Role of the Vam3p Transmembrane Segment in Homodimerization and SNARE Complex Formation†

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ABSTRACT: Intracellular membrane fusion in eukaryotic cells is mediated by SNARE (soluble *N*-ethylmaleimide sensitive factor (NSF) attachment protein receptor) proteins and is known to involve assembly of cognate subunits to heterooligomeric complexes. For synaptic SNAREs, it has previously been shown that the transmembrane segments drive homotypic and support heterotypic interactions. Here, we demonstrate that a significant fraction of the yeast vacuolar SNARE Vam3p is a homodimer in detergent extracts of vacuolar membranes. This homodimer exists in parallel to the heterooligomeric SNARE complex. A Vam3p homodimer also formed from the isolated recombinant protein. Interestingly, homodimerization depended on the transmembrane segment. In contrast, formation of the quaternary SNARE complex from recombinant Vam3p, Nyv1p, Vti1p, and Vam7p subunits did not depend on the transmembrane segment of Vam3p nor on the transmembrane segments of its partner proteins. We conclude that Vam3p homodimerization, but not quaternary SNARE complex formation, is promoted by TMS−TMS interaction. As the transmembrane segments of Vam3p and other SNARE homologues were previously shown to be critical for membrane fusion downstream of membrane apposition, our results may shed light on the functional significance of SNARE TMS−TMS interactions.

SNARE[1] (soluble *N*-ethylmaleimide sensitive factor (NSF) attachment protein receptor) proteins are essential for most types of intracellular membrane fusion reactions in eukaryotic cells (*1*−*3*). Supported and regulated by tethering factors and other accessory proteins, SNAREs that are localized to cognate membranes form a stable quaternary trans complex that leads to membrane apposition and thus paves the way to actual membrane fusion (*4*, *5*). In synapses, this complex is composed of synaptobrevin II, syntaxin 1A, and SNAP-25. Trans complex formation of these proteins depends on a coiled coil structure of the interacting cytoplasmic SNARE domains (*6*). According to the identity of the amino acid at the central hydrophilic zero layer of the coiled-coil, SNAREs are classified as Q- and R-SNAREs (*6*). Unfortunately, the crystal structure of the complete SNARE complex including the transmembrane segments (TMSs) of synaptobrevin II and syntaxin 1A is not known. In vivo, a fraction of the R-SNARE synaptobrevin II apparently does not participate in SNARE complex formation; rather, it appears to exist as a homodimer as suggested by cross-linking experiments performed on brain fractions (*7*−*10*) or visualization of fluorescently tagged molecules (*11*). Evidence for homodimerization has also been obtained for recombinant synaptobrevin II in detergent solution (*10, 12*−*14*) although the interaction appears to be of low affinity (*15, 16*). Based on mutagenesis and molecular modeling, synaptobrevin II dimerizes via sequence-specific interaction of its TMS that appears to form a tightly packed interface in the helix−helix dimer (*12*−*14, 17*). TMS-mediated homodimerization has also been shown for the Q-SNARE syntaxin 1A (*13, 14*), and the affinity of this interaction appears to exceed that of synaptobrevin II (*16*).

In yeast, a set of SNARE proteins is required for postmitotic fusion of fragmented vacuoles. Prior to fusion, the Q-SNAREs Vam3p, Vam7p, and Vti1p and the R-SNARE Nyv1p form a cis complex (*18*). Upon dissociation by the α-SNAP analogue Sec17p and the NSF analogue Sec18p, the subunits reassociate to a trans complex. Previous work has addressed the potential role of the Vam3p TMS in complex formation and membrane fusion. We have shown that Vam3p with an isoprenoid anchor in place of its TMS efficiently associated with its cognate partners in cis and in trans. On the other hand, isoprenylated Vam3p did not support vacuole−vacuole fusion to the extent of contents mixing (*19*) thus indicating a functional role of the TMS downstream of membrane apposition.

Here, we examined homodimeric and heterooligomeric complexes containing Vam3p in vivo and in vitro. Our current results reveal the existence of a Vam3p homodimer

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[1] Abbreviations: CD, circular dichroism; GSH, glutathione; GST, glutathione-*S*-transferase; SNARE, soluble *N*-ethylmaleimide sensitive factor attachment protein receptor; TMS, transmembrane segment.

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in detergent extracts of vacuolar membranes. Homodimerization is preserved with recombinant Vam3p and depends on the TMS. In contrast to that, the TMS appears not to play a role in homodimerization of the other vacuolar SNAREs nor in complex formation.

EXPERIMENTAL PROCEDURES

Analysis of Native SNARE Complexes by Glycerol Gradient Centrifugation. Vam3p was cloned with a GST-tag into a pRS317 CEN vector under the control of a NOP1 promotor, and expressed in BJ3505 (MATa pep4::HIS3 prb1-Δ1.6R HIS3 lys2-208 trp1Δ101 ura3-52 gal2 can) wild-type yeast cells. Vacuoles were isolated by DEAE lysis and Ficoll gradient centrifugation and incubated in reaction buffer (20 mM PIPES/KOH, pH 6.8, 125 mM KCl, 5 mM $MgCl_2$) in the absence or presence of an ATP-regenerating system as indicated (*20*). Vacuoles were then detergent solubilized in lysis buffer (0.5% (w/w) Triton X-100, 20 mM HEPES, pH 7.4, 150 mM NaCl, 1x PIC, 1 mM PMSF), incubated for 10 min at 4 °C on a nutator, and centrifuged for 10 min at 20000*g*. The cleared supernatant was applied to a linear 10−30% glycerol gradient (in lysis buffer) and centrifuged for 18 h at 40 000 rpm in an SW40 rotor. Fractions (1 mL) were collected from the top of the gradient. 10% of each fraction was removed as a loading control, and the rest was incubated for 2 h at 4 °C with 30 μL of GSH beads. The resin was washed three times in lysis buffer with decreasing Triton X-100 concentrations (0.5%, 0.1%, 0.025%, all w/v), and proteins were eluted by boiling for 5 min in SDS sample buffer.

Cloning of Vacuolar SNARE Proteins and Their Mutants for Prokaryotic Expression. Vam3p, Vti1p, Vam7p (pET28a plasmid, Novagen), and Nyv1p (pET15b) were kindly provided by Thomas Söllner (*21*). T7 promoter sequencing primer was used as a 5′-primer for all PCR. Vam3pΔTM (codons 1−263) was amplified using this primer and the 3′-primer 5′-CAGGGTCTCGAGACCGCATTTGTTACGGTCCCT-3′ by PCR and inserted between *Nco*I and *Xho*I sites into pET28a. Vti1pΔTM (codons 1−188) and Nyv1pΔTM (codons 1−226) were amplified with the 3′-primers 5′-ATTAGCGAATTCCCTTCTAGTCATTGTTTTTAG-3′ or 5′-AATTTTGGATCCTTCTGCCACCACATTATTTC-3′ and inserted between *Bam*HI and *Eco*RI sites into pET28a, or between *Nde*I and *Bam*HI sites into pET15b, respectively. Vam3pA4 was prepared by mutating codons 265, 269, 273, and 277 of Vam3p to alanine by site-directed mutagenesis using a Bio-Rad T7 mutagenesis kit according to the manufacturer's instruction. All constructs were verified by dideoxy sequencing.

Expression of Proteins in Escherichia coli. Plasmids encoding Vam3p or its mutants, Vti1p or Vti1pΔTM or Vam7p were transformed into Rosetta (DE3) cells (Novagen) for expression. The cells were grown in LB medium at 37 °C with chloramphenicol (30 μg/mL) and kanamycin (35 μg/mL) until $OD_{600} = 0.5-0.8$, when they were put on ice for 30−45 min. Subsequently, they were induced with 1 mM IPTG and shaken at 16 °C overnight (20 h). Rosetta (DE3) cells with Nyv1p or NyvlpΔTM were grown in LB medium at 37 °C with chloramphenicol (30 μg/mL) and ampicillin (200 μg/mL) until $OD_{600} = 0.5-0.8$ prior to induction with 1 mM IPTG and shaken at 37 °C for 3 h. Sec17p and Sec18p plasmids (pQE-9) were transformed into M15 cells (Qiagen) and expressed similar to Nyv1p.

Purification of the Proteins. The cells were lysed in buffer containing 25 mM HEPES, pH 7.6, 1 M NaCl, 2% (v/v) Triton X-100, and 2% (v/v) Thesit, 10 mM β-mercaptoethanol, and 20% (v/v) glycerol by sonication. Insoluble material was separated by centrifugation (19500*g* for 30 min at 4 °C). All proteins contain either an N-terminal or a C-terminal His_6-tag. For purification, the clear lysates were loaded onto HiTrap chelating HP columns (Pharmacia). The columns were washed with buffer containing 25 mM HEPES, pH 7.6, 1 M NaCl, 1% (v/v) Thesit, 10 mM β-mercaptoethanol, and 10% (v/v) glycerol with an imidazole concentration gradient (0−100 mM) and the proteins were eluted with the same buffer containing 500 mM imidazole. Proteins were dialyzed against 25 mM HEPES, pH 7.6, 1 M NaCl, 1% (v/v) Thesit, 10 mM β-mercaptoethanol, and 10% (v/v) glycerol to remove imidazole and stored at 4 °C at approximate protein concentrations of 20 μM.

SNARE Assembly and Disassembly. For homotypic assembly, individual SNARE proteins (∼5 μM, each) were carefully loaded onto 15−60% sucrose gradients containing 25 mM HEPES, pH 7.6, 1 M NaCl, 1% (v/v) Thesit or Triton X-100, and 10 mM β-mercaptoethanol and centrifuged for 15 h at 4 °C at 240000*g* in an SWTi 60 rotor in a Beckman ultracentrifuge. Twelve fractions were collected from the bottom with a syringe and precipitated with trichloroacetic acid. Proteins were subjected to 12.5% SDS−PAGE and visualized by Western blotting with respective antibodies and the chemiluminescence method. For SNARE complex assembly, Vam3p, Vti1p, Nyv1p, and Vam7p (each ∼5 μM, final concentration) or the respective mutants were mixed and incubated at 4 °C overnight in a 50 μL reaction volume. The protein mixture was subjected to sucrose gradient centrifugation as described above. Each fraction was immunoprecipitated with Vam3p antibody coupled to protein A agarose (*20*). Precipitated proteins were eluted from the pellet with 100 mM glycine, pH 2.5, and 0.5% (v/v) Thesit followed by TCA precipitation, SDS−PAGE, and Western blotting. For SNARE complex disassembly, the SNARE protein mixture was incubated at 4 °C with Sec18p (∼1.2 μM) and Sec17p (∼3 μM) plus 2 mM $MgCl_2$, 0.5 mM ATP or 0.5 mM ATPγS in a final volume of 100 μL for 1 h (*4*). The protein mixtures were then subjected to sucrose gradient centrifugation, TCA precipitation, and Western blotting.

Circular Dichroism Spectroscopy. All spectra were recorded on a JASCO J-715 model CD spectrometer with a scan speed of 50 nm/min and an average of 10−20 scans per sample. The spectra were recorded from 200 to 260 nm at 20 °C using a 0.1 cm path length quartz cuvette. Prior to analysis, proteins were dialyzed against 10 mM Tris-HCl pH 7.4, 150 mM NaCl, and 1% (v/v) Thesit since high salt concentrations give high background absorbance in the UV region. Spectra were recorded at equal protein concentrations (∼3 μM) for each type of protein as judged by comparing the optical density of Coomassie-blue stained bands on SDS−PAGE.

RESULTS

Native Vam3p Forms a Homodimer. To determine the oligomeric state of Vam3p protein on vacuole membranes,

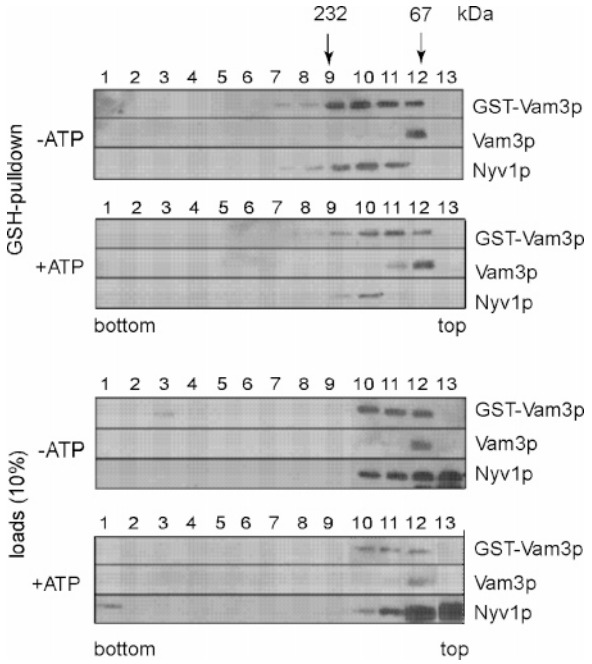

FIGURE 1: Analysis of the oligomeric Vam3p state on vacuoles. Purified vacuoles from the BJ3505 strain expressing wild-type and GST-Vam3p (180 μg) were incubated in the presence or absence of an ATP-regenerating system, then centrifuged (10 min, 12000*g*, 4 °C), and solubilized in 500 μL of lysis buffer for 10 min at 4 °C. The clarified supernatant was applied onto a 10−30% glycerol gradient and centrifuged for 18 h at 40000 rpm in an SW40 rotor. Fractions (1 mL) were then collected, 10% were removed as a loading control (bottom panels), and the remaining fractions were incubated with GSH beads for 2 h on a nutator. Beads were washed three times and proteins were eluted by boiling in SDS-sample buffer. Proteins were analyzed by SDS−PAGE and Western blotting. The experiments were done three times with similar results.

we isolated vacuoles from yeast strains expressing both wild-type Vam3p and a version where glutathione-*S*-transferase is fused to the N-terminus of the protein (GST-Vam3p). Both proteins are found on vacuoles as determined by subcellular fractionation, and GST-Vam3p can functionally complement the wild-type protein (*22*). Previously, it has been shown that Vam3p resides in a *cis*-SNARE complex together with Vam7p, Vti1p, and Nyv1p (*18, 20, 22*) and that Vam3p can self-associate (*23*). However, the oligomeric state of Vam3p was not resolved in the latter study. We decided to determine the molecular mass of the Vam3p/GST-Vam3p complex by separating the detergent-solubilized proteins by glycerol gradient centrifugation followed by GSH pulldown. As shown in Figure 1, protein complexes containing GST-Vam3p are distributed between the 67 kDa and the 232 kDa markers under these conditions. The major fraction of coprecipitating Vam3p is found in a ~67 kDa complex while coprecipitating Nyv1p is part of a complex peaking at ~170 kDa. Thus, GST-Vam3p appears to form a mixed homodimer (calculated molecular mass = 97 kDa) with Vam3p and, alternatively, be part of the four-membered *cis*-SNARE complex (calculated molecular mass = 163 kDa) together with Vam7p, Vti1p, and Nyv1p (*22*). As wild-type Vam3p does not coprecipitate together with Nyv1p, *cis*-SNARE complexes contain only one copy of Vam3p protein. Importantly, addition of ATP significantly reduced both the fraction of GST-Vam3p that is part of the ~170 kDa *cis*-complex and the amount of coprecipitated Nyv1p. The total protein amount of Nyv1p was not affected by ATP as shown in the loading control. Dissociation of the quaternary SNARE complex is therefore a result of priming and disassembly, mediated by endogenous Sec17p/Sec18p in agreement with earlier findings demonstrating partial complex disassembly in the presence of ATP (*20, 24*). On the other hand, preincubation of vacuoles with ATP did not affect coprecipitation of Vam3p and GST-Vam3p, revealing that homodimerization is not subject to Sec17p/Sec18p-mediated disassembly. Addition of known membrane fusion inhibitors (BAPTA, Gdi1p, GTPγS) or protein antibodies that block membrane fusion (α-Vam3p, α-Sec18p) also did not affect the interaction of the GST-Vam3p with wild-type Vam3p (data not shown). Under our conditions, we did not detect higher molecular weight complexes containing Vam3p that have previously been reported (*23*).

Homodimerization of Vam3p and of Other Vacuolar SNAREs. Here, we examined whether the ability to homodimerize in vivo is preserved with isolated recombinant Vam3p and whether homodimerization depends on the TMS. For comparison, self-assembly was also examined for other vacuolar SNARES, i.e., Vti1p, Nyv1p, and Vam7p.

All proteins were tagged with a hexa-histidine sequence, expressed in *E. coli*, solubilized with the detergent Thesit (or Triton X-100, were indicated) and purified by immobilized metal ion affinity chromatography. First, individual purified proteins were tested for self-assembly by SDS−PAGE under mild conditions. This method was previously used to demonstrate homodimerization of synaptic SNAREs (*10, 12, 14*). In case of vacuolar SNAREs, however, SDS−PAGE analysis did not reveal significant self-assembly (data not shown), which may be due to inhibition of protein−protein interactions by bound SDS molecules. Therefore, we investigated potential self-assembly of our proteins under nondenaturing conditions by sucrose gradient centrifugation. Migration of full-length proteins was compared to that of mutant versions, where the TMSs had been deleted or mutated. Gradient fractions were analyzed by Western blot analysis using specific antisera. Approximate molecular masses of the proteins in the gradients were estimated with reference to marker proteins run in parallel. As shown in Figure 2A, the distribution of recombinant Vam3p (36 kDa) peaks at the peak position of the 66 kDa marker, which is indicative of a homodimer. In addition, some of the protein migrates to higher molecular weight fractions that may correspond to low amounts of oligomeric species. Interestingly, the cytoplasmic fragment Vam3pΔTM (residues 1−263) migrates at ~30 kDa, which indicates a monomeric structure. A monomer was also seen with the Vam3pA4 mutant where residues V265, I269, V273, and V277 within the predicted TMS had been mutated to Ala. Densitometric analysis of the Western blot shown in Figure 2A reveals the distribution of the proteins at a more quantitative level (Figure 2B). A similar picture emerged, when the detergent Triton X-100 was used in place of Thesit (Figure 2C). Full-length Vti1p (29 kDa) as well as Vti1pΔTM (residues 1−188) proteins also peak at 66 kDa (Figure 2D). Thus, Vti1p also appears to form a homodimer, which, however, does not depend on the TMS. Full-length Nvy1p (33 kDa) and Nyv1pΔTM (residues 1−226) give broad distributions that peak at the position of the 29 kDa marker but extend into the high molecular mass region of the gradients (Figure

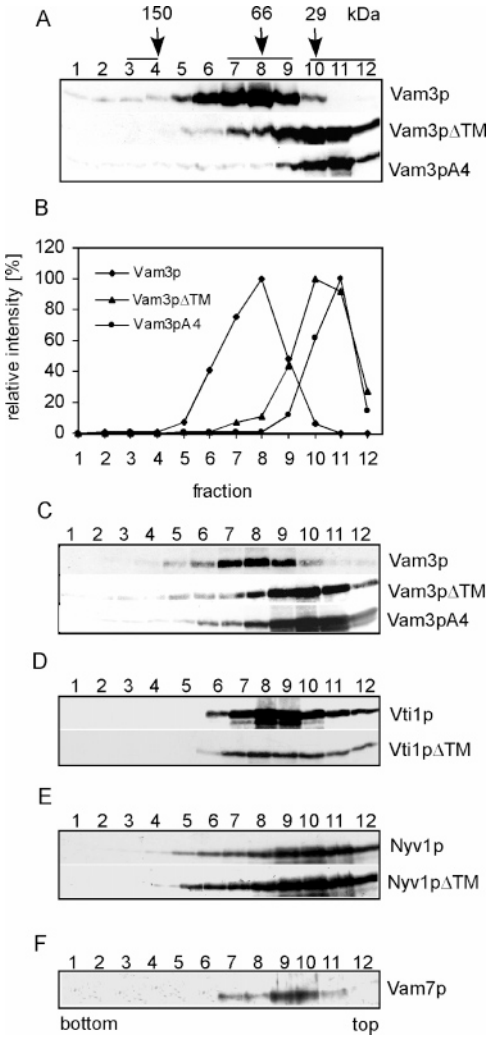

FIGURE 2: Homotypic assembly of recombinant SNAREs. Purified Vam3p, Vam3pΔTM, Vam3pA4, Vti1p, Vti1pΔTM, Nyv1p, Nyv1pΔTM, or Vam7p (~5 μM, each) were separately subjected to sucrose gradient (15−60% sucrose) centrifugation in 25 mM HEPES, pH 7.6, 1 M NaCl, 1% (v/v) Thesit (or Triton X-100 in C), and 10 mM β-mercaptoethanol. Fractions were analyzed by Western blotting with the respective antibodies. Fraction numbers are given at the top. For part B, the film corresponding to the Western blot shown in part A was evaluated by densitometry and the intensities of the bands relative to the intensities of the strongest bands (=100%) of each protein are shown. Vertical arrows indicate the fractions where the marker proteins (carbonic anhydrase, 29 kDa; serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa) predominated while horizontal lines denote all fractions containing significant amounts of the respective marker. All gradients run in the presence of Thesit were done eight times and the one with Triton X-100 was done four times with similar results.

2E). Nyv1p is thus mainly monomeric under these conditions but may assemble to homo-oligomers with low affinity. Vam7p does not contain a TMS but is anchored to the vacuolar membrane by binding to phosphoinositides via its N-terminal PX domain (*25, 26*). The corresponding Vam7p protein (40 kDa) runs at ~40 kDa, suggesting its monomeric nature (Figure 2F). It should be noted that using some other buffer salts, detergents, and lower ionic strength led to significant aggregation, especially in case of Vti1p.

We conclude that homodimerization of Vam3p, but not of Vti1p, depends on the TMS. Further, the disruptive effect of the A4 mutations reveals that the Vam3p TMS−TMS interaction is sequence-specific and suggests that the mutated

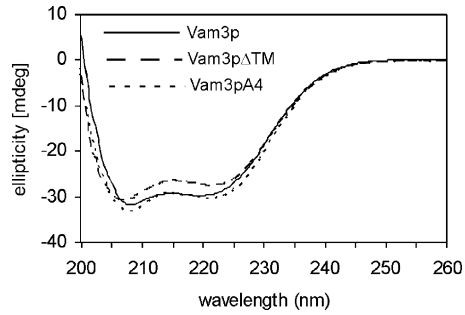

FIGURE 3: CD spectra of recombinant Vam3p, Vam3pΔTM, and Vam3pA4 proteins recorded in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Thesit. Protein concentrations were comparable (~3 μM, each) as judged by the Coomassie-staining intensities of protein bands in SDS−PAGE.

residues are part of the helix−helix interface. SNARE TMSs are believed to be α-helical (*14, 17, 27*). In principle, their deletion or mutation could lead to unfolding of the α-helical cytoplasmic assembly domains. To exclude such an indirect effect, we compared the secondary structures of the proteins tested here by circular dichroism (CD) spectroscopy at protein concentrations that were similar for each variant of a given SNARE subtype. The spectra of all Vam3p proteins, irrespective of deleting or of mutating the TMS, exhibit the characteristic line shapes of α-helices with strong negative molar ellipticities at 208 and 222 nm (Figure 3) (*28*). The secondary structures of Nyv1p, Vti1p, and Vam7 were also investigated by CD spectroscopy and also shown to correspond to highly α-helical structures with or without their respective TMSs (Supplementary Figure 1, Supporting Information). Thus, the secondary structures of vacuolar SNAREs are stable under the conditions used here and are not significantly influenced by the TMSs.

The SNARE Complex Does Not Depend on Transmembrane Segments. Here, we examined whether the Vam3p TMS influences the stability and/or multimerization of the SNARE complex. Recombinant Vam3p, Nyv1p, Vti1p, and Vam7p proteins were mixed at a stoichiometric ratio and analyzed for self-assembly. Significant complex formation could not be observed by SDS−PAGE analysis, even when sample boiling was omitted (data not shown). This is in contrast to the known stability of the synaptic SNARE complex in the presence of SDS (*29*) but in line with the SDS lability of the yeast exocytotic SNARE complex (*30*).

Therefore, vacuolar SNARE complex formation was examined by sucrose gradient centrifugation. To exclusively monitor Vam3p associated partner proteins, the gradient fractions were immunoprecipitated with a Vam3p antibody prior to Western blotting. As shown in Figure 4A, a significant part of all proteins migrated to gradient fractions 3−5 where none of the individual proteins had previously been seen (see Figure 3). These high molecular mass fractions are centered around the 150 kDa marker protein and thus likely to contain the quaternary complex with a 1:1:1:1 stoichiometry (calculated molecular mass: 138 kDa). This agrees with the in vitro assembly of the vacuolar SNARE complex as previously shown by coprecipitation experiments (*31*). Proteins seen in fractions 6 and 7 are likely to correspond to binary and ternary complexes of Vam3p bound to Vti1p, Nyv1p, and/or Vam7p. In a control experiment, we verified that the recombinant complex is an

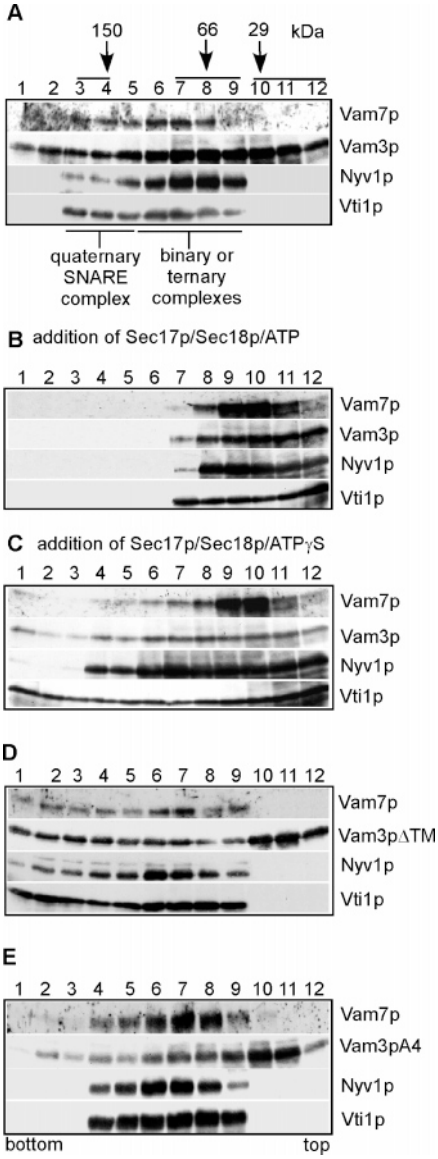

FIGURE 4: Assembly of recombinant SNARE complex. (A) Purified Vam3p, Vti1p, Nyv1p, and Vam7p (~5 μM, each) were mixed, incubated overnight, and subjected to sucrose gradient centrifugation in 25 mM HEPES, pH 7.6, 1 M NaCl, 1% (v/v) Thesit, 10 mM β-mercaptoethanol. The fractions were immunoprecipitated with Vam3p antibody, and the precipitates were analyzed by Western blotting with respective antibodies. Note the formation of high molecular weight complexes that are not seen with the individual proteins (see Figure 2). Vam7p, Vti1p, and Nyv1p are not present in fractions 10−12 as these proteins are not co-immunoprecipitated with Vam3p in these fractions. (B) Complexes formed under the same conditions as in part A were incubated with recombinant Sec17p (~3 μM) and Sec18p (~1.2 μM) plus 0.5 mM ATP and 2 mM $MgCl_2$ prior to sucrose gradient centrifugation. Note the disappearance of the high molecular weight fractions under this condition. (C) Complexes were formed and treated as in B except that ATP was replaced by ATPγS. Fractions were not immunoprecipitated with Vam3p antibody for B and C. (D) Complexes were formed as in A except that Vam3p was replaced by Vam3pΔTM. (E) Complexes were formed as in A except that Vam3p was replaced by Vam3pA4. The lack of proteins in fraction 3 is not a systematic observation and thus does not indicate reduced complex formation in this case. Fraction numbers and the positions of marker proteins are given at the top. All these experiment were done twice with similar results.

authentic counterpart of the SNARE complex found in vacuoles, rather than an unspecific aggregate. To this end, we tested whether it can be dissociated by Sec17p and Sec18p in an ATP-dependent way. Indeed, all complexes below fraction 7 are dissociated upon incubation with Sec17p/Sec18p in the presence of ATP (Figure 4B) but not of its nonhydrolyzable analogue ATPγS (Figure 4C). Thus, quaternary complex formation by our recombinant proteins is specific and reversible. To visualize all proteins irrespective of their association with Vam3p, gradient fractions were not co-immunoprecipitated prior to Western blot analysis in these experiments. Therefore, the gradients shown in Figure 4B,C also contain Vam7p, Nyv1p, and Vti1p in low molecular mass fractions 10−12. In a separate experiment we found that the recombinant Vam3p homodimer is not dissociated by Sec17p/Sec18p/ATP (results not shown); this agrees with the resistance of the native homodimer to ATP treatment (see: Figure 1).

To examine whether the Vam3p TMS influences SNARE complex formation, we assembled complexes that contained Vam3pΔTM or Vam3pA4 in place of wild-type Vam3p. In both cases, the complex at ~150 kDa and some lower molecular mass assemblies are as abundant as with wild-type Vam3p (Figure 4D,E). Additional high molecular mass forms were seen in fractions 1 and 2 when complexes contained Vam3pΔTM; this suggests that the SNARE complex tends to aggregate in this situation. In addition, we also analyzed complexes that do not contain any TMSs upon mixing Vam3pΔTM, Vti1pΔTM, Nyv1pΔTM, and Vam7p. This complex is indistinguishable from the complex containing Vam3pΔTM and its full-length partners (results not shown).

We conclude that recombinant vacuolar SNARE proteins assemble to a quaternary complex that is authentic as defined by its sensitivity to Sec17p/Sec18p. Further, complex formation does not depend in a detectable way on the TMS of Vam3p or of any other SNARE tested here.

DISCUSSION

Our results demonstrate that Vam3p in vacuolar membranes exists in at least two different noncovalent complexes. It assembles to a quaternary complex with Nvy1p, Vti1p, and Vam7p as known from previous co-immunoprecipitation experiments (*31*). Moreover, we show that a significant fraction of endogenous Vam3p is associated with coexpressed GST-tagged Vam3p in a complex that appears to correspond to a homodimer. Previously, it has been proposed that a fraction of Vam3p is associated with the GTPase Vps1p in a very high molecular mass (>2 MDa) complex and that Vam3p/Vam3p interaction depends on the presence of Vps1p (*23*). Under our experimental conditions, we were unable to detect this high molecular mass complex.

A mostly homodimeric form of Vam3p also forms from the purified recombinant protein. This dimer dissociates upon deletion or mutation of the TMS. Since these sequence alterations did not affect the secondary structure of the protein, these results imply that the TMS mediates homodimerization. Analyzed under the same conditions, homodimerization could also be shown for Vti1p, but not for Vam7p. Nyv1p is mainly monomeric but exhibits a tendency to self-assemble with low affinity. In contrast to Vam3p, the TMS did not contribute in a detectable way to self-assembly of either Vti1p or Nyv1p. Homodimerization via sequence-

specific TMS−TMS interaction was previously shown for the synaptic Q- and R-SNAREs synaptobrevin II and syntaxin 1A (*10, 12−14*). Our current results extend these observations in that they reveal conservation of TMS−TMS interaction in an evolutionarily distant member of the SNARE family, the Q-SNARE Vam3p. However, they also indicate that TMS-driven homodimerization may not be a universal feature pertaining to all SNARE proteins.

Furthermore, we examined whether the Vam3p TMS would stabilize the quaternary SNARE complex, e.g. via heterotypic interactions with TMSs from its cognate partners. The complex was reconstituted from recombinant proteins and detected by velocity gradient centrifugation followed by co-immunoprecipitation. The recombinant complex proved to be an authentic correlate of the native complex since it was readily dissociated by Sec17p/Sec18p/ATP. Since deleting the TMSs of Vam3p and its partners did not reduce the abundance of the quaternary complex, the TMSs apparently do not stabilize the heterotypic interactions. This is in line with previous observations where replacement of the Vam3p TMS by an isoprenoid anchor did not reduce the abundance of *cis*- or *trans*-SNARE complexes in vacuoles as examined by co-immunoprecipitation experiments in detergent solubilizates (*19*). Similar findings were reported for yeast exocytotic SNAREs Sncp and Ssop as coprecipitation of these proteins was unaffected upon TMS replacement by isoprenoid chains (*32*). These exocytotic SNAREs were suggested to form multimeric SNARE complexes based upon coprecipitation of epitope-tagged and wild-type SNAREs from high molecular mass velocity gradient fractions. Multimerization indeed appears to be important for exocytosis driven by synaptic (*33−37*) and endosomal mammalian SNAREs (L. Mascia and D.L., unpublished work). On the other hand, the molecular mass distribution of native or recombinant vacuolar SNAREs in the velocity gradients shown in the present study does not support the existence of multimeric complexes. We cannot exclude, however, that low affinity multimerization in vacuolar membranes is disrupted upon detergent treatment.

One unexpected result of our study is that all vacuolar SNAREs investigated here appear to be mainly α-helical in the isolated state as judged from the line shapes of the corresponding CD spectra. When we estimate secondary structure contents using the approximate protein concentrations (see Experimental Procedures), our data suggest that they exhibit ~80% α-helicity. This is in marked contrast to synaptic or yeast exocytotic SNAREs. There, cytoplasmic domains of syntaxin orthologues were shown to be only partially folded in isolation while SNAP-25 and synaptobrevin orthologues were completely unstructured. Binary assembly of exocytotic Q-SNAREs induces helicity and is thus thought to precede R-SNARE binding which results in a fully helical ternary complex (*30, 38*). This directed assembly process has been proposed to begin at domains that are more distal to the membrane surface and to proceed toward the membrane (*39, 40*). We propose that formation of the vacuolar SNARE complex proceeds from already folded subunits. We may speculate that this is a property that distinguishes symmetric fusion reactions, like vacuole−vacuole fusion, from asymmetric fusion, like in exocytosis. While R- and Q-SNAREs are simultaneously present on both membranes in the former case, different SNARE subunits reside on opposing membranes in the latter case.

What could be the functional role of Vam3p homodimerization driven by TMS−TMS interactions? Replacing the TMSs of Ssop, Sncp, or of Vam3p by isoprenoid anchors drastically affected the ability of these proteins to mediate membrane fusion (*19, 32*). Since trans complex formation and, by implication, membrane apposition were not disturbed in these experiments, the SNARE TMSs appear to be required for a downstream reaction. Interestingly, the fusion defect of isoprenylated Sncp and Ssop could be rescued by addition of lysophosphatidyl choline to the distal monolayers (*32*). This observation implied that fusion by isoprenylated proteins was trapped at the hemifusion intermediate where only the outer, but not the inner, bilayer leaflets mix. When synaptic SNAREs were expressed on the surface of eukaryotic cells ("flipped-SNAREs"), they induced fusion of the plasma membranes that, however, was arrested at hemifusion if the TMSs were replaced by glycosyl phosphatidylinositol anchors (*41*). Furthermore, the ratio of hemifusion to full fusion increased with decreasing concentrations of full-length synaptic (*42*) or yeast exocytotic (*27*) SNAREs in a liposome fusion assay. This concentration dependence is consistent with SNARE TMS−TMS interactions in the transition of hemifusion to complete bilayer merger. It is not clear whether SNARE homodimers act in parallel to heteromeric SNARE complexes. It is conceivable, however, that trans complex formation mediates membrane docking while the homodimers are required for complete fusion. In support of this hypothesis, a recent study suggests that synaptic SNAREs that are reconstituted at very low protein/lipid ratios do accelerate lipid mixing of liposomal membranes. Interestingly, this acceleration was observed even without concurrent trans complex formation, provided that the liposomes were aggregated by poly(ethylene glycol) (*43*).

NOTE ADDED IN PROOF

Very recently, evidence for TMS−TMS interaction was also obtained for the yeast secretory syntaxin homologue Sso1p by electron paramagnetic resonance spectroscopy (Zhang, Y., Shin, Y.-K. (2006) Transmembrane Organization of Yeast Syntaxin-Analogue Sso1p, *Biochemistry 45*, 4173−4181.)

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SUPPORTING INFORMATION AVAILABLE

Supplementary Figure 1 depicting CD spectra of recombinant Nvy1p and Ny1pΔTM (A), Vti1p and Vti1pΔTM (B), or Vam7p (C) in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Thesit. Protein concentrations were comparable as judged by the Coomassie-staining intensities of protein bands in SDS−PAGE. This material is available free of charge via the Internet at http://pubs.acs.org.

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