophilic attack of the thiolate of the target protein on the α-carbon of bound Pal-CoA allows the formation of a thioester bond. After this reaction, the CoA might remain bound to the transfer protein.

It is tempting to speculate that, similar to the modulation of cysteines by intramolecular effects, protein–protein interactions could affect the pK_a of a cysteine *in trans*. In this context, it is interesting to note that the SNARE SNAP25 is acylated approximately 100-fold more efficiently when it is bound to the SNARE syntaxin (Veit, 2000) and that G_α palmitoylation is favoured in the presence of $G_{\beta\gamma}$ (Duncan & Gilman, 1996). It is possible that interaction with a protein partner might influence protein stability and, therefore, the accessibility of the sulphydryl group, or it might change the local pK_a of the target cysteine. It is also noteworthy that not only palmitate (C_{16}) but also palmitoleate ($C_{16:1}$), stearate (C_{18}) and oleate ($C_{18:1}$) have been found on target cysteines (Hallak *et al*, 1994; Schroeder *et al*, 1996; Liang *et al*, 2002, 2004). These findings indicate that the respective enzyme must be less specific in its substrate recognition *in vivo* than, for example, the cytosolic *N*-myristoyl transferase (Bhatnagar *et al*, 1997, 1998).

The second level at which spontaneous acylation might be regulated is the availability of Pal-CoA in the cell (Fig 2). Eukaryotic cells maintain a low free Pal-CoA concentration in the cytoplasm; the conserved acyl-CoA binding protein (ACBP) binds free Pal-CoA and maintains the intracellular concentration within the nanomolar range (Faergeman & Knudsen, 1997). This buffering effect of ACBP might be crucial in preventing uncontrolled palmitoylation. Consistent with this idea, ACBP has been used as a tool to quench autoacylation. Interestingly, PAT-mediated acylation is largely resistant to ACBP (Leventis *et al*, 1997; Dunphy *et al*, 2000), which indicates that a factor can compete with ACBP for Pal-CoA and then transfer the palmitate moiety to the target cysteine. For this reaction to occur, the factor does not necessarily need to form a thioester intermediate (Fig 1B). It would be sufficient to bind and present Pal-CoA to the thiolate of the target protein, thereby facilitating an efficient nucleophilic attack (transfer protein-assisted acylation; Fig 1C).

A crucial difference between the two models is the nature of palmitate binding to the PAT. Whereas a thioester intermediate constitutes a covalent interaction, a transfer protein might noncovalently associate with the palmitate and CoA moiety. After the transfer of palmitate, CoA could remain associated with the PAT, thereby preventing the incorporation of another Pal-CoA molecule for a further round of fusion. This is consistent with our observation that Ykt6 binds not only Pal-CoA but also CoA alone (Dietrich *et al*, 2004). Furthermore, the transfer-protein model would provide a possible explanation for our intriguing finding that, *in vitro*, Ykt6 is required in a 1:1 ratio with its substrate Vac8 for efficient acylation. *In vivo,* further control mechanisms might allow an efficient release of CoA from Ykt6.

In this context, it is notable that Erf2 and Akr1, even though they are assumed to be enzymes, have not been shown to act as such—kinetics and titrations have not been presented so far. The strongest arguments for their specificity are based on mutations in the DHHC box that inactivate the proteins (Lobo *et al*, 2002; Roth *et al*, 2002), which indicate that crucial amino acids might be involved in the formation of a potential thioester intermediate during the reaction. The autoacylation of DHHC proteins, as reported for Erf2 (Lobo *et al*, 2002), might point to such an intermediate.

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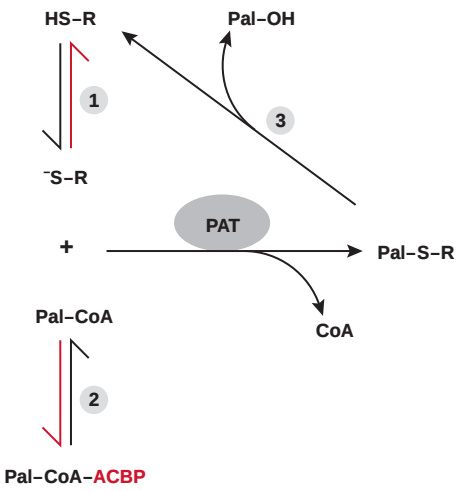

Fig 2 | Regulation of palmitoylation: thiolate formation and the availability of palmitoyl-CoA. (1) Thiolate formation on the substrate protein (R) could be counteracted by changes in the redox environment; that is, the formation of disulphides or oxidation of the sulphydryl group. (2) The available palmitoyl-CoA (Pal-CoA) pool is buffered by the acyl-CoA binding protein (ACBP). (3) Pal transfer protein (PAT)-mediated protein palmitoylation is counteracted by a thioesterase.

However, these residues and, consequently, autoacylation could also be required for protein stability, as the DHHC box has similarity to a zinc-finger domain. Taking into consideration the fact that the autoacylation of some proteins is strongly increased in the presence of their interaction partners (Duncan & Gilman, 1996; Veit, 2000), it is conceivable that the binding of Erf2 to Ras2, or of Akr1 to Yck2, might facilitate autoacylation.

These speculations on the regulation of protein palmitoylation are consistent with findings on another cysteine-based reversible protein modification: *S*-nitrosylation. This post-translational process is implicated in the control of several physiological reactions, such as smooth-muscle relaxation (Davis *et al*, 2001; Hess *et al*, 2001; Stamler *et al*, 2001; Boehning & Snyder, 2003). *S*-nitrosylation occurs on the sulphydryl groups of many target proteins and is not catalysed by enzymes. It might therefore be predicted that nitrosylation targets cysteine residues randomly (Davis *et al*, 2001). However, only certain cysteine side chains in target proteins are modified by *S*-nitrosylation (Lander *et al*, 1995; Xu *et al*, 1998; Sun *et al*, 2001). This indicates that the local environment of the target sequence controls the availability of sulphydryl groups (Hess *et al*, 2001), which is similar to our view of the availability of certain cysteines for palmitoylation. Interestingly, *S*-nitrosylation usually occurs in the vicinity of hydrophobic surfaces, either at membranes or in proteins (Stamler *et al*, 2001), and the autoacylation of peptides is facilitated under the same conditions (Quesnel & Silvius, 1994).

It is important to note that our discussion of *S*-acylation might not reflect the mechanism of palmitoylation for the signalling molecules Hedgehog and Wnt (reviewed by Linder & Deschenes, 2004; Mann & Beachy, 2004). The modification of these proteins occurs in the oxidizing environment of the Golgi and, at least for Hedgehog, results in an N-amide linkage (Mann & Beachy, 2004). It is therefore unlikely to reflect the mechanism of cytosolic *S*-acylation.

Our transfer protein-based model of *S*-acylation integrates several conflicting observations that have been made on the palmitoylation of proteins. It proposes that a PAT mediates specific acylation (consistent with an enzymatic mechanism) in a manner that is not dependent on a thioester intermediate (as predicted by studies on autoacylation). As an enzymatic function has not yet been shown, we propose that the protein that mediates this reaction should be known as a Pal transfer protein (and might therefore retain the acronym PAT).

We speculate that the specificity of palmitoylation is determined by the target sequence; that is, the formation of the thiolate anion. The function of a Pal transfer protein is to present Pal-CoA to the thiolate of the target protein. Ykt6 fulfils the criteria for such an activity: on vacuoles, Ykt6 is present in close proximity to Vac8 and is able to bind Pal-CoA through its longin domain (Dietrich *et al*, 2004). On the basis of our present knowledge, we consider it unlikely that Ykt6 contains an active site, and it remains possible that Erf2 and Akr1 act by a similar mechanism. As the activities of all three yeast PATs can be reconstituted *in vitro*, we are now in a good position to address directly many of the unresolved questions and to arrive at a general mechanism of protein palmitoylation.

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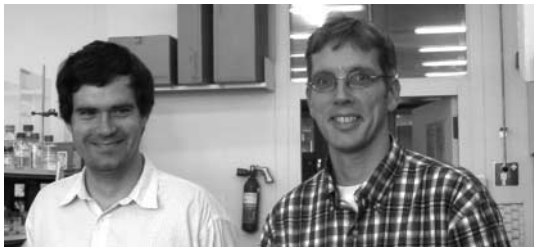

Lars E.P. Dietrich & Christian Ungermann, who is the recipient of an EMBO Young Investigator Award

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