The vacuolar DHHC-CRD protein Pfa3p is a protein acyltransferase for Vac8p

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PALMITOYLATION OF THE VACUOLAR MEMBRANE PROTEIN Vac8p is essential for vacuole fusion in yeast (Veit, M., R. Laage, L. Dietrich, L. Wang, and C. Ungermann. 2001. EMBO J. 20:3145–3155; Wang, Y.X., E.J. Kauffman, J.E. Duex, and L.S. Weisman. 2001. J. Biol. Chem. 276:35133–35140). Proteins that contain an Asp-His-His-Cys (DHHC)–cysteine rich domain (CRD) are emerging as a family of protein acyltransferases, and are therefore candidates for mediators of Vac8p palmitoylation. Here we demonstrate that the DHHC-CRD proteins Pfa3p (protein fatty acyltransferase 3, encoded by YNL326c) and Swf1p are important for vacuole fusion. Cells lacking Pfa3p had fragmented vacuoles when stressed, and cells lacking both Pfa3p and Swf1p had fragmented vacuoles under normal growth conditions. Pfa3p promoted Vac8p membrane association and palmitoylation in vivo and partially purified Pfa3p palmitoylated Vac8p in vitro, establishing Vac8p as a substrate for palmitoylation by Pfa3p. Vac8p is the first N-myristoylated, palmitoylated protein identified as a substrate for a DHHC-CRD protein.

Introduction

Yeast vacuoles are organelles with roles in degradation and storage. In the budding yeast Saccharomyces cerevisiae, vacuoles undergo cycles of fragmentation and fusion (Jones et al., 1993). The fusion of vacuole fragments, termed homotypic vacuole fusion, has been a model system for studying membrane fusion. Vacuole fusion is a SNARE-mediated event that proceeds in three steps: disassembly of cis-SNARE complexes, docking of vacuoles, and membrane fusion (Wickner and Haas, 2000).

Modification of proteins with the fatty acid palmitate has an important role in membrane fusion events. Both intra-Golgi transport and vacuole fusion are stimulated by palmitoyl-Coenzyme A (palm-CoA) and are inhibited by the palmitylation inhibitor 2-bromo-palmitate (Pfanner et al., 1990; Haas and Wickner, 1996; Veit et al., 2001). An important target of palmitoylation in vivo and partially purified Pfa3p palmitoylated Vac8p in vitro, establishing Vac8p as a substrate for palmitoylation by Pfa3p. Vac8p is the first N-myristoylated, palmitoylated protein identified as a substrate for a DHHC-CRD protein.

Vac8p is present on vacuole membranes (Fleckenstein et al., 1998; Pan and Goldfarb, 1998; Wang et al., 1998) and both N-myristoylation and palmitoylation of Vac8p are important for this localization (Wang et al., 1998). During in vitro vacuole fusion assays, Vac8p is palmitoylated on purified vacuoles after disassembly of the cis-SNARE complexes (Veit et al., 2001). Although Vac8p is palmitoylated after priming, Vac8p does not appear to be required until later in the fusion reaction. Vacuoles lacking Vac8p proceed through docking and formation of trans-SNARE pairs, but do not fuse (Wang et al., 2001). In cells this translates to a fragmented vacuole phenotype (Pan and Goldfarb, 1998; Wang et al., 1998; Seeley et al., 2002). The specific role of Vac8p in fusion is unclear, but is likely to involve protein–protein interactions mediated by its many armadillo repeats. Because Vac8p still partially localizes to the vacuole membrane in the absence of palmitoylation, it has been proposed that palmitoylation may influence the function of Vac8p by directing it to subdomains within the vacuole membrane (Wang et al., 2001).

The importance of Vac8p palmitoylation for membrane fusion has generated interest in identifying the proteins that regulate this modification. Ykt6p is an essential SNARE protein.
involved in many membrane trafficking events including vacuole fusion (Ungermann et al., 1999). Inclusion of α-Ykt6p antibodies in vacuole fusion assays blocks both membrane fusion and palmitoylation of Vac8p (Dietrich et al., 2004). In vitro, the NH2-terminal longin domain of Ykt6p promotes Vac8p palmitoylation in a nonenzymatic manner (Dietrich et al., 2004). The role of Ykt6p in Vac8p palmitoylation in vivo has not been explored.

A family of proteins characterized by a conserved DHHC-CRD (Bohm et al., 1997; Putilina et al., 1999) has recently been linked to protein palmitoylation. Two yeast DHHC-CRD proteins, Erf2p (in complex with Erf4p) and Akr1p, palmitoylate COOH-terminal cysteine residues of the small GTPase Ras2p and the kinase Yck2p, respectively (Lobo et al., 2002; Roth et al., 2002). Both Erf2p and Akr1p require the DHHC-CRD for protein acyltransferase (PAT) activity suggesting that it is a PAT domain. We sought to determine whether the other yeast DHHC-CRD proteins also function as PATs. In this study we present genetic and biochemical evidence that the gene product of the uncharacterized open reading frame YNL326c is a PAT for Vac8p. Accordingly, we have assigned the name PFA3 (protein fatty acyltransferase 3), as this is the third member of the DHHC-CRD family found to have PAT activity. Pfa3p is the first DHHC-CRD protein found to palmitoylate a cysteine motif near a myristoylated NH2 terminus.

Results

DHHC-CRD proteins are required for vacuole fusion

As a first step toward identifying palmitoylation substrates of DHHC-CRD proteins, we generated yeast strains with the five small DHHC-CRD genes deleted (ERF2, PFA3/YNL326c, YOL003c, YDR459c, and SWF1) in various combinations. When examining the vacuole morphology of these strains by staining with the lipophilic dye FM 4–64, we found that pfa3Δswf1Δ cells had highly fragmented vacuoles (Fig. 1). The accumulation of small clustered vacuoles indicates that these cells have a defect in vacuole fusion. This is a synthetic phenotype, because pfa3Δ or swf1Δ cells have normal vacuole morphology when grown in rich media. The observed phenotype is a consequence of the gene deletions, because vacuole fusion is rescued by expression of either Pfa3p-2xFlag or Swf1p-myc (unpublished data).

Further examination of the pfa3Δ strain revealed that aberrant vacuole morphology was induced when the cells were stressed. In the presence of DTT, pfa3Δ vacuoles became highly fragmented whereas vacuole morphology in the wild-type (WT) strain did not change (Fig. 1). The same phenotype was detected when the cells were grown under conditions of glucose limitation (Fig. 1). We also observed DTT-induced vacuole fragmentation in the pfa3Δ strain from the Saccharomyces genome deletion project (Winzeler et al., 1999) and in pfa3Δ cells derived from YPH499 (unpublished data). Vacuole morphology of swf1Δ cells was not perturbed by DTT treatment (unpublished data). This suggests that both PFA3 and SWF1 are important for vacuole fusion, but PFA3 has a primary role that is apparent under stressful conditions in the single deletion strain.

As both Pfa3p and Swf1p contain DHHC-CRDs that are hypothesized to have PAT activity (Linder and Deschenes, 2003), the vacuole fragmentation in the deletion strains could be related to palmitoylation of one or more substrates. The frag-
mented vacuoles observed in pfa3Δswf1Δ or stressed pfa3Δ cells are strikingly similar to a reported phenotype of cells lacking the palmitoylated armadillo repeat-containing protein Vac8p. Interestingly, the vacuole fragmentation in vac8Δ cells increases when the cells are stressed by placement into hypotonic media (Wang et al., 2001). We created a vac8Δ strain and examined vacuole morphology. In rich media, some fragmentation was observed (Fig. 1). However, upon treatment with DTT, fragmentation increased dramatically, mimicking the shift observed in pfa3Δ cells. Given that Vac8p palmitoylation is required for vacuole fusion (Veit et al., 2001; Wang et al., 2001), we hypothesized that the phenotype observed in pfa3Δ or pfa3Δswf1Δ cells may be related to loss of Vac8p palmitoylation.

Pfa3p localizes to the vacuole membrane

To examine the localization of Pfa3p, a 3xGFP tag was integrated into the PFA3 locus, resulting in a COOH-terminally tagged protein. Fluorescence microscopy revealed association of Pfa3p-3xGFP with the vacuolar membrane, as indicated by codistribution with FM 4–64 (Fig. 2). This is consistent with the localization reported in the Yeast GFP Fusion Localization Database (yeastgfp.ucsf.edu; Huh et al., 2003). However, the images in the database show diffuse fluorescence in the vacuole lumen, whereas in our strain the fluorescence is concentrated on the limiting membrane. Although both proteins are COOH-terminally tagged, the database strain is the result of a single GFP integration that may account for the different localizations. The Pfa3p-3xGFP is functional because the vacuoles in this strain retained WT morphology upon DTT treatment (unpublished data). The vacuolar localization of both Pfa3p and Vac8p further supports the idea that these proteins are linked functionally. By contrast, Swf1p has been localized to the ER (Valdez-Taubas and Pelham, 2005).

Vacuole fusion depends on PAT activity of Pfa3p

If Pfa3p promotes vacuole fusion through its predicted PAT activity then the DHHC-CRD is likely to play an important role in Pfa3p function. To examine the role of the Pfa3p DHHC-CRD in vacuole fusion, we sought to inactivate the DHHC-CRD within the context of the full-length protein. In Erf2p and Akr1p, mutation of the cysteine within the DHHC motif disrupts function in vivo by abolishing substrate palmitoylation (Lobo et al., 2002; Roth et al., 2002). Using this as a model for making an inactive Pfa3p, we mutated the DHHC cysteine, Cys134, to serine. To explore the ability of PFA3 alleles to function in vacuole fusion, constructs using the endogenous PFA3 promoter and a COOH-terminal 2xFlag tag were created. WT PFA3 was placed in a centromeric plasmid to mimic endogenous expression levels. C134S Pfa3p-2xFlag could not be detected when expressed from a centromeric plasmid, so it was expressed from a high copy plasmid. Expression of both constructs was confirmed by Western blotting of whole cell lysates of pfa3Δ transformants (Fig. 3 B). When expressed from a centromeric plasmid, Pfa3p-2xFlag rescued the vacuole fusion defect of DTT-treated pfa3Δ cells, as shown in Fig. 3 A. In contrast, C134S Pfa3p-2xFlag expression did not rescue the vacuole fusion defect. Thus we conclude that Cys134 of Pfa3p, and by extension a functional DHHC-CRD, is required for vacuole fusion.

Pfa3p promotes Vac8p palmitoylation and membrane association in vivo

Inactivation of the Pfa3p DHHC-CRD and loss of Vac8p palmitoylation have the same consequence: a defect in vacuole fusion. If Pfa3p palmitoylates Vac8p, we would expect to find a decrease in Vac8p palmitoylation in pfa3Δ cells. To evaluate the role of PFA3 in Vac8p palmitoylation in vivo, metabolic labeling with [3H]palmitate was performed. In pfa3Δ cells Vac8p-myc-GFP palmitoylation was decreased by 80% (Fig. 4 A), indicating that Pfa3p is required for a large portion of Vac8p palmitoylation. Surprisingly, Vac8p-myc-GFP palmitoylation was decreased to a similar level in pfa3Δ and pfa3Δswf1Δ cells. Considering SWF1 does not appear to influence Vac8p palmitoylation, it may promote vacuole fusion in some other manner. A reported consequence of the loss of Vac8p palmitoylation (through mutation of the three NH2-terminal cysteine residues)
is an increase in cytosolic Vac8p (Wang et al., 1998). Because Vac8p palmitoylation was decreased in the absence of PFA3, we expected a corresponding increase in cytosolic Vac8p. This prediction was tested by examining the subcellular distribution of Vac8p in WT and pfa3Δ cells. Differential centrifugation was used to separate internal membranes (Fig. 4 B, IM), the plasma membrane (Fig. 4 B, PM), and cytosolic proteins (Fig. 4 B, C). As shown in Fig. 4 B, Vac8p-myc-GFP was concentrated in the internal membrane fraction in WT cells. In pfa3Δ cells, a substantial cytosolic fraction was detected, indicating that the decrease in palmitoylation we observed was sufficient to disrupt Vac8p membrane association.

Pfa3p autoacylates in vitro

Erf2p and Akr1p are palmitoylated in vitro when incubated with [3H]palm-CoA, and Akr1p is palmitoylated in vivo (Lobo et al., 2002; Roth et al., 2002). Mutations that block Erf2p and Akr1p palmitoylation also block substrate palmitoylation, indicating that the two events are linked (Lobo et al., 2002; Roth et al., 2002). To determine if Pfa3p shares this characteristic, Pfa3p-Flag was overexpressed in yeast. Membranes prepared from these yeast were evaluated for protein palmitoylation by incubation with [3H]palm-CoA followed by fluorography, as shown in Fig. 5. Pfa3p-Flag incorporated palmitate (lane 1). C134S Pfa3p-Flag was expressed at a similar level to WT Pfa3p-Flag, but did not autoacylate (Fig. 5, lane 2). The lack of autoacylation suggests that this mutant will also be deficient in PAT activity, and validates our use of this mutant as a loss of function allele in the rescue experiments described above.

Pfa3p promotes Vac8p palmitoylation in vitro

To determine whether Pfa3p palmitoylates Vac8p in vitro, we developed an assay based on the ability of Pfa3p-Flag to autoacylate in membranes. We hypothesized that overexpressed Pfa3p-Flag may be able to promote palmitoylation of a substrate that was also present in the membranes. To this end, Pfa3p-Flag and Vac8p-myc were coexpressed. When membranes were incubated with [3H]palm-CoA, incorporation of palmitate into Vac8p-myc was observed only in the presence of Pfa3p-Flag (Fig. 5, lane 4). Palmitoylation of Vac8p depended on Pfa3p autoacylation, as coexpression of C134S Pfa3p did not promote Vac8p palmitoylation (Fig. 5, lane 5). When coexpressed with Pfa3p-Flag, C4,5,7S Vac8p-myc (Cys\(^{-}\) ) was not palmitoylated (Fig. 5, lane 7), indicating that the reaction is occurring on the NH\(_2\)-terminal cysteine residues. Both in vitro palmitoylation events occurred via the expected thioester linkage, as the incorporated palmitate was cleaved by treatment with hydroxylamine (unpublished data). In lanes 1 and 7, a weak palmitoylation signal is observed just below where the Vac8p-myc band is observed in lane 4. This likely represents palmitoylation of endogenous Vac8p present in the membranes.

The vacuole morphology phenotype and reduced palmitoylation of Vac8p in pfa3Δ cells suggest that Pfa3p is a PAT for Vac8p in yeast. To determine whether Vac8p palmitoylation is specific to Pfa3p, we tested Vac8p as a substrate for other DHHC-CRD proteins. The DHHC-CRD proteins were expressed in yeast as Flag-tagged constructs using a galactose-inducible promoter. Pfa3p, Akr1p, Erf2p (with its binding partner Erf4p), Yol003p, and Ydr459p expressed well, but Swf1p and Akr2p were expressed at low or undetectable levels and were not analyzed further (unpublished data). Membranes con-
taining the overexpressed DHHC-CRD proteins were incubated with purified N-myristoylated Vac8p-myc-6xHis (myr-Vac8p) in a PAT assay. As shown in Fig. 6, autoacylation of the DHHC-CRD proteins was apparent in each case. However, Pfa3p-Flag membranes were the only ones to promote robust Vac8p palmitoylation. Low levels of Vac8p palmitoylation were observed with the Erf2p/Erf4p membranes. To test whether Erf2p/Erf4p contributes to palmitoylation of Vac8p in vivo, we performed radiolabeling studies in a pfa3/H9004 erf2/H9004 strain. There was no further reduction in radioactive palmitate incorporation in Vac8p from that seen with pfa3/H9004 cells (Fig. 4 A), suggesting that Erf2p does not account for residual palmitoylation of Vac8p in these cells.

To further characterize the enzymology of Vac8p palmitoylation by Pfa3p, we sought to perform PAT assays with purified components. Pfa3p-Flag was expressed in yeast and purified by affinity-chromatography from membrane detergent extracts. A mock purification using detergent extracts from cells transformed with vector (pESC) was performed in parallel. PAT activity for Vac8p was enriched 36-fold in the Pfa3p preparation after Flag-immunoaffinity chromatography (Table I). No activity was detected in Flag peptide eluates from the vector control preparation. The silver stain shown in Fig. 7 A revealed a prominent 37-kD protein present only in the Pfa3p-Flag preparation. Except for an 82-kD protein, contaminating proteins were present in equivalent amounts in both the Pfa3p-Flag and the control preparations. The more abundant 82-kD protein was not reproducibly observed in silver stains of other Pfa3p preparations. Fig. 7 B shows that Flag immunoreactivity comigrated with the prominent 37-kD band in the silver stain.

When incubated with [3H]palm-CoA in vitro, only the Pfa3p preparation exhibited a 37-kD protein that autoacylated (Fig. 7 C). Western blotting of a Pfa3p-Flag preparation demonstrated that Ykt6p did not copurify, although it was detected in the detergent extract (Fig. S1 http://www.jcb.org/cgi/content/full/jcb.200507048/DC1). These results establish that Vac8p palmitoylation by Pfa3p occurred independently of Ykt6p.

The time course of Pfa3p-Flag autoacylation and myr-Vac8p-myc-6xHis palmitoylation is shown in Fig. 7 D. Autoacylation of Pfa3p-Flag occurred rapidly, reaching maximal levels within minutes (Fig. 7 D, inset). The amount of Pfa3p-Flag protein present in each reaction was 0.5 pmol. The amount of [3H]palm-CoA in the indicated time and analyzed by fluorography (inset; 4 d exposure) and scintillation counting (curve). Data are representative of two independent experiments.

Table I. Purification of Flag-Pfa3p

| Protein | Total Activity | Specific Activity | Purification |
|---------|----------------|------------------|--------------|
|         | mg             | U                | U/mg         | fold         |
| Pfa3p   | Membranes      | 10.0             | 88.0         | 8.8*         | 1.0          |
|         | Extract        | 6.0              | 64.0         | 10.7         | 1.2          |
|         | Elution        | 0.005            | 1.6          | 320.0        | 36.0         |
|         | pESC Membranes | 10.0             | 1.0          | 0.12         | 0.12         |
|         | Extract        | 8.3              | 1.0          | 0.12         | 0.12         |
|         | Elution        | 0.001            | 0            | 0            |              |

* One unit of activity is pmol of [3H]palm-Vac8p produced per minute.
Palmitoylation of myr-Vac8p-myc-6xHis occurred with a slower time course; the assay was linear to 10 min and reached saturation at ~60 min (Fig. 7 D, inset and curve). Palmitoylation of myr-Vac8p-myc-6xHis was dependent on substrate concentration (unpublished data). Maximal labeling of Vac8p occurred at a substoichiometric ratio of Pfa3p to Vac8p (0.5 pmol Pfa3p/15 pmol myr-Vac8p). Under these conditions, 1.1 pmol palmitate was incorporated into Vac8p, which is approximately twice the amount of Pfa3p–Flag protein in the assay. The ratio of pmol palmitate incorporated into Vac8p to pmol of PAT may be an underestimate if autoacylated Pfa3p–Flag represents the fraction of Pfa3p that can transfer palmitate to a substrate.

**Discussion**

We have identified a novel regulator of membrane fusion in yeast, the DHHC-CRD protein Pfa3p, and demonstrated that Pfa3p exerts its effects via palmitoylation of Vac8p. This is the first palmitoylation event assigned to Pfa3p, a vacuolar DHHC-CRD. In addition, we provide evidence that another DHHC-CRD protein, Swf1p, is involved in vacuole fusion through an unknown mechanism that may be independent of Vac8p. Pfa3p is the first DHHC-CRD protein reported to palmitoylate cysteine residues in the context of a NH2-terminal myristoylated motif. Our findings predict that members of the DHHC-CRD protein family will modify other N-myristoylated, palmitoylated proteins such as heterotrimeric G protein α subunits and nonreceptor tyrosine kinases.

Vacuoles in pfa3Δ cells are normal when the cells are grown in rich media, explaining why PFA3 was not identified in the genome-wide screen for vacuole morphology mutants (Seeley et al., 2002). In the absence of PFA3, there is still a low level of Vac8p palmitoylation that is apparently sufficient for the normal rate of vacuole fusion in a rapidly growing cell. Our results with pfa3Δ and vac8Δ cells suggest that cells increase vacuole fragmentation and fusion under stressful conditions. In pfa3Δ cells the low levels of palmitoylated Vac8p may not be able to support the increase in fusion, resulting in the accumulation of small vacuoles.

Whereas the fragmented vacuole phenotype of pfa3Δ cells is observed only under stressful conditions, Pfa3p is responsible for Vac8p palmitoylation under nonstressful conditions as well. Experiments performed under normal growth conditions show disruption of Vac8p palmitoylation and membrane association (Fig. 4). It is unknown if activity of DHHC-CRD proteins is regulated, but it would be of great interest to determine if Pfa3p activity is activated in response to stress to cope with a potentially increased demand for palmitoylated Vac8p.

Vacuole inheritance is another Vac8p function that has been reported to depend on palmitoylation (Wang et al., 1998). It is notable that pfa3Δ cells do not have a vacuole inheritance defect, although we did detect the defect in our vac8Δ strain (unpublished data). This suggests that a decreased amount of Vac8p palmitoylation is sufficient to support vacuole inheritance, or that the Vac8p cysteine residues have a palmitoylation-independent function. Alternatively, different cysteine residues may be important for the different functions of Vac8p. The Vac8p palmitoylation observed in pfa3Δ cells may be on a cysteine that is important for function in inheritance but not fusion.

The difference in vacuole morphology between pfa3Δ and pfa3Δ swf1Δ cells indicates that Swf1p has a role in vacuole fusion. The similar level of Vac8p palmitoylation in pfa3Δ and pfa3Δ swf1Δ cells suggests that Swf1p promotes vacuole fusion in a manner independent of Vac8p palmitoylation. The presence of a DHHC-CRD suggests that Swf1p is a PAT. It has recently been reported that deletion of SWF1 results in the loss of palmitoylation of the SNARE proteins Sec1, Syn8, and Tlg1 (Valdez-Taubas and Pelham, 2005). Swf1p is likely to be the enzyme responsible for palmitoylation of proteins modified at cysteine residues near their transmembrane domains like the aforementioned SNAREs as well as Vam3, a vacuolar t-SNARE (Valdez-Taubas and Pelham, 2005). Swf1p may exert its influence on vacuole fusion through palmitoylation of SNARE proteins that are involved in trafficking through the endosomal and Golgi compartments.

It has been reported that palmitoylation of Vac8p is mediated by the NH2-terminal longin domain of Ykt6p (Dietrich et al., 2004). Ykt6p is not present in our partially purified Pfa3p–Flag preparations that have activity toward Vac8p in vitro (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200507048/DC1). Therefore, we have discovered a PAT activity toward Vac8p that is independent of Ykt6p. A comparison of the properties of Ykt6p and Pfa3p suggest that they palmitoylate Vac8p by different mechanisms. The longin domain of Ykt6p binds palm-CoA (and CoA) through a noncovalent interaction and transfers it to Vac8p (Dietrich et al., 2004). The reaction does not appear to be enzymatic because it saturates at equimolar concentrations of longin domain and Vac8p (Dietrich et al., 2004). A direct comparison of the relative potencies of Ykt6p and Pfa3p is not possible because the amount of Vac8p palmitoylated by Ykt6p was not reported. In the case of Pfa3p, Vac8p palmitoylation is optimal at substoichiometric concentrations of Pfa3p to Vac8p. Maximal palmitoylation was observed at a 30:1 ratio of Vac8p/Pfa3p, suggesting that the reaction is catalytic. Similar to Erf2p and Akr1p, Pfa3p becomes acylated during the reaction and both autoacylation and palmitate transfer to Vac8p are dependent upon Cys134 in the DHHC-CRD motif. Future experiments will be directed at testing whether Pfa3p palmitoylation represents an acyl-intermediate.

Palmitoylation of Vac8p by Pfa3p–Flag occurs on a slow time scale, reaching saturation after 60 min. This may reflect suboptimal assay conditions. Pfa3p is an integral membrane protein and may function more effectively if reconstituted into liposomes. However, a slow turnover number for lipid-modifying enzymes is not unprecedented. Farnesyltransferase has a turnover number on the order of 1 min−1 (Reiss et al., 1990; Pompliano et al., 1993). It is also possible that the reaction we are observing is slow because we are missing another protein component. In the case of Erf2p, the Erf4p binding partner is required for both autoacylation and PAT activity (Lobo et al., 2002; and unpublished data). No proteins copurified with Pfa3p–Flag in stoichiometric quantities, however this could be because the Pfa3p–Flag was overexpressed. Erf4p is not likely to be a
binding partner for Pfa3p because it is localized to the ER membrane (Zhao et al., 2002) whereas Pfa3p is on the vacuole.

Our genetic and biochemical data strongly argue that Pfa3p is a PAT for Vac8p. However, the residual Vac8p palmitoylation in pfa3Δ, pfa3Δswf1Δ, and pfa3Δerf2Δ cells argues for a second activity. The radiolabeling experiments were performed under conditions where Vac8p-myc-GFP was overexpressed. That in turn might allow Vac8p to encounter PATs that it would not associate with under physiological circumstances. However, vac8Δ cells have a more dramatic vacuolar fragmentation phenotype under nonstressful conditions than pfa3Δ cells (Fig. 1), indicating that some endogenous Vac8p found in pfa3Δ cells is functional and therefore likely to be palmitoylated. Ykt6p and the DHHC-CRD proteins other than Pfa3p, Swf1p, and Erf2p are candidates for a second activity that palmitoylates Vac8p.

To date, a Vac8p homologue has not been identified in mammalian systems. However, the requirement for palm-CoA for mammalian Golgi fusion indicates that protein palmitoylation is important, even though the targets have not been identified (Glick and Rothman, 1987; Pfanner et al., 1990). Our results suggest that a DHHC-CRD protein may be involved in promoting Golgi fusion. Two mammalian DHHC-CRD proteins, HIP14 and GODZ, are present in the Golgi and are candidates for this role (Singaraja et al., 2002; Uemura et al., 2002). Continued efforts to localize and find substrates for DHHC-CRD proteins will further elucidate the roles of palmitoylation in membrane trafficking.

Materials and methods

Strains and constructs

Yeast culture and genetic manipulation were performed by standard methods (Adams et al., 1997). Yeast transformations were performed as described previously (Chen et al., 1992). Yeast transformations were performed as described elsewhere (Duronio et al., 1990). Our genetic and biochemical data strongly argue that DHHC-CRD PALMITOYLATION OF VAC8P • SMOTRYS ET AL. 1097

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Table II. Plasmids used in this study

| Name    | Description          |
|---------|----------------------|
| pML395  | pESC-TRP, GAL promoter, PFA3-Flag |
| pML724  | pESC-TRP, GAL promoter, C134S pfa3-Flag |
| pML547  | pBS, PFA3-3xGFP-TRP1 |
| pML734  | pVT102U, ADH promoter, VAC8-myc |
| pML775  | pVT102U, ADH promoter, C4,5,7S vac8-myc |
| pML735  | pVT102U, ADH promoter, VAC8-myc-GFP |
| pML796  | pRT102U, ADH promoter, C4,5,7S vac8-myc-GFP |
| pML780  | pRS313, PFA3, PFA3-2xFlag |
| pML782  | pRS423, PFA3 promotor, C134S pfa3-2xFlag |
| pML810  | pVT102U, ADH promoter, SWF1-myc |
| pML658  | pQE60, VAC8-myc-6xHis |
| pB131   | NMT1 (Duronio et al., 1990) |
| pML477  | pESC-TRP, GAL promoter, AKR1-Flag |
| pML393  | pESC-TRP, GAL promoter, YOLO003c-Flag |
| pML394  | pESC-TRP, GAL promoter, YDK459c-Flag |
| Flag-ERF2| pESC-TRP, GAL promoter, Flag-ERF2 (Lobo et al., 2002) |
| GST-ERF4| pEG(KG), GAL promoter, GST-ERF4 (Lobo et al., 2002) |

YML195. Dissection of YML195 generated a pfa3Δ haploid YML232. The haploid vac8Δ and swf1Δ strains (YML216 and YML236 respectively) were generated from SWY595 in the same manner. pfa3Δswf1Δ cells (YML237) and pfa3Δerf2Δ (YML249) were recovered from dissection of YML200 (PFA3/pfa33 SWF1/swf1Δ ERF2/erf2Δ YOLO003c/yol003Δ YDK459c/ydk459Δ), which was created by repeated rounds of dissection and mating of strains carrying individual deletions. Integrations of deletion cassettes were confirmed by colony PCR. All haploids used in this study are MATa. All PCR products were ligated into pCR2.1-TOPo before subsequent cloning steps (Invitrogen). The PFA3, YOLO003c, and YDK459c coding sequences were amplified from genomic DNA and cloned into pESC-TRP (Stratagene) as EcoRI-Nhel fragments to generate pML395, pML393, and pML796 respectively. The AKR1 coding sequence was amplified from genomic DNA and cloned into pESC-TRP as a SpeI-Spel fragment to generate pML477. Flag-ERF2 and GST-ERF4 expression constructs are described elsewhere (Lobo et al., 2002). For the plasmid rescue experiments, PFA3 plus 350 base pairs of the promoter region was amplified from genomic DNA and cloned into pK3313 (WT.pml790) or pK3423 (C134s, pml782) as a XhoI-BamHI fragment (Sikorski and Hieter, 1989). The 3’ primer used to amplify the region added a 2xFlag tag. Quikchange mutagenesis (Stratagene) was used to make the C134s pfa3 mutations in pML724 and pML782. The SWF1 coding region was amplified from genomic DNA and cloned into a modified Baculovirus vector (pBlueBac4.5B[--]) as a SpeI-Spel fragment to add a COOH-Terminal myc tag. SWF1-myc was then cut out of the pBlueBac construct (Xhol-Sall) and inserted into pVT102U at the Xhol site to create pML810 (Vernet et al., 1987). VAC8 was amplified from genomic DNA with primers that added a 5’ BamHI site and a 3’ myc tag followed by a Xhol site, a stop codon, and a HindIII site. This product was cloned into pVT102U as a BamHI/HindIII fragment. GFP was then inserted into the COOH-Terminal XhoI site to generate VAC8-myc-GFP (pML735). C4,5,7S vac8 was made by amending VAC8 from pML734 with a mutagenic S’ primer followed by cloning into pVT102U to generate pML775. Addition of GFP to pML775 resulted in pML796. All constructs and mutations were verified by sequencing. Integration of a COOH-Terminal 3xGFP tag into the PFA3 locus was made possible by modification of the pBS-3xGFP-TRP1 vector (Lee et al., 2003). A 3’ fragment of PFA3 (nucleotides 352–1005) was inserted into a BamHI site upstream of the 3xGFP to create PM457. This plasmid was digested within the PFA3 sequence at a unique restriction site, BsmI, and integrated into SWY595. Dissection yielded YML163 [MATa PFA3-3xGFP-TRP]. Colony PCR and Western blotting confirmed proper integration. The Vac8p-myc-6xHis bacterial expression plasmid was made by amplification of VAC8 from genomic DNA and cloning into pQE60 (QIAEGEN) as an Ncol-BgIII fragment. The 3’ primer added a COOH-Terminal myc tag. The N-myristoyltransferase expression construct pBB131 is described elsewhere (Duronio et al., 1990).

Cell fractionation

WT and pfa3Δ cells were transformed with Vac8p-myc-GFP. Cells were grown in selective media and harvested in mid-log phase. Glass bead lysis and fractionation by differential centrifugation were performed as described (Wang et al., 1998). Whole cell lysates were adjusted to equal protein concentration before centrifugation. Equal percentages of the internal membranes (P13), plasma membrane (P100), and cytosol (S100) fractions were separated by SDS-PAGE and Western blotted with a-myc ascites.

Microscopy

Cells were grown and labeled in YPD unless treated with DTT or low glucose, in which case they were grown in minimal media. Rapidly growing cells were labeled with 20 μM FM 4–64 (Invitrogen) for 30 min and then collected and suspended in fresh media lacking FM 4–64 for 60 min (Vida et al., 1999).

Table III. Strains used in this study

| Strain | Relevant genotype |
|--------|-------------------|
| SWY518 | WT                |
| YML232 | pfa3Δ             |
| YML236 | swf1Δ             |
| YML237 | pfa3Δswf1Δ        |
| YML249 | pfa3Δerf2Δ        |
| YML216 | vac8Δ             |
| YML163 | PFA3-3xGFP-TRP    |
and Emr, 1995). Cells were washed twice with nonfluorescent media (NF; 8 mM KPO4, pH 5.4, 2 mM MgSO4, 27 mM [NH4]2SO4, 2% glucose, 0.04× complete amino acids) before imaging. For DTT-treated samples, 2 mM DTT was added to cultures at the start of FM 4–64 labeling 2 h before microscopy. 2 mM DTT was maintained in the media after the labeling period and in the NF media washes. Low glucose samples were shifted to minimal media with 0.05% glucose 4 h before microscopy, and were washed and visualized in NF media containing 0.05% glucose.

Images were collected at room temperature with an Olympus IX-81 inverted microscope with a 100× objective, a camera (model CCD-300T-RC; Dage MTI) and NIH Image software. The only image processing was adjustment of brightness and/or contrast in Adobe Photoshop.

**Metabolic palmitate labeling**

Yeast cells were transformed with Vac8-myc-6xHis constructs. Equal numbers of rapidly growing cells (25 OD600 units) were treated with 3 μg/ml cerulein for 1 h and subsequently labeled with [3H]palmitate (45.0 Ci/mmole, NEN Life Science Products) at a final concentration of 0.2 mCi/ml for 15 min at 30°C. Cells were lysed as described (Wang et al., 1998). Whole cell lysates were incubated on ice for 1 h in lysis buffer containing 1% Triton X-100 (Roche), and then centrifuged at 15,000 g for 10 min. Vac8-myc-GFP was immunoprecipitated from extracts with affinity-purified α-GFP antibody coupled to protein G-conjugated beads (GE Health care; Harlow and Lane, 1988). The GFP antibodies were generated and affinity purified as described (Geerd et al., 1999). Immunoprecipitated proteins were resolved by SDS-PAGE and detected with SYPRO Ruby protein gel stain (Molecular Probes) or estimated by comparison of Western blot signals to a Flag-tagged protein of known concentration. The extract and elutions were also probed with α-Ykt6 antibody, which was kindly provided by Dr. James McNew (Rice University, Houston, TX; McNew et al., 1997).

For in vitro membrane PAT assays presented in Fig. 5, WT and C134S (pML724) Pfa3p-Flag constructs were cotransformed into E. coli DH5α to generate Vac8p-myc-6xHis produced in Escherichia coli by coexpression with Nmyristoyltransferase (Duronio et al., 1990; Fig. S2). Details of the purification can be found in Online supplemental material. The protein concentration of the final preparation was determined by the method of Bradford. Approximately 50% of the final preparation was full-length myristoylated Vac8p-myc-6xHis, as estimated by examination of the Coomassie blue-stained gel (Fig. S2).

**Online supplemental material**

In Fig. S1, Ykt6p does not copurify with Pfa3p-Flag. Fig. S2 shows that recombinant Vac8-myc-6xHis is myristoylated. A description of the procedure for purification of myr-Vac8-myc-6xHis is available online. Online supplemental materials are available at http://www.jcb.org/cgi/content/full/jcb.200507048/DC1.

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