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 defect.

Membrane fusion along the secretory pathway requires the specific interaction of SNAREs 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 bypairing 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 substep of fusion. We show that the TMD is essential for cis-SNARE.
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 (77 Mutagen kit, Bio-Rad). All mutations were verified by dideoxynucleotide chain-termination sequencing. Plasmids carrying the VAM3 promoter and open reading frame with the respective mutations and the URA3 marker were digested with BsrBI and transformed into BJ3505 and DKY6281 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 and DKY6281 strains 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 strain as described above. Similarly, pRS406-NYV1 containing the promoter and open reading frame of NYV1 was integrated into the DKY6281 strain to generate DKY6281NYV1 strain.

**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-alanine phosphatase due to a lack of the processing protease Pep4p. Vacuoles were reisolated by brief centrifugation and washed three times in lysis buffer containing 0.1% Triton X-100. Elution of the bound proteins was performed with trichloroacetic acid (13% v/v). The remaining detergent was removed 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.

**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. 1A).

We first determined whether lipid anchoring or coiled-coil mutations affected vacuole morphology (Fig. 1B). As expected, Vam3-231–250 cells had fragmented vacuoles, suggesting an inactivation of Vam3 function (Fig. 1B, 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. 2A). 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 coimmunoprecipitation, regardless of the vacuole type (Fig. 3A, lanes 1 and 5). However, Ykt6 and Vti1 were clearly enriched in SNARE complexes from Vam3-CCIIM vacuoles (Fig. 3A, 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 complex...
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**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 centrifuged (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 SDS-PAGE and Western blotting. Vacuoles were found exclusively in the 8000 × g pellet, while Golgi and endoplasmic reticulum are recovered in the 16,000 × g pellet (data not shown). 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. 3A, black bars). This dependence on exogenous Sec18 is not due to a lack of endogenous Sec18 on vacuoles (Fig. 3C). 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. 3D, lane 8). This suggested the poor disassembly observed for Vti1 and Vam3-CCIIM in Fig. 3A could also include efficient reassembly...
of trans-SNARE complexes. In agreement with this assumption, addition of the late fusion inhibitor BAPTA that allows vacuole docking (37) to the CIIM vacuoles did not influence the association of Nyv1 with Vti1 (Fig. 3D, 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 nyv1Δ strain. Complexes between Nyv1 and Vam3 only form if the vacuoles dock or fuse and can be detected by coimmunoprecipitation of Nyv1 and Vam3-wt or the respective mutants and Vam3-CCIIM. In contrast, vacuoles lacking Nyv1 are enlarged (53). This suggested that a portion of vacuoles dock or fuse and can be detected by coimmunoprecipitation of Nyv1 and Vam3-wt or the respective mutants and Vam3-CCIIM (Fig. 4A). A fraction (5%) of the detergent extract that was removed and proteins were precipitated by 13% trichloroacetic acid. C, fusion of the deletion strains. B, SNARE complexes were analyzed by coimmunoprecipitation with protein A-immobilized antibodies to Vam3 (for details see “Experimental Procedures”). A fraction (5%) of the detergent extract was fused against the Dkyvamv3Δ strain in the presence of ATP, cytosol, Sec18, and CoA for 90 min at 26°C. Fusion of Dkyvamv3Δ and Bnyv1Δ 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.

Fig. 4. Analysis of Nyv1 deletion strains containing Vam3 TMD mutants. A, vacuolar morphology. B, SNARE complexes were analyzed by coimmunoprecipitation with protein A-immobilized antibodies to Vam3 (for details see “Experimental Procedures”). A, fraction (5%) of the detergent extract was fused against the Dkyvamv3Δ strain in the presence of ATP, cytosol, Sec18, and CoA for 90 min at 26°C. Fusion of Dkyvamv3Δ and Bnyv1Δ 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.

The trans-SNARE complexes were analyzed by incubating vacuoles from vam3Δ and the respective nyv1Δ 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. 4B). However, fusion was poor for both mutant Vam3 proteins (Fig. 4C). This was not due to a lack of known vacuolar SNAREs on the CIIM vacuoles (Fig. 4D). 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. 5A). 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 CIIM and wild-type Vam3 over time (Fig. 5B). 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. 5C, 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 CIIM mutant vacuoles, although the overall signal of Vam3-CCIIM vacuoles is reduced 5-fold (Fig. 6, A and B, and Fig. 2A). 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 [3H]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. 6C). 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-
lulation 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 Vam3-wt, which in turn may 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.

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
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 fused 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-CC11M 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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