Functional Analysis of Conserved Structural Elements in Yeast Syntaxin Vam3p*

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Vam3p, a syntaxin-like SNARE protein involved in yeast vacuole fusion, is composed of a three-helical N-terminal domain, a canonical SNARE motif, and a C-terminal transmembrane region (TMR). Surprisingly, we find that the N-terminal domain of Vam3p is not essential for fusion, although analogous domains in other syntaxins are indispensable for fusion and/or protein-protein interactions. In contrast to the N-terminal domain, mutations in the SNARE motif of Vam3p or replacement of the SNARE motif of Vam3p with the SNARE motif from other syntaxins inhibited fusion. Furthermore, the precise distance between the SNARE motif and the TMR was critical for fusion. Insertion of only three residues after the SNARE motif significantly impaired fusion and insertion of 12 residues abolished fusion. As judged by co-immunoprecipitation experiments, the SNARE motif mutations and the insertions did not alter the association of Vam3p with Vam7p, Vti1p, Nyv1p, and Ykt6p, other vacuolar SNARE proteins implicated in fusion. In contrast, the SNARE motif substitutions interfered with the stable formation of Vam3p complexes with Vam7p and Vti1p, although Vam3p complexes with Vam7p and Ykt6p were still present. Our data suggest that in contrast to previously characterized syntaxins, Vam3p contains only two domains essential for fusion, the SNARE motif and the TMR, and these domains have to be closely coupled to function in fusion.

Extensive evidence suggests that intracellular membrane fusion reactions in eukaryotic cells are performed via similar mechanisms that are conserved from yeast to humans (reviewed in Refs. 1–4). At least four classes of proteins appear to be essential for all intracellular fusion reactions: SNARE proteins, the SNARE chaperones (NSF and p97 with adaptor molecules), Sec1/munc18-like proteins (SM proteins), and Rab proteins. SNARE proteins are identified by a signature sequence of 55 residues, the SNARE motif, which is followed in most SNAREs by a C-terminal transmembrane region (TMR) (5, 6). Subtle differences among SNARE motifs allow identification of subfamilies of SNARE proteins, such as syntaxin-like SNAREs. Extensive information on the function of SNARE proteins was derived from studies on mammalian synaptic transmission in which the biochemistry of these proteins was initially elucidated (reviewed in Ref. 7), and in yeast genetics, which allowed definition of the generality of SNARE function (reviewed in Refs. 2, 3).

At the mammalian synapse, three SNAREs mediate synaptic vesicle exocytosis: the vesicle protein synaptobrevin/VAMP and the plasma membrane proteins syntaxin 1 and SNAP-25. The SNARE motifs of these proteins (one each in syntaxin and synaptobrevin; two in SNAP-25) form a stable complex, the so-called core complex, that is composed of a four-helical bundle (8–12). Similar complexes are probably formed by SNARE proteins in all membrane fusion reactions. Because the SNARE motifs are generally adjacent to TMRs, which in turn are anchored in opposing membranes, formation of core complexes by SNARE proteins may bring the two membranes into close proximity and thereby force membrane fusion (reviewed in Ref. 4). In yeast, one of the best studied example of membrane fusion is the vacuole fusion reaction (reviewed in Refs. 13–15). Five SNARE proteins have been implicated in vacuole fusion: the syntaxin-like SNARE Vam3p, the SNAP-25-like SNAREs Vam7p and Vti1p, and the synaptobrevin/VAMP-like SNAREs Nyv1p and Ykt6p (16–22). These five vacuolar SNAREs probably function analogously to SNAREs in other fusion reactions, but it is currently unclear if Nyv1p and Ykt6p contribute to the formation of separate four-helical core complexes, or if a novel type of core complex is assembled.

Syntaxin 1 is considered as the central SNARE protein in synaptic vesicle exocytosis because its SNARE motif participates in core complex formation, and its N-terminal domain is required for binding the SM protein munc18-1 (Ref. 23, also called n-sec 1, Ref. 24 or rb-sec1, Ref. 25). The three-helical N-terminal domain of syntaxin 1 (26, 27) spontaneously folds onto the SNARE motif to generate a closed conformation, which is incompatible with core complex formation, but required for munc18-1 binding (28, 29). Because munc18-1 is essential for fusion (28, 30), syntaxin 1 may couple SNARE and SM protein function during synaptic vesicle exocytosis. Interestingly, two other syntaxin-like SNAREs were found to contain independently folded three-helical N-terminal domains, the yeast plasma membrane syntaxin Sso1p (31, 32) and yeast vacuolar syntaxin Vam3p (33). This suggests that three-helical N-terminal domains can be considered a hallmark of syntaxins that may have an evolutionary conserved function. Indeed, similar to syntaxin 1, the N-terminal domain of Sso1p also folds back onto the SNARE motif into a closed conformation that is essential for yeast viability (32). However, surprisingly the closed conformation of Sso1p does not appear to bind the SM protein Sec1p (34), although this result has been questioned (35). Equally surprisingly, the yeast vacuolar syntaxin Vam3p appears to be constitutively open, and the N-terminal domain of
Vam3p (different from the syntaxin 1 N-terminal domain) is not required for binding to the corresponding SM protein Vps33p (33).

These results raise the question of whether syntaxins perform general functions in membrane fusion via their conserved three-helical N-terminal domains coupled to the SNARE motifs or if these domains are specialized for a given fusion reaction. To address this, we have now studied the functional importance of the conserved structural domains of the yeast vacuolar syntaxin Vam3p using the in vitro vacuole fusion reaction that allows a combination of yeast genetics with an in vitro assay (36). We now show that, as expected, the SNARE motif of Vam3p is essential for vacuolar fusion. Unexpectedly, however, we find that the N-terminal domain is dispensable. The precise sequence of the SNARE motif is important for fusion, as is the distance of the motif from the TMR. Our results suggest that syntaxins only share general functions mediated by the SNARE motif and TMR, but not by the N-terminal domains.

EXPERIMENTAL PROCEDURES

Plasmids and Yeast Strains—The yeast strains and the plasmid vectors used for transgenic expression of wild-type and mutant Vam3ps in this study are shown in Table I. Plasmids were constructed by standard methods; mutagenesis was performed with the QuikChangeTM site-directed mutagenesis kit (Stratagene). The Vam3 deletion mutants of yeast (YWD1 and YWD2) were generated by transforming pBNeoaVam3 into the DKY6218 and TVY1 strains (37, 38) with neomycin selection; transformants were selected with G418 (200 mg/l). Deletion of the Vam3 gene in the transformants by homologous recombination was confirmed by polymerase chain reaction using primers from outside of the Vam3 gene. YWD1 and YWD2 were then transformed with the expression vectors listed in Table I containing the indicated coding sequences under control of the yeast Vam3 promoter in the episomal single copy expression vector pRS416. All plasmids were confirmed by sequencing; protein expression in all yeast strains was confirmed by immunoblotting.

Vacuole Fusion Assays—Vacuoles were purified by Ficoll density gradient centrifugation in vacuole purification buffer (10 mM PIPES/KOH, pH 6.8, 0.2 mM sorbitol), and fusion assays were carried out essentially as described (36, 39) with some modifications. Fusion reactions (volumes: 30 μl) contained 4.5 μg of protein of vacuoles from the DKY6218 and TVY1 strains expressing the same wild-type or mutant Vam3p in 23.3 μl 2.2 mM of 10 mM salt adjustment buffer (100 mM PIPES/KOH, pH 6.8, 0.5 mM sorbitol, 1 mM potassium acetate, 0.5 μM KCl, 50 mM MgCl2), 3 μl of a 10 mM ATP-regenerating system (creatine kinase 10 g/l, 0.4 mM creatine phosphate, 5 mM ATP, 10 mM MgCl2), 0.5 μM of protease inhibitor mixture PICT (50× PIC: 10 μM leupeptin (0.5 μl), 50 μl of 1,10-phenanthroline (500 mM in ethanol), 25 μl of pepstatin A (1 mg/ml in methanol), 50 μl of Pefabloc (100 μM)), 1 μl of yeast cytosolic protein (50 μl of the appropriate antibody on ice for 1 h. 40 μl of protein A or protein G-Sepharose beads (50%) were added, beads were incubated overnight at 4 °C with agitation, washed three times with IP buffer, and bound proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE and immunoblotting with antibodies to Vam3p, Vam7p, Vti1p, Nyv1p, and Ykt6p (the last three antibodies were a generous gift from Dr. W. Wickner, Dartmouth).

Analysis of In Vivo Vacuole Morphology by FM4-64 Staining—Analysis was performed essentially as described (40). Briefly, yeast cells of the various genotypes were grown overnight in selection medium (–URA). Cells were then diluted to an A600 of 0.2 in YPD medium and grown at 30 °C to A600 of 0.8, pelleted, and resuspended in 16 μl FM4-64 (Molecular Probes) in YPD medium. Afterward, cells were incubated for 15 min at 30 °C with agitation, washed, immobilized on concanavalin A-coated slides, and viewed in a fluorescent microscope.

RESULTS AND DISCUSSION

Strategy—The strategy pursued in this study was based on the in vitro vacuole fusion assay developed by Wickner and co-workers (36, 39). This assay is based on the proteolytic activation of vacuolar pro-alkaline phosphatase by vacuolar protease A (38). Vacuoles from yeast cells lacking either protease A or pro-alkaline phosphatase have no enzymatically active alkaline phosphatase. When these vacuoles are fused in vitro, alkaline phosphatase is activated and can be easily monitored. Our overall goal was to employ the in vitro vacuole fusion assay to examine the effect of Vam3p mutations on fusion. To pursue this goal, we first generated yeast strains lacking Vam3p and either protease A or pro-alkaline phosphatase (YWD1 and YWD2, Table I). We then expressed wild-type and mutant Vam3ps in these strains from single-copy episomal vectors containing the normal Vam3 promoter to avoid overexpression of the protein. All expressed Vam3ps carried an N-terminal FLAG epitope to allow protein detection independent of antigenic sites in Vam3p that may have been deleted in the mutants. In this manner we could verify by immunoblotting that the various Vam3p mutants were present on the purified vacuoles that were used for the vacuole fusion assays. In addition, we examined the Vam3p mutants for their ability to form heteromeric complexes with the other vacuolar SNARE proteins, Vam7p, Vti1p, Ykt6p, and Nyv1p, as an indication of the structural integrity and proper folding of the Vam3p mutants on the vacuoles. Finally, we tested these mutants in an independent assay for vacuole fusion competence, a fluorescence assay in which vacuoles are visualized in vivo by uptake of the dye FM4-64 (40).

Role of the Vam3p N-terminal Domain and SNARE Motif in Vacuole Fusion—We first examined whether the two major cytoplasmic regions of Vam3p, the N-terminal three-helical domain, and the SNARE motif, are essential for fusion (Fig. 1A). In purified vacuoles isolated from the mutant yeast cells, both mutant Vam3ps were present at levels similar to those of wild-type Vam3p (Fig. 1B). Deletion of the N-terminal domain of Vam3p had no effect on overall fusion between vacuoles, whereas deletion of the SNARE motif abolished fusion (Fig. 1C). Co-immunoprecipitation experiments of Vam3p and Vam7p from the yeast cells used for the in vitro fusion assays demonstrated that wild-type FLAG-tagged Vam3p was efficiently co-immunoprecipitated with Vam7p using either monoclonal FLAG tag or polyclonal Vam7p antibodies (Fig. 2, lanes 3–6). No co-immunoprecipitation of Vam7p and Vam3p was observed with mutant Vam3p lacking the SNARE motif using either Vam3p or Vam7p antibodies for immunoprecipitations (Fig. 2, lanes 4–6). In contrast, Vam3p lacking the N-terminal domain was as efficiently co-immunoprecipitated with Vam7p as wild-type Vam3p (Fig. 2, lanes 7–9). Furthermore, the Vam3p immunoprecipitates from yeast cells expressing wild-type and N-terminally deleted Vam3p, but not SNARE-motif
TABLE I

Yeast strains and yeast expression vectors

| Strain   | Genotype                          | Source                      |
|----------|-----------------------------------|-----------------------------|
| DDKY6281 | MATα leu2–3, 112 ura3–52 his3–99 200 trp1–901 ade2–101 suc2–200 | ref. 38                     |
| TVY1     | MATα MATa ura3–52 his3–19 GAL200 trp1–901 leu2–3, 112 lys2–801 suc2–901 pep4::LEU2 | ref. 37                     |
| SEY6210  | MATα MATa ura3–52 his3–91 GAL1200 trp1–901 leu2–3, 112 lys2–801 suc2–901 pho8::TRP1 vam3 | this study                   |
| YWD2     | MATα ura3–52 his3–901 leu2–3, 112 lys2–801 suc2–901 | ref. 37                     |

Vector name | Encoded protein/residue numbers | Source                      |
|------------|---------------------------------|-----------------------------|
| pRS416     | None (episomal expression vector) | Dr. B. Horazdovsky          |
| pBNeo      | None (Neomycin resistance cassette vector) | Dr. B. Horazdovsky          |
| pBNeo Vam3 | Vam3 gene with coding region replaced by neomycin resistance cassette | this study                   |
| pRS416FlagVam3 | Full-length Vam3p-(1–283) | this study                   |
| pRS416FlagVam3N | Vam3p N-terminal deletion (136–283) | this study                   |
| pRS416FlagVamSNARE | Vam3p with SNARE motif-deletion (187–252); junction sequence: ELPDDQ | this study                   |
| pRS416FlagVam3 (Pep12) | Vam3p with SNARE motif replaced by Pep12p SNARE motif (residues 1–196 and 255–283 of Vam3p; 202–259 of Pep12p) | this study                   |
| pRS416FlagVam3QA | Vam3p zero-layer Q mutated to A (Q224A) | this study                   |
| pRS416FlagVam3QR | Vam3p zero-layer Q mutated to R (Q224R) | this study                   |
| pRS416FlagVam3–9 | Vam3p with 9 aa insertion between SNARE motif and TMR after R257 (inserted sequence: ASGSSGSSG) | this study                   |
| pRS416FlagVam3–12 | Vam3p with 12 aa insertion between SNARE motif and TMR after R257 (inserted sequence: ASGSSGSSG) | this study                   |

All Vam3p proteins contain an N-terminal FLAG tag.

Structure/Function Analysis of Vam3p

FIG. 1. Role of the N-terminal domain and the SNARE motif of Vam3p in vacuole fusion in vitro. A, schematic view of the domain structures of wild-type Vam3p (WT), the deletion mutant lacking the N-terminal domain (N-del) of Vam3p, and the deletion mutant lacking the SNARE motif (SNARE-del). The positions of the N-terminal FLAG tag (FLAG), the three N-terminal α-helices (HA, HB, and HC), the SNARE motif, and the transmembrane region (TMR) are indicated. Residue numbers of the domain boundaries are shown below the wild-type structure.

B, immunoblotting analysis of wild-type and mutant Vam3ps in purified vacuoles used for in vitro fusion assays. Purified vacuoles (4.5 μg of protein per lane) from YW1 (left lane) and YW2 yeast cells (right lane) expressing wild-type Vam3p, the N-terminal-deleted Vam3p, or Vam3p with a SNARE motif deletion were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using antibodies to the FLAG-epitope. Numbers on the left indicate positions of molecular size markers. C, fusion assays of the vacuoles from yeast cells expressing wild-type or mutant Vam3ps (see panels A and B).

Fusion was measured as the alkaline phosphatase activity after 90 min 30 °C incubation in vitro after subtraction of the control values obtained with reactions kept on ice, with the wild-type control set at 100%. Data shown are the mean ± S.E. from multiple independent experiments (n = 4).
deleted Vam3p, contained all other SNARE proteins that are currently implicated in vacuole fusion (22). Vit1p, Nyv1p, and Ykt6p were efficiently co-immunoprecipitated with wild-type- and N-terminal-deleted Vam3p, but were absent from immunoprecipitates of SNARE motif-deleted Vam3p (Fig. 3, lanes 1–9). These results suggest that the SNARE motif of Vam3p is essential for vacuole fusion and for SNARE complex formation, whereas removal of the N-terminal domain of Vam3p has no major effect on either fusion or SNARE complexes. The necessity of the SNARE motif for fusion is not unexpected given the role of SNARE motifs in mediating assembly of core complexes during fusion (reviewed in Ref. 4).

The dispensability of the N-terminal domains of Vam3p, however, is surprising in view of the similarity of this domain to the N-terminal domains of Sso1 that is essential for exocytosis (32), and of syntaxin 1 that is essential for binding the SM protein Munc18 (23, 24).

To corroborate with independent methods that the N-terminal domain of Vam3p is not required for fusion, we used antibody inhibition experiments (Fig. 4) and an in vivo assay of vacuole fusion that employs the fluorescent dye FM4-64 (Fig. 5). First, as described previously (22), an antibody to Vam3p that reacts with the entire molecule including the SNARE motif (anti-Vam3p) inhibited vacuole fusion whereas preimmune serum (Fig. 5). Second, when vacuoles were visualized in yeast cells expressing wild-type Vam3p or mutant Vam3ps with deletions of either the SNARE motif or the N-terminal domain, the morphology of the vacuoles in cells with wild-type and N-
terminal-deleted Vam3p appeared normal (Fig. 5, panels A', A'', C', and C''). In contrast, cells expressing Vam3p with a deletion of the SNARE motif contained a fragmented staining pattern typical for a vacuole fusion defect (Fig. 5, panels B' and B''). These results are consistent with the conclusion that the N-terminal domain of Vam3p is not directly involved in the fundamental fusion reaction, whereas the SNARE motif is essential.

The Precise Sequence of the SNARE Motif of Vam3p Is Essential—Syntaxin-like SNAREs exhibit a characteristic pattern of sequence similarity in their SNARE motifs (6). Overexpression, but not physiological levels of expression of the endosomal syntaxin Pep12p, rescues vacuolar Vam3p deficiency; conversely, overexpressed Vam3p can rescue the Pep12p deficiency, suggesting that there is limited functional redundancy among these syntaxins (18, 41). The specificity of a syntaxin for a given step in membrane traffic could be caused by their sequences outside the SNARE motif, a possibility that would be consistent with the distinct functions of the N-terminal domains of syntaxins revealed in the experiments described in Figs. 1–3. To test this, we replaced the SNARE motif of Vam3p with the SNARE motif of two other yeast syntaxins, Pep12p and Sed5p (Fig. 6). The Vam3p mutants containing the SNARE motifs from Sed5p and Pep12p expressed as well as wild-type Vam3p and were present on the vacuoles used for the in vitro fusion assays at levels similar to wild-type Vam3p (data not shown). However, both SNARE motif replacement mutants were unable to support in vitro fusion similar to the SNARE motif deletion mutant, suggesting that at physiological levels, even the SNARE motif of Pep12p is not competent to function like the SNARE motif of Vam3p (Fig. 6A). Furthermore, the FM4-64 assay confirmed for both SNARE replacement mutants that vacuolar fusion was impaired as evidenced by a fractured morphology of the vacuoles (Fig. 5, panels D'–E').

We then tested if the SNARE motif replacement mutants still formed heteromeric complexes with other SNARE proteins as assayed by co-immunoprecipitation. In contrast to the SNARE motif deletion mutant, immunoprecipitation of Vam7p brought down the mutant Vam3p although less efficiently as with wild-type Vam3p (Fig. 6B). However, full SNARE complexes were not formed in the SNARE motif replacement mutants because immunoprecipitations failed to co-isolate Vti1p and Nyv1p with Vam3p, although the synaptobrevin-like SNARE Ykt6p was still complexed with Vam3p (Fig. 3, lanes 10–15). Together these experiments suggest that when proteins are not overexpressed, the precise SNARE motif of a syntaxin is of central importance to its function in membrane trafficking. These data are consistent with the notion that incomplete SNARE complex formation caused the fusion deficiency in the SNARE motif replacement mutants, although they do not prove this point because other activities of the SNARE motifs could have contributed.

The Role of the Zero-layer Residues in the Vam3p SNARE Motif in Vacuole Fusion—A striking feature of the synaptic core complex is the central “zero-layer” contact in the four-helical bundle. The zero-layer is formed by hydrogen bonds between one arginine residue and three glutamine residues (12) and represents the only hydrophilic interaction in the interior of the core complex, all other interactions being hydrophobic. Zero-layer contacts are probably conserved in all SNARE core complexes (5), but studies on the potential role of the zero-layer in membrane fusion have led to conflicting results (42–44). The critical role of the Vam3p-SNARE motif in vacuole fusion provides an opportunity to test the role of the zero-layer in an in vitro system with physiological membranes.

We produced yeast cells expressing mutant Vam3p in which the zero-layer glutamine is replaced by an alanine or arginine residue in the context of the full-length protein. These mutants were expressed at wild-type levels and copurified with the vacuoles (data not shown). However, in vitro analysis of fusion between vacuoles from the mutant yeast strains uncovered a major deficit (Fig. 7). A moderate but significant inhibition of fusion was already obtained with the glutamine → alanine substitution, whereas the glutamine → arginine substitution had a much more severe effect (Fig. 7A), as was previously observed for the syntaxin-like SNARE Sso1p in exocytosis (42, 43). In the FM4-64 assay, both mutants exhibited only a slight abnormality in vacuole morphology (Fig. 5, panels F'–G''), whereas the FM4-64 assay confirmed for both SNARE replacement mutants that vacuolar fusion was impaired as evidenced by a fractured morphology of the vacuoles (Fig. 5, panels D'–E').

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Coupling of the SNARE Motif to the TMR—Although it is debatable whether formation of the core complex causes complete membrane fusion, partial fusion, or precedes fusion, it is clear that it brings the fusing membranes into close proximity (Ref. 11; reviewed in Ref. 4). If this proximity was directly involved in fusion, the distance between the SNARE motif and TMR in the core complex should be important for the efficiency of the fusion reaction. The requirement for the TMRs in SNAREs for fusion is well documented (see for example for
arginine residue (Vam3p(QR); lanes 19–21); mutants in which the glutamine residue in the zero layer of the SNARE motif was mutated into an alanine (Vam3p(QA); Vam3p(Pep12); lanes 10–12; markers. Numbers on the left indicate positions of molecular size markers.

Vacuolar proteins were harvested from yeast cells harboring wild-type Vam3p (WT; lanes 1–3); Vam3p mutants in which the SNARE motif (SNARE-del; lanes 4–6) or the N-terminal domain (N-del; lanes 7–9) were deleted; Vam3p mutants in which the Vam3p SNARE motif was replaced with the SNARE motif from Pep12p (Vam3p(Pep12); lanes 10–12) or from Sed5p (Vam3p(Sed5); lanes 13–15); Vam3p mutants in which the glutamine residue in the zero layer of the SNARE motif was mutated into an alanine (Vam3p(QA); lanes 16–18) or an arginine residue (Vam3p(QR); lanes 19–21); and a Vam3p mutant in which 12 amino acids were inserted between the SNARE motif and the TMR (Vam3p(12aa); lanes 22–24). Vam3p was immunoprecipitated from the vacuolar proteins by virtue of the N-terminal epitope tag attached to all Vam3ps, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with antibodies to Vti1p, Nyv1p, and Ykt6p as indicated on the right. For each Vam3p protein, the input is compared with the specific immunoprecipitate (IP) and a control in which immunoprecipitates were performed with beads without antibodies (Control). Numbers on the left indicate positions of molecular size markers.

Vam3p,Refs. 45, 46). However, the necessity of the coupling between the SNARE motif and the TMR for membrane fusion has not been demonstrated in a physiological fusion reaction. The potential role of such coupling was studied using a reconstituted liposome assay by introducing flexible linkers between the SNARE motifs and TMRS of syntaxin or synaptotagmin. Surprisingly, only a gradual decrease in fusion efficiency with the length of the linkers was observed (47). Significant fusion still remained even with linkers of 33 residues (>100 Å in an extended conformation), and multiple rounds of fusion had to be calculated to detect a major effect of these insertions.

To probe the role of SNARE motifs/TMR coupling in a physiological membrane fusion system, we examined whether mutant Vam3p carrying insertions of a linker between the TMR and SNARE motif still functions in fusion (Fig. 8A). In these experiments, the linker contained 3, 6, 9, and 12 small, non-charged amino acid residues to avoid artifacts due to the inserted sequences, for example because of positive charges. Immunoblots showed that the mutant Vam3ps were expressed at wild-type levels and were present on purified vacuoles (data not shown). Insertion of three amino acids between the SNARE motif and the TMR already inhibited fusion significantly (Fig. 8B). The longer linkers caused a progressive decrease in fusion efficiency, with 12 amino acids reducing fusion to the background levels observed when the SNARE motif is eliminated (Fig. 1C). The effect of the 12 amino acid insertion on fusion was confirmed in the FM4-64 assay (Fig. 5, panels H′ and H″). Co-immunoprecipitations demonstrated that the mutant Vam3ps retained the ability to bind to Vam7p (Fig. 8C). Even the 12 residue insertion had no effect on the formation of the vacuolar SNARE complexes because all SNAREs were co-immunoprecipitated from the yeast cells with Vam3p containing the insertion (Fig. 3, lanes 22–24). Overall, these results indicate that the coupling between the SNARE motif and the TMR of Vam3p is critical for membrane fusion but not for SNARE complex formation. This provides strong support to the notion that at least one of the functions of the core complex is to approximate the two membranes together.

SUMMARY

The mutational analysis of Vam3p described here yielded expected and unexpected results. The observation that the SNARE motif of Vam3p is essential for vacuolar fusion confirms extensive evidence for the general importance of SNARE motifs for intracellular membrane fusion and supports a critical role for Vam3p in vacuole fusion. Unexpectedly, however, we found that the N-terminal three-helical domain of Vam3p is not required for fusion. A minimal syntaxin composed of the SNARE motif and the TMR was sufficient to support full-fledged fusion. This finding was unexpected because the N-terminal domain of Vam3p resembles similar essential domains in Sso1p and syntaxin 1 (33), giving rise to the expectation that this domain must have a critical role in fusion. The fact that the N-terminal domain of Vam3p does not perform such a critical role extends previous observations from this and other laboratories that the SM protein Vps33p (which is essential for vacuole fusion) is coupled to the vacuolar SNARE machinery not via the N-terminal epitope tag attached to all Vam3ps, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with antibodies to Vti1p, Nyv1p, and Ykt6p as indicated on the right. For each Vam3p protein, the input is compared with the specific immunoprecipitate (IP) and a control in which immunoprecipitates were performed with beads without antibodies (Control). Numbers on the left indicate positions of molecular size markers.

![Image](http://www.jbc.org/)

- **Fig. 3.** Immunoprecipitation analysis of Vam3p complexes with the vacuolar SNARE proteins Vti1p, Nyv1p, and Ykt6p in the various Vam3p mutants. Vacuolar proteins were harvested from yeast cells harboring wild-type Vam3p (WT; lanes 1–3); Vam3p mutants in which the SNARE motif (SNARE-del; lanes 4–6) or the N-terminal domain (N-del; lanes 7–9) were deleted; Vam3p mutants in which the Vam3p SNARE motif was replaced with the SNARE motif from Pep12p (Vam3p(Pep12); lanes 10–12) or from Sed5p (Vam3p(Sed5); lanes 13–15); Vam3p mutants in which the glutamine residue in the zero layer of the SNARE motif was mutated into an alanine (Vam3p(QA); lanes 16–18) or an arginine residue (Vam3p(QR); lanes 19–21); and a Vam3p mutant in which 12 amino acids were inserted between the SNARE motif and the TMR (Vam3p(12aa); lanes 22–24). Vam3p was immunoprecipitated from the vacuolar proteins by virtue of the N-terminal epitope tag attached to all Vam3ps, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with antibodies to Vti1p, Nyv1p, and Ykt6p as indicated on the right. For each Vam3p protein, the input is compared with the specific immunoprecipitate (IP) and a control in which immunoprecipitates were performed with beads without antibodies (Control). Numbers on the left indicate positions of molecular size markers.

- **Fig. 4.** Antibody inhibition of in vitro vacuole fusion. Vacuoles from protease- and alkaline phosphatase-deficient yeast cells were incubated without antibodies (None), or in the presence of preimmune serum (PIS), of antibodies raised to the entire cytoplasmic sequence of Vam3p (Anti-Vam3p), of antibodies specific for the N-terminal domain of Vam3p (Anti-Vam3pN), and of an irrelevant control antibody (Control). Data shown are from a single representative experiment.

The mutational analysis of Vam3p described here yielded expected and unexpected results. The observation that the SNARE motif of Vam3p is essential for vacuolar fusion confirms extensive evidence for the general importance of SNARE motifs for intracellular membrane fusion and supports a critical role for Vam3p in vacuole fusion. Unexpectedly, however, we found that the N-terminal three-helical domain of Vam3p is not required for fusion. A minimal syntaxin composed of the SNARE motif and the TMR was sufficient to support full-fledged fusion. This finding was unexpected because the N-terminal domain of Vam3p resembles similar essential domains in Sso1p and syntaxin 1 (33), giving rise to the expectation that this domain must have a critical role in fusion. The fact that the N-terminal domain of Vam3p does not perform such a critical role extends previous observations from this and other laboratories that the SM protein Vps33p (which is essential for vacuole fusion) is coupled to the vacuolar SNARE machinery not via the N-terminal epitope tag attached to all Vam3ps, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with antibodies to Vti1p, Nyv1p, and Ykt6p as indicated on the right. For each Vam3p protein, the input is compared with the specific immunoprecipitate (IP) and a control in which immunoprecipitates were performed with beads without antibodies (Control). Numbers on the left indicate positions of molecular size markers.
In the previous structural studies. These data thus show that N-terminal domains of syntaxins are unlikely to generally form closed conformations. Furthermore, our findings strongly suggest that the similar N-terminal domains of different syntaxins perform distinct functions. A possible explanation for the differences between syntaxins is that the various fusion reactions are subject to specializations that evolved in response to biological necessities and that these specializations are determined, among others, by the non-conserved properties of syntaxins. This speculation would suggest, for example, that synaptic vesicle fusion is specialized for speed, whereas the absence of leakiness may be a critical requirement for vacuole fusion.

How do the SNARE motif and the TMR function together in vacuole fusion? The replacement and the zero-layer mutants of the Vam3p SNARE motif severely inhibited fusion, demonstrating that the precise primary sequence of the SNARE motif must be important. The SNARE motif point mutants were still...
capable of forming heteromeric complexes with all of the other SNAREs that have been implicated in vacuole fusion (Vam7p, Vti1p, Nyv1p, and Ykt6p) and did not inhibit fusion as severely as the SNARE motif replacements. The most plausible explanation for these findings is that the zero-layer mutations partially destabilized the core complex and that a highly stable core complex is required for full fusion. However, the alternative explanation that the SNARE motif may be involved in additional activities besides core complex formation, e.g. interactions with other proteins that may be dependent on the zero layer, is not excluded by these data. Such an alternative explanation would agree with previous evidence indicating that SNARE core complex assembly alone may not be sufficient to mediate a full fusion reaction (22). Independent of which explanation will prove to be correct, it is interesting that the conserved zero layer of the core complex appears to be less important for fusion than the specific primary sequence of a SNARE motif, despite the fact that SNARE motifs, which do not function together in vivo, form stable core complexes in vitro (48, 49). Furthermore, our data revealed that the short distance between SNARE motif and TMR in Vam3p is critical for vacuole fusion, supporting a role for core complex formation in forcing fusion of the membranes. Viewed together, these results provide strong evidence that forcing the membranes...
into close proximity is required for fusion but does not prove that such proximity is sufficient for fusion.

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REFERENCES
1. Ferro-Novick, S., and Jahn, R. (1994) Nature 370, 191–193
2. Götte, M., and Fischer von Mollard, G. (1998) Trends Cell Biol. 8, 215–218
3. Nichols, B. J., and Pelham, H. R. B. (1998) Biochim. Biophys. Acta 1404, 9–31
4. Jahn, R., and Sudhof, T. C. (1999) Annu. Rev. Biochem. 68, 863–911
5. Fasshauer, D., Sutton, R. B., Brunger, A. T., and Jahn, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15781–15786
6. Weimbs, T., Mostov, K. E., Low, S. H., and Hofmann, K. (1998) Trends Cell Biol. 8, 260–262
7. Sudhof, T. C., and Scheller, R. H. (2000) in Synapses (Cowan, M. W., Sudhof, T. C., and Stevens, C. F., eds), pp. 177–216, The Johns Hopkins Univ. Press
8. Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Germonos, S., Tempt, P., and Rothman, J. E. (1993) Nature 362, 318–324
9. Sollner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H., and Rothman, J. E. (1993) Cell 75, 409–418
10. Hayashi, T., McMahon, H., Yamashita, S., Binz, T., Hata, Y., Sudhof, T. C., and Niemann, H. (1994) EMBO J. 13, 5051–5061
11. Hanson, P. I., Roth, R., Morisaki, H., Jahn, R., and Heuser, J. E. (1997) Cell 90, 523–535
12. Sutton, R. B., Fasshauer, D., Jahn, R., and Brunger, A. T. (1998) Nature 395, 347–353
13. Conibear, K., and Scheller, T. H. (1998) Biochim. Biophys. Acta 1404, 211–230
14. Wendland, B., Emr, S. D., and Riezman, H. (1998) Curr. Opin. Cell Biol. 10, 513–522
15. Wickner, W., and Haas, A. (2000) Annu. Rev. Biochem. 69, 247–275
16. Wada, Y., Ohsumi, Y., and Nomoto, Y. (1992) J. Biol. Chem. 267, 18665–18670
17. Wada, Y., Nakamura, N., Ohsumi, Y., and Hirata, A. (1997) J. Cell Sci. 110, 1299–1306
18. Darsow, T., Reider, S. E., and Emr, S. D. (1997) J. Cell Biol. 138, 517–529
19. Fischer von Mollard, G., and Stevens, T. H. (1999) Mol. Biol. Cell 10, 1719–1732
20. Sato, T. K., Darsow, T., and Emr, S. D. (1998) Genetics 148, 85–98
21. Sato, T. K., Darsow, T., and Emr, S. D. (1998) Mol. Cell. Biol. 18, 5308–5319
22. Ungerermann, C., von Mollard, G. F., Jensen, O. N., Margolies, N., Stevens, T. H., and Wickner, W. (1999) J. Cell Biol. 145, 1435–1442
23. Hata, Y., Slaughter, C. A., and Sudhof, T. C. (1995) Nature 366, 347–351
24. Garcia, P. P., Gatti, E., Butler, M., Burton, J., and De Camilli, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2003–2007
25. Persner, J., Hsu, S.-C., and Scheller, R. H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1445–1449
26. Fernandez, L., Ubach, J., Dulaubova, I., Zhang, X., Sudhof, T. C., and Rizo, R. (1998) Cell 94, 841–849
27. Lerman, J. C., Rubble, J., Fairman R., and Hughson, F. M. (2000) Biochemistry 39, 8470–8479
28. Dulaubova, I., Sugita, S., Hill, S., Hosaka, M., Fernandez, L., Sudhof, T. C., and Rizo, J. (1999) EMBO J. 18, 4372–4382
29. Misura, K. M., Scheller, R. H., and Weis, W. I. (2000) Nature 404, 355–362
30. Verhage, M., Maia, A. S., Plomp, J. J., Bruusgaard, A. B., Heeroma, J. H., Vermeer, H., Toonen, R. F., Hammer, R. E., van den Berg, T. K., Missler, M., Geuze, H., and Sudhof, T. C. (2000) Science 287, 864–869
31. Fiebig, K. M., Rice, L. M., Pollock, E., and Brunger, A. T. (1999) Nat. Struct. Biol. 6, 117–123
32. Munson, M., Chen, X., Cocina, A. E., Schultz, S. M., and Hughson, F. M. (2000) Nat. Struct. Biol. 7, 894–902
33. Dulaubova, I., Yamaguchi, T., Wang, Y., Sudhof, T. C., and Rizo, J. (2001) Nat. Struct. Biol. 8, 258–264
34. Carr, C. M., Grote, E., Munson, M., Hughson, F. M., and Novick, P. J. (1999) J. Cell Biol. 146, 333–344
35. Yang, B., Steegmaier, M., Gonzalez, L. C., and Scheller, R. H. (2000) J. Cell Biol. 148, 247–252
36. Haas, A., Conradt, B., and Wickner, W. (1994) J. Cell Biol. 126, 87–97
37. Gerhardt, B., Kordas, T. J., Thompson, C. M., Patel and Vida, T. (1998) J. Biol. Chem. 273, 15818–15829
38. Klionsky, D. J., and Emr, S. D. (1989) EMBO J. 8, 2241–2250
39. Haas, A., Conradt, B., and Wickner, W. (1995) Methods Cell Sci. 17, 283–292
40. Vida, T. A., and Emr, S. D. (1995) J. Cell Biol. 128, 779–792
41. Gotte, M., and Gallwitz, D. (1997) FEBS Lett. 411, 48–52
42. Katz, L., and Brenwald, P. (2000) Mol. Biol. Cell 11, 3849–3858
43. Ossig, R., Schmitz, H. D., deGroot, B., Riedel, D., Keranen, S., Ronne, H., Grubmuller, H., and Jahn, R. (2000) EMBO J. 19, 6000–6010
44. Chen, Y. A., Scales, S. J., Patel, S. M., Doung, Y.-C., and Scheller, R. H. (1999) Cell 97, 165–174
45. Piper, R. C., Bryant, N. J., and Stevens, T. H. (1997) J. Cell Biol. 138, 531–545
46. Gerrard, S. R., Mecklen, A. B., and Stevens, T. H. (2000) Traffic 1, 45–55
47. McNew, J. A., Weber, T., Engelman, D. M., Sollner, T. H., and Rothman, J. E. (1999) Mol. Cell 4, 415–421
48. Fasshauer, D., Antonin, W., Margittai, M., Pabst, S., and Jahn, R. (1999) J. Biol. Chem. 274, 15440–15446
49. Yang, B., Gonzalez, L. J., Prekeris, R., Steegmaier, M., Advani, R. J., and Scheller, R. H. (1999) J. Biol. Chem. 274, 5649–5653
50. Robinson, J. S., Klionsky, D. J., Banta, L. M., and Emr, S. D. (1998) Mol. Cell. Biol. 18, 5813–5824
