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ATP-independent Control of Vac8 Palmitoylation by a SNARE Subcomplex on Yeast Vacuoles*

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Yeast vacuole fusion requires palmitoylated Vac8. We previously showed that Vac8 acylation occurs early in the fusion reaction, is blocked by antibodies against Sec18 (yeast *N*-ethylmaleimide-sensitive fusion protein (NSF)), and is mediated by the R-SNARE Ykt6. Here we analyzed the regulation of this reaction on purified vacuoles. We show that Vac8 acylation is restricted to a narrow time window, is independent of ATP hydrolysis by Sec18, and is stimulated by the ion chelator EDTA. Analysis of vacuole protein complexes indicated that Ykt6 is part of a complex distinct from the second R-SNARE, Nyv1. We speculate that during vacuole fusion, Nyv1 is the classical R-SNARE, whereas the Ykt6-containing complex has a novel function in Vac8 palmitoylation.

The post-translational modification of a protein can change its conformation, subcellular localization, and function. Thus, such highly consequent alterations must be tightly controlled in time and space. Protein palmitoylation, or thiol (*S*)-acylation, is a reversible lipid modification, defined as the transfer of an activated fatty acid, such as palmitoyl coenzyme A (Pal-CoA),[1] to a cysteine residue via a thioester linkage (reviewed in Refs. 1 and 2). In this way, a cytosolic protein can be stably anchored to membranes, an association that is made reversible by thioesterases (2, 3).

Protein palmitoylation is involved in various viral and intracellular fusion reactions, including synaptic vesicle fusion and the homotypic fusion of yeast vacuoles (3–5). The latter requires palmitoylation of the vacuolar protein Vac8 (6–8). Vac8 consists of three domains: (i) a myristoylated N-terminal Src homology 4 domain with three cysteines that can be palmitoylated, (ii) a long armadillo repeat, and (iii) a C-terminal asparagine-rich stretch. Besides fusion, Vac8 is required for vacuole inheritance, the maintenance of nucleus-vacuole junctions, and the cytosol-to-vacuole transport of the aminopeptidase I, Ape1 (5, 6, 9–13).

Recently, we found that the R-SNARE (arginine-soluble NSF attachment protein (SNAP) receptor) Ykt6 mediates Vac8 acylation (14). Ykt6 is a ubiquitous SNARE found on multiple membranes of the secretory and endocytic pathways (15–20). Purified Ykt6 is sufficient to promote the transfer of Pal-CoA to Vac8 (14). On yeast vacuoles, Ykt6 was found in association with the glutamine (Q)-SNAREs Vam3, Vam7, and Vti1, the R-SNARE Nyv1, and the AAA-ATPase Sec18 and its co-factor Sec17 (yeast NSF/α-SNAP), indicating that that all these proteins form one complex, the *cis*-SNARE complex (16), which is the putative result of a previous round of fusion (21). Vac8 is also found in this complex (8), proximal to Ykt6 (14).

Fusion of isolated vacuoles is initiated when Sec18/17 disassemble the *cis*-SNARE complex in an ATP hydrolysis-dependent manner (22, 23). This priming reaction allows the vacuolar SNAREs to interact in *trans* (docking), which leads to lipid mixing. We previously observed that priming and Vac8 palmitoylation occur simultaneously. Since both reactions were also sensitive to antibodies against Sec18, we speculated that Vac8 acylation might be dependent on the priming reaction (8). However, the situation turned out to be more complicated, since antibodies to Sec17, which also inhibit priming, do not affect Vac8 acylation (14). We therefore proposed that, at the onset of *in vitro* vacuole fusion, two Sec18-dependent reactions are triggered: (*a*) together with Sec17, Sec18 mediates priming; and (*b*) in a reaction independent (or upstream) of Sec17, Sec18 regulates Vac8 acylation (14). The fact that all proteins involved in these two reactions are part of, or associated to, the *cis*-SNARE complex suggested to us that Vac8 acylation is a regulated event dependent on specific components of a SNARE subcomplex. With this study we have characterized the regulation of Vac8 palmitoylation during *in vitro* vacuole fusion and the composition of vacuolar SNARE complexes in more detail.

EXPERIMENTAL PROCEDURES

Biochemical Reagents and Antibodies—[9,10-^{3}H]Palmitic acid (50 Ci/mmol) was obtained from Hartmann Analytic (Braunschweig, Germany). [^{3}H]Pal-CoA was synthesized with [^{3}H]palmitate and acyl-CoA synthetase as described (24). All other biochemical reagents were purchased from Sigma or Roth (Karlsruhe, Germany), unless indicated. All reagents added to vacuoles were prepared in, or dialyzed into, PS buffer (10 mM PIPES/KOH, pH 6.8, 200 mM sorbitol) unless indicated other-

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[1] The abbreviations used are: Pal-CoA, palmitoyl coenzyme A; GFP, green fluorescent protein; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-*O*-(thiotriphosphate); HA, hemagglutinin epitope; MCLR, microcystin-LR; NSF, *N*-ethylmaleimide-sensitive fusion protein; PIPES, 1,4-piperazinediethanesulfonic acid; SNAP, soluble NSF attachment protein; SNARE, soluble NSF attachment protein receptor; Q-SNARE, glutamine-SNARE; R-SNARE, arginine-SNARE.

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TABLE I
S. cerevisiae strains used in this study

Strain	Genotype	Reference
CUY001	BJ3505: *MATa pep4Δ::HIS3 prb1-Δ1.6R HIS3 lys 2–208 trp1-Δ101 ura3–52 gal2 can*	31
CUY002	DKY6281: *MATa leu2–3 leu2–112 ura3–52 his3-delta200 trp 1-Δ101 lys 2–801 suc 2-Δ9 pho8Δ::TRP1*	31
CUY009	CUY001; *nyv1Δ::HIS3*	25
CUY010	CUY001; *vam3Δ::TRP1*	25
CUY011	CUY002; *nyv1Δ::HIS3*	25
CUY012	CUY002; *vam3Δ::TRP1*	25
CUY024	CUY001; *vam7Δ::TRP1*	36
CUY297	CUY001; *NYV1::6xHA-TRP1*	This study
CUY333	CUY010; *pRS406-NOP1pr-GST-VAM3*	This study
CUY334	CUY012; *pRS406-NOP1pr-GST-VAM3*	This study
CUY1250	CUY002; *ykt6Δ; pRS423-Ykt6pr-GFP$_{int}$Ykt6*	This study

wise. Antibodies used were anti-hemagglutinin epitope (HA) monoclonal antibody (BabCO) and rabbit polyclonal antibodies against Vam3, Nyv1, Vti1, Vam7, Ykt6, Sec17 (14, 25), and His-tagged GFP (this study). Unless indicated, Nyv1-HA was detected with anti-HA antibody. Antibodies to GFP were raised in New Zealand White rabbits and affinity-purified as described (14) using CNBr-Sepharose (Amersham Biosciences) immobilized pure protein. Preparations of IgGs and affinity-purified antibodies were as described (26).

Yeast Strains and Molecular Biology—Saccharomyces cerevisiae strains are listed in Table I. Yeast were cultured in yeast extract-peptone-glucose. Nyv1 was C-terminally tagged with 6xHA by transformation of a PCR fragment containing the tag and a TRP1 marker into BJ3505 (CUY001) (27). Construction of the yeast strain CUY1250 containing internally GFP-tagged Ykt6 was as follows. DKY6281 (CUY002) was transformed with *pRS416-YKT6pr-YKT6^{WT}* (47), and endogenous *YKT6* was deleted by replacement of the open reading frame with the *KANMX4* selection marker (28); the resulting strain was unable to grow on synthetic medium containing 5′-fluoroorotic acid, confirming the *ykt6* deletion. This strain was then transformed with the plasmid *pRS423-YKT6pr-GFP$_{int}$YKT6* (47), and loss of *pRS416-YKT6pr-YKT6^{WT}* was induced by growth on 5′-fluoroorotic acid. For N-terminal tagging of Vam3 with glutathione *S*-transferase (GST), the genomic *VAM3* open reading frame without start codon was PCR-amplified and inserted into the GST-containing vector pGEX4T-3 (Amersham Biosciences). The new *GST-VAM3* open reading frame was then amplified by PCR and inserted into the yeast integrative vector pRS406 (29) containing the *NOP1* promoter. The plasmid pRS406-NOP1pr-GST-VAM3 was linearized in the *URA3* marker and transformed into BJ3505 *vam3Δ* (CUY010) and DKY6281 *vam3Δ* (CUY012) (25). Subsequent sequencing revealed that the Vam3 sequence has the point mutation K96E, affecting a non-conserved residue in helix C of the N-terminal extension. This does not affect the functionality of the protein, as detailed under "Results."

Recombinant Proteins—Purifications of Vac8-GST, His$_6$-Sec18 E350Q, and His$_6$-Sec18 from *Escherichia coli* were as described (8, 30). His-tagged GFP, used for antibody production and affinity purification, was expressed from the plasmid *pET15b-eGFP* (kindly provided by W. Nickel, Biochemie-Zentrum der Universität Heidelberg) and purified from *E. coli*.

In Vitro Palmitoylation—BJ3505 vacuoles were incubated under fusion conditions at 26 °C. Incubation times and time of [^{3}H]Pal-CoA or [^{3}H]palmitate addition varied between experiments, as indicated in the figure legends (Fig. 1 and 2). Where indicated Vac8-GST (15 μg) was added. Vacuoles were pelleted (10 min, 12,000 × *g*), resuspended in PK buffer (20 mM PIPES/KOH, pH 6.8, 120 mM KCl) containing 0.5× protease inhibitor mixture (1× protease inhibitor mixture = 7.5 μM Pefabloc SC, 7.5 ng/ml leupeptin, 3.75 μM *o*-phenanthroline, and 37.5 ng/ml pepstatin (31)), and pelleted again. Vacuoles were resuspended in SDS sample buffer without 2-mercaptoethanol and analyzed by SDS-PAGE and fluorography.

For extract preparation, membranes from a 200-μl reaction (as described above but without the addition of radioactive palmitate) were solubilized in 100 μl of Triton buffer (0.25% Triton X-100, 120 mM KCl, 20 mM PIPES/KOH pH 6.8, 1× protease inhibitor mixture) and incubated for 1 h at 4 °C with agitation. Insoluble material was removed by centrifugation (15 min, 20,000 × *g*, 4 °C), and the supernatant (10 μl, about 5 μg of vacuoles) was incubated with [^{3}H]Pal-CoA in a total volume of 100 μl (adjusted with PK buffer) at 26 °C. Proteins were precipitated with trichloroacetic acid (13% v/v) and acetone and analyzed by SDS-PAGE and fluorography.

Vacuole Isolation and in Vitro Fusion—Vacuoles were isolated from the yeast strains BJ3505 (*pep4Δ prb1Δ*) and DKY6281 (*pho8Δ*) by DEAE-dextran lysis and Ficoll gradient centrifugation. Fusion between these vacuoles was measured by a biochemical complementation assay as described (31). Briefly, 2–3 μg each of *pep4Δ* and *pho8Δ* vacuoles were incubated together at 26 °C in 30 μl of PS buffer containing protease inhibitor mixture (0.02×/30 μl), reaction salts (0.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 125 mM KCl), and CoA (10 μM) in the presence or absence of an ATP-regenerating system (0.5 mM ATP, 40 mM creatine phosphate, 0.1 mg/ml creatine kinase). A colorimetric substrate, *p*-nitrophenyl phosphate, for alkaline phosphatase was added to detergent-solubilized reactions, and the absorbance at 400 nm was measured. One unit of fusion activity is defined as the production of 1 μmol of *p*-nitrophenol/min/μg *pep4Δ* vacuoles.

Where indicated, reactions also contained cytosol (0.5 mg/ml (30)), His$_6$-Sec18 (200 ng/ml (30)), microcystin-LR (MCLR; 10 μM; Alexis Biochemicals), or the recombinant Rab GDP-dissociation inhibitor, Gdi1 (10 μM (31)). Large scale fusion reactions contained the same proportions of all reagents as above.

Affinity Purification—All experiments were with protease-deficient vacuoles (BJ3505 background) unless written otherwise. After incubation in a large scale fusion reaction (where indicated in the figures), vacuoles were diluted to 1.5 ml with PSK buffer (10 mM PIPES/KOH, pH 6.8, 200 mM sorbitol, 150 mM KCl), pelleted (5 min, 12,000 × *g*, 4 °C), washed once with 500 μl of PSK buffer, and pelleted again. Membranes were detergent-solubilized in 1 ml of lysis buffer composed of 0.5% Triton X-100, 20 mM HEPES/KOH, pH 7.4, and 150 mM KCl (see below for exceptions). The following protease inhibitors were used: 1× protease inhibitor mixture, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml $α_2$-macroglobulin (Roche Applied Science). Lysis was for 10 min on a nutator at 4 °C. A fraction of the cleared extracts (10 min, 20,000 × *g*, 4 °C) was removed and trichloroacetic acid/acetone-precipitated. The remaining detergent extract was incubated with the appropriate affinity matrix overnight on a nutator at 4 °C, and beads were washed in lysis buffer containing decreasing amounts of detergent (0.5%, 0.1%, 0.025% Triton X-100) before elution.

For Fig. 3*B*, vacuoles were solubilized in lysis buffer containing 0.5% Triton X-100, 20 mM PIPES/KOH, pH 6.8, and 150 mM KCl; in Fig. 3*C*, solubilization buffer was 0.1% Triton X-100, 20 mM HEPES/KOH pH 7.4, and 150 mM NaCl. Anti-GFP, anti-Sec17 or anti-Vti1 antibodies (0.1–1 μl) were cross-linked to protein A-Sepharose (Amersham Biosciences), and proteins were eluted with 0.1 mM glycine, pH 2.5, and 0.025% Triton X-100 and trichloroacetic acid/acetone-precipitated.

Vacuolar *trans*-SNARE complexes were analyzed (Fig. 4*D*) by immunoprecipitation of Nyv1-HA with anti-HA antibodies (0.1–1 μl) bound to protein G-Sepharose (10 μl; Amersham Biosciences); elution was by boiling in SDS sample buffer containing 2-mercaptoethanol. For tandem isolation of *trans*-SNARE complexes (Fig. 4*E*), fusion reactions were diluted into 5 ml of PSK buffer, and vacuoles were collected, resuspended in 1 ml of PSK buffer, and isolated again prior to detergent lysis. Vacuole extracts were incubated for 2 h with glutathione-Sepharose 4B (15–40 μl; Amersham Biosciences), and bound proteins were eluted with 1.5 ml of elution buffer (50 mM HEPES/KOH, pH 7.4, 150 mM KCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 mM reduced GSH). Eluates were then incubated with protein G-Sepharose/anti-HA antibody overnight at 4 °C. Beads were washed twice for 10 min with lysis buffer containing 0.5% and then 0.1% Triton X-100 and then for 10 min with lysis buffer containing 300 mM KCl and 0.025% Triton X-100. Proteins were eluted by boiling in reducing sample buffer.

For size analysis of SNARE complexes (Fig. 4*F*), vacuole detergent extracts were centrifuged on continuous glycerol density gradients (10–30%, 18 h, 250,000 × *g*, 4 °C, SW40 rotor). 1-ml fractions were collected, incubated with GSH-Sepharose overnight on a nutator at 4 °C, and

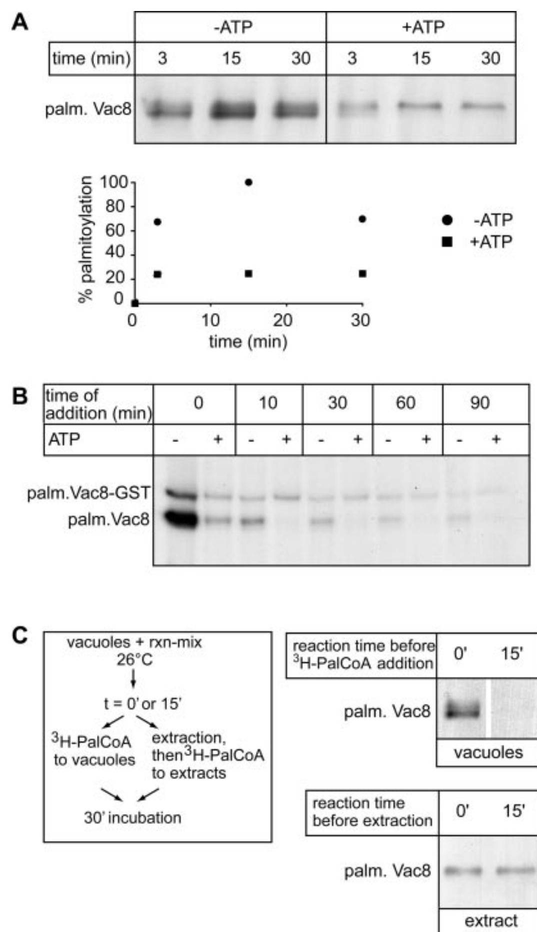

FIG. 1. **Restriction of palmitoylation activity.** *A*, time course of palmitoylation. Isolated vacuoles (see "Experimental Procedures") were incubated at 26 °C in fusion reaction buffer in the presence of [^{3}H]Pal-CoA for the indicated times. Reactions were stopped by shift to ice, and vacuoles were re-isolated and resuspended in SDS sample buffer without 2-mercaptoethanol. Palmitoylated Vac8 (*palm. Vac8*) was identified by SDS-PAGE and fluorography. *B*, addition of Vac8-GST and [^{3}H]Pal-CoA to the ongoing reaction. Vacuoles were incubated at 26 °C in the absence or presence of an ATP in fusion reaction buffer. At the indicated time points, purified Vac8-GST and [^{3}H]Pal-CoA were added, samples were incubated for 30 min, and then the reaction was placed on ice. Vacuoles were processed as described in *A*, to detect palmitoylated Vac8. *C*, comparison of palmitoylation on vacuoles and after extraction, as schematized in the *left panel*. Vacuoles were incubated at 26 °C in fusion reaction buffer without ATP. At the indicated time points, [^{3}H]Pal-CoA was added, and samples were incubated for another 30 min and then processed as described in *A* (*top right panel*). A parallel vacuole incubation was extracted prior to incubation with [^{3}H]Pal-CoA as described under "Experimental Procedures" (*bottom right panel*).

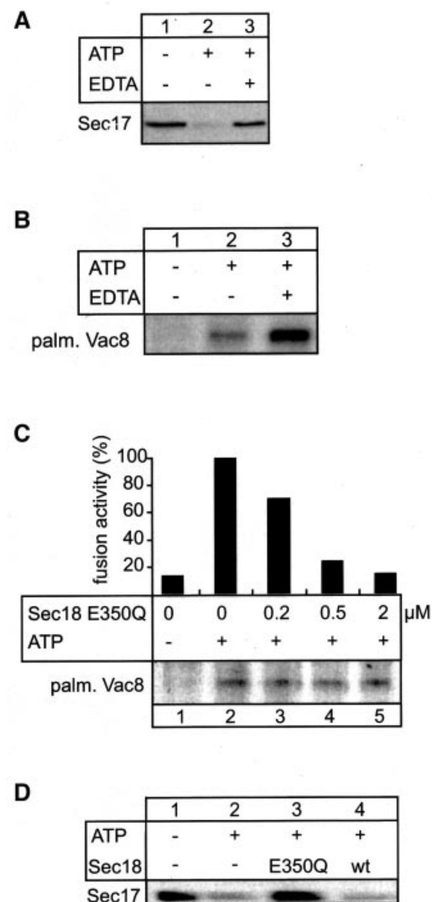

FIG. 2. **Different functions of Sec18 in palmitoylation and priming.** *A*, EDTA blocks ATP-dependent release of Sec17 from vacuoles. BJ3505 vacuoles were incubated for 10 min at 26 °C in a 150-μl reaction containing cytosol, CoA, and His$_6$-Sec18 in the presence or absence of ATP-regenerating system and EDTA (1 mM). Vacuoles were re-isolated, washed twice with 500 μl of PSK buffer, and analyzed by immunoblotting with anti-Sec17 antibodies. *B*, EDTA enhances Vac8 palmitoylation. The experiment was identical to that described in *A*, except that it was twice as large and contained [^{3}H]palmitate (150 μCi), and if present, ATP was without the regenerating system. Palmitoylated Vac8 was identified by non-reducing SDS-PAGE and fluorography. *C*, ATPase-deficient Sec18 E350Q blocks fusion but not palmitoylation. The indicated concentrations of Sec18 E350Q were added to fusion reactions, and fusion was determined after 90 min (see "Experimental Procedures"). The corresponding concentrations (as in *B*) of Sec18 E350Q were included in palmitoylation reactions. Quantification of the relative incorporation of radioactivity by laser densitometry shows no significant difference in *lanes 2–5*. *D*, Sec18 E350Q inhibits ATP-dependent Sec17 release. Reactions were performed as described in *A*. Where indicated, 2 μM Sec18 E350Q or wild type was added to the reaction.

washed and eluted by boiling in SDS sample buffer. Proteins were analyzed by 12% SDS-PAGE and Western blotting (see figure legends for methods not discussed under "Experimental Procedures").

RESULTS

Vac8 Palmitoylation on Isolated Vacuoles Does Not Require Exogenous ATP and Is Restricted to a Narrow Time Window during Vacuole Fusion—On isolated yeast vacuoles, Vac8 becomes palmitoylated in a reaction parallel to the Sec18/17- and ATP-dependent priming reaction (14). Vac8 palmitoylation is sensitive to antibodies against Sec18 but is insensitive to Sec17 antibodies (14). Since Sec17 is required for Sec18 to hydrolyze ATP (32), we asked whether Vac8 acylation needs ATP. In previous experiments, we used [^{3}H]palmitate for Vac8 labeling and thus had to add ATP for its activation to [^{3}H]Pal-CoA (8). Here, we directly added [^{3}H]Pal-CoA to vacuoles and analyzed the kinetics and ATP dependence of Vac8 labeling by SDS-PAGE and fluorography. Surprisingly, Vac8 acylation was more efficient in the absence of ATP (Fig. 1*A*), even though ATP-dependent priming and fusion were inhibited (Fig. 2*A*, *C*). This shows that ATP addition is not necessary for Vac8 acylation and, together with the Sec17 antibody data, indicates that palmitoylation is independent of the Sec18 ATPase activity. In addition, our results show that the palmitoylation reaction is regulated, as it occurs within a narrow time frame; with or without ATP, maximal palmitoylation was achieved early in the reaction (Fig. 1*A*) and was restricted to this interval (Fig. 1*B*). The temporal restriction of palmitoylation could be due to either a decreasing availability of substrate (Vac8, Pal-CoA) or inactivation of the palmitoylating activity. To distinguish between these possibilities, we incubated vacuoles with or without ATP and,

at defined time points, added recombinant Vac8-GST and [^{3}H]Pal-CoA for an additional incubation of 10 min. When Vac8-GST and [^{3}H]Pal-CoA were added after 10 min, the labeling of both endogenous and exogenous Vac8 was significantly decreased (Fig. 1*B*). This indicates that the palmitoylating activity becomes down-regulated during the incubation. We previously showed that this activity can be recovered from detergent-solubilized vacuoles (14, 24). In contrast to our observations using intact vacuoles (Fig. 1*A*, *B*), these extracts do not lose their ability to mediate Vac8 acylation over time (24). We therefore asked whether we could use detergent extraction to rescue the ability of Vac8 to be palmitoylated at a later time point. For this, we incubated vacuoles for 0 and 15 min at 26 °C and then either labeled them directly with [^{3}H]Pal-CoA (Fig. 1*C*, *top right panel*) or first solubilized the vacuoles with detergent and then added [^{3}H]Pal-CoA (Fig. 1*C*, *bottom right panel*). As expected, intact vacuoles lost their ability to acylate Vac8 after 15 min. In contrast, Vac8 was efficiently labeled from extracts prepared at either time point. The fact that temporal control of Vac8 acylation is removed by extraction indicated to us that this activity is regulated by structural rearrangements on the vacuole. The task was then to find out more about the nature of this vacuole-specific control mechanism. We previously demonstrated that antibodies to Sec18 inhibit Vac8 acylation on isolated vacuoles (8), whereas Sec18 is not involved in acylation after detergent extraction (14). This suggests Sec18 as a potential regulator of spatiotemporally controlled Vac8 acylation. Furthermore, Sec18, Vac8, and the SNARE Ykt6, which we identified as responsible for Vac8 acylation, are found in association with the *cis*-SNARE complex (24), suggesting that a better understanding of this complex might lend insight into the control of Vac8 acylation. Therefore, we examined the possible involvement of Sec18 and the *cis*-SNARE complex in regulating palmitoylation.

ATP Hydrolysis by Sec18 Is Not Required for Vac8 Acylation—The priming reaction of *in vitro* vacuole fusion (*i.e. cis*-SNARE disassembly and Sec17 release) (22, 23) requires Sec17 and added ATP (22). However, Sec18-dependent acylation of Vac8 is insensitive to anti-Sec17 antibodies (14) and does not require the addition of ATP (Fig. 1, *A* and *B*). To directly test whether the Sec18 function in Vac8 acylation is independent of ATP hydrolysis, we used two established methods to inhibit the ATPase activity of Sec18. First, we incubated vacuoles in the presence of the divalent cation chelator EDTA, which blocks the ability of Sec18 to disassemble SNARE complexes and the corresponding release of Sec17 from the vacuole (Fig. 2*A*) (22). Strikingly, EDTA did not inhibit Vac8 palmitoylation but, rather, stimulated it (Fig. 2*B*). Stimulation by EDTA might be due to chelation of Zn^{2+} ions that are known to interact with sulfhydryl groups (33), although additional experiments are necessary to clarify this point. Importantly, this observation suggested that the palmitoylation activity does not require, or is even more robust without, ATP hydrolysis by Sec18. We therefore hypothesized that a Sec18 mutant homologous to the *Drosophila melanogaster* comatose mutant (Sec18 E350Q (34, 35)), which cannot hydrolyze ATP, would interfere with vacuole fusion but not palmitoylation. Vacuole fusion is assayed by a biochemical complementation assay that measures content mixing (see "Experimental Procedures"). When we titrated purified Sec18 E350Q into the assay, fusion was completely prevented, and indeed, palmitoylation of Vac8 was unaffected (Fig. 2*C*). Inhibition of fusion was due to a block in priming, because Sec18 E350Q inhibited Sec17 release from the vacuole (Fig. 2*D*). The presence of Sec18 E350Q, in contrast to EDTA, did not stimulate palmitoylation.

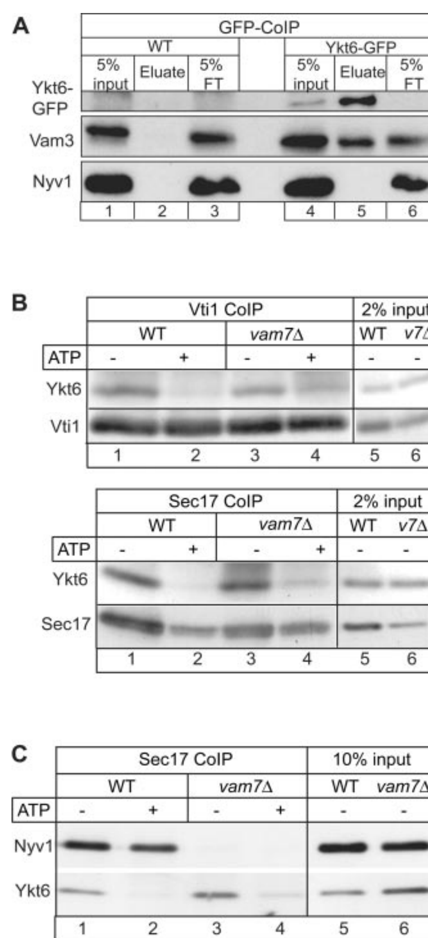

FIG. 3. **Analysis of Ykt6 and Nyv1 complexes.** *A*, analysis of the Ykt6 SNARE complex. Vacuoles from a strain containing only endogenous untagged Ykt6 (*WT*, wild type; CUY002) and a strain carrying internally GFP-tagged Ykt6 (CYU1250) as the sole Ykt6 copy (120 μg each) were processed for anti-GFP co-immunoprecipitation (*CoIP*) as described under "Experimental Procedures." Equal amounts of vacuolar extract (*input*) and flow-through (*FT*) (5% each) are shown. Western blots were decorated with antibodies to Vam3, Nyv1, and Ykt6. *B*, analysis of Vti1 and Sec17 complexes. Wild-type and *vam7*Δ vacuoles (60 μg each) were incubated in fusion reactions with or without ATP for 10 min at 26 °C, re-isolated, and processed as described in *A*. Half of the lysate was analyzed by anti-Vti1 co-immunoprecipitation and the other half with anti-Sec17 co-immunoprecipitation. An aliquot (2% of total) was removed prior to loading onto beads. Western blots were decorated with antibodies against Ykt6 and Vti1 or Sec17 as indicated. *C*, because the amounts of Nyv1 and Sec17 are reduced on *vam7*Δ vacuoles, the anti-Sec17 co-immunoprecipitation in *B* was repeated with normalized protein amounts; 10% of the lysate was removed prior to loading on beads, and Western blots were decorated with antibodies against Nyv1 and Ykt6.

Positioning of the Ykt6 Protein in a Novel Complex on Vacuoles—We previously showed that Ykt6 is required for palmitoylation of Vac8 (14). This is supported by the proximity of Vac8 and Ykt6 on vacuoles and a function of the Ykt6 *longin* domain early in the fusion reaction (14). Ykt6 was found to be associated with the vacuole *cis*-SNARE complex (16); however, this complex does not seem to be homogeneous. We analyzed *cis*-SNARE complexes on isolated vacuoles and found that although GFP-tagged Ykt6 interacts with the vacuolar Q-SNARE Vam3, it is not detectable with the second vacuolar R-SNARE, Nyv1 (Fig. 3*A*). Moreover, in mutants lacking the Q-SNARE Vam7, Nyv1 is missing from the *cis*-SNARE complex, although it still contains the Q-SNAREs Vam3 and Vti1 (36). This is not merely a Q-SNARE complex, however, as it also contains the R-SNARE Ykt6 (Fig. 3, *B* and *C*). These data demonstrate that the *cis*-SNARE complex is more heterogene-

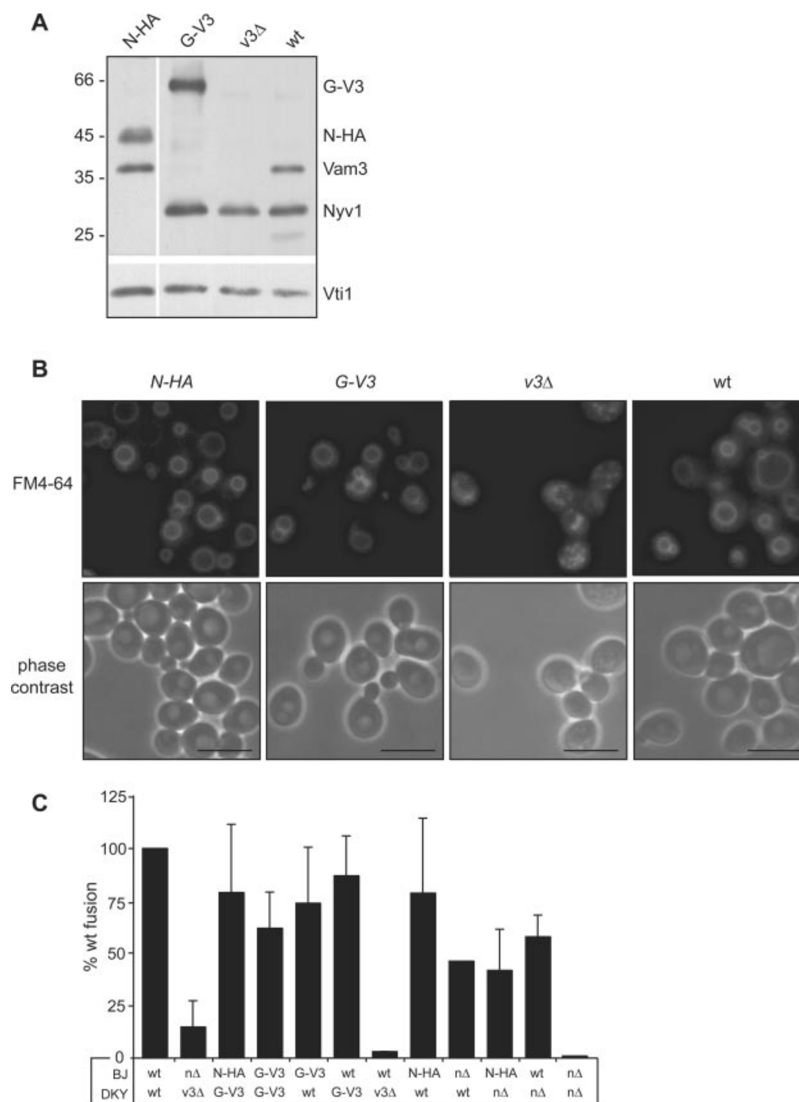

Fig. 4. **Analysis of the Nyv1-containing *trans*-SNARE complexes.** *A*, vacuole localization of Nyv1-HA (*N-HA*) and GST-Vam3 (*G-V3*). Vacuoles (6 μg) from the respective strains were lysed in SDS sample buffer, and SNAREs were identified by Western blotting with antibodies to Nyv1, Vam3, and Vti1; *v3Δ*, *vam3Δ*; *wt*, wild type (parent strain). *B*, *in vivo* analysis. Vacuoles in the indicated BJ3505 strains (see Table I) were analyzed by pulse chase with FM4-64 (46) and fluorescence or phase contrast microscopy using a Zeiss Axiovert 35 fluorescence microscope. *C*, functionality of tagged SNAREs in vacuole fusion. Vacuoles from the indicated strains were fused in standard fusion reactions (see "Experimental Procedures") for 60–90 min with ATP, with the addition of cytosol or recombinant Sec18p (ratio of tag to wild-type fusion was invariant). In each experiment, fusion from a reaction without ATP was subtracted and fusion was compared with wild type (set to 100%; average wild-type units = 2.6). The graph represents values averaged from multiple experiments. *Error bars* are standard deviations among several independent experiments; *n*Δ = *nyv1*Δ. *D*, specificity of *trans*-complex formation confirmed by inhibitors. Vacuoles were incubated in the presence of cytosol with either no ATP, ATP alone, or ATP with the late-stage fusion inhibitor MCLR or the tethering inhibitor Gdi1, as described under "Experimental Procedures." The graph shows *in vitro* fusion between BJ Nyv1-HA/DKY GST-Vam3 vacuoles (3 μg each). Control reactions with ATP/no inhibitor were set to 100%. *Error bars* are standard deviations among n experiments: −ATP = 10; +ATP (no inhibitor control) = 10; MCLR (10 μM) = 7; recombinant Gdi1 (9 μM) = 2. In the Western, large scale fusion reactions containing 40 μg each of Nyv1-HA and GST-Vam3 (both BJ3505) and the reaction components described above were incubated for 30 min at 26 °C. Vacuoles were re-isolated and detergent-solubilized, and 2% of them were removed and trichloroacetic acid-precipitated (the *+ATP* sample is shown), and protein complexes were analyzed by anti-HA co-immunoprecipitation (*co-IP*) (see "Experimental Procedures" for details). *Lanes* are from a single representative gel but are arranged in the figure as indicated by *white spaces*. Blots were decorated with anti-Vam3 followed by anti-HA antibodies. *E*, affinity isolation of the *trans*-SNARE complex. A schematic diagram of the assay is shown. Fusion reactions (2.25 ml) containing 225 μg each of GST-Vam3 and Nyv1-HA vacuoles were incubated with 10 μM MCLR −/+ ATP for 30 min. Lysis of vacuoles and sequential isolation of complexes by GSH pull-down, and anti-HA immunoprecipitation was as described under "Experimental Procedures." Proteins were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. *F*, sizing of wild-type *cis*- and *trans*-SNARE complexes. Vacuoles were treated as described in *E* through the solubilization step, and extracts were fractionated by ultracentrifugation on 10–30% continuous glycerol density gradients (18 h, 250,000 × *g*, 4 °C, SW40 rotor; 1 = top of gradient) and then subjected to GSH pull-downs (see "Experimental Procedures"). Complexes were analyzed by Western blotting with the indicated antibodies. Sizes were determined by a parallel marker-mixture gradient (Amersham Biosciences). The sizes (in kDa) of marker proteins and their corresponding gradient fractions are indicated.

ous than previously appreciated. Since priming is not required for Ykt6-mediated palmitoylation of Vac8 (Figs. 1 and 2) and the *cis*-SNARE complex appears to be heterogeneous, we hypothesized that the *cis*-SNARE complex present on isolated vacuoles provides R-SNAREs for two pathways: 1) Ykt6, with the help of Sec18 in a function independent of priming, mediates Vac8 palmitoylation; 2) Nyv1, following Sec18/17-dependent priming, enters into the *trans*-SNARE complexes that drive homotypic vacuole fusion.

To determine the involvement of vacuolar R-SNAREs in the transition from *cis*- to *trans*-SNARE complexes, we decided to devise a new assay to compare these two complexes (Fig. 4).

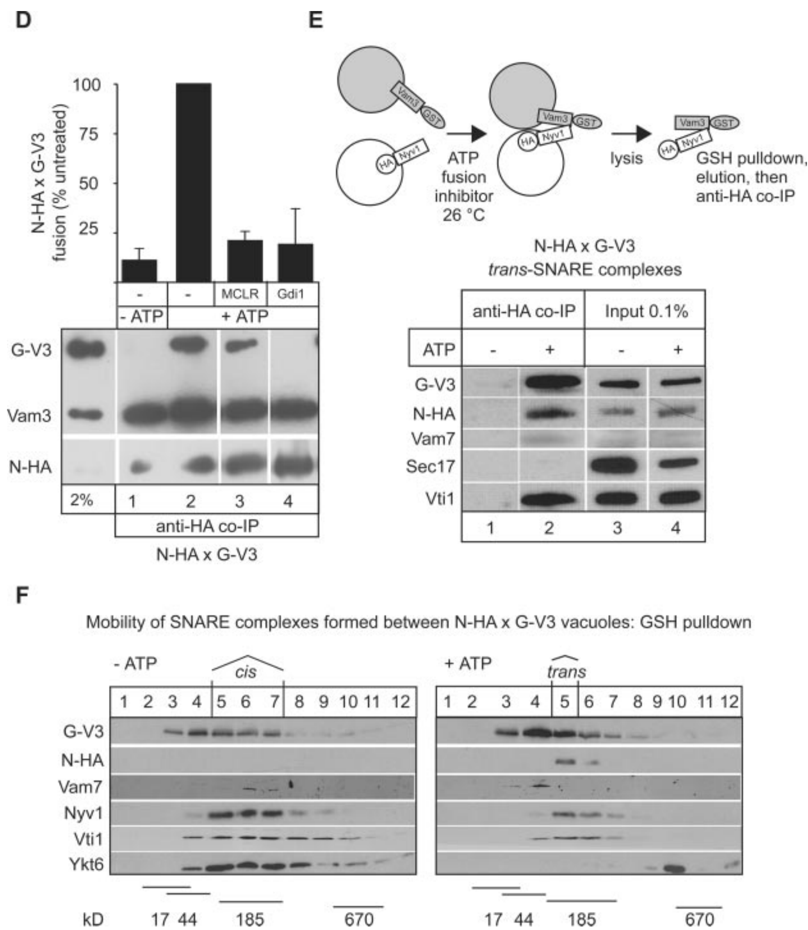

FIG. 4—*continued*

The existing assay of *trans*-SNARE pairing is based on the establishment of Vam3/Nyv1 interactions, in *trans*, between *nyv1*Δ and *vam3*Δ vacuoles (37). Vam3 is the "heavy chain" of the vacuolar Q-SNARE complex containing Vam3, Vam7, and Vti1 and is required for fusion of vacuoles (25, 37) and liposomes (38); in both assays, Nyv1 was identified as the valid R-SNARE (25, 37, 38). Because SNARE mutants were used, however, this assay precludes a comparison of *trans*-SNAREs with *cis*-SNAREs and permits analysis of only binary interactions between Vam3 and Nyv1; complete *trans*-SNARE complexes between biological membranes have been uncharacterized until now.

For our new assay, we tagged Vam3 in one strain and Nyv1 in the other, so that a mixture of vacuoles purified from these strains would form doubly tagged *trans*-SNARE complexes. Vam3 was tagged at its N terminus with GST, and Nyv1 was C-terminally 6xHA-tagged. Both proteins behave like their untagged counterparts, based on the criteria of vacuole localization (Fig. 4*A*), vacuole morphology (Fig. 4*B*), and *in vitro* vacuole fusion (Fig. 4*C*), indicating that neither tag interferes with SNARE function. Thus, our new assay uses vacuoles with wild-type characteristics.

Nyv1-HA/GST-Vam3 SNARE pairs are detected when vacuoles carrying the respective tags are incubated together under normal fusion conditions, solubilized, and subject to affinity precipitation with anti-HA antibodies or GSH beads (Fig. 4*D* and data not shown). Complexes are prevented when priming or tethering are blocked by withholding, respectively, ATP or Gdi1 inactivation of the Rab GTPase Ypt7 (39), showing that they form in line with the fusion reaction and prior to solubilization. Authentic *trans*-SNARE pairs, *i.e.* formed in *trans* prior to fusion rather than as a result of fusion, are observed when vacuoles are incubated with the late-stage fusion inhibitors MCLR (40, 41), 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) (7, 42), or GTPγS (40, 43) (Fig. 4*D* and data not shown).

Using our newly established assay, we determined the composition of *trans*-SNARE complexes and compared it with the vacuolar *cis*-complex. To detect complete *trans*-SNARE complexes rather than binary SNARE interactions, we took advantage of the reversible association of GST-Vam3 with GSH beads. SNARE complexes were enriched on GSH beads, released by the addition of reduced glutathione to maintain complex integrity, and re-isolated with HA antibodies, so that only those proteins that stably associate with *trans*-interacting GST-Vam3 and Nyv1-HA were recovered (Fig. 4*E*). Vti1 and Vam7 co-purified with the *trans*-SNARE complex, whereas Sec17 and Ykt6 are absent (Fig. 4*E* and data not shown).

In order to confirm the absence of Ykt6 from Nyv1-containing *trans*-SNARE complexes, we determined the size of complexes containing *trans*-interacting GST-Vam3 and Nyv1-HA. Vacuoles were incubated with ATP and a fusion inhibitor (as described above), to allow for *trans*-SNARE formation, and then detergent-solubilized. The extract was then centrifuged through a 10–30% glycerol gradient, and complexes between Nyv1-HA and GST-Vam3 were analyzed by GSH pull-downs of the individual fractions. As shown in Fig. 4*F*, Nyv1-HA/GST-Vam3 complexes were isolated from fractions corresponding to a size of 10 S. This suggests that Vam3, Vam7, Vti1, and Nyv1 form the *trans*-SNARE complex core, in keeping with the requirement of these four SNAREs for liposome fusion (38). Ykt6 was not found in the Nyv1-containing *trans*-SNARE complex

(Fig. 4*F*, *right*). This is consistent with our observation that vacuolar *cis*-SNARE complexes do not contain both Nyv1 and Ykt6 (Fig. 3*A*). Multimerization of *cis*-SNARE complexes, or the segregation of higher order complexes into different membrane domains, might have allowed previous co-purification of Nyv1 and Ykt6 under less stringent conditions (16).

We suggest, based on the current data, that there are different vacuolar subcomplexes containing one or the other core R-SNARE. Nyv1 appears to form classical, four-helix bundle SNARE complexes, whereas Ykt6 has a unique function with Sec18 in coordinating palmitoylation of the fusion factor Vac8.

DISCUSSION

We previously reported that Vac8 is palmitoylated during an early stage of vacuole fusion (8). This reaction is mediated by the SNARE Ykt6 (14). Vac8 association with vacuolar SNAREs and inhibition of palmitoylation by Sec18 antibodies suggested that priming might be required for palmitoylation. However, since Sec17 antibodies did not inhibit Vac8 palmitoylation, we suspected that Sec18 has a novel role in the palmitoylation reaction (14). Here, we have shown that this potential role of Sec18 is indeed independent of *cis*-SNARE disassembly, as it does not require ATP hydrolysis. EDTA and an ATP hydrolysis-defective Sec18 protein (E350Q) both blocked fusion but did not inhibit palmitoylation. One possible explanation for this is that Sec18 is not directly involved in the regulation of Vac8 palmitoylation and that the inhibition by anti-Sec18 is steric. This would be intriguing because other antibodies that recognize the *cis*-complex do not have this effect, and it might give insights into the architecture of the palmitoylation complex. Alternatively, Sec18 could be directly implicated in promoting Vac8 acylation in an ATP-independent manner. This would occur in parallel or prior to the ATP- and Sec17-dependent function of Sec18 in *cis*-SNARE complex disassembly (14). As neither Sec18-dependence (14) nor temporal regulation of the palmitoylating activity was observed for vacuolar extracts (Fig. 1), we speculate that Sec18 is implicated in rearrangements of vacuole protein complexes that link palmitoylation to the rest of the fusion reaction. Early studies on vacuole fusion revealed a salt- and temperature-dependent “stage I” that occurs independent of and prior to the ATP-dependent priming stage (44). Vac8 palmitoylation requires salt and incubation at a physiological temperature (24) but does not need ATP; perhaps, then, Vac8 palmitoylation corresponds to stage I, which may involved Sec18 in an unconventional role. This would raise the possibility that completion of stage I is the ATP- and priming-independent signal that terminates Vac8 palmitoylation on vacuoles.

How do we define the *cis*-complex, with which Ykt6, Sec18, and Vac8 are associated, that is responsible for the regulation of palmitoylation? A previous study reported that Ykt6, under mild purification conditions, is in a complex with multiple vacuolar SNAREs, including the R-SNARE Nyv1 (16). Our present analysis of SNARE complexes on wild-type and mutant membranes (Figs. 3 and 4), however, reveals a different picture. We found that SNARE complexes isolated from vacuoles contain either Nyv1 or Ykt6, consistent with the findings from studies with liposomes that Nyv1 and Ykt6 are mutually exclusive in SNARE complexes (38). We conclude that the previous stoichiometric co-isolation of Ykt6 and Nyv1 was most likely a result of the lateral association of distinct complexes and that the vacuolar *cis*-complex is more heterogeneous than thus far appreciated.

If Nyv1 forms the *trans*-SNARE complexes that drive homotypic vacuole fusion, why does vacuole fusion require an additional R-SNARE complex? We previously showed that vacuole fusion first gains resistance to Ykt6 antibodies and only later (at docking) does it become resistant to antibodies against the other vacuolar SNAREs. Ykt6 itself is a palmitoylated protein, both in humans (20) and in yeast (47). We also recently found that Ykt6 undergoes priming-dependent release from vacuoles, by a mechanism that might require its depalmitoylation. Thus, it appears that during homotypic vacuole fusion, Ykt6 does not behave as a classical SNARE. Instead, it might take advantage of its association with vacuolar SNAREs (indeed, it is required as a SNARE for traffic to the vacuole (17, 45)) to participate in other fusion subreactions such as Vac8 palmitoylation.

We are beginning to understand how palmitoylation at the vacuole is controlled. We propose the following working model for Ykt6-mediated palmitoylation of Vac8. On isolated vacuoles, Vac8 is associated with a *cis*-SNARE subcomplex that contains Ykt6. Ykt6, with the help of Sec18, transfers its palmitate to Vac8. This is a priming-independent reaction (parallel or prior to priming) that, nevertheless, is under strict spatiotemporal control. Ykt6 remains tightly associated with the vacuole because it is part of a *cis*-SNARE complex four-helix bundle. Following priming, Ykt6 dissociates from its SNARE partners and, having lost its stable lipid anchor, falls off the membrane.

In conclusion, we have found that Vac8 palmitoylation must be limited to a specific window of vacuole fusion, which is controlled by a specialized, Ykt6-containing *cis*-SNARE subcomplex. A detailed analysis of this Ykt6 complex will be an important task for future research.

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