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Palmitoylation Plays a Role in Targeting Vac8p to Specific Membrane Subdomains

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Vac8p is a multifunctional yeast protein involved in several distinct vacuolar events including vacuole inheritance, vacuole homotypic fusion, nucleus–vacuole junction formation and the cytoplasm to vacuole protein targeting pathway. Vac8p associates with the vacuole membrane via myristoylation and palmitoylation. Vac8p has three putative palmitoylation sites, at Cys 4, 5 and 7. Here, we show that each of these cysteines may serve as a palmitoylation site. Palmitoylation at Cys 7 alone provides partial function of Vac8p, whereas palmitoylation at either Cys 4 or Cys 5 alone is sufficient for Vac8p function. In the former mutant, there is a severe defect in the localization of Vac8p to the vacuole membrane, while in the latter mutants, there is a partial defect in the localization of Vac8p. In addition, our studies provide evidence that palmitoylation targets Vac8p to specific membrane subdomains.

Key words: membrane subdomain, organelle inheritance, palmitoylation, Vac8p, yeast

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During cell division, most cytoplasmic organelles are actively distributed to the new daughter cells. In budding yeast, the distribution of vacuoles initiates early in the cell cycle (1); a portion of the mother vacuole most proximal to the incipient bud site moves along actin filaments and is drawn into the emerging bud (2). Vacuole movement requires the class V myosin, Myo2p (2–4). The globular tail of Myo2p binds directly Vac17p, which in turn binds directly to Vac8p (5,6). Vac8p is attached to the vacuole membrane. The Vac17p–Vac8p complex forms the vacuole-specific Myo2p receptor and specifically regulates Myo2p attachment to the vacuole (5,6). Myo2p attachment to its other cargoes occurs through interaction with other receptors [reviewed by Pruyne et al. and Pashkova et al. (7,8)].

Vac8p is an armadillo-repeat-containing protein (9–11). In addition to its role in vacuole inheritance, Vac8p is required for at least four other vacuole-related processes: homotypic vacuole fusion (12–14), the cytoplasm to vacuole protein targeting pathway (15), formation of the nucleus–vacuole junction (16) and caffeine resistance (17). Each of these functions involves a unique Vac8p binding partner; the armadillo repeats of Vac8p serve as docking sites for these proteins (17).

Vac8p is both myristoylated and palmitoylated; the former modification occurs at Gly 2, and the latter at one or more of the nearby residues, Cys 4, Cys 5 and/or Cys 7 (11). Exactly which site(s) is palmitoylated has not yet been determined. The covalent attachment of myristate to the amino-terminal Gly occurs cotranslationally and facilitates the subsequent post-translational addition of palmitate to the nearby Cys residues [reviewed by Linder and Deschenes (18)]. Myristoylation and palmitoylation are critical for Vac8p association with the vacuole membrane (11).

Palmitoylation is a common reversible modification of eukaryotic proteins that increases their membrane affinity and reversibly affects both protein localization and function (19). In addition to playing a general role in membrane association, studies on proteins such as Src kinases and Gα subunits suggest that palmitoylation specifically targets these proteins to lipid rafts, subdomains on the plasma membrane that are enriched in sphingolipids and cholesterol (19). Targeting to a membrane subdomain would increase the local concentration and likely facilitates protein sorting and signaling (20–22).

A number of mutational studies have shown that the precise position of the palmitoyl moieties and the sequence of the surrounding amino acids can determine the specific membrane to which a protein will be targeted. For example, for the receptor tyrosine kinase Lck, mutation of Cys 5 but not of Cys 3 results in mislocalization of the protein from the plasma membrane to the Golgi region (23). In the neuronal protein postsynaptic density-95 (PSD-95), deletion of the amino acid between the palmitoylated Cys 3 and 5 results in mistargeting of the protein from the postsynaptic membrane to the axonal membrane. In contrast, mutation of basic amino acids near the palmitoylation sites at Cys 3 and 4 of growth-associated protein-43 (GAP-43) results in its mislocalization from the axonal membrane to dendrites (24).

Palmitoylation of Vac8p is important for its localization and its function in vacuole inheritance (11) and homotypic vacuole fusion (12–14). Notably, Vac8p is uncommon among myristoylated and palmitoylated proteins. For example, Vac8p is localized on the yeast vacuole; only one other protein that is myristoylated and palmitoylated, Meh1p, is found on the vacuole. Other known myristoylated and palmitoylated proteins are found at the plasma membrane (25). Moreover, Vac8p possesses three cysteines near its myristoylation site; each cysteine is a potential palmitoylation site. This contrasts with other studied myristoylated and palmitoylated proteins, which have one or at most two palmitoylation sites. Importantly, the three cysteines found at the amino terminus of Vac8p are highly conserved in Vac8p proteins from other yeasts and fungi. So far, Vac8p has been sequenced from ten species. In six of the sequences, the spacing of Gly 2 and the three cysteines is identical (Figure 1A). This degree of conservation strongly suggests that the three cysteines are important. These findings raise several questions: (1) Are all three cysteines palmitoylated? (2) How many palmitoylation sites are required for full function of Vac8p? (3) Does palmitoylation serve a role in addition to its function to attach Vac8p to the vacuole membrane? (4) Do different sites have different functions?

Here, we show, by analysis of all possible single and double mutants of Cys 4, 5 and 7, that all three of the putative palmitoylation sites in Vac8p play roles in its association with the vacuole membrane and its vacuole-membrane-related functions. In addition, we present evidence that palmitoylation may target Vac8p to specialized lipid subdomains.

Results

Palmitoylation of either Cys 4 or Cys 5 is required for Vac8p association with the vacuole membrane

Our previous studies demonstrated that Vac8p is myristoylated and palmitoylated and strongly suggested that Cys 4, 5 and 7 are each sites of palmitoylation (11). In order to determine whether each of these cysteines was a site of palmitoylation and to determine the role of acylation in Vac8p localization, all possible single and double mutants of Cys 4, 5 and 7 were constructed. Analysis of these mutants showed that acylation is not required for Vac8p expression or its stability. As measured by immunoblot analysis, Vac8p mutants that are not myristoylated or not palmitoylated are present in cells at similar steady state levels as wild-type Vac8p (Figure 1B).

Acylation of Vac8p is critical to its proper localization. Wild-type Vac8p was readily observed on the vacuole membrane by conventional immunofluorescence microscopy (Figure 2). However, using the same technique, neither the *vac8-G2A* nor the *vac8-C4G/C5T/C7S* mutants could be detected (data not shown). Therefore, we used a more sensitive 'sandwich' immunofluorescence microscopy technique (see *Materials and Methods*). Use of this technique revealed that a fraction of the Vac8p mutant that cannot be myristoylated, *vac8-G2A*, was still associated with the vacuole membrane. This mutant is not palmitoylated to the same extent as wild type (11). Therefore, this finding suggests that myristoylation and/or normal levels of palmitoylation are required for full association of Vac8p with the vacuole membrane. In contrast, in the absence of any myristoylation and palmitoylation, Vac8p cannot associate

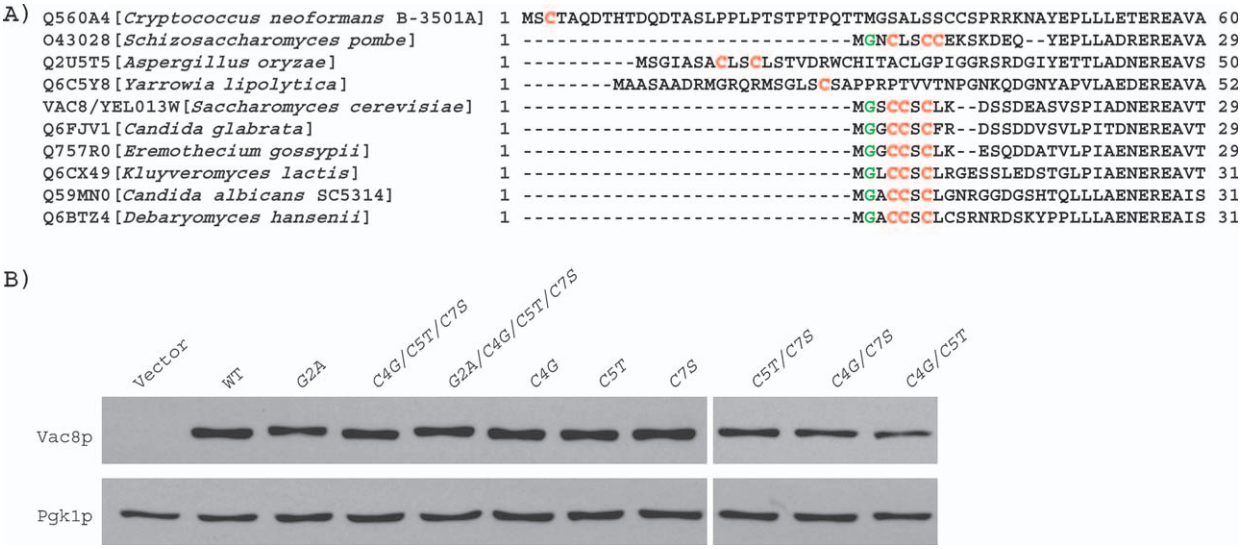

Figure 1: Acylation of Vac8p is not required for protein stability. A) Sequence alignment of Vac8p homologues in fungi. Potential myristoylation and palmitoylation sites are shown in green and red, respectively. B) Immunoblot analysis of the levels of Vac8p in wild-type (WT) and mutant cells. Equal OD_{600} of crude cell extracts from mid-log cells were subjected to immunoblot analysis using anti-Vac8p and anti-Pgk1p antibodies.

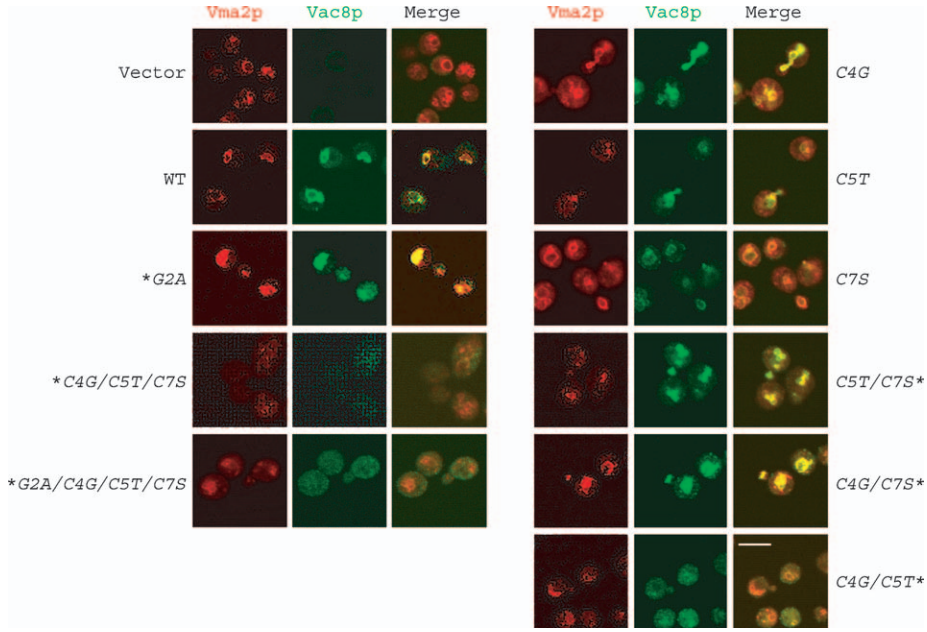

Figure 2: Palmitoylation at either Cys 4 or Cys 5 is required for vacuole membrane association of Vac8p. The vacuole membrane (red) was visualized with mouse anti-Vma2p and rhodamine-red-conjugated donkey anti-mouse IgG. Vac8p (green) was labeled with rabbit anti-Vac8p and Alexa-488-conjugated goat anti-rabbit IgG. * indicates that a 'sandwich' technique was used. For the sandwich technique, Vac8p was labeled with rabbit anti-Vac8p, followed by goat anti-rabbit IgG and Alexa-488-conjugated donkey anti-goat IgG. Scale bar is 5 μm.

with the vacuole membrane. The *vac8-G2A/C4G/C5T/C7S* mutant, which cannot be acylated, was detected only as small, dispersed spots in the cytoplasm. Loss of only one putative palmitoylation site had little effect on Vac8p localization: all three of the single mutants (*vac8-C4G*, *C5T* and *C7S*) had levels of vacuole membrane-associated Vac8p that were detectable by conventional immunofluorescence microscopy. Loss of two potential palmitoylation sites resulted in decreased localization of Vac8p to the vacuole membrane, such that detection of the protein required the use of a sandwich technique. Two of the double mutants (*vac8-C5T/C7S* and *vac8-C4G/C7S*) were found to be localized to both the vacuole membrane and the cytoplasm. The remaining double mutant (*vac8-C4G/C5T*) had a more severe phenotype. Some Vac8p was detected on the vacuole membrane, but most of the protein was present as small spots dispersed in the cytoplasm. This indicates that either Cys 4 or Cys 5 is crucial for the proper localization of Vac8p to the vacuole membrane. These immunofluorescence localization studies parallel those observed using subcellular fractionation (26).

Palmitoylation of either Cys 4 or Cys 5 is required for vacuole inheritance, vacuole fusion and caffeine resistance

Palmitoylation is required for Vac8p function (11). Simultaneous mutation of both the myristoylation and the palmitoylation sites abolishes Vac8p function in vacuole inheritance (11), homotypic vacuole fusion (13) (also see Figure 3A) and caffeine resistance (Figure 3B). Because Vac8p-G2A is not palmitoylated to the same extent as wild-type Vac8p (11), it is difficult to test the role of myristoylation in Vac8p function. Consistent with a partial defect in palmitoylation, the *vac8-G2A* mutant, while capable of supporting vacuole inheritance, had a modest defect in homotypic vacuole fusion as measured by vacuole fragmentation (11,13) (also see Figure 3A). Moreover, myristoylation alone is not sufficient for Vac8p function: the palmitoylation-minus mutant *vac8-C4G/C5T/C7S* was defective in vacuole inheritance, homotypic vacuole fusion and caffeine resistance (11,13) (Figure 3A–B).

Each of the single Cys point mutants functioned similarly to wild-type *VAC8* in vacuole inheritance (Figure 3A). These mutants were virtually normal for homotypic vacuole fusion (Figure 3A) and caffeine resistance (Figure 3B). Likewise, two of the double mutants *vac8-C5T/C7S* and *vac8-C4G/C7S* behaved the same as the single mutants (Figure 3A–B). Importantly, however, the remaining double mutant *vac8-C4G/C5T* was severely defective in these functions: less than 20% of cells displayed normal vacuole inheritance and less than 40% showed normal vacuole morphology (Figure 3A). This double mutant also was less resistant to caffeine and had a clear growth defect on 0.2% caffeine plates (Figure 3B). These results indicate that palmitoylation at either Cys 4 or Cys 5 is required for vacuole inheritance, homotypic vacuole fusion and caffeine resistance. Palmitoylation at Cys 7 alone results in a partial defect in these Vac8p functions.

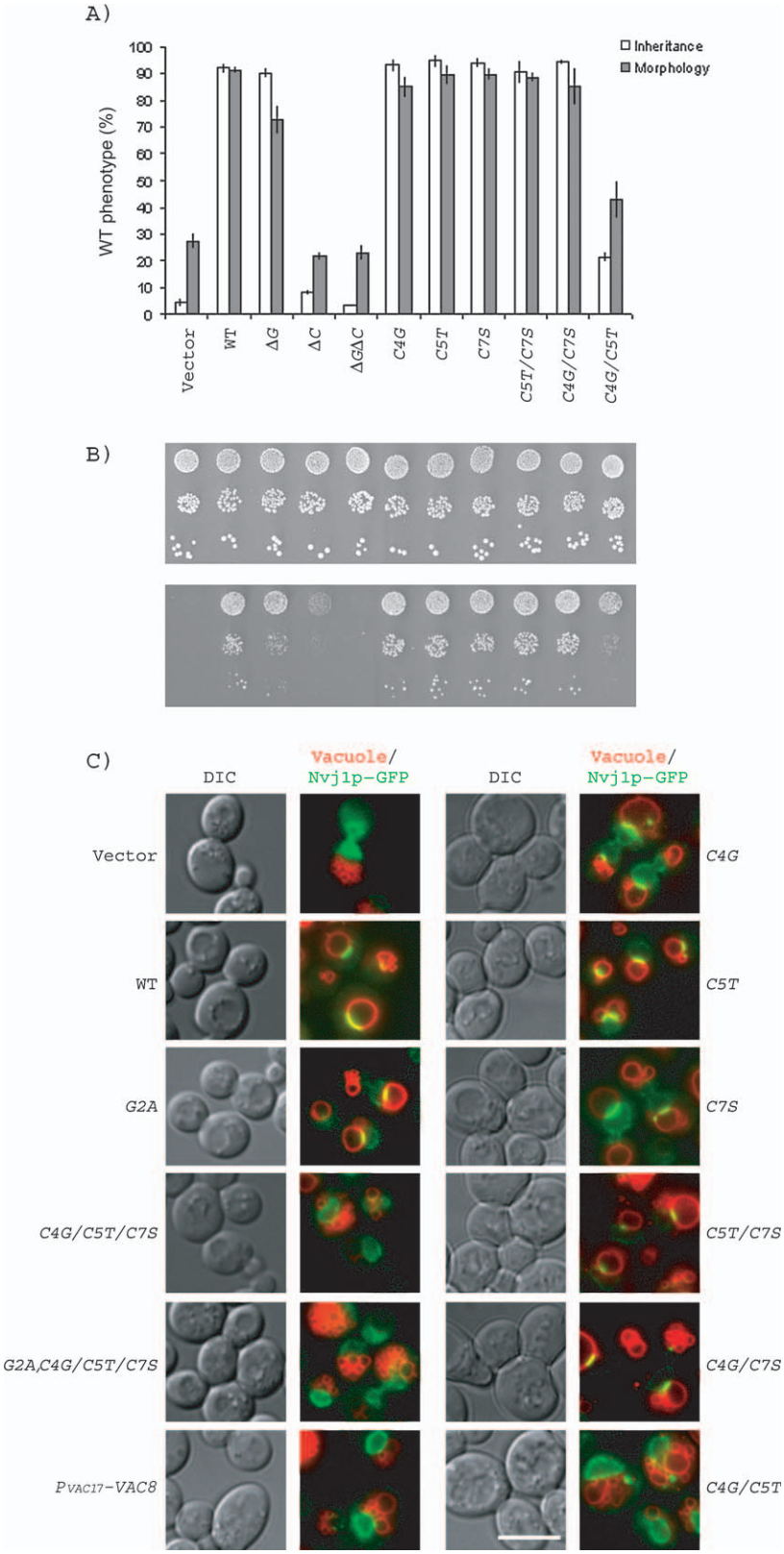

Figure 3: Functional analysis of Vac8p palmitoylation mutants. A) Vacuole inheritance and vacuole homotypic fusion in wild-type and mutant *vac8* cells. The indicated plasmids were transformed into a *vac8Δ* strain. Transformants were labeled with FM4-64 for 1.5 h and chased for one cell doubling. For each strain, more than 100 cells were scored for vacuole inheritance and for vacuole fusion. The vacuole of a wild-type (WT) cell usually contains three to six lobes. Cells that contained more than six lobes were scored defective in homotypic vacuole fusion. The average and standard deviations from three independent experiments are shown. *ΔG*: *G2A*; *ΔC*: *C4G/C5T/C7S* and *ΔGΔC*: *G2A/C4G/C5T/C7S*. B) Caffeine resistance of WT and mutant *vac8* cells. *vac8Δ* cells carrying the indicated plasmids were spotted onto SC-URA (Synthetic Complete-Uracil) and SC-URA + 0.2% caffeine plates and incubated at 24°C for 3 days (SC-URA plates) or 4 days (SC-URA + 0.2% caffeine plates). C) Localization of Nvj1p–GFP in wild-type and mutant *vac8* cells. p*NVJ1–GFP* was cotransformed with the indicated *vac8* mutants. Transformants were labeled with FM4-64 for 1.5 h and chased for one cell doubling time. Scale bar is 5 μm.

The levels of Vac8p on the vacuole membrane are crucial for formation of the nucleus–vacuole junction

Vac8p–green fluorescent protein (GFP) is enriched in at least two regions of the vacuole membrane: the nucleus–vacuole junction (10) and vacuole–vacuole junctions (vertices) (10,14). The nucleus–vacuole junction is the site of piecemeal microautophagy of the nucleus, whereby a portion of the nuclear membrane is engulfed by the vacuole and degraded by vacuolar hydrolases (27). Nvj1p, an outer nuclear membrane protein, is a component of the nucleus–vacuole junction and interacts with Vac8p in a yeast two-hybrid test (16). The proper localization of Nvj1p at the nucleus–vacuole junction requires enrichment of Vac8p at the same region (16).

Consistent with the above studies, in the presence of wild-type *VAC8*, Nvj1p–GFP concentrated at the nucleus–vacuole junction (Figure 3C). In the *vac8-G2A* mutant or in cells containing any of the single Cys mutants, Nvj1p–GFP was predominantly localized to the nucleus–vacuole junctions, with a small amount of Nvj1p–GFP dispersed around the nuclear envelope (Figure 3C). Two of the double mutants (*vac8-C5T/C7S* and *vac8-C4G/C7S*) were also nearly normal with most of the Nvj1p–GFP at the nucleus–vacuole junction. In contrast, in the double mutant *vac8-C4G/C5T* and the mutants in which all three Cys residues are replaced, Nvj1–GFP was dispersed throughout the outer nuclear membrane (Figure 3C). These findings suggest that palmitoylation of Vac8p at either Cys 4 or Cys 5 is required for localization of Vac8p and Nvj1p to the nucleus–vacuole junction.

Defects observed in nucleus–vacuole junction formation in the Vac8p mutant that cannot be palmitoylated at both Cys 4 and Cys 5 could be due directly to a loss of palmitoylation or due to the fact that levels of the mutant on the vacuole membrane are significantly lower than those of wild-type Vac8p. To distinguish between these possibilities, we compared the *vac8-C4G/C5T* mutant with a second *vac8* mutant P_{VAC17}-*VAC8*, which is composed of the wild-type *VAC8* open-reading frame driven by the *VAC17* promoter. The levels of *VAC8* expressed from the *VAC17* promoter are ~10-fold lower than those of wild type (17). As measured by immunoblot analysis of isolated vacuoles, P_{VAC17}-*VAC8* and *vac8-C4G/C5T* mutants have similar levels of vacuole membrane-associated Vac8p (Figure 4A). Notably, this low level of Vac8p is not sufficient for formation of the nucleus–vacuole junction; Nvj1p–GFP was mislocalized in both mutants (Figure 3C). The above results show that the levels of Vac8p on the vacuole membrane are critical for formation of the nucleus–vacuole junction. Thus, we could not test whether palmitoylation plays a direct role.

Palmitoylation plays a role in targeting Vac8p to microdomains on the vacuole membrane

To further test the roles of Vac8p palmitoylation, we compared additional functions of the P_{VAC17}-*VAC8* and *vac8-C4G/C5T* mutants. These two mutants demonstrated functional differences in vacuole fusion and vacuole inheritance. P_{VAC17}-*VAC8* was significantly better at these two functions than *vac8-C4G/C5T* (Figure 4B). To analyze the partial defect of *vac8-C4G/C5T* in vacuole inheritance, we compared the localization of Myo2p relative to the vacuole in wild-type, *vac8-C4G/C5T/C7S*, *vac8-C4G/C5T*, P_{VAC17}-*VAC8* and *vac8Δ* cells. In wild-type cells, a portion of the vacuole extends to the bud tip, where it colocalizes with Myo2p–GFP. Likewise in the P_{VAC17}-*VAC8* mutant, which contains all three potential palmitoylation sites, a portion of the vacuole colocalizes with Myo2p–GFP. In contrast, in the absence of Vac8p, the vacuole remains in the mother cell, while Myo2p–GFP is concentrated at the bud tip. The *vac8-C4G/C5T/C7S* mutant shows the same phenotype. Notably, the *vac8-C4G/C5T* mutant has an intermediate phenotype. In approximately 50% of the cells, a small portion of the vacuole moves into the bud and colocalizes with Myo2p–GFP (Figure 4C). These results strongly support the hypothesis that Cys 7 can serve as a palmitoylation site. It is tempting to speculate that a single palmitoyl moiety at Cys 7 is not sufficient to fully anchor Vac8p on the vacuole membrane.

The fact that Vac8p is palmitoylated raises the possibility that vacuole microdomains may exist and, furthermore, may share common properties with the better characterized plasma membrane microdomains, specifically lipid rafts. Palmitoylated plasma membrane proteins, such as G proteins and Src kinases, reside in lipid rafts that are rich in sphingolipids and cholesterol (18). Sphingolipids and ergosterol (the yeast equivalent of cholesterol) are found in very low levels in the vacuole membrane (28). Intriguingly, a block in the biosynthesis of ergosterol (*erg6Δ*) or very long chain fatty acids (*sur4Δ*), sphingolipid precursors, results in fragmented vacuoles (29), a phenotype similar to that observed in the *vac8Δ* mutant. Moreover, like Vac8p, ergosterol accumulates at vacuole–vacuole vertices (30). These results suggest that ergosterol, sphingolipids and additional lipids form specific microdomains on the vacuole membrane and that Vac8p is enriched in these microdomains. Thus, we conducted the following experiments.

Plasma membrane lipid rafts are insoluble in Triton X-100. We tested the behavior of Vac8p in Triton X-100 and in other more mild detergents, Brij 98, Lubrol WX and Tween-20. These detergents have been successfully used to separate distinct types of plasma membrane rafts in mammalian and yeast cells (31–33). Each of these plasma membrane rafts has a unique protein and lipid composition (33). Moreover, 'Lubrol rafts' and 'Brij 98 rafts' have distinct physiological roles compared to 'Triton rafts' *in vivo* (34–37).

The solubility of Vac8p was compared with that of Pma1p, a plasma membrane protein that resides in Triton-insoluble rafts (28), and that of Pgk1p, a cytosolic protein. We found that regardless of the detergent, Pma1p remained

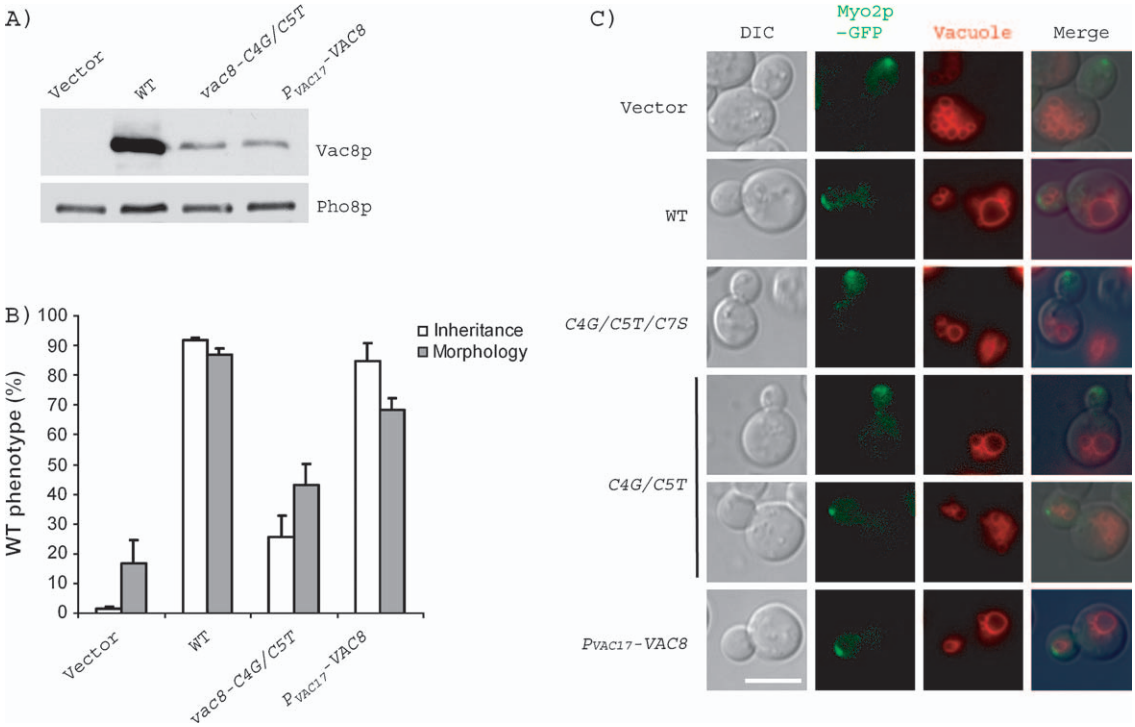

Figure 4: Palmitoylation of Vac8p is required for vacuole inheritance and homotypic vacuole fusion. A) Comparison of vacuole membrane association of Vac8p-C4G/C5T and P_{VAC17}-Vac8p. Vacuoles from a *vac8Δ* strain carrying the indicated plasmids were isolated and analyzed by immunoblotting using anti-Vac8p and anti-Pho8p antibodies. B) Functional differences of Vac8p-C4G/C5T and P_{VAC17}-Vac8p. Vacuole inheritance and vacuole morphology were measured as described in Figure 3. The average and standard deviations from three independent experiments are shown. C) Localization of vacuoles and Myo2p–GFP in wild-type and *vac8* mutant cells. The indicated plasmids were introduced into a *vac8Δ* strain that contains genomically integrated *MYO2–GFP* (LWY7814). Cells were labeled with FM4-64 for 1.5 h and chased for one cell doubling time. More than 15 cells were scored in each sample. The representative examples are shown. Scale bar is 5 μm.

associated with lipid and floated in the low-density (membrane-containing) fraction, while Pgk1p appeared with the soluble components (Figure 5A). The behavior of Vac8p in detergent was more complex. In the absence of detergent, most of Vac8p floated and cofractionated with Pma1p (Figure 5A). In the presence of Triton X-100 (Figure 5A) or Brij 98 (data not shown), Vac8p was completely soluble. In contrast, in the presence of the milder detergents, Lubrol WX or Tween-20, a significant amount of Vac8p cofractionated with Pma1p (Figure 5A), suggesting that a fraction of the Vac8p resides in 'Lubrol' rafts or 'Tween 20 rafts'.

Next, we tested whether the Lubrol WX- or Tween 20-insoluble portion of Vac8p accounted for the functional difference between the *vac8-C4G/C5T* and the *P_{VAC17}-VAC8* mutants. We applied the same gradient centrifugation to the mutants in the presence of Lubrol WX (Figure 5B) or Tween-20 (data not shown). A modest but reproducible difference was observed. We consistently detected more P_{VAC17}-Vac8p in the same fractions as Pma1p.

In order to further test whether fully palmitoylated Vac8p resides in subdomains on the membrane, we compared the localization of P_{VAC17}-Vac8p with Vac8p-C4G/C5T. By immunoblot analysis, both proteins were found to be at similar levels on the vacuole membrane (Figure 4A). However, when detected by immunofluorescence microscopy, P_{VAC17}-Vac8p was observed as many spots on the vacuole membrane, whereas the Vac8p-C4G/C5T mutant had far fewer spots, presumably because it is more diffusively distributed (Figure 5C). Interestingly, this distribution of P_{VAC17}-Vac8p to small spots on the vacuole membrane is similar to what has been observed for its binding partner Vac17p (6). We postulate that these small spots are Vac8p microdomains that facilitate the interaction between Vac8p and Vac17p.

Discussion

In this study, a series of Vac8p mutants missing the myristoylation and/or palmitoylation sites were generated and the effects of these mutations on the localization and functions of Vac8p were assessed. Point mutations that abolish myristoylation and/or palmitoylation do not affect the stability of Vac8p but greatly affect its distribution. Importantly, the different behaviors exhibited by point

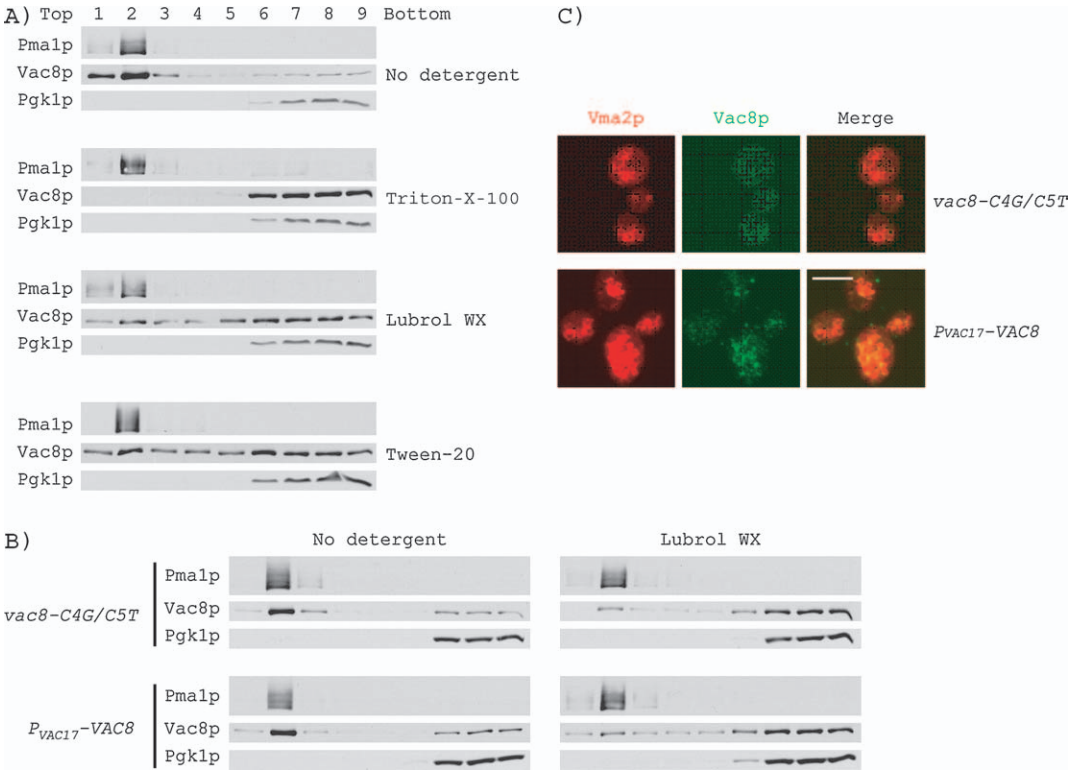

Figure 5: Palmitoylation plays a role in targeting Vac8p to specific microdomains. A) Solubility of Vac8p in the absence or presence of Triton X-100, Lubrol WX and Tween 20. Wild-type cells were lysed and fractionated in a 0–40% (w/v) Optiprep gradient in the absence or presence of 1% (w/v) Triton X-100, 0.5% (w/v) Lubrol WX or 1% (w/v) Tween 20. Samples were collected from the top (fraction 1) to the bottom (fraction 9) of the gradient, and proteins were subjected to immunoblot analysis for Pma1p, Vac8p and Pgk1p. B) Solubility of Vac8p-C4G/C5T and P_{VAC17}-Vac8p in the absence or presence of 0.5% Lubrol WX. C) The localization of Vac8p-C4G/C5T and P_{VAC17}-Vac8p by immunofluorescence microscopy. Vac8p (green) was detected by a 'sandwich' technique. Vac8p spots are much less frequent in the *vac8-C4G/C5T* mutant. Vacuole-localized Vac8p 'spots' were not detected in 36 randomly chosen *vac8-C4G/C5T* cells, while vacuole-localized Vac8p spots were present in 31% of P_{VAC17}-*VAC8* cells ($n = 88$). Scale bar is 5 μm.

mutants that affect two of the three palmitoylation sites indicate that these sites are not equivalent. Mutants that have point mutations at two of the three palmitoylation sites, but are palmitoylated either at Cys 4 or Cys 5, localize to the vacuole membrane although partial mislocalization of these mutant proteins to the cytoplasm occurs. In contrast, the *vac8* mutant that contains Cys 7 as its sole site of palmitoylation localizes to small dots, some on the vacuole, but many are elsewhere in the cell.

The genetic analysis presented here suggests that Cys 4, 5 and 7 are each palmitoylated. Two lines of evidence suggest that Cys 7 is palmitoylated. First, comparison of *vac8-C4G/C5T* and *vac8-C4G/C5T/C7S* shows that the former mutant is more functional than the latter in vacuole inheritance and vacuole fusion (Figure 3A). Second, in the *vac8-C4G/C5T* mutant, approximately 50% of the vacuoles colocalize with Myo2p on the bud tip (Figure 4C), suggesting that Vac8p is still associated with vacuole membrane in these cells. In contrast, in the *vac8-C4G/C5T/C7S* mutant, vacuoles are not drawn by Myo2p to the bud (Figure 4C). This defect is likely due to the fact that Vac8p is not retained on the vacuole membrane. Our results contrast with the studies using acyl–biotin exchange, where it had been proposed that Cys 7 is not palmitoylated (26). However, in that analysis, only membrane-bound proteins were included. Because most of the Vac8p-C4G/C5T is soluble, the acyl–biotin exchange method likely is not sensitive enough to detect palmitoylation at Cys 7.

Palmitoylation at Cys 7 alone was not sufficient for targeting Vac8p to the nucleus–vacuole junction. It was not possible to test whether palmitoylation of Vac8p is specifically required for Vac8p targeting to the nucleus–vacuole junction because palmitoylation of Vac8p is important for its overall attachment to the vacuole membrane. When compared with wild type, the *vac8-C4G/C5T* mutant has 10-fold less Vac8p on the vacuole membrane (Figure 4 A). We found that a 10-fold drop in Vac8p levels, either through lower expression of Vac8p using the P_{VAC17}-*VAC8* mutant or through loss of palmitoylation due to the *vac8-C4G/C5T* mutant, causes a mislocalization of Nvj1p from the nucleus–vacuole junction (Figure 3C), a sign that Vac8p is also not targeted there.

Our studies show that palmitoylation of Vac8p is directly required for Vac8p function in homotypic vacuole fusion. P_{VAC17}-*VAC8* and *vac8-C4G/C5T* mutants have similar levels of Vac8p on the vacuole membrane; yet, the former mutant supports vacuole fusion better than the latter (Figure 4B). Vac8p is targeted to vacuole–vacuole vertices (14,27), where it functions in homotypic vacuole fusion (12,13). The localization of Vac8p to vacuole–vacuole vertices is regulated and does not occur at the interface of vacuoles that have been physically forced into contact by sedimentation (14). Along with Vac8p, docked vacuoles are also enriched for ergosterol at the vacuole–vacuole vertices (30). Thus, it is tempting to speculate that reversible palmitoylation of Vac8p regulates its movement into lipid raft-like, ergosterol-containing vacuole–vacuole vertices where fusion occurs. In support of this hypothesis, Vac8p is reversibly palmitoylated during vacuole fusion (12,13). Furthermore, ergosterol is required for vacuole–vacuole fusion. Absence of *ERG6*, one of the ergosterol biosynthetic genes, results in fragmented vacuoles (38), and conversely, overexpression of *ERG6* increases vacuole fusion (39).

Palmitoylation of Vac8p may also localize it to microdomains related to vacuole inheritance. Similar to vacuole fusion, P_{VAC17}-*VAC8* supports vacuole inheritance better than *vac8-C4G/C5T*. Myo2p and Vac17p are concentrated at small patches on the vacuole membrane (4,6). Whether ergosterol specifically colocalizes with the Vac8p–Vac17p–Myo2p complexes remains to be tested. Fillipin, which is fluorescent and binds ergosterol-containing membranes *in vivo* and *in vitro*, has been used to observe ergosterol on isolated vacuoles. However, when yeast are treated with fillipin *in vivo*, the fluorescence at the plasma membrane is too strong to detect the ergosterol that is present on internal membranes (40).

Lipid rafts were identified first on the plasma membrane (41). A number of recent studies have demonstrated that rafts also exist on intracellular membrane organelles along the secretory pathway including the Golgi complex (42), the endoplasmic reticulum (43) and endosomes (34). Likewise, functional lipid microdomains likely exist on the yeast vacuole membrane. It is likely that the lipid components of these microdomains are different from those of the lipid rafts characterized thus far. Characterization of the putative Vac8p microdomains may require development of new techniques to probe its lipid components.

Materials and Methods

Yeast strains and plasmids

The following strains were used in this study: LWY7235 (*MATα leu2, 3-112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*) (44), LWY2887 (*MATα leu2, 3-112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vac8Δ::HIS3*) (11) and LWY7814 (*MATα leu2, 3-112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vac8Δ::HIS3 myo2::MYO2-GFP*).

vac8-G2A, *vac8-C4G/C5T/C7S*, *vac8-G2A/C4G/C5T/C7S*, *NVJ1-GFP*, P_{VAC17}-*VAC8* and *VAC17-GFP* plasmids were described previously (11,17).

vac8-C4G, *vac8-C5T* and *vac8-C7S* plasmids were constructed using the Transformer Site-Directed Mutagenesis Kit (ClonTech, Mountain View, CA, USA). *vac8-C5T/C7S*, *vac8-C4G/C7S* and *vac8-C4G/C5T* plasmids were constructed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The residues substituted at each cysteine were chosen based on the original well-characterized mutants, *vac8-C4G/C5T/C7S* and *vac8-G2A/C4G/C5T/C7S* (11,17). Comparison of the phenotypes of the above point changes with those reported for mutants where cysteine was substituted with alanine (26) revealed that the *vac8-C4G*, *vac8-C5T* and *vac8-C7S* and *vac8-C4A*, *vac8-C5A* and *vac8-C7A* mutants behave similarly.

Immunoblot analysis

To detect Vac8p in total cell extracts, mid-log phase cells (OD_{600} 0.4–0.8) were lysed by vigorous agitation with 0.5-mm glass beads in 100 μL of lysis buffer [0.3 M sorbitol, 10 mM Tris pH 7.5, 0.1 M NaCl, 1 mM MgCl2, 1.0 mM ethylenediaminetetraacetic acid (EDTA) and 1× yeast protease inhibitor cocktail (Sigma, St. Louis, MO, USA)]. Cell extracts were mixed with 36 μL of 4× SDS loading buffer and heated at 70°C for 10 min. Five microliters of each sample (equal to 0.037 OD_{600} units of cells) was loaded onto a 10% SDS–PAGE. Proteins were transferred to nitrocellulose membranes and subjected to immunoblotting.

Fluorescence microscopy

Observation of vacuole inheritance and morphology with FM4-64 (N-(3-triethylammoniumpropyl-)-4-(6(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide) (Molecular Probes, Inc. Eugene, OR, USA) was performed as described (11). Images were obtained using an Axioscope 2 (Carl Zeiss MicroImaging, Jena, Germany) or the DeltaVision RT Restoration Microscopy System (Applied Precision, Issaquah, WA, USA).

Localization of Vac8p in yeast cells by indirect immunofluorescence microscopy was performed as described (6). Briefly, for cells with Vac8p levels similar to wild type, spheroplasts were labeled with rabbit anti-Vac8p (1:100) and Alexa-488-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:200). For cells with low levels of Vac8p, spheroplasts were labeled with rabbit anti-Vac8p (1:100), goat anti-rabbit IgG (1:240), and Alexa-488-conjugated donkey anti-goat IgG (1:200). Vacuole membranes were visualized with a mouse monoclonal anti-vacuolar adenosine triphosphatase (60 kDa subunit) antibody (1:100; Molecular Probes) and rhodamine-red-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA, USA). Images were obtained with a laser confocal system (MRC-1024; Bio-Rad, Hercules, CA, USA).

All images were processed by MetaMorph Imaging 4.5 Software (Universal Imaging, Buckinghamshire, UK) or Adobe Photoshop (Adobe, San Jose, CA, USA).

Comparison of vacuole-associated Vac8p in the vac8-C4G/C5T *and* P_{VAC17}-VAC8 *mutants*

Vacuoles were isolated (13) from *vac8Δ* cells containing pRS416 (vector), p*VAC8*, p*vac8-C4G/C5T* or pP_{VAC17}-*VAC8*. The total protein concentration of the vacuole fraction was measured with a protein assay kit (Bio-Rad) using BSA as a standard. Five micrograms of total proteins from each membrane fraction was analyzed by immunoblotting for Vac8p and Pho8p.

Biochemical isolation of lipid rafts

Lipid rafts from yeast cells were isolated as described (41). About 20 OD_{600} units of mid-log phase wild-type cells (LWY7235) were collected and washed once with water. The cell pellet was lysed in 1 mL of ice-cold TNE (Tris-NaCl-EDTA) buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA and 1× Sigma yeast protease inhibitor cocktail) by vortexing with 50-mm glass beads for 5 min at 4°C. The lysate was cleared of unbroken cells and cell debris by centrifugation at 500 × ***g*** for 5 min. Three hundred

microliters of the cleared lysate was mixed with 30 μL of 10% (w/v) Triton-X-100 (Fisher, Hanover, IL, USA), 5% (w/v) Brij 98 (Sigma), 5% (w/v) Lubrol WX (Serva, Heidelberg, Germany), 10% (w/v) Tween-20 (Fisher) or water. The reactions were incubated at 4°C for 30 min. Two hundred and fifty microliters of the treated lysate was adjusted to 40% (w/v) Optiprep by addition of 500 μL of 60% Optiprep (Accurate, Westbury, NY, USA). About 1.2 mL of 30% and 200 μL of 0% Optiprep were layered on top. The gradients were subjected to centrifugation (2.5 h at 160 000 x ***g*** in a TLS-55 rotor) at 4°C. Ten fractions (200 μL each) were collected from the top. Five microliters of each fraction was subjected to immunoblot analysis for detection of Pma1p, Vac8p and Pgk1p.

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