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The Canonical DHHC Motif Is Not Absolutely Required for the Activity of the Yeast *S*-acyltransferases Swf1 and Pfa4*[S]

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Background: Mutations in the DHHC motif of *S*-acyltransferases are thought to result in lack of activity.
Results: The yeast *S*-acyltransferases Swf1 and Pfa4 are partially active in the absence of an intact DHHC motif.
Conclusion: *S*-acylation may occur by alternative mechanisms.
Significance: These results contribute to understanding the mechanism of protein *S*-acylation and suggest that proteins with divergent DHHC motifs might possess *S*-acyltransferase activity.

Protein *S*-acyltransferases, also known as palmitoyltransferases (PATs), are characterized by the presence of a 50-amino acid domain called the DHHC domain. Within this domain, these four amino acids constitute a highly conserved motif. It has been proposed that the palmitoylation reaction occurs through a palmitoyl-PAT covalent intermediate that involves the conserved cysteine in the DHHC motif. Mutation of this cysteine results in lack of function for several PATs, and DHHA or DHHS mutants are used regularly as catalytically inactive controls. In a genetic screen to isolate loss-of-function mutations in the yeast PAT Swf1, we isolated an allele encoding a Swf1 DHHR mutant. Overexpression of this mutant is able to partially complement a *swf1*Δ strain and to acylate the Swf1 substrates Tlg1, Syn8, and Snc1. Overexpression of the palmitoyltransferase Pfa4 DHHA or DHHR mutants also results in palmitoylation of its substrate Chs3. We also investigated the role of the first histidine of the DHHC motif. A Swf1 DQHC mutant is also partially active but a DQHR is not. Finally, we show that Swf1 substrates are differentially modified by both DHHR and DQHC Swf1 mutants. We propose that, in the absence of the canonical mechanism, alternative suboptimal mechanisms take place that are more dependent on the reactivity of the acceptor protein. These results also imply that caution must be exercised when proposing non-canonical roles for PATs on the basis of considering DHHC mutants as catalytically inactive and, more generally, contribute to an understanding of the mechanism of protein palmitoylation

Palmitoylation or *S*-acylation is a widespread posttranslational modification of proteins that involves the addition of a long-chain fatty acid to a cysteine residue of a protein through thioestherification. Palmitoylation is involved in many important biological processes by controlling the function, localization, and stability of numerous proteins such as ion channels, receptors, SNAREs, and signaling molecules like Ras or G proteins (reviewed in Refs. 1–6).

S-acylation is mediated by a family of polytopic membrane proteins containing between four and six transmembrane domains. This family is characterized by the presence of a 50-residue-long domain called the DHHC domain, which in most but not all cases is also cysteine-rich (DHHC-CRD)[3] and gets its name from a highly conserved DHHC signature tetrapeptide. Outside of this domain, two other short motifs, TT*X*E and DPG, are also highly conserved (4). Finally, the palmitoyltransferase conserved C-terminal (PaCCT) motif is present in most PATs (7).

Several conserved residues within the DHHC-CRD were mutated for the yeast PATs Erf2 and Pfa3, the PATs responsible for the palmitoylation of Ras2 and Vac8, respectively, resulting in lack of function in most but not all cases (8, 9). A thorough mutational analysis of the DHHC-CRD domain of Swf1, the PAT for transmembrane SNARE proteins in yeast (10), allowed us to propose a model for the structure of the Swf1 DHHC-CRD in which a role in zinc coordination is assigned to specific cysteines and histidines (11). Besides being responsible for catalysis, the DHHC domain might have additional functions. We have shown that DHHC domains are not interchangeable among different PATs and proposed that these domains might also be involved in the determination of enzyme specificity (12).

Mutations in the conserved cysteine of the DHHC motif have been obtained for several PATs, resulting in loss of activity. This led to the hypothesis that this motif is crucial for catalysis (4, 10, 13–16). Less clear is why these mutants act as dominant negative when overexpressed in cultured mammalian cells (17–19).

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[3] The abbreviations used are: CRD, cysteine-rich domain; YPD, yeast extract, peptone, dextrose; PaCCT, palmitoyltransferase conserved C terminus; PAT, palmitoyltransferase; ABE, acyl-biotinyl exchange; NEM, *N*-ethylmaleimide; CW calcofluor-white; EV, empty vector; *OD*, optical density.

This effect is not reproduced for yeast Swf1.[4] It has been proposed that PATs form oligomers both *in vitro* and *in vivo* and that mutations in the cysteine of the DHHC motif favor their formation. These oligomers are thought to be at least less active or inactive altogether (18, 20).

Regarding the mechanism of protein palmitoylation, the involvement of a covalent PAT-palmitate intermediate has been postulated on the basis of the fact that several PATs have been shown to autoacylate (14, 16). Recently, using yeast Erf2 as a model, it has been shown that the palmitoylation reaction occurs in two steps, the first one involving the formation of a palmitoyl-Erf2 intermediate, followed by the transfer of the palmitoyl moiety to the substrate protein (9). For mammalian DHHC2 and DHHC3, a single-turnover assay showed that radiolabeled acyl groups are transferred from the PAT to the substrate, confirming a ping-pong mechanism for these enzymes (22). Because mutations in the cysteine from the DHHC motif render PATs inactive for both transacylation and autoacylation, the evidence pointed to this cysteine as the target for autoacylation, although this has not been formally demonstrated. A recent study on the yeast PAT Akr1 has indicated that the protein can still be acylated in *trans* when this cysteine is mutated, indicating that other sites for *S*-acylation are possible (23). Moreover, a recent proteomics study has shown that the human DHHCs 5, 6, and 8 are autoacylated outside of the DHHC-CRD in three cysteines located in the C-terminal region in a CCX^{7-13}C(S/T) motif (24). However, this motif is not present in other human or yeast PATs.

In this work, we describe a partial loss-of-function allele of SWF1 that, when overexpressed, can partially complement a *swf1*Δ strain. This allele carries a mutation of the conserved cysteine in the DHHC motif for an arginine, and the resulting protein allows the modification of several Swf1 substrates *in vivo*. This is intriguing because if this residue is involved in forming the covalent PAT-palmitate intermediate, then it should be essential for function.

We extended these observations to Pfa4, the PAT for Chs3 and amino acid permeases (13, 15), and discovered that the pivotal cysteine in the DHHC motif of Pfa4 can be replaced both by arginine and alanine, resulting in a protein that still displays palmitoylation activity toward its substrate. Finally, we studied the role of the first histidine in the DHHC and showed that a Swf1 DQHC mutant is also partially active.

Experimental Procedures

Strains

The strains used in this study are BY4742 or derivatives containing complete deletions of SWF1, PFA4, or PFA3 ORFs from the EUROSCARF consortium.

Plasmids

The plasmids encoding Swf1, Pfa3, and Pfa4 under the control of the TPI1 promoter have been described previously (10, 12). Swf1-DHHA mutant construction is described in Ref. 10. Swf1-DHHR, Swf1-DQHC, and Swf1-DHHG mutants were obtained in the screen described in Ref. 11.

Swf1 Mutants in the DHHC Motif—Plasmid pJV362, which encodes a TPI1 promoter-driven SWF1 lacking the whole DHHC domain, is described in Ref. 12. To generate Swf1-DHHK, Swf1-DHHL, Swf1-DHHD, and Swf1-DQHR mutants, appropriate synthetic DNA fragments encoding mutated DHHC domains were ordered from Genscript, and the mutants were generated *in vivo* by gap repair on plasmid pjv362.

Pfa4 Mutants in Cysteine 108—The region encoding the Pfa4 C terminus downstream of the DHHC motif was amplified using oligonucleotides oPfa4 06 (5′-TTGTCCATGGACGATGAATTGCGTCG-3′) and oPGK-R (5′-TTAGCGTAAAGGATGGGG-3′). Oligonucleotide oPfa4 06 introduces an NcoI site through a silent mutation after the DHHC motif. The resulting PCR product was cloned in the NcoI and HindIII sites in the pRSET-A plasmid to generate plasmid pbPFA4CT. The region encoding the Pfa4 N-terminal domain was amplified by PCR using oligos oPfa4 01 (5′-AAAGGATCCATGCCAGTAAAGTTAAGG-3′) and 05 (5′-CATCGTCCATGGCCTATGATGATCCATCATTAGG-3′) for the DHHR mutant and oligos oPfa4 01 and 07 (5′-CATCGTCCATGGCGCATGATGATCCATCATTAGG-3′) for the DHHA mutant. The PCR products were digested with BamHI/NcoI and inserted into pPFA4CT. The resulting plasmids were digested with BamHI/HindIII to generate fragments encoding mutant Pfa4, which were then transfered to a YcpLac33-based vector containing a triple HA epitope, the TPI1 promoter, and the PGK1 terminator. All constructs were verified by DNA sequencing.

Protein Electrophoresis and Western Blots

SDS-PAGE and Western blots for the detection of palmitoyltransferases were carried out as described previously (12). For the detection of PAT oligomers, samples were heated for 10 min at 37 °C before loading.

For the detection of palmitate shifts, samples were prepared as described for the detection of PATs and run in 22.5% SDS-polyacrylamide gels. The bands were quantified using ImageJ software, and the plot represents mean ± S.E. of three independent experiments. Anti-Swf1 antibody is described in Ref. 12. Anti-Tlg1 antibody is described in Ref. 25. Anti-Chs3 antibody was a gift from Dr. Randy Sheckman.

Acyl Biotinyl Exchange (ABE)

ABE for the detection of Snc1 was carried out as described in Ref. 15. For the detection of palmitoylated Chs3 and Pfa4, the protocol was modified to include only membrane proteins. For this, 30 OD units of cells at OD = 1 were lysed by glass bead disruption in ABE lysis buffer containing 10 mM *N*-ethylmaleimide (NEM). The lysate was centrifuged for 4 min at 300 × *g*, and then the supernatant was centrifuged for 20 min at 17,000 × *g* to collect the membrane fraction. This pellet was resuspended in 500 μl of lysis buffer with 10 mM NEM, Triton X-100 was added to 1.7% final concentration, and the samples were incubated with rotation for 1 h at 4 °C. The samples were then centrifuged for 5 min at 17,000 × *g*, the supernatants were precipitated with chloroform:methanol, and the pellets were air-dried and resuspended in 30 μl of ABE buffer SB (4%

[4] A. González Montoro, S. Chumpen Ramirez, and J. Valdez Taubas, unpublished observations.

SDS, 50 mM Tris HCl pH 7.4, 5 mM EDTA) with 10 mM NEM. 120 μl of lysis buffer with 1 mM NEM was added, and the samples were incubated overnight at 4 °C. The rest of the protocol was performed exactly as described in Ref. 15 but using the whole amount of protein for each sample.

For the detection of palmitoylated Swf1-protein A, a pull-down assay was performed before the ABE to increase sensitivity. 90 OD of cells at OD = 1 were collected and lysed exactly as described for Pfa4 and Chs3. After Triton X-100 extraction and centrifugation, the supernatant was incubated with 50 μl of IgG-Sepharose beads for 2 h. The beads were washed three times with lysis buffer, and the proteins were eluted with 120 μl of ABE SB buffer by heating at 50 °C for 10 min with agitation. The eluted proteins were mixed with the equivalent of 20 OD units of the flow-through as carrier protein, and the volume was adjusted to 600 μl with lysis buffer containing 1 mM NEM. The proteins were precipitated with chloroform:methanol, and the rest of the protocol was carried out as described for Pfa4 and Chs3.

Bioinformatics

For analysis of the occurrence of divergent DHHC motifs, we used the sequences recognized by Pfam DHHC hidden Markov model (PF01529) for the representative proteomes (26) at 75% comembership. Proteins with point mutations in the aspartic acid, the first histidine, the cysteine of the DHHC motif, or combinations of them were separated. Sequences that had been removed from Uniprot, that were deemed to be of poor quality, or that lacked an identifiable DHHC domain were removed. The proteins were aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation) (27) and then aligned using the profile-profile alignment option against a highly curated alignment of DHHC PATs from model organisms. Finally the alignment was curated manually using Jalview (28). The sequences of the regions that align with the DHHC, DPG, TT*X*E, and PaCCT motifs were included in the table.

Results

A Swf1 DHHR Mutant Maintains Palmitoylation Activity—Mutants in the cysteine from the DHHC motif have been used as standard lack of function controls in PAT activity assays. Changing this residue to either alanine or serine resulted in lack of both auto- and transacylation activity for all PATs tested (4, 29, 30). In agreement, a Swf1 DHHA mutant has been shown to be inactive (10). We carried out a genetic screen to isolate loss-of-function mutations in Swf1. Most mutations mapped to the DHHC domain region and corresponded to residues involved in zinc coordination. Mutations in the DHHC motif were also found, including a DHHG mutant (11). We also isolated a small set of partial lack of function alleles. One of them corresponds to a substitution of the conserved cysteine in the DHHC motif (Cys-164) by an arginine (DHHR).

The functionality of this mutant was assayed in growth tests on solid medium containing lactate as a carbon source or glucose plus 0.85 M NaCl, in which the *swf1*Δ strain is unable to grow. Under these conditions, a *swf1*Δ strain complemented with WT SWF1 grows, whereas a DHHA mutant, our standard loss-of-function allele, does not. However, we consistently observed growth of *swf1*Δ strains complemented with the DHHR mutant (Fig. 1*A*). It should be stressed that all of these constructs are driven by a TPI1 promoter and are therefore overexpressed.

To investigate whether other residues were able to rescue Swf1 function, we generated a set of mutations in which Cys-164 was replaced by an aspartic acid, a leucine, and a lysine. Fig. 1*A* shows that changing Cys-164 to either alanine, lysine, aspartic acid, leucine, or glycine produces non-functional proteins.

The activity of the DHHR mutant was confirmed by directly assessing the palmitoylation status of the Swf1 substrate Snc1 by the gel shifts resulting from the incorporation of the palmitate moiety. Fig. 1*B* shows that although no palmitoylated Snc1 is detectable in a *swf1*Δ strain transformed with an empty vector, a fraction of Snc1 is modified upon overexpression of the DHHR mutant (Fig. 6).

Several mutations within the DHHC domain of Swf1 result in unstable proteins (11). To investigate whether the phenotypes of the mutants in Cys-164 are due to gross changes in protein levels, we analyzed the expression level of the different mutants by Western blot analysis. Fig. 1*C* shows that these mutant proteins are present at similar levels. Under these conditions, the amount of DHHR mutant is 60% lower than that of WT and DHHA Swf1, but this actually reflects the degree of aggregation when protein samples are boiled in sample buffer (see below and Fig. 4). All of these mutants have monomer levels well above endogenous Swf1, which is not detected under these conditions. These results indicate that Swf1 can function as an active PAT, albeit inefficiently, in the absence of the conserved cysteine in the DHHC motif.

Pfa4 DHHA and DHHR Mutants Are Active when Overexpressed—To analyze whether the partial activity of the DHHR mutant was restricted to Swf1, we generated DHHA and DHHR mutants in Pfa4. Pfa4 is the PAT responsible for *S*-acylation of Chs3 (13) and several amino acid permeases (15). Lack of Pfa4 activity leads to the retention of non-palmitoylated Chs3 in the endoplasmic reticulum and reduced levels of chitin on the cell wall (13). The *pfa4*Δ strain is therefore resistant to calcofluor-white (CW), a toxic dye that binds to chitin. Fig. 2*A* shows that although a *pfa4*Δ strain complemented with WT Pfa4 does not grow on YPD medium containing 100 μg/ml CW, the strain transformed with an empty vector is able to grow in this medium. The strain transformed with a Pfa4 DHHR mutation, however, displays an intermediate phenotype. Surprisingly, the strain expressing the DHHA mutant also displays an intermediate phenotype, indicating that this protein is also active to some degree. The *pfa4*Δ strain complemented with Pfa4 DHHA appears to grow slightly more than when complemented with DHHR, suggesting that the DHHA protein is less active. We next evaluated the palmitoylation status of the Pfa4 substrate Chs3 with a modified version of the ABE protocol (15). Fig. 2*B* shows that Chs3 is not palmitoylated in the *pfa4*Δ strain and is palmitoylated when the strain is complemented with WT Pfa4. In a *pfa4*Δ strain expressing Pfa4 DHHR and Pfa4 DHHA, palmitoylated Chs3 is detected, in agreement with the growth tests. Fig. 2*C* shows the quantification of the *S*-acylation status of Chs3 by WT and mutant Pfa4 from three inde-

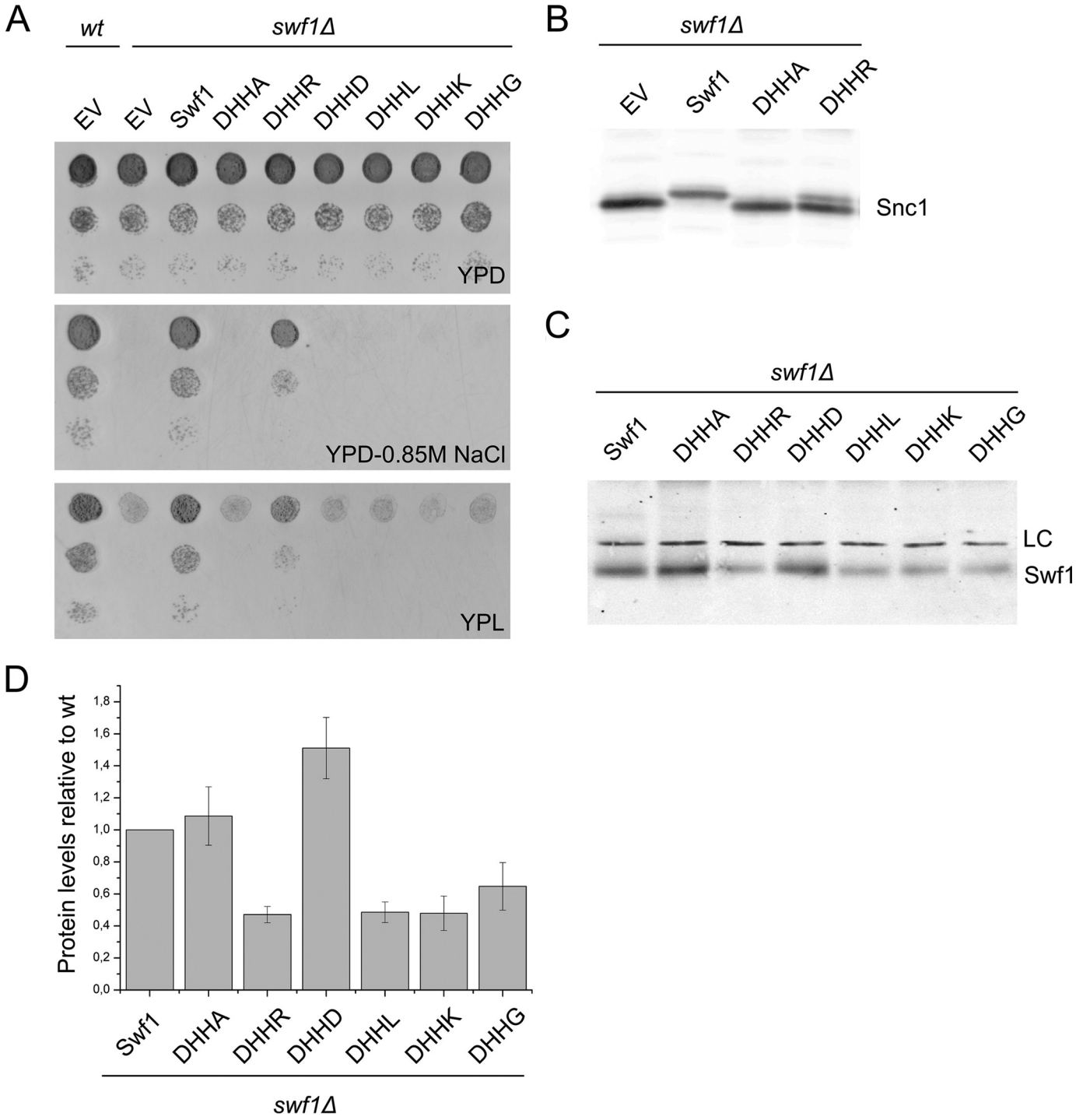


FIGURE 1. **A Swf1 DHHR mutant has palmitoyltransferase activity.** *A*, serial dilution of WT and *swf1*Δ strains transformed with empty vectors (EV), WT Swf1, or DHHC motif cysteine (Cys-164) mutants were grown in YPD, YPD plus 0.85 M NaCl, or YP with 2% lactate (*YPL*) as the sole carbon source. *B*, Snc1 palmitoylation by Swf1 mutants. Membrane proteins from a *swf1*Δ strain transformed with EV, WT Swf1, or the indicated mutants in combination with myc-tagged Snc1 were subjected to SDS-PAGE and Western blot analysis using anti-myc antibodies. Palmitoylated Snc1 shifts to slightly higher molecular weights. *C*, to evaluate the protein levels of overexpressed Swf1 and Swf1 DHHC mutant proteins, membrane proteins from *swf1*Δ strains expressing WT or Swf1 DHHC mutants were boiled with sample buffer, subjected to SDS-PAGE and Western blot analysis, and developed with anti-Swf1 antibody. *LC*, loading control. *D*, quantification of three independent experiments as in *C*. *Error bars* represent values relative to the loading control and WT sample (mean ± S.E.).

pendent experiments. The amount of palmitoylated Chs3 detected by ABE in the presence of the Pfa4 DHHR mutant is two-thirds of that obtained with WT Pfa4, and with the DHHA mutant one-third, in agreement with the growth tests. We next evaluated the amount of WT and mutated Pfa4 by Western blot analysis and found that the DHHA and DHHR mutants levels are 55% and 70% lower that WT levels. This, however, reflects the fact that they form more oligomers or aggregates that are not observed in the gel when samples are heated (see below and Fig. 4).

Swf1 and Pfa4 are specifically involved in the palmitoylation of transmembrane proteins. We decided to investigate whether

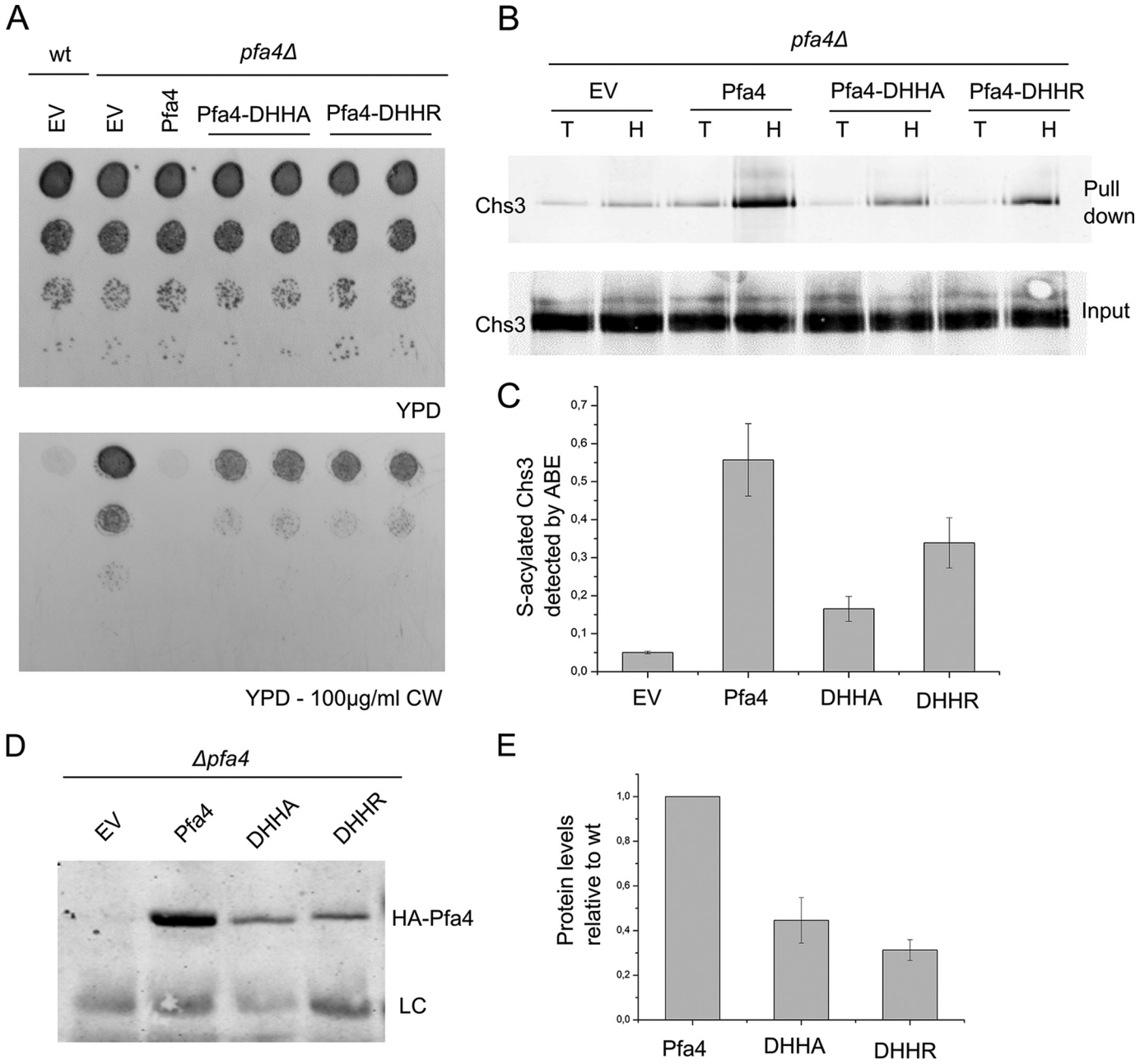


FIGURE 2. **Palmitoylation activity of Pfa4 DHHA and DHHR mutants.** *A*, serial dilutions of WT or *pfa4*Δ strains transformed with EV or the indicated mutant alleles were grown in YPD or in YPD with 100 μg/ml of CW. *B* and *C*, palmitoylation status of the Pfa4 substrate Chs3 in a *pfa4*Δ strain complemented with WT Pfa4 or DHHA and DHHR mutants as determined by ABE (see "Experimental Procedures"). Shown are a representative Western blot (*B*) and quantification (*C*) of three independent experiments. For quantification, each pulldown was normalized by its respective input, and, for each sample, the value of the Tris-treated fraction (*T*) was subtracted from the hydroxilamine-treated fraction (*H*). *Error bars* represent mean ± S.E. *D* and *E*, protein levels of overexpressed Pfa4 and Pfa4 DHHC mutant proteins. Membrane proteins from *pfa4*Δ strains transformed with WT or mutated DHHC alleles were boiled with sample buffer, subjected to SDS-PAGE and Western blot analysis, and developed with a polyclonal anti-HA antibody. Endogenous Tlg1 was used as loading control (*LC*). *D*, representative Western blot. *E*, quantification of four independent experiments. *Error bars* represent values relative to the loading control and WT sample (mean ± S.E.).

DHHC mutants in another yeast PAT, such as Pfa3, which palmitoylates extrinsic membrane proteins such as Vac8 (31), shows this same residual PAT activity when overexpressed. The palmitoylation status of Vac8 in a *pfa3*Δ strain, complemented with WT Pfa3 or a DHHR mutant, was assessed by ABE. We found no detectable palmitoylation of Vac8 by the Pfa3 DHHR mutant (data not shown). These results are in agreement with those in Ref. 8.

Swf1 and Pfa4 Mutants in the DHHC Cysteine Are Not Acylated—All PATs tested have been shown to autoacylate, and this activity was lost when the cysteine in the DHHC motif was mutated (4, 14, 16). However, this always coincided with situations in which there was no measurable substrate acylation activity. Therefore, the autoacylation of the Swf1 DHHR mutant was assayed. This was carried out using a protein A-tagged version of Swf1, and the acylation status was assessed

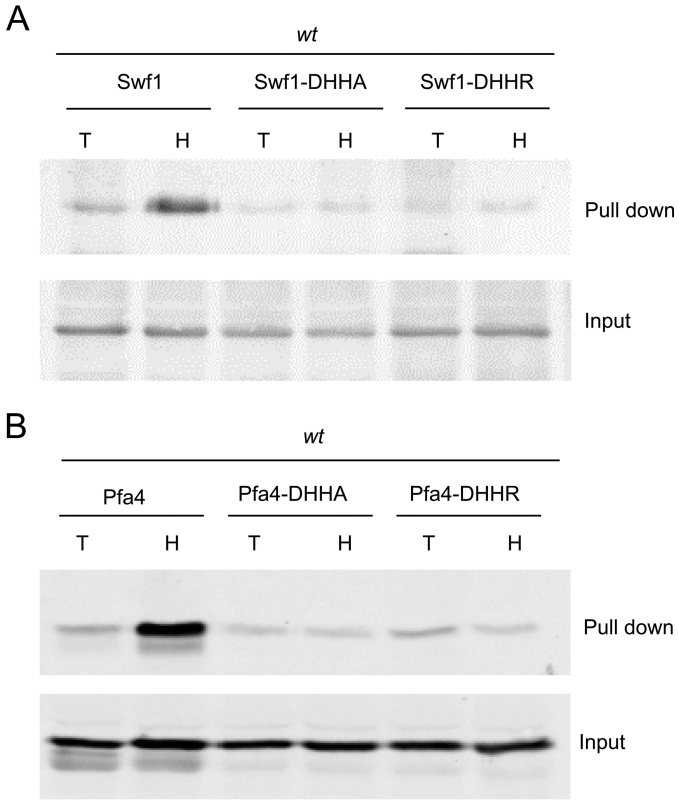


FIGURE 3. **Autoacylation of mutants in the cysteine of the DHHC motif.** *A*, the acylation status of protein A-tagged Swf1 or its DHHA and DHHR mutants in a WT background was determined by ABE. *T*, Tris; *H*, hydroxylamine. *B*, the acylation status of HA-tagged versions of Pfa4 or its DHHA and DHHR mutants in a WT background was determined by ABE. Similar results were obtained when the same experiments were carried out in strains lacking the corresponding endogenous PATs.

by a modified version of the ABE protocol that allows for more sensitivity (see "Experimental Procedures"). Fig. 3*A* shows that we were able to detect autoacylation of WT Swf1, but we did not detect autoacylation of the DHHA or DHHR mutants.

We carried out the same set of experiments for Pfa4. Fig. 3*B* shows that WT Pfa4 is palmitoylated but DHHA and DHHR are not, despite their residual PAT activity. Because some PATs have been shown to autoacylate in *trans* (23), these experiments were performed both in WT and *pfa4*Δ or *swf1*Δ strains with similar results, suggesting that there are no palmitoylated cysteines in these proteins other than the cysteine in the DHHC motif.

Oligomerization of DHHC Motif Mutants—Mammalian DHHC3 has been shown to form dimers and trimers, and their formation was favored in a DHHS mutant (18). These oligomers have been shown later to occur in DHHC2 and DHHC3 both *in vitro* and *in vivo* by bioluminescence resonance energy transfer (20). Again, the formation of oligomers was favored by mutation in the conserved cysteine of the DHHC motif. Treatment with the palmitoylation inhibitor 2-bromopalmitate suggested that it is the inactive state of the enzyme that favors the formation of oligomers. Activity measurements indicate that, at least for WT DHHC3, monomers are more active than dimers (20).

The oligomerization of yeast PATs has not been reported. However, an appealing possibility is that the Swf1 DHHR mutant has residual activity because it forms fewer oligomers than inactive mutants. Swf1 oligomers quickly become evident in SDS-PAGE when samples are not boiled. Fig. 4*A* shows that oligomer formation is increased substantially in the DHHA mutant and even more so in the DHHR mutant. The oligomers are independent of the presence of a thiol reducing agent, indicating that they do not involve the formation of disulfide bridges (data not shown).

A Swf1 DQHC mutant, which is also partially active (see below), displays higher oligomer levels than the WT protein (Fig. 4*A*). This mutant, unlike the DHHR mutant, is autoacylated (Fig. 4*B*), indicating that it is the inactive/partially active state rather than the acylation state of the PAT that correlates with oligomer formation. We then looked at Pfa4 mutants. When samples are boiled, there is a clear reduction in the amount of mutant monomeric Pfa4 proteins detected in a Western blot (Fig. 2, *D* and *E*). When samples were heated at 37 °C, the levels of monomeric WT or mutant Pfa4 are comparable, with bands corresponding to dimers and trimers evident for the mutants (Fig. 4*C*).

To assess oligomerization of PATs by an independent method that does not involve SDS denaturation, we carried out pulldown assays. Lysates of cells coexpressing Swf1-protein A and HA-Swf1 in the WT, DHHA, and DHHR versions were incubated with IgG-Sepharose beads. The material bound to the beads was subjected to SDS-PAGE and Western blot analysis using anti-HA antibodies. Fig. 4*D* shows that HA-tagged WT Swf1 is not copurified with protein A-tagged WT Swf1. When this was carried out using the mutant versions, both DHHA and DHHR mutants were pulled down. Again, dimers and trimers appeared on the pulldown fraction of the mutants. The simplest interpretation of these results is that WT Swf1 does not form oligomers whereas both DHHR and DHHA Swf1 mutants do, and to similar levels. These results are in line with the increased oligomerization of mutant PATs that has been described previously (20). These results indicate that the partial activity of Swf1 DHHR is not due to, nor does it result in, less oligomer formation with respect to a DHHA mutant.

Hetero-oligomerization of PATs has been reported previously (18). Given the increased tendency to form oligomers of the mutant proteins, a possibility was that the palmitoylation of the substrates we observed *in vivo* was mediated by the formation of hetero-oligomers with other PATs. To test this hypothesis, we assayed whether the complementation of Swf1 DHHR and Pfa4 DHHA and DHHR was also observable in a strain lacking all three endoplasmic reticulum-residing PATs: Erf2, Swf1, and Pfa4 (10, 13, 32). Fig. 5 shows that the mutants were still able to suppress the phenotypes of the deletion of their respective genes in this genetic background, indicating that their activity is not mediated by hetero-oligomers with other endoplasmic reticulum-resident PATs.

A Swf1 DQHC Mutant Has Palmitoylation Activity but a DQHR Does Not—One possible explanation for the partial activity of Swf1 DHHR and Pfa4 DHHR and DHHA mutants is that the interaction of these enzymes with the protein substrate and palmitoyl-CoA might favor a direct nucleophilic attack of the acceptor cysteines on palmitoyl-CoA, allowing the reaction to proceed without the formation of the covalent PAT-palmi-

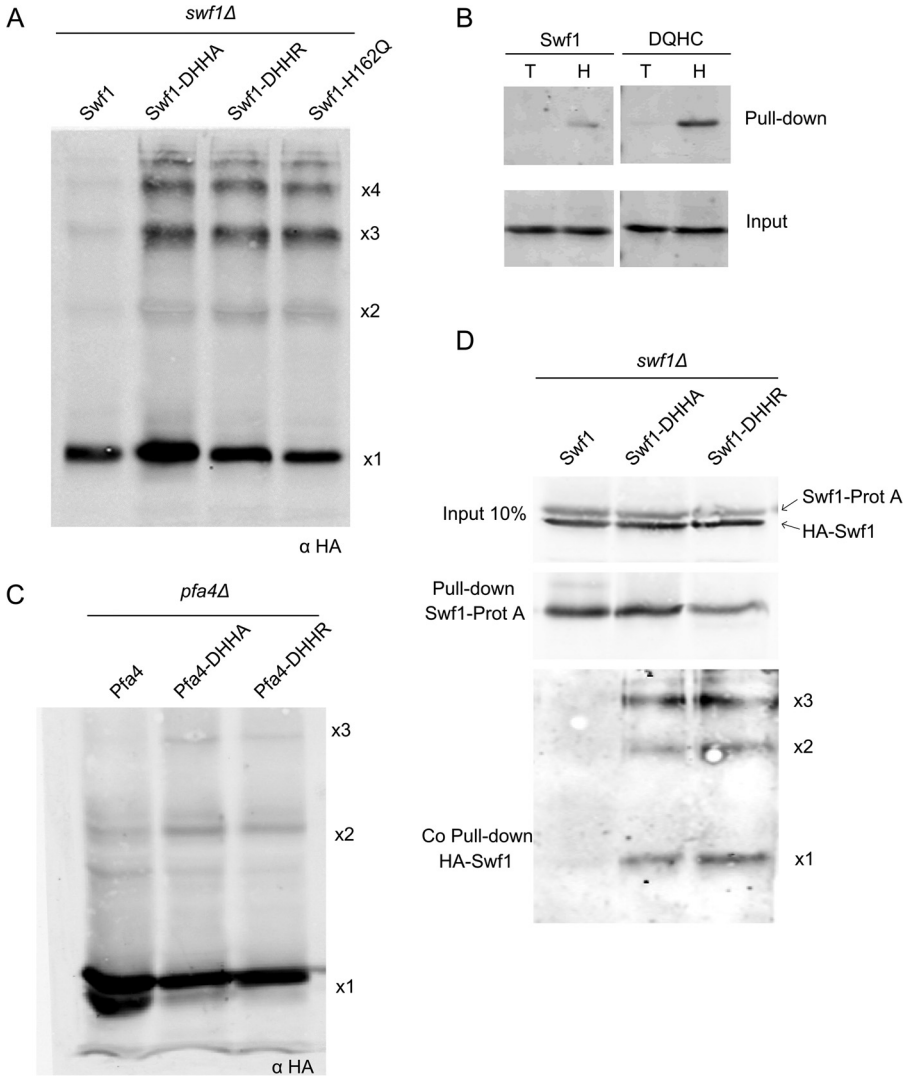


FIGURE 4. **Oligomer formation by DHHC mutants.** *A*, membrane proteins from *swf1*Δ strains expressing HA-tagged versions of WT Swf1 or the indicated mutant alleles were incubated with sample buffer and heated at 37 °C for 10 min and subjected to SDS-PAGE and Western blot analysis. *B*, the autoacylation of the Swf1 DQHC mutant was assessed by ABE. *T*, Tris; *H*, hidroxylamine. *C*, oligomer formation by WT Pfa4 and its DHHA and DHHR mutants. Membrane proteins from *pfa4*Δ strains expressing HA-tagged versions of WT Pfa4 or the indicated mutant alleles were incubated with sample buffer, heated at 37 °C for 10 min, and subjected to SDS-PAGE and Western blot analysis. *D*, copurification of HA-Swf1 with Swf1-protein A (*Prot A*). Cells coexpressing Swf1-ProtA and HA-Swf1 in their WT, DHHA, or DHHR mutant versions were lysed and incubated with IgG-Sepharose beads. The material bound to the beads was subjected to SDS-PAGE and Western blot analysis.

tate intermediate. A direct transfer reaction would be possible because of the high reactivity of palmitoyl-CoA and would be highly dependent on the rate of formation of a thiolate in the acceptor cysteines.

An Erf2 DAHC mutant is able to autoacylate but not to transfer palmitate to its substrate Ras2 *in vitro* (9). This could mean that histidine 201 is not necessary for the autoacylation step but for the second step of the reaction, possibly by aiding in the generation of the thiolate in the substrate.

In our screen, we isolated a DQHC mutant of Swf1 that is partially active both in growth tests and in the acylation of Tlg1, Syn8, and Snc1 substrates (Fig. 6). The DQHC mutant is acylated in a *swf1*Δ strain, indicating that it is able to autoacylate (Fig. 4*B*). The steady-state level of acylation observed is higher than that of the WT protein, in agreement with the idea that this histidine is necessary for the second step of the reaction. Interestingly, human DHHC13 has a naturally occurring DQHC motif and has been shown to be active toward Huntingtin (33) and ClipR59 (34).

We reasoned that if the first histidine in the DHHC motif is indeed involved in the formation of the acceptor cysteine thiolate, then mutation of that histidine in the context of the DHHR mutant would result in complete lack of activity. Indeed, a double mutant, DQHR, results in complete loss of Swf1 activity, as assessed by growth tests (Fig. 6*A*) or palmitoylation level of Swf1 substrates (Fig. 6, *B*–*G*).

Swf1 DHHR and DQHC Mutants Display Different Efficiencies of Palmitoylation Depending on the Acceptor Substrate—If the activity observed for Swf1 DHHR is due to a direct transfer of palmitate from palmitoyl-CoA, then one might expect that the efficiency of palmitoylation would depend on the nucleophilicity of the acceptor cysteines in the substrates. Likewise, if the activity of the Swf1 DQHC mutant is due to the formation of a thioester intermediate that is a better palmitate donor than

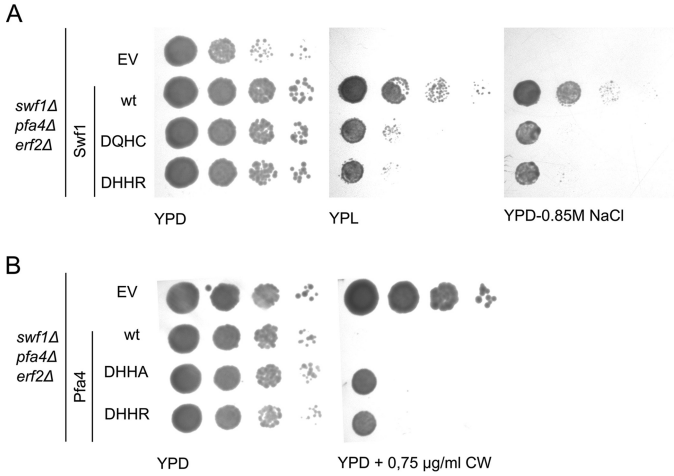


FIGURE 5. **The activity of the mutants in the DHHC motif is not mediated by hetero-oligomers with other endoplasmic reticulum PATs.** *A,* serial dilution of WT and *swf1Δ pfa4Δ erf2Δ* strains transformed with EV, WT Swf1, or DHHC motif cysteine (Cys-164) mutants were grown in YPD, YPD plus 0.85 M NaCl, or YP with 2% lactate (*YPL*) as the sole carbon source. *B*, serial dilutions of WT or *swf1Δ pfa4Δ erf2Δ* strains transformed with EV or the indicated mutant alleles of PFA4 were grown in YPD or in YPD with 75 μg/ml of CW.

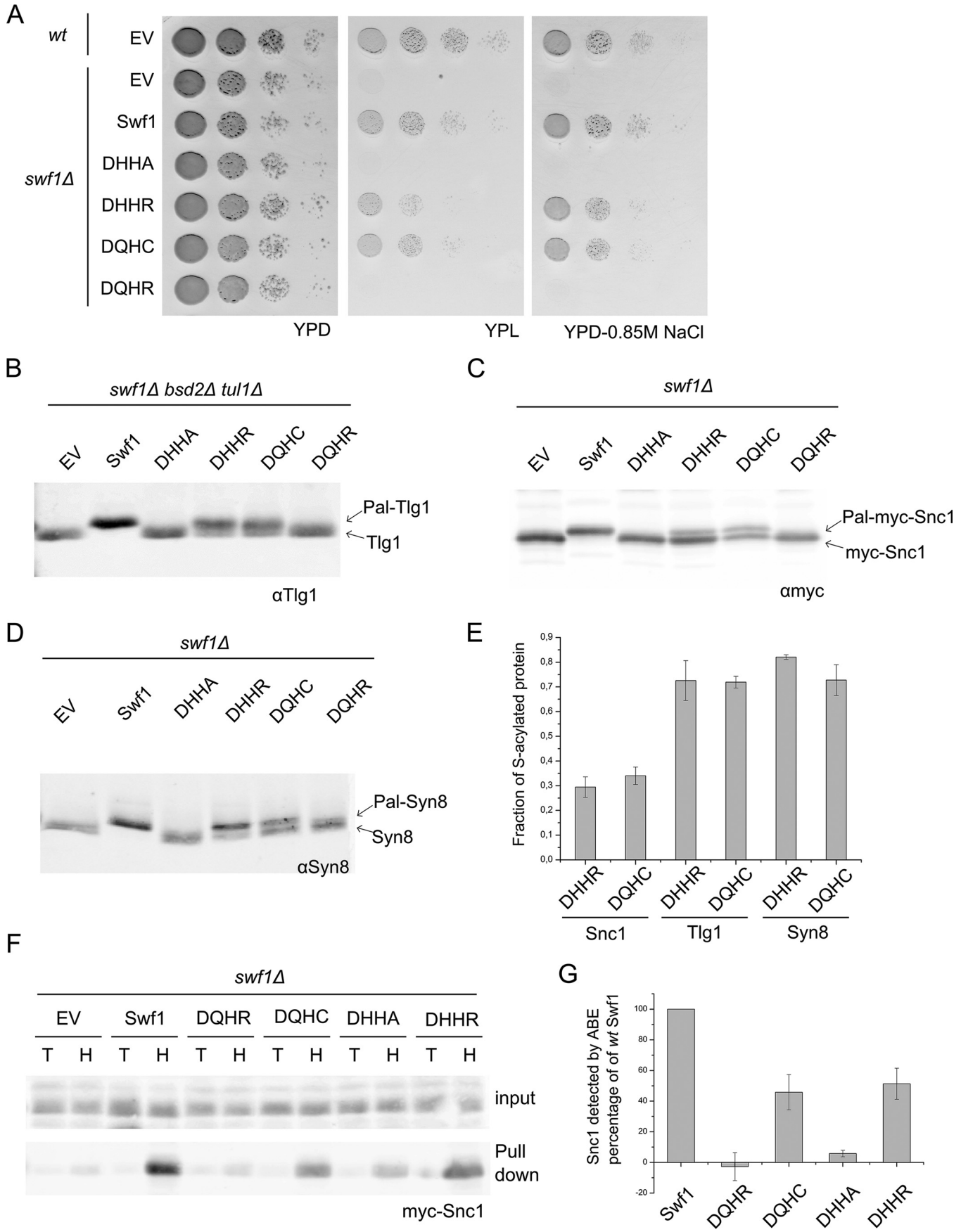
palmitoyl-CoA, then this activity would depend on the intrinsic formation rate of the thiolate in the substrate because the histidine involved in aiding its formation is not present. Therefore, we could expect these mutants to display different palmitoylation efficiencies depending on the substrate.

We analyzed the palmitoylation efficiency of Swf1 DHHR and DQHC toward three different substrates: Tlg1, Snc1, and Syn8. The acylation of these substrates was assessed by the gel shifts produced by the presence of palmitate because this allows the simultaneous observation of the acylated and non-acylated protein (Fig. 6, *B–E*). For the experiments to be comparable, the Tlg1 experiments were carried out in a *bsd2*Δ*tul1*Δ strain to protect non-acylated Tlg1 from degradation (10). These experiments show that the fraction of acylated substrate in the presence of Swf1 DHHR and DQHC mutants is between 70–85% acylated protein for Tgl1 and Syn8, and for Snc1 it is between 25–35%, indicating that palmitoylation of these different substrates is not affected to the same extent by the mutations in the DHHC motif (Fig. 6*E*). Note that all of these substrates are fully palmitoylated in the presence of WT Swf1. We carried out an ABE assay of Snc1 for the different Swf1 mutants, and the results are in good agreement with those obtained by gel shifts (Fig. 6, *F* and *G*).

Incidence of Divergent DHHC Motif-containing Proteins—If mutants in the canonical DHHC motif are able to retain some activity, then it is possible to speculate that proteins with a naturally occurring divergent DHHC motif could be active PATs. Mammalian DHHC13, which bears a DQHC motif, has been shown to be active as a PAT (33, 34). PATs with divergent DHHC motifs could play a role in the palmitoylation of highly reactive substrates. To explore this possibility, we analyzed the occurrence of divergent DHHC motifs in the sequences recognized by Pfam DHHC hidden Markov model in the representative proteomes (26). Supplemental Table S1 shows proteins with point mutations in the aspartic acid, the first histidine, the cysteine of the DHHC motif, or combinations of them and the sequence of the regions that align with the DHHC, DPG, TT*X*E, and PaCCT motifs (4, 7). The second histidine was not considered because it is likely part of the zinc finger (11), and, therefore, its conservation correlates with the conservation of the zinc binding structure. Of these sequences, 12 have highly divergent motifs with all three amino acids mutated. 35 additional sequences have mutations in the cysteine, of which nine also have a mutation in the first histidine, and nine have a mutation in the position corresponding to the aspartic acid. 26 additional sequences have mutations in the first histidine, of which 11 also contain a mutation in the aspartic acid. Within these proteins, there are several examples of proteins with an appropriate number of transmembrane domains, a conserved DHHC domain, and conserved DPG, TT*X*E, and PaCCT motifs, indicating that they could represent active PATs (supplemental Table S1). Orthologs of human DHHC13, containing ankyrin repeats and a DQHC motif, are present in all mammalian proteomes analyzed. These proteins have a very high degree of identity, indicating that they are most likely the result of a single mutation event that was conserved. The DQHC mutant proteins of *Xenopus tropicalis* (F7EAF1) and *Latimeria chalumnae* (H3B897) also possess ankyrin repeats and a high degree of similarity with the mammalian orthologs of DHHC13, indicating that the mutation may have occurred before the divergence of mammals. The *Sorghum bicolor* DQHC-containing protein (C5X9Z3), however, has a much lower degree of conservation and does not contain ankyrin repeats, indicating that it has probably arisen from an independent mutation event. Within *Brachycera* insects, orthologs of the *Drosophila melanogaster* protein GABPI (Q9VQK9), which contains a DHHS mutation, are conserved, and there is a subgroup of *Drosophila* that seems to have acquired an additional mutation, resulting in an SHHS-divergent motif.

Discussion

In this work, we have shown that, at least for two yeast palmitoyltransferases, Swf1 and Pfa4, palmitate transfer to the substrate can occur even in the absence of the conserved cysteine in their DHHC motifs. This cysteine is thought to form the covalent PAT-palmitate intermediate from which the palmitate is transferred to the substrates, and it is therefore expected to be essential for PAT activity. There are several possible interpretations of these results. First, palmitoylation of the cysteine in the DHHC motif of PATs could be important for protein folding and activity but not represent the primary site from which palmitate is transferred. Another possibility is that although this cysteine is the primary site from which palmitate is transferred, a secondary site exists. Analyses of the partially active Swf1 and Pfa4 mutants indicated that autoacylation was not detected, neither in *cis* nor in *trans*. This suggests that the cysteine in the DHHC motif is the only site of PAT autoacylation and that there are no detectable secondary cysteines from which palmitate could be transferred by a ping-pong mechanism. Nevertheless, this was determined using ABE assays, which are rather inefficient, so we cannot rule out that the detection of a secondary palmitoylation site has been hindered by lack of sensitivity, particularly if this intermediate is short-lived. Another issue with this possibility is that all remaining


A
wt
EV
swf1Δ
EV
Swf1
DHHA
DHHR
DQHC
DQHR
YPD
YPL
YPD-0.85M NaCl
B
swf1Δ bsd2Δ tul1Δ
EV
Swf1
DHHA
DHHR
DQHC
DQHR
Pal-Tlg1
Tlg1
αTlg1
C
swf1Δ
EV
Swf1
DHHA
DHHR
DQHC
DQHR
Pal-myc-Snc1
myc-Snc1
αmyc
D
swf1Δ
EV
Swf1
DHHA
DHHR
DQHC
DQHR
Pal-Syn8
Syn8
αSyn8
E
Fraction of S-acylated protein
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9
DHHR
DQHC
DHHR
DQHC
DHHR
DQHC
Snc1
Tlg1
Syn8
F
swf1Δ
EV
Swf1
DQHR
DQHC
DHHA
DHHR
T H T H T H T H T H T H
input
Pull down
myc-Snc1
G
Snc1 detected by ABE percentage of of wt Swf1
-20
0
20
40
60
80
100
Swf1
DQHR
DQHC
DHHA
DHHR

cysteines within the Swf1 DHHC motif appear to have a structural role in the coordination of zinc (11).

We investigated whether the oligomerization status of PATs was linked to the partial activity of Pfa4 and Swf1 mutants and showed that there is no clear correlation between their oligomerization status and activity. These experiments indicate that both inactive and partially active PATs have an increased tendency to form oligomers.

The fact that only the substitution of Swf1 DHHC cysteine by an arginine results in partial activity is intriguing. We initially thought that the positive charge may be required but that a DHHK mutant is inactive. Additionally, in the case of Pfa4, both DHHA and DHHR showed some degree of functionality, although the DHHR mutant was 2-fold more active than the DHHA mutant (Fig. 2*C*). Current structural and mechanistic knowledge of the *S*-acylation reaction is insufficient to allow speculations about the observed effects of these particular mutations on Swf1 or Pfa4 at the molecular level.

The results of Jennings and Linder (22) and Mitchell *et al.* (9) suggest that palmitoylation proceeds through a thioester intermediate involving the cysteine in the DHHC motif. The environment of the catalytic site could enhance the electrophilicity of this thioester, therefore favoring the nucleophilic attack by the thiolate of the substrate protein. The experiments by Mitchell *et al.* (9) with Erf2 show that the first histidine in the DHHC motif is not necessary for the formation of the palmitoylated-PAT intermediate, and, therefore, that it does not play a role in the formation of the nucleophile in the PAT. Conversely, mutation of this histidine is necessary for the transfer of the palmitoyl moiety to the substrate, indicating that the role of the first histidine would probably be the deprotonation of the thiol of the substrate protein, making it more nucleophilic. In this context, the activity of the Swf1 DHHR mutant and the Pfa4 DHHR and DHHA mutants could be explained by a direct nucleophilic attack of the substrate on palmitoyl-CoA. The first histidine of the motif, which is present in these mutants, would favor the formation of thiolate in the substrate protein. This would allow the reaction to proceed, albeit less efficiently, in the absence of the palmitoyl-PAT intermediate, therefore involving a less electrophilic carbonyl. This reaction would occur through a transfer protein-assisted palmitoylation mechanism, as the one proposed previously (35). The high reactivity of palmitoyl-CoA would make this transference possible.

In the presence of a DHHC motif without the first histidine, the palmitoyl-PAT intermediate would be formed, as has been shown for Erf2 (9), DHHC13 (34, 36), and Swf1 (this study). The presence of this electrophilic carbonyl together with the PAT binding to both substrates, holding them in the vicinity of each other, could permit modification at observable rates even when the histidine is not present to aid in the formation of the thiolate of the substrate protein. This could explain the partial activity we observe for the Swf1 DQHC mutant and the activity observed for DHHC13.

Both of these suboptimal reaction mechanisms could be more dependent on the acceptor protein reactivity than the canonical mechanism. It has been shown that cysteines in proteins can differ significantly in their acidity and nucleophilicity depending on their molecular environment (37, 38). Indeed, we see different efficiencies of palmitoylation for the three substrates analyzed which, under WT conditions, are fully palmitoylated. The reactivity of cysteines can sometimes be assessed *in vitro* (39). In this case, where cysteines are at the cytosolic border of the transmembrane domains, the reactivity could be influenced by the lipid environment and, therefore, by the subcellular localization or by the way in which the transmembrane domains are inserted in the membrane. Therefore, such measurements would probably lack biological relevance.

It has been shown that, under certain conditions, the uncatalyzed palmitoylation reaction requires only five times the activation energy than the PAT-catalyzed reaction (40). This could mean that just approaching the two substrates could produce reaction rates that allow the detection of palmitoylated substrates *in vivo*. Also, the low availability of free palmitoyl-CoA has been proposed as a mechanism to prevent the uncatalyzed reaction *in vivo* (35), and the ability of the mutant proteins to displace Acyl-CoA binding protein and make palmitoyl-CoA available for an attack may account for their partial activity.

It is important to point out that we have observed activity of mutants lacking either the first histidine or the cysteine in the DHHC motif for PATs that palmitoylate integral membrane proteins. In this case, both enzyme and substrates are restricted to the plane of the membrane and may be available to the PAT for longer times, allowing palmitate transfer by less effective mechanisms. Although we were unable to detect residual activity of Pfa3 *in vivo*, this may be due to sensitivity limitations, and we cannot rule out that this residual activity is present in PATs that modify extrinsic membrane proteins.

There are several proteins recognized by the Pfam DHHC hidden Markov model (PF01529) that have substitutions of one or more amino acids of the DHHC motif. Among them is human DHHC13, which presents a DQHC motif and has been shown to palmitoylate Hungtintin and Clip59R (33, 34). The presence of this type of PATs could allow for the specific palmitoylation of substrates that bind the PAT with high affinity or that have more nucleophilic cysteines. Clearly this PAT has relevant biological functions because DHHC13 knockout mice

FIGURE 6. **Palmitoyltransferase activity of Swf1 DHHC mutants toward different substrates.** *A*, suppression of a *swf1*Δ strain growth defect by Swf1 DHHR, DQHC, and DQHR mutants. Serial dilutions of a WT strain transformed with EV or a *swf1*Δ strain complemented with EV and either WT Swf1 or the indicated mutant alleles were grown in YPD, YPD plus 0.85 M NaCl, or YP with lactate (*YPL*) as the sole carbon source. *B–E*, acylation of Swf1 substrates by DHHC motif mutants. Membrane proteins from *swf1*Δ strains transformed with EV, WT Swf1, or the indicated mutant alleles were subjected to SDS-PAGE in 22.5% acrylamide gels and Western blot analysis to detect the molecular weight shifts generated by palmitoylation. Tlg1 and Syn8 were detected using antibodies against the endogenous proteins. For the detection of Snc1, the strains were cotransformed with a myc-tagged version of this SNARE. *B*, *C*, and *D* are representative gels, and *E* depicts the quantification of the fraction of acylated protein from three independent experiments. *Error bars* represent mean ± S.E. It should be noted that all three proteins are fully acylated in the presence of WT Swf1, and no acylation is detectable for s*wf1*Δ strains. *F*, acyl-biotinyl exchange assay to evaluate palmitoylation of Snc1 in the presence of the different mutants of Swf1. *G*, quantification of the three independent experiments in *F*. The value obtained for the strain transformed with the empty vector was subtracted from the values obtained in the others strains, and values are represented as the percentage of the value obtained for WT Swf1. *Error bars* represent mean ± S.E.

develop neuropathological and behavioral features of Huntington disease (41), and mice with a nonsense mutation in this gene present with alopecia, osteoporosis, and systemic amyloidosis (42). Most other PATs with the divergent DHHC motif that we identified have not yet been studied. The fact that some of them seem to be conserved within certain clades supports the possibility that they are functional.

An additional consequence of the results shown here is that care must be exercised when using DHHC mutants as complete lack-of-function alleles. There are several examples in the literature where the effects observed upon expression of mutated PATs have been assigned to non-canonical functions of these proteins when there might actually be effects of reduced palmitoylation activity (21, 43). It might be advisable to use more than one mutant or combinations of mutations (*i.e.* DAHA) to make sure that the effects observed are indeed additional functions of the PATs that are not related to their palmitoylation activity. Overall, the data presented here add another layer of complexity to the mechanism of protein palmitoylation and highlight the need for further research on this topic.

Author Contributions—A. G. M. and S. C. R. conducted the experiments. A. G. M. and J. V. T. designed the experiments and wrote the paper. J. V. T. supervised the whole project.

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