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The Transmembrane Domain of Vam3 Affects the Composition of *cis*- and *trans*-SNARE Complexes to Promote Homotypic Vacuole Fusion*

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It is presently not clear how the function of SNARE proteins is affected by their transmembrane domains. Here, we analyzed the role of the transmembrane domain of the vacuolar SNARE Vam3 by replacing it by a lipid anchor. Vacuoles with mutant Vam3 fuse poorly and have increased amounts of *cis*-SNARE complexes, indicating that they are more stable. As a consequence efficient *cis*-SNARE complex disassembly that occurs at priming as a prerequisite of fusion requires addition of exogenous Sec18. *trans*-SNARE complexes in this mutant accumulate up to 4-fold over wild type, suggesting that the transmembrane domain of Vam3 is required to transit through this step. Finally, palmitoylation of Vac8, a reaction that also occurs early during priming is reduced by almost one-half. Since palmitoylated Vac8 is required beyond *trans*-SNARE complex formation, this may partially explain the fusion deficiency.

Membrane fusion along the secretory pathway requires the specific interaction of SNAREs[1] that are localized to vesicles and target organelles (1). Fusion reactions are controlled by Sec18/NSF and α-SNAP/yeast Sec17 that disassemble preexisting SNARE complexes or activate t-SNAREs (2, 3). Upon docking of a vesicle with its target membrane, SNAREs from opposite membranes form *trans*-SNARE complexes, which are a prerequisite for complete fusion (4). *In vitro* studies with recombinant SNAREs, reconstituted into liposomes, indicated that SNAREs can drive bilayer mixing by pairing in a cognate fashion (5, 6).

t-SNAREs, also termed syntaxins, consist of three domains: the regulatory N terminus, the coiled-coil domain required for SNARE complex formation (7–11), and the transmembrane domain (TMD), by which most SNAREs are anchored to membranes (12). Transmembrane domains of SNAREs are important for SNARE function. Alterations in synaptobrevin or syntaxin TMDs in *Caenorhabditis elegans* cause strong neurotransmission defects (13). *C. elegans* and vertebrates contain syntaxin isoforms that differ only by their TMDs (14–16). Furthermore, TMDs of syntaxin 1A and synaptobrevin II drive homo- as well as heterodimerization (17–19). Moreover, the interaction of syntaxin 1A with synaptobrevin, but not SNAP-25, depends on the TMD of the t-SNARE (20). In addition, *in vitro* fusion of liposomes requires anchoring of SNAREs via transmembrane domains (21) and tight coupling between the coiled-coil and the TMD (22). Replacing the TMDs of exocytic SNAREs in yeast arrested secretion (23). Interestingly, TMD peptides alone are sufficient to drive fusion of liposomes (24), suggesting that they constitute autonomous fusogenic domains. However, the evidence suggesting that SNAREs themselves may act as catalysts relies mostly on minimal systems. It does not exclude the possibility that SNAREs regulate other factors that could act as fusogens on cellular membranes or enhance SNARE-mediated fusion (25–27).

Yeast vacuole fusion occurs in a cascade of priming, docking, and fusion (28). Five SNAREs, the t-SNARE-like proteins Vam3, Vam7, and Vti1, and the v-SNAREs Nyv1 and Ykt6 are found in the vacuolar *cis*-SNARE complex (29). Vam3, Vti1, and Nyv1 carry TMDs, Ykt6 is anchored via a prenyl anchor, and Vam7 binds to membranes via its PX domain (28, 30, 31). During priming, Sec18 and Sec17 disassemble *cis*-SNARE complexes in an ATP-dependent manner. Docking requires the Rab GTPase Ypt7, the HOPS tethering complex, and two Rho GTPases, Rho1 and Cdc42 (32–34), as well as the Vtc complex (35) and remodeling of actin (36). This is followed by the formation of *trans*-SNARE complexes (26). The final step of yeast vacuole fusion requires protein phosphatase 1, calmodulin, the proteolipid (V0 sector) of the vacuolar ATPase (37–39), and the palmitoylated fusion factor Vac8 (40–46). Acylation of Vac8 is an essential subreaction of vacuole fusion and occurs at priming (44). Although Vac8 functions after *trans*-SNARE pairing (42), its acylation, a requirement for the function of Vac8, occurs already at priming. Due to its acylation Vac8 therefore connects priming and fusion.

Here, we analyzed the function of the TMD of the t-SNARE Vam3. For this we replaced the TMD with a prenyl anchor and determined the consequences of the TMD alteration for fusion activity and for defined SNARE complex formation at substeps of fusion. We show that the TMD is essential for *cis*-SNARE

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[1] The abbreviations used are: SNARE, soluble NSF attachment protein receptor (where NSF is *N*-ethylmaleimide-sensitive factor); SNAP, soluble NSF attachment protein; TMD, transmembrane domain; PIPES, 1,4-piperazinediethanesulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTPγS, guanosine 5′-*O*-(thiotriphosphate).

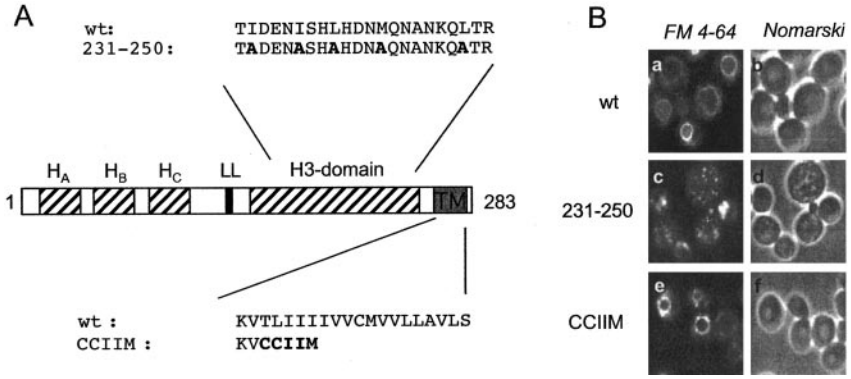

FIG. 1. **Vam3 mutants and vacuole morphology.** *A*, Vam3 mutants used in this study. The coiled-coil domains (*hatched boxes*; H_A, H_B, H_C, and H3) were predicted with the *coils* program (63). *Gray boxes* indicate the dileucine sorting motif (*LL*) and the transmembrane domain (*TM*). The H3 domain was mutagenized at five positions to alanine. Vam3-CCIIM contains the farnesylation site of yeast Ykt6 instead of the TMD. *B*, vacuole morphology. Log phase grown cells from the respective BJ strains were incubated in 50 μl of YPD with FM4–64 (30 μM, Molecular Probes, Eugene, OR) for 20 min at 30 °C (64). Cells were reisolated by centrifugation (1 min, 5000 × *g*), washed twice with 1 ml of YPD medium, and chased for 15 min at 30 °C. Cells were analyzed using a standard fluorescence microscope. *Panels a* and *b*, wild type; panels *c* and *d* (VAM3-231-50), panels *e* and *f*, VAM3-CCIIM.

complex function and for the transition from *trans*-SNARE complex formation to full fusion.

EXPERIMENTAL PROCEDURES

Reagents and Strains—Reagents were purchased from Sigma, unless stated otherwise. All *VAM3* constructs were generated using site-directed oligonucleotide mutagenesis (47) on a single-stranded template of pRS406-VAM3 (T7 Mutagene kit, Bio-Rad). All mutations were verified by dideoxynucleotide chain-termination sequencing. Plasmids carrying the *VAM3* promotor and open reading frame with the respective mutations and the *URA3* marker were digested with *Bst*BI and transformed into BJ3505*vam3*Δ and DKY6281*vam3*Δ strains. Clones that grew on uracil-deficient plates were analyzed for Vam3 production by immunoblotting using antibodies against Vam3. Strains used for the *trans*-SNARE assay were generated as follows: *NYV1* was deleted in BJ3505*vam3*Δ and DKY6281*vam3*Δ by transformation of a PCR fragment containing a KAN marker and distal regions of the open reading frame of *NYV1*. Colonies growing on YPD (1% yeast extract, 2% peptone, 2% glucose) + Geneticin were restreaked and analyzed for deletion of *NYV1* by immunoblotting. Then the pRS406-vectors carrying mutations in *VAM3* were genomically integrated into the *ura3* locus of the BJ3505*nyv1*Δ*vam3*Δ strain as described above. Similarly, pRS406-NYV1 containing the promotor and open reading frame of *NYV1* was integrated into the DKY6281-*nyv1*Δ*vam3*Δ strain to generate DKY6281*vam3*Δ.

Vacuole Fusion Assay—Vacuoles are prepared from BJ3505 and DKY6281 strains as described previously (48). Fusion is measured by a biochemical complementation assay. BJ3505 vacuoles contain pro-alkaline phosphatase due to a lack of the processing protease Pep4p. Vacuoles from DKY6281 lack alkaline phosphatase. After fusion of the membranes, Pep4p matures pro-alkaline phosphatase to the catalytically active protein, which can be assayed spectrophotometrically (48).

Standard fusion reactions were performed with 3 μg of each vacuole type in reaction buffer (5 mM $MgCl_2$, 125 mM KCl, 20 mM PIPES/KOH, pH 6.8, and 200 mM sorbitol) supplemented with an ATP regenerating system (0.5 mM ATP, 40 mM creatine phosphate, 0.1 mg/ml creatine kinase), 10 μM CoA, cytosol (0.5 μg/μl), His-Sec18 (200 ng/ml), and a protease inhibitor mixture (49) in a 30-μl volume.

Coimmunoprecipitation—After the fusion reaction, vacuoles were pelleted (20,000 × *g*, 5 min, 4 °C), washed once with 1 ml of reaction buffer containing 150 mM KCl, and reisolated as before. Vacuoles were detergent-solubilized in 1 ml of lysis buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.4 × protease inhibitor mixture, 10 μg/ml α_2-macroglobulin) and incubated for 10 min on a nutator at 4 °C. Insoluble material was removed by centrifugation (20,000 × *g*, 5 min, 4 °C). A fraction (5%) of the clarified supernatant was removed, and proteins were precipitated by the addition of trichloroacetic acid (13% v/v). The remaining detergent extract was added to protein A-Sepharose beads containing the coupled antibodies (50) and incubated on a nutator overnight at 4 °C. The beads were reisolated by brief centrifugation and washed three times in lysis buffer containing 0.1% Triton X-100. Elution of the bound proteins was by addition of 1 ml of 100 mM glycine, pH 2.5, and 0.025% Triton X-100. Proteins were precipitated with trichloroacetic acid (13% v/v), washed with 1 ml of ice-cold 100% acetone, briefly dried, and resolved in SDS sample buffer. Protein complexes were analyzed by SDS-PAGE and Western blotting.

RESULTS

Functional Characterization of Vam3 Mutants—To analyze the role of the TMD of the SNARE Vam3 in vacuole fusion, we replaced it by the isoprenylation sequence of Ykt6 (51). As a control, we inactivated the coiled-coil domain (231–250) by mutating five critical residues (Fig. 1*A*).

We first determined whether lipid anchoring or coiled-coil mutations affected vacuole morphology (Fig. 1*B*). As expected, Vam3-(231–250) cells had fragmented vacuoles, suggesting an inactivation of Vam3 function (Fig. 1*B*, *panels c* and *d*; Ref. 52). In contrast, vacuoles with lipid-anchored Vam3 were as wild-type vacuoles, suggesting functionality *in vivo*. Second, we asked whether the lack of a transmembrane domain would influence fusion efficiency. Interestingly, fusion of Vam3-CCIIM vacuoles is strongly reduced in an *in vitro* fusion assay (see "Experimental Procedures"; Ref. 48). Since Vam3 is essential for homotypic vacuole fusion (53), vacuoles deleted for Vam3 or carrying the coiled-coil mutant fused poorly (Fig. 2*A*). Vam3-CCIIM was expressed and localized to vacuoles comparable with wild-type Vam3 and was not recovered from other compartments or from the cytosol of the yeast cell (Fig. 2, *B* and *C*). Thus, sorting and membrane attachment did not depend on the presence of the TMD, but presumably on the previously defined dileucine motif within the cytoplasmic domain (54).

Analysis of cis- and trans-SNARE Complexes—We then questioned whether removal of the Vam3 TMD had affected the composition of *cis*-SNARE complexes. Both wild-type and Vam3-CCIIM vacuoles contained comparable amount of vacuolar SNAREs (not shown), indicating that sorting of other membrane proteins to vacuoles with Vam3-CCIIM was not impaired. *cis*-SNARE complexes were analyzed by immunoprecipitation with antibodies to two subunits of the vacuolar t-SNAREs Vti1 and Vam3 (55). Comparable results were obtained with either antibody, indicating that they are indeed part of the same complex (see below). Similar amounts of Nyv1 were isolated with Vam3 by coimmunoprecipiation, regardless of the vacuole type (Fig. 3*A*, *lanes 1* and *5*). However, Ykt6 and Vti1 were clearly enriched in SNARE complexes from Vam3-CCIIM vacuoles (Fig. 3*A*, *lanes 1* and *5*), suggesting that the SNARE complex becomes more stable or is altered in its composition in the absence of the Vam3-TMD. Fewer complexes were observed for the coiled-coil mutant of Vam3, as reported previously (not shown; Ref. 52).

To analyze the consequences of the altered SNARE com-

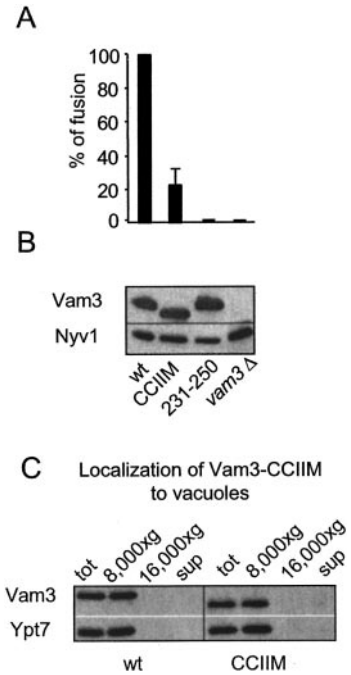

FIG. 2. **Fusion and localization of lipid anchored Vam3.** *A*, fusion of BJ and DKY strains containing the indicated mutations. Vacuoles (3 μg of each) were incubated in a 30-μl reaction with fusion buffer in the presence of ATP, cytosol, Sec18 (3 μg/ml), and CoA (10 μM). Fusion was assayed after 90 min at 26 °C. Fusion of the wild-type vacuoles was set to 100%, and fusion-independent alkaline phosphatase activity was subtracted. The results represent averages of five independent experiments (+S.D.). *B*, expression and localization of Vam3 mutants. Vacuoles (10 μg) of the indicated strains were analyzed by SDS-PAGE and Western blotting. Immunoblots were decorated with antibodies to Vam3 and Nyv1. *C*, subcellular localization of Vam3-CCIIM. Log-phase-grown yeast cells were prepared for lysis as during the vacuole preparation. After lysis with lyticase the yeast pellet was resuspended in 20 mM PIPES/KOH, pH 6.8, 200 mM sorbitol and 40 μg/ml DEAE-dextran were added. Cells were briefly incubated on ice for 2 min, then heat shocked for 2 min at 30 °C. Unbroken cells and debris were removed by centrifugation (400 × *g*, 5 min, 4 °C). Lysates containing 50 mg of protein each were centrifuged for 10 min at 8000 × *g*, then the supernatant was spun again at for 10 min at 16,000 × *g*. Proteins of the remaining supernatant were trichloroacetic acid-precipitated. Proteins of the untreated lysate, each pellet fraction, and the supernatant were analyzed by SDS-PAGE and Western blotting. Vacuoles are found exclusively in the 8000 × *g* pellet, while Golgi and endoplasmic reticulum are recovered in the 16,000 × *g* pellet (34).

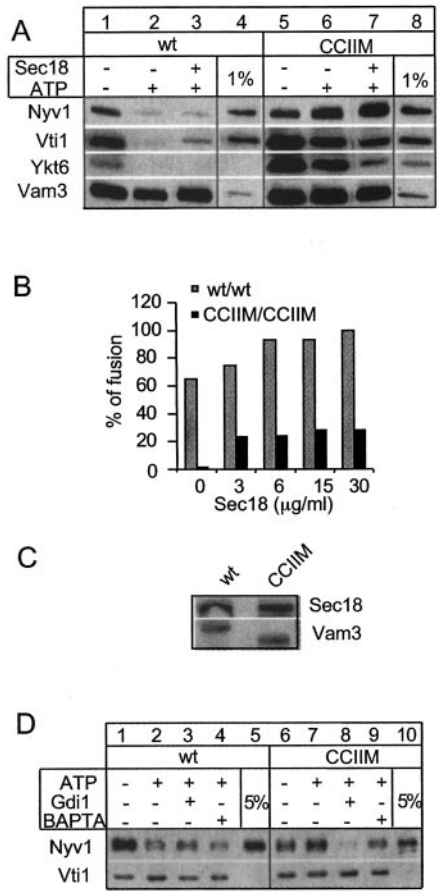

FIG. 3. **Alterations of *cis*-SNARE complex affect priming.** *A*, altered *cis*-SNARE complex composition and priming. Vacuoles (60 μg) were incubated with or without ATP for 10 min at 26 °C in the presence of cytosol, CoA, and Sec18, reisolated (5 min, 20,000 × *g*, 4 °C), and detergent-solubilized. For comparison, a portion (1%) of the total detergent extract was removed and precipitated with trichloroacetic acid (13% v/v). Protein complexes were analyzed by coimmunoprecipitation with protein A-immobilized antibodies to Vam3 (for details see "Experimental Procedures"). Proteins were released from the beads by addition of 1 ml of 0.1 M glycine, pH 2.6, and precipitated with trichloroacetic acid, washed with ice-cold acetone, analyzed by SDS-PAGE, and immunoblotted with the respective antibodies. *B*, Sec18-dependent fusion of Vam3-CCIIM vacuoles. Wild-type and Vam3-CCIIM vacuoles were incubated for 90 min at 26 °C in the presence of ATP, cytosol, CoA, and the indicated amounts of recombinant Sec18. Alkaline phosphatase activity was determined. A representative example ($n = 3$) is shown. *C*, localization of Sec18 to vacuoles. Purified vacuoles (10 μg) were solubilized in sample buffer, and proteins were analyzed by SDS-PAGE. Immunoblots were decorated with the indicated antibodies. *D*, accumulation of *trans*-complexes in the Vam3-CCIIM mutant. Vacuoles were incubated for 30 min at 26 °C with or without ATP in the presence of cytosol, CoA, and Sec18. Where indicated, Gdi1 (30 μg/ml) or BAPTA (1 mM) was added. Then vacuoles were sedimented, detergent-solubilized, and processed for coimmunoprecipitation with antibodies to Vti1. A fraction of the detergent extract (5%) is shown for comparison.

plexes we investigated Sec18/ATP-dependent priming that causes disassembly of SNARE complexes as shown previously (50). Disassembly was most efficient for the coiled-coil mutant (not shown). While wild-type SNARE complexes also readily dissociated when vacuoles were preincubated with ATP only (Fig. 3*A*, *lanes 1* and *2*), additional Sec18 was required to cause partial disassembly of Vam3-CCIIM containing *cis*-SNARE complexes as indicated by reduced coimmunoprecipitation of Vti1 and Ykt6 with Vam3 (compare *lanes 6* and *7*).

We therefore asked whether vacuoles carrying Vam3-CCIIM required Sec18 also for fusion. In all previous fusion experiments we routinely added recombinant Sec18 to the reactions. When we omitted Sec18, vacuoles carrying Vam3-CCIIM were completely inactive, but fused as soon as recombinant Sec18 was included (Fig. 3*B*, *black bars*). This dependence on exogenous Sec18 is not due to a lack of endogenous Sec18 on vacuoles (Fig. 3*C*). Thus, the absence of the Vam3 TMD causes enrichment of more stable *cis*-SNARE complexes that require higher Sec18 concentrations for activation.

Even more striking in contrast to wild-type SNARE complexes, where all SNAREs separated from Vam3 during priming, Nyv1 associated more tightly with Vam3-CCIIM when vacuoles were preincubated with Sec18 and ATP. One possibility was an association of Nyv1 with Vam3-CCIIM in *trans* between vacuoles. We used inhibitors to analyze this in more detail. The docking inhibitor Gdi1 extracts the Rab GTPase Ypt7 and blocks vacuole-vacuole contact (56, 57). If the interaction of the t-SNARE complex (which contains Vam3 and Vti1) and the v-SNARE Nyv1 would indeed be a result of efficient *trans*-SNARE complex formation, Gdi1 addition to the priming reaction should block this association. Indeed, Nyv1 was completely displaced from Vti1 if Gdi1 had been added to the preincubation of Vam3-CCIIM vacuoles (Fig. 3*D*, *lane 8*). This suggested the poor disassembly observed for Vti1 and Vam3-CCIIM in Fig. 3*A* could also include efficient reassembly

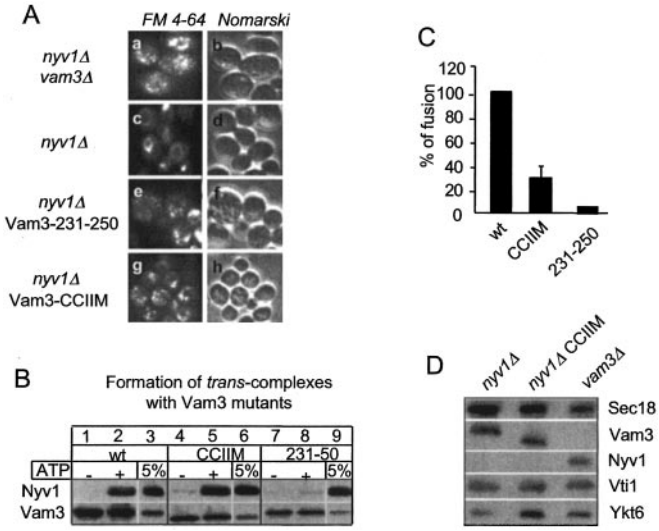

FIG. 4. **Analysis of Nyv1 deletion strains containing Vam3 TMD mutants.** *A*, vacuolar morphology. BJ*nyv1*Δ *vam3*Δ with or without genomically encoded mutant or wild-type Vam3 were labeled with 30 μM FM4–64 (Molecular Probes) and visualized by fluorescence microscopy (64). *Panels a* and *b*, BJ*nyv1*Δ *vam3*Δ; *panels c* and *d*, BJ*nyv1*Δ; *panels e* and *f*, BJ*nyv1*Δ *VAM3-231-50*; *panels g* and *h*, BJ*nyv1*Δ *VAM3CCIIM*. *B*, accumulation of *trans*-complexes in the mutants. Vacuoles from BJ*nyv1*Δ with Vam3-wt or the respective mutants and DKY*vam3*Δ (65 μg each) were incubated together at 26 °C for 40 min in a 600-μl reaction with or without ATP in the presence of cytosol and CoA. Protein complexes were analyzed by coimmunoprecipiation with protein A-immobilized antibodies to Vam3 (for details see "Experimental Procedures"). A fraction (5%) of the detergent extract was removed and proteins were precipitated by 13% trichloroacetic acid. *C*, fusion of the deletion strains. BJ*nyv1*Δ, BJ*nyv1*Δ *VAM3CCIIM*, and BJ*nyv1*Δ *VAM3-231-50* vacuoles (3 μg each) were fused against the DKY*vam3*Δ strain in the presence of ATP, cytosol, Sec18, and CoA for 90 min at 26 °C. Fusion of DKY*vam3*Δ and BJ*nyv1*Δ vacuoles was set to 100%. *D*, analysis of protein composition of vacuoles. Isolated vacuoles (10 μg) from the respective strains were solubilized in sample buffer and analyzed as before.

of *trans*-SNARE complexes. In agreement with this assumption, addition of the late fusion inhibitor BAPTA that allows vacuole docking (37) to the CCIIM vacuoles did not influence the association of Nyv1 with Vti1 (Fig. 3*D*, *lanes 7* and *8*). Similar results were obtained when SNARE complexes were isolated with anti-Vam3 antibodies (not shown). Thus, the association of Nyv1 and Vti1 occurred after docking and could reflect increased *trans*-SNARE complex formation.

To confirm that replacement of the TMD of Vam3 by a lipid anchor supports the formation of *trans*-SNARE complexes, we employed an established assay to measure *trans*-SNARE complexes in the fusion reaction (26). In brief, we fused vacuoles from one strain deleted for Vam3 with vacuoles isolated from a *nyv1*Δ strain. Complexes between Nyv1 and Vam3 only form if the vacuoles dock or fuse and can be detected by coimmunoprecipitation with antibodies to Vam3 (26). Even though *vam3*Δ but not *nyv*Δ vacuoles are fragmented, fusion of *vam3*Δ and *nyv1*Δ vacuoles requires the same proteins and factors like wild-type vacuoles (26). Thus, results obtained with mutant vacuoles can be related to the wild-type situation.

Replacing wild-type Vam3 by Vam3-CCIIM in the *nyv1*Δ cells dramatically changed the vacuole morphology (Fig. 4*A*). Vacuoles lacking Nyv1 are enlarged (53). This suggested that another SNARE can replace Nyv1 *in vivo*, although Nyv1 is essential for *in vitro* fusion (50). In contrast, vacuoles fragment when Vam3 is anchored via a lipid anchor in the *nyv1*Δ background (Fig. 4*A*, *panels g* and *h*). On those vacuoles, only Vti1 has a transmembrane domain within the SNARE complex, which is apparently not sufficient to drive vacuole fusion *in vivo*. TMDs of Nyv1 and of Vam3 could also be critical for the fusion reaction itself, either as catalysts of fusion or as interaction partners of other fusion factors.

trans-SNARE complexes were analyzed by incubating vacuoles from *vam3*Δ and the respective *nyv1*Δ cells in the presence or absence of ATP. Detergent extracts were then immunoprecipitated with anti-Vam3 antibodies. The amount of coprecipitated Nyv1 is a measure of formed *trans*-SNARE complexes (26). Lipid-anchored Vam3 formed *trans*-SNARE complexes at least as efficiently as the wild-type Vam3 did, while the coiled-coil mutant did not (Fig. 4*B*). However, fusion was poor for both mutant Vam3 proteins (Fig. 4*C*). This was not due to a lack of known vacuolar SNAREs on the CCIIM vacuoles (Fig. 4*D*). Thus, replacement of the Vam3 TMD by a lipid anchor did permit *trans*-SNARE complex formation, but affected a late step in the fusion reaction. Furthermore, *trans*-SNARE complexes of Vam3-CCIIM vacuoles were authentic and not a result of *in vitro* assembly in detergent, since the docking inhibitor Gdi1 did block formation of *trans*-SNARE complexes, while GTPγS, a fusion inhibitor, did not (Fig. 5*A*). We repeatedly observed that *trans*-SNARE complexes were more stable and resisted higher salt concentrations when Vam3-CCIIM vacuoles were used. Therefore, we compared *trans*-SNARE formation with CCIIM and wild-type Vam3 over time (Fig. 5*B*). We also used higher concentrations of salt for the immunoprecipitation to analyze the stability and accumulation of *trans*-SNARE complexes. Low amounts of recombinant Sec18 were included to allow efficient priming of Vam3-CCIIM containing vacuoles. These concentrations are not sufficient to disrupt *trans*-SNARE complexes, while incubation with high amounts of Sec18 do disassemble *trans*-SNARE complexes containing Vam3-CCIIM (not shown), as shown previously for *trans*-SNARE complexes containing wild-type Vam3 (26). At these low Sec18 concentrations, a 4-fold stimulation of *trans*-SNARE complex formation was seen in the absence of the Vam3 TMD compared with wild-type (Fig. 5*C*, *lane 5 versus lane 11*, and *D*). Thus, formation of *trans*-SNARE complexes is strongly favored in the absence of the Vam3 TMD, but fusion is strongly reduced. This indicates that the Vam3 TMD itself may have a direct role in fusion or influence in addition downstream reactions.

Interestingly, even though *trans*-SNARE complexes accumulated, we did not see an effect on the fusion kinetics (not shown). Acquisition of resistances to priming or docking inhibitors was similar for wild-type and CCIIM mutant vacuoles, although the overall signal of Vam3-CCIIM vacuoles is reduced 5-fold (Fig. 6, *A* and *B*, and Fig. 2*A*). Thus, fusion must have been blocked at a late stage. The palmitoylation of Vac8 occurs early during vacuole fusion (44), but acylated Vac8 is required late in the fusion reaction, presumably after *trans*-SNARE pairing (42). Since a portion of Vac8 is associated with the SNARE complex and palmitoylation requires Sec18 (44), a defect in Vac8 acylation could explain why fusion is reduced and how this could be triggered by a priming defect. When vacuoles were labeled with [^{3}H]palmitate, Vac8, being present equally on both vacuole types, was reproducibly labeled almost twice as efficient on wild-type than on Vam3-CCIIM vacuoles (Fig. 6*C*). Since palmitoylated Vac8 is required in a reaction after formation of *trans*-SNARE pairing (42), this 45% reduction could contribute to the reduced fusion observed.

DISCUSSION

Our data shed light on the possible function of a SNARE TMD during an authentic fusion reaction in a eukaryotic cell. Three stages of vacuole fusion are clearly affected by alterations in the TMD: the dissociation of *cis*-SNARE complexes during the priming step, *trans*-SNARE pairing, and palmitoy-

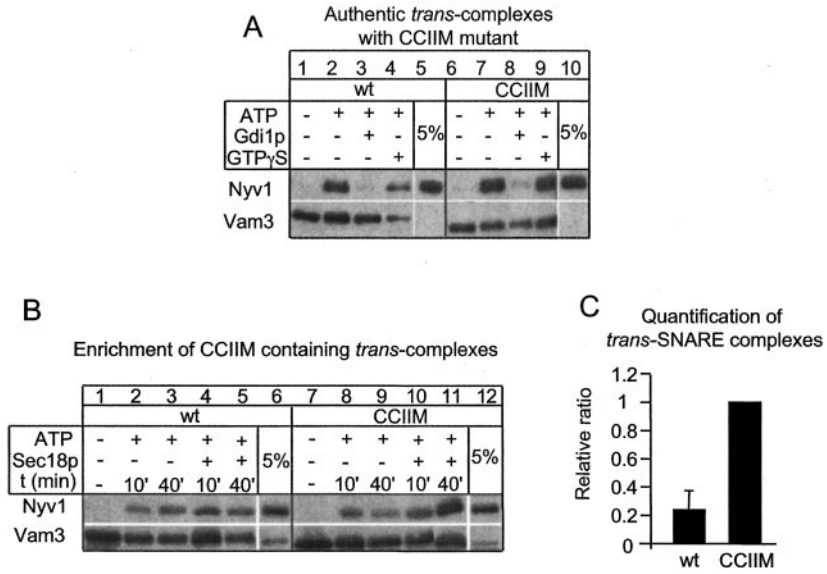

FIG. 5. **Accumulation of *trans*-SNARE complexes in the absence of the Vam3 TMD.** *A*, authentic *trans*-SNARE complexes of Vam3-CCIIM vacuoles. Vacuoles (65 μg each) from BJ*nyv1*Δ containing Vam3-wt or Vam3-CCIIM were incubated with DKY*vam3*Δ at 26 °C in a 600-μl reaction with or without ATP in the presence of cytosol, CoA, Sec18, and the indicated docking inhibitor Gdi1 (30 μg/ml) or fusion inhibitor GTPγS (2 mM) for 40 min. Protein complexes were analyzed by coimmunoprecipitations with antibodies to Vam3 as described under "Experimental Procedures." *B*, accumulation of *trans*-SNARE complexes of Vam3-CCIIM triggered by Sec18 addition. The experiment was done as described in the legend to *B*. Sec18 (3 μg/ml) was added where indicated. At the times indicated samples were set on ice before processing for coimmunoprecipitation. *C*, quantification of *trans*-SNARE complexes. *trans*-SNARE complex accumulating in the presence of Sec18 after 40 min were quantified by laser densitometry (n = 8). Ratios of Nyv1 and Vam3 signals of each precipitation with Vam3-CCIIM were set to 100%.

lation of the fusion factor Vac8.

cis-SNARE complexes are altered and more stable in the absence of the Vam3 TMD. This is most obvious for the interaction of Vam3-CCIIM with Vti1 and Ykt6. Addition of exogenous Sec18 is essential to obtain a low level of fusion of vacuoles containing Vam3-CCIIM. In agreement with this, increased Sec18 concentrations are also required to disassemble stable *cis*-SNARE complexes. Removal of the TMD of Vam3 might influence the conformation of the cytoplasmic domain and thus the interaction with Vti1 and Ykt6 (2, 20), thereby interfering with priming. In addition, it is possible that the altered *cis*-SNARE complex may lack certain factors necessary for efficient priming. We did, however, not detect reduced levels of Sec18, nor of the other SNAREs or of Sec17 (not shown). It is noteworthy that a similar priming defect is also observed on vacuoles lacking subunits of the Vtc-complex, a membrane integral protein complex that associates with V0 and with the v-SNARE Nyv1 (35). Vacuoles from *vtc1*Δ and *vtc4*Δ cells also require Sec18 addition to overcome their deficiency in priming and to partially rescue fusion. Vtc4 is present on CCIIM and wild-type vacuoles in equal amounts.[2] Our data therefore suggest a more tightly associated SNARE complex due to a lack of the Vam3 TMD rather than an effect caused by a lack of Vtc proteins.

Vacuoles containing Vam3-CCIIM appeared intact *in vivo* and had a residual fusion activity of ~20–30% *in vitro*. This activity may suffice *in vivo* to maintain vacuolar structure. Deletion of the vacuolar v-SNARE Nyv1 alone does not lead to vacuolar fragmentation (53), suggesting partial redundancy with another SNARE *in vivo*. However, deletion of Nyv1 in Vam3-CCIIM cells did cause a synthetic phenotype and resulted in vacuolar fragmentation. This demonstrates on the one hand that Nyv1 is involved in vacuolar fusion also *in vivo*. On the other hand it confirms that Vam3-CCIIM is not fully functional *in vivo*. It is important to note that vacuoles contain three t-SNAREs, Vti1, Vam7 and Vam3, of which Vti1 and Vam3 have TMDs. We thus show that a single SNARE TMD per t-SNARE complex is not sufficient to drive homotypic vacuole fusion.

Our data confirm previous observations in several fusion systems that SNAREs depend on an intact coiled-coil domain to function in fusion (4). The observed fusion defect of the coiled-coil mutant in vacuole fusion as also observed by Wang *et al.* (52) is a direct result of the poor assembly of *trans*-SNARE complexes. This straightforward explanation does not hold for the fusion defect observed with lipid-anchored Vam3. Here, *trans*-SNARE complexes containing Vam3-CCIIM accumulated to about 4-fold higher levels than in the corresponding wild type, although fusion between Vam3-CCIIM vacuoles was strongly reduced. We conclude that the formation of the *trans* complex during docking does not automatically induce fusion. *trans*-SNARE complexes may not be required throughout fusion. This observation is in contrast to a requirement of SNAREs to induce fusion of liposomes (5, 6), but agrees with findings that *trans*-SNARE complexes are dispensable for the transition from docking to fusion during homotypic vacuole fusion and cortical vesicle exocytosis (25, 26). In fact, *trans*-SNARE complexes form during the fusion reaction and can be detected, even if fusion is blocked by fusion inhibitors (this study; Refs. 26 and 58). In addition, the TMD may play a functional role downstream of docking. Previously, it was reported that SNAREs with long, but not with short, prenyl anchors induced fusion in the reconstituted proteoliposome system (21). This prompted the suggestion that bilayer-spanning membrane anchors of SNAREs present on both membranes stress lipid bilayers sufficiently upon *trans*-SNARE pairing to induce fusion (21). A caveat is the instability of the proteoliposomes used as they could not sequester 5-kDa oligonucleotides (21); this indicated a severely disturbed bilayer structure and question the validity of this approach. It is possible that the SNARE TMD is required at the final step of membrane fusion, *i.e.* at the stage of actual lipid merger. Indeed, Grote *et al.* (23) observed that replacement of the TMDs

[2] J. Rohde, L. Dietrich, D. Langosch, and C. Ungermann, unpublished results.

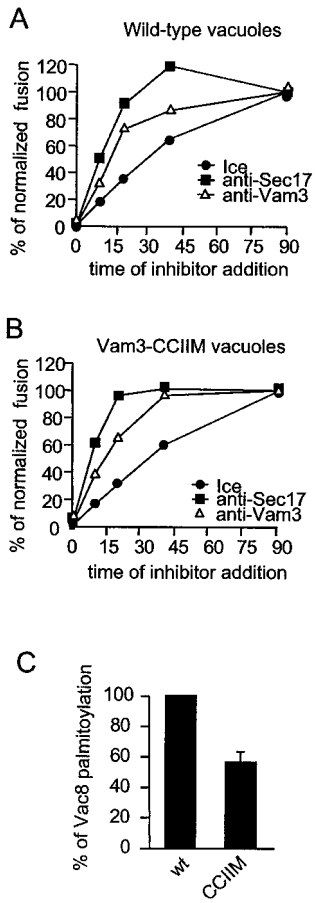

FIG. 6. **A late block in fusion of Vam3-CCIIM vacuoles caused by Vac8 acylation defect.** *A* and *B*, time of addition experiment. A 30× scale fusion reaction was started in the presence of ATP, Sec18, CoA (10 μM), and cytosol at 26 °C. Aliquots (30 μl) were removed at the indicated times, and antibodies (200 ng/μl) to Sec17 or Vam3 were added or left on ice. Then samples were incubated at 26 °C for a total of 90 min before being assayed for fusion. To allow comparison of wild-type and Vam3-CCIIM vacuoles the final fusion was set to 100% in each graph. *C*, reduced palmitoylation of Vac8 on Vam3-CCIIM vacuoles. Vacuoles from both strains were incubated for 10 min at 26 °C with ATP, CoA (10 μM), Sec18, and [^{3}H]palmitate. Vacuoles were recovered by centrifugation (5 min, 8000 × *g*) and solubilized in sample buffer. Proteins were analyzed by SDS-PAGE and fluorography as described previously (44). Pal-Vac8 on films was quantified by laser densitometry. An average of 10 experiments is shown (±S.E.).

of other yeast SNAREs (Snc2p or Sso2p) by prenyl anchors also affected membrane fusion. Lysolipid addition to cells partially rescued this defect, possibly to overcome a stage of exocytosis arrested at hemifusion. Structurally, TMD-TMD interactions may drive fusion by supporting and finishing the "zippering up" of the coiled-coil domains (59) into the lipid bilayer itself (18, 19). Alternatively, conformational flexibility of SNARE TMDs may support fusion. In fact, liposomes fuse efficiently when just TMD peptides corresponding to synaptic SNAREs or viral fusion proteins were incorporated into the bilayer (24, 60). Since sequence variants displaying more stable α-helical conformations in isotropic solution were less fusogenic, fusogenicity may depend on the conformational flexibility of the α-helical SNARE TMDs in the membrane.

Finally, the Vam3 TMD could be critical to direct or bind to other proteins that function after docking. In fact, several factors have been reported that act downstream of the SNAREs in yeast vacuole fusion, including the protein phosphatase 1, calmodulin, a subunit of the Vtc complex, the V0 proteolipid of the vacuolar ATPase (35, 37–39), and Vac8 (41–46, 61). Vac8 becomes palmitoylated coincident with priming and requires Sec18 (44). The level of Vac8 palmitoylation was reduced on vacuoles containing the Vam3-CCIIM mutant. The acylated Vac8 is, however, required after *trans*-SNARE pairing (42), possibly to coordinate proteins at the fusion site. It may thus couple priming and fusion. Even though its function is poorly understood it is clear that Vac8 function is associated with its palmitoylation state (41, 42, 44, 46, 61, 62). It is likely that a more tightly associated and altered SNARE complex is the main cause of the reduced Vac8 palmitoylation. The accumulation of *trans*-SNARE complexes may be associated with the reduction in palmitoylation, but cannot be the only reason, since *trans*-SNARE complexes did not accumulate between vacuoles lacking Vac8 entirely (42). The identification of a direct binding partner of the Vam3 TMD may be informative to fully elucidate the role of SNAREs and in particular their TMDs in fusion.

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