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Trans-SNARE Complex Assembly and Yeast Vacuole Membrane Fusion

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cis-SNARE complexes (anchored in one membrane) are disassembled by Sec17p (α-SNAP) and Sec18p (NSF), permitting the unpaired SNAREs to assemble in trans. We now report a direct assay of trans-SNARE complex formation during yeast vacuole docking. SNARE complex assembly and fusion is promoted by high concentrations of the SNARE Vam7p or Nyv1p or by addition of HOPS (homotypic fusion and vacuole protein sorting), a Ypt7p (Rab)-effector complex with a Sec1/Munc18-family subunit. Inhibitors that target Ypt7p, HOPS, or key regulatory lipids prevent trans-SNARE complex assembly and ensuing fusion. Strikingly, the lipid ligand MED (myristoylated alanine-rich C kinase substrate effector domain) or elevated concentrations of Sec17p, which can displace HOPS from SNARE complexes, permit full trans-SNARE pairing but block fusion. These findings suggest that efficient fusion requires trans-SNARE complex associations with factors such as HOPS and subsequent regulated lipid rearrangements.

homotypic fusion and vacuole protein sorting | Rab/Ypt

Regulated membrane fusion is essential for cell compartmentation. Intracellular fusion requires Rab-family GTPases, Rab-effector complexes, Sec1/Munc18 proteins, key regulatory lipids, and SNARE proteins (1). Most SNARE proteins are membrane-bound by a C-terminal apolar region or by a prenyl tail. SNARE proteins have membrane-proximal heptad repeat sequences, termed the SNARE motif. These proteins associate in alpha-helical, coiled-coil bundles as heteromeric SNARE complexes (2). Three glutamine residues and one arginine residue at the center of the four associated SNARE motifs, termed the zero layer, have a conserved role in SNARE function and categorize each SNARE as either Q- or R-SNARE (3). SNARE complexes are in cis when their apolar anchors are all in the same membrane bilayer or in trans when these anchors are in closely apposed membranes, poised for fusion (4). SNAREs and SNARE complexes associate with other factors, including Sec1/Munc18 proteins (5, 6), Ca^{2+}-sensors such as synaptotagmin (7–9), and others (10–12), for fusion. trans-SNARE pairs may promote membrane fusion by inducing local physical stress on the bilayer (13), destabilizing bilayer structure through their slanted transmembrane domains (14), or enriching membrane destabilizing lipids such as diacylglycerol at the fusion site (15). Despite their importance, there have been few reports of direct physical assay of trans-SNARE pairs (4, 16, 17).

We study membrane fusion with vacuoles from *Saccharomyces cerevisiae*. Vacuole fusion requires the Rab GTPase Ypt7p, its hexameric effector HOPS (homotypic fusion and vacuole protein sorting) complex, three Q-SNAREs (Vam3p, Vti1p, and Vam7p), one R-SNARE (Nyv1p), and key "regulatory" lipids (ergosterol, diacylglycerol, and phosphoinositides). At the start of *in vitro* vacuole fusion reactions, the chaperones Sec18p (yeast NSF) and Sec17p (α-SNAP) disassemble cis-SNARE complexes, freeing the SNAREs for association in trans. Vacuoles tether, supported by Ypt7p (18) and HOPS (19), and are drawn together until each pair of tethered vacuoles has disk-like regions of "boundary" membrane that are tightly apposed (20). Each of the key fusion factors (Ypt7p, HOPS, the SNAREs, and the regulatory lipids) become enriched at a ring-shaped microdomain surrounding the boundary membrane, termed the vertex ring (15, 20). SNARE pairing follows some time later and leads to complete fusion.

Yeast vacuoles isolated from *vam3*Δ or *nyv1*Δ strains cannot undergo homotypic fusion (21). However, vacuoles from *vam3*Δ strains fuse slowly with vacuoles from *nyv1*Δ strains, suggesting that these SNAREs pair in trans (21). Assays of the physical association of Vam3p and Nyv1p from vacuoles from *nyv1*Δ and *vam3*Δ strains, respectively, offered a direct assay of trans-SNARE pairing (4). However, these studies did not adequately distinguish SNARE pairs that were truly in trans from those that may have been trans but had been rendered cis by fusion, or from those that were formed *de novo* in cis after fusion. Engineering epitope tags on different SNAREs may permit the distinction between cis and trans complexes with vacuoles that are otherwise wild type in their fusion activity (17). We now report an assay of trans-SNARE complex formation with vacuoles that undergo normal rates and extents of fusion. Each vacuolar constituent that is needed for vertex ring enrichment is needed for trans-SNARE pairing, but either the phosphoinositide ligand myristoylated alanine-rich C kinase substrate effector domain (MED) or an excess of the SNARE chaperone Sec17p permit trans-SNARE complex formation while blocking the progression to fusion.

Results

We now combine an assay of trans-SNARE pairing with our standard fusion assay (22), in which proteases in vacuoles prepared from a *pho8*Δ strain gain access to the proPho8p in vacuoles from a *pep4*Δ strain and convert it to active Pho8p phosphatase. For epitope-tagged SNAREs to be useful in trans-SNARE pairing assays, the tag must be stable, *in vivo* and during vacuole isolation and incubation. Fusion must proceed at normal rates when the tagged SNAREs are the only forms of those SNAREs in a fusion reaction; otherwise, the fusion of vacuoles from X-*vam3*, *NYV1* with vacuoles from *VAM3*, Y-*nyv1*, where X and Y are fused epitopes, might be mediated by the wild-type Vam3p and Nyv1p. It is therefore necessary to assay the fusion of X-*vam3*, Y-*nyv1*, *pep4*Δ and X-*vam3*, Y-*nyv1*, *pho8*Δ vacuoles to ensure that the fusion functions of the tagged SNAREs are intact. Finally, to assay SNARE physical associations, a tag should allow for the specific isolation or detection of the tagged SNARE. We surveyed many fusions to the N or C terminus of Vam3p and Nyv1p and found that none fulfilled all of these criteria. We therefore surveyed sites of insertion of a short (25-residue) calmodulin-binding peptide (CBP) sequence (23), and found that its insertion into the coding sequence of Vam3p

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The authors declare no conflict of interest.

Abbreviations: HOPS, homotypic fusion and vacuole protein sorting; CBP, calmodulin-binding peptide; MED, myristoylated alanine-rich C kinase substrate effector domain.

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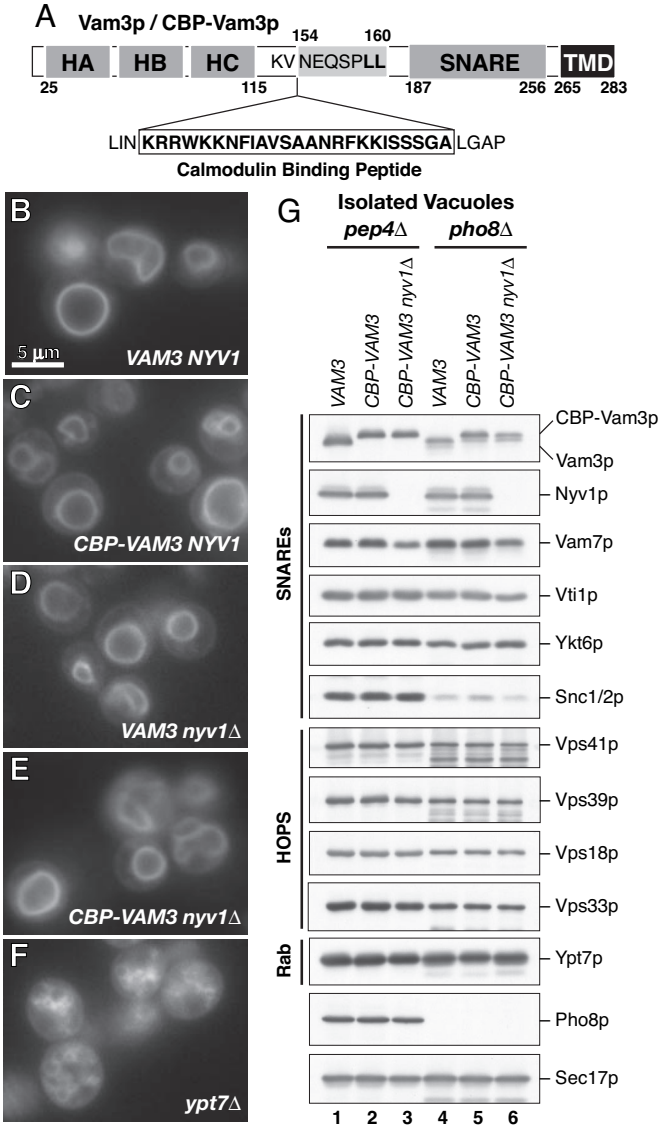


Fig. 1. Vam3p bearing an internal epitope tag is functional *in vivo*. (*A*) Vam3p domain structure. A CBP was inserted between the helices ABC N-terminal domain and the SNARE domain. The CBP was inserted N-terminal to the dileucine sorting motif. (*B–F*) Vacuole morphology in yeast cells expressing wild-type Vam3p or CBP-Vam3p. Overnight cultures of BJ3505 yeast cells and derivatives were stained in YPD with FM 4-64 (5 μM). Cells were concentrated by centrifugation, applied to glass slides, and overlaid with a coverslip. Micrographs of stained vacuoles were acquired as described (6). (*G*) Protein composition of isolated yeast vacuoles. Vacuoles from the indicated strains were heated in sample buffer. Proteins were separated by SDS/PAGE (5 μg per lane), transferred to nitrocellulose, and incubated with the indicated antibodies. Because vacuoles prepared from DKY6281 have active proteases, some protein degradation (e.g., Snc1/2p and Vps41p) occurred during isolation.

(Fig. 1*A*) did not disturb vacuole morphology, either in an otherwise wild-type strain (Fig. 1 *B* vs. *C*) or in the *nyv1*Δ background (Fig. 1 *D* vs. *E*). Nyv1p is the major R-SNARE that mediates vacuole fusion, but the ability of other R-SNAREs to substitute for its function (24) allows *nyv1*Δ strains to have normal vacuole morphology (Fig. 1*D*) as previously reported (21). Vacuoles from *pep4*Δ or *pho8*Δ strains expressing wild-type Vam3p or CBP-Vam3p shows normal levels of other SNAREs, HOPS subunits, or the Rab Ypt7p (Fig. 1*G*). Although we do see a synthetic defect in Vam7p localization to the vacuole when the CBP-Vam3p is paired with *nyv1*Δ (Fig. 1*G*, compare lanes 2 and 3), we can add recombinant Vam7p to fusion assays.

trans-SNARE pairing can be assayed with a mixture of vacuoles bearing CBP-Vam3p, *nyv1*Δ, and vacuoles with wild-type SNAREs, where the CBP-Vam3p from one fusion partner associates with Nyv1p from the other, whereas the wild-type Vam3p faces no Nyv1p at all on its fusion partner. We therefore systematically tested the functionality of the CBP-Vam3p in the presence or absence of Nyv1p on vacuoles from our *pep4*Δ and *pho8*Δ tester strains (Fig. 2*A*). Fusion required physiological temperature, was blocked by Gdi1p (which extracts Ypt7p), and required Nyv1p in at least one fusion partner, as previously reported (21). The presence of CBP-Vam3p on either or both fusion partners had a modest effect on fusion, reducing fusion to ≈50% (Fig. 2*A*). In the absence of Nyv1p on one fusion partner, further reduction of fusion was seen. However, fusion activity is restored in all cases by the addition of the Vam7p SNARE to the assays (Fig. 2*A*, open bars). Thus, CBP-Vam3p can mediate fusion when it provides the only source of Vam3p.

To determine whether CBP-Vam3p could directly interact with Nyv1p from acceptor membranes, vacuoles bearing CBP-Vam3p and deleted for Nyv1p were incubated with vacuoles with wild-type Vam3p and Nyv1p. Vacuole membranes were sedimented, solubilized in detergent, and the CBP-Vam3p was recovered with immobilized Ca^{2+}/calmodulin. CBP-Vam3p bound ≈1% of the Nyv1p from the acceptor vacuoles and reached a steady-state level of associations by 30 min (Fig. 2*B*, lanes 5–6) while fusion continued beyond this time. The other vacuolar SNAREs, Vam7p and Vti1p, showed coincident increase in CBP-Vam3p associations. Because these CBP-Vam3p vacuoles lack cis-SNARE complexes with Nyv1p, our findings suggest that association with Nyv1p in trans may stabilize Vti1p and Vam7p interactions with CBP-Vam3p. When detergent extracts from reactions containing only a single vacuole type were mixed, CBP-Vam3p showed little association with other SNAREs (Fig. 2*B*, lane 10). Thus Nyv1p is required for stable SNARE complex assembly, but it remained unclear whether these SNARE associations occur before fusion, form merely as a consequence of fusion, or some combination of the two.

We therefore tested various vacuole fusion inhibitors on the assembly of SNARE complex between Nyv1p and CBP-Vam3p. Vacuole fusion occurs in four functionally distinct stages: priming, tethering, docking, and fusion. Initially, vacuoles have unpaired Nyv1p, Vam3p, and Vti1p, bound by their C-terminal apolar anchor domains; however, the Vam7p SNARE, which has no apolar domain, is not stably bound to the vacuole without association with other SNAREs (6, 24). Priming is only needed to release free Vam7p for trans-SNARE complex assembly, as the addition of recombinant Vam7p allows fusion when priming is blocked (24). Although antibodies to Sec17p or to Sec18p prevent Nyv1p association with CBP-Vam3p and fusion (Fig. 3*A*, lanes 5 and 6), even low levels of added Vam7p stimulate fusion (lane 8 vs. 4) and relieve the ability of priming inhibitors to block fusion and Nyv1p associations with CBP-Vam3p (lanes 9 and 10). Elevated concentrations of Sec17p can block SNARE complex assembly (Fig. 3*A*, lane 7), but even in the presence of recombinant Vam7p, added Sec17p blocks fusion while permitting Nyv1p associations with CBP-Vam3p (lane 11). Thus normal cis-SNARE complex disassembly is required for subsequent SNARE complex assembly, and Nyv1p can enter a prefusion trans-SNARE complex with CBP-Vam3p.

Tethering, which requires Ypt7p and HOPS, is needed for SNARE pairing (Fig. 3*B*). The extraction of Ypt7p by added Gdi1p (Fig. 3*B*, lane 6), the premature activation of its GTPase activity by recombinant Gyp1–46p (lane 7), their combined actions (lane 8), or the addition of an antibody to Ypt7p (lane 9) prevents SNARE complex assembly and vacuole fusion. Antibody inhibition of both fusion and SNARE pairing is relieved by preincubating the antibody with its cognate peptide (Fig. 3*B*, lane 10). The HOPS complex is also needed for

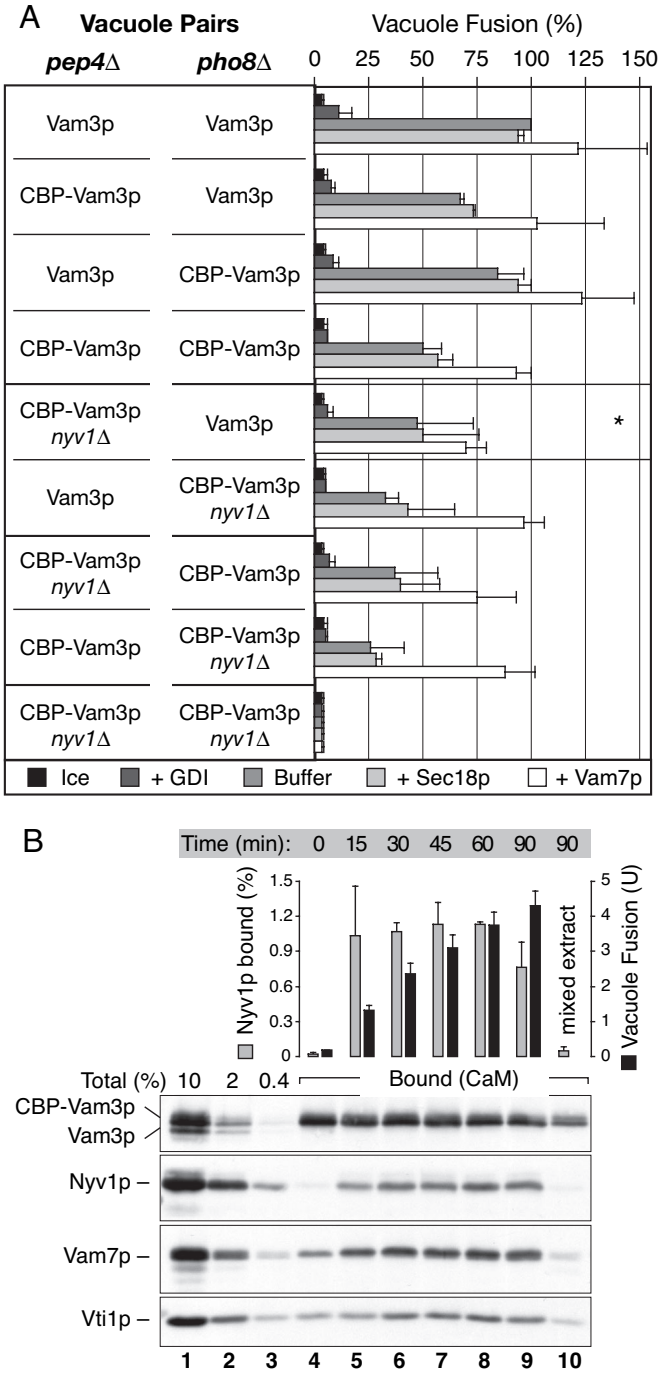


Fig. 2. CBP-Vam3p promotes vacuole fusion and SNARE complex assembly *in vitro*. (*A*) CBP-Vam3p, as the sole source of Vam3p, can mediate vacuole fusion. Vacuoles were isolated from the indicated strains (*pep4*Δ is BJ3505 and *pho8*Δ is DKY6281) expressing the indicated SNAREs and assayed under standard fusion conditions. Reactions were incubated at 27°C (or held on ice) for 90 min. Where indicated, GDI (1 μM), Sec18p (1.5 nM), or Vam7p (40 nM) were added from the start. Bars represent the average fusion activity of two independent experiments in which reactions bearing wild-type Vam3p and no fusion activator or inhibitor (middle gray bar) was set to 100%. Error bars indicate the range of the values in the two experiments. The asterisk highlights that combination of yeast vacuoles used for subsequent trans-SNARE interactions in this study. (*B*) Assay of SNARE complex assembly between CBP-Vam3p and Nyv1p. Vacuoles bearing CBP-Vam3p and deleted for Nyv1p were mixed with vacuoles from DKY6281. Reactions were incubated at 27°C for the indicated times after which vacuole fusion (black bars) and the associations of CBP-Vam3p with Nyv1p from the acceptor vacuoles (gray bars) and other SNAREs was measured by affinity purification from detergent extracts using immobilized calmodulin on agarose beads (see *Materials and Methods*). Two detergent extracts were also prepared from reactions containing a single vacuole type, a CBP-Vam3p *nyv1*Δ vacuole reaction, or a DKY6281 vacuole reaction. These two extracts were mixed and the CBP-Vam3p interactions with Nyv1p that occur in solution were examined (lane 10). The experiment shown was performed in duplicate and is representative of three independent trials. Bars indicate the average fusion and Nyv1p associations as determined by densitometry of the duplicates, and error bars represent the range of values between them.

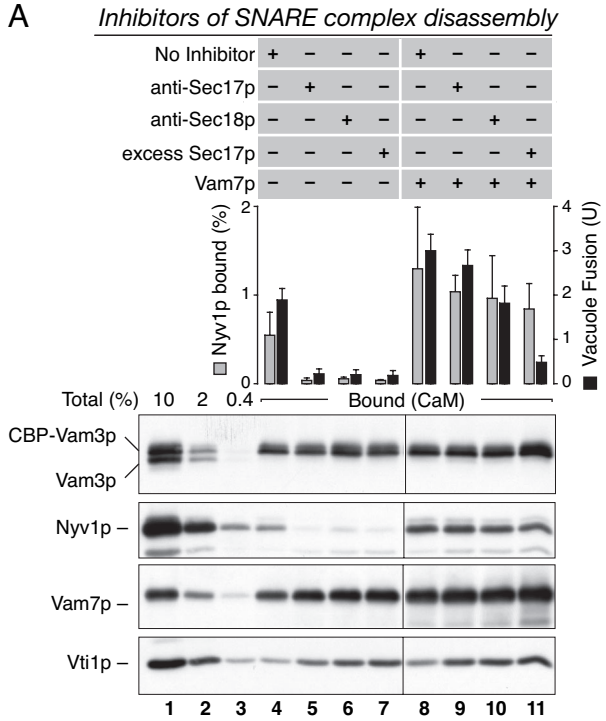
tethering (19), and antibody to its Vps33p subunit blocks SNARE pairing and fusion (Fig. 3*B*, lane 11). These results confirm that GTP-bound Ypt7p and its effector HOPS have functions before SNARE complex assembly.

Key regulatory lipids (ergosterol, diacylglycerol, and 3- and 4-phosphatidylinositides) are required for normal enrichment of fusion components at the vertex ring (15, 25). HOPS binds to phosphoinositides directly and through interaction with the PI (3)P-binding PX domain of Vam7p (19). We explored whether these lipids play a role during trans-SNARE complex assembly. MTM-1, a PI (3)P-specific phosphatase, SigD, a PI(4,5)P_2-specific PI (5)P phosphatase, FAPP PH domain, a ligand of PI (4)P, and ENTH domain, a ligand of PI(4,5)P_2, all inhibit trans-SNARE pairing (Fig. 4). We have previously reported that the MED polypeptide, which interacts with PI(4,5)P_2 and PI(3,4)P_2 (26), will prevent fusion but allow added Vam7p to bind to vacuoles in association with the other SNAREs and HOPS (6). We now find that this association includes trans-SNARE complex formation, as fusion is effectively blocked (Fig. 4, lane 5). MED inhibition of fusion can be reversed by added PI(4,5)P_2 (27), which suggests that MED is not irreversibly perturbing the vacuole membrane. Because we find that several inhibitors that affect PI(4,5)P_2 or its precursors also block trans-SNARE complex assembly and fusion while MED only prevents fusion, MED inhibition of vacuole fusion may reflect other interactions in addition to PI(4,5)P_2.

The levels of vacuolar trans-SNARE complex might be limited by the stoichiometry or availability of one of its constituents, such as one of the four SNAREs or perhaps HOPS, which associates with SNARE complexes that assemble late in the fusion reaction (6). As a result, trans-SNARE complex may be kinetically favored by elevated concentrations of HOPS or any of the SNAREs. We find that elevated concentrations of Vam7p promote SNARE complex formation and fusion (Fig. 5*A*). Added HOPS further stimulates trans-SNARE complex assembly and fusion; this stimulation was not inhibited by MED and thus occurs before fusion (Fig. 5*A*, lanes 7 and 8) while incubation with GDI and Gyp1–46p prevented this assembly (data not shown). Furthermore, although Nyv1p is present on vacuoles in stoichiometric excess over Vam3p (20), elevation of its level through genetic overexpression on the acceptor vacuole membranes (27) also enhanced trans-SNARE pairing and fusion (Fig. 5*B*, lanes 3 and 9; Y. Jun and W.T.W., unpublished data). This ability of enhanced levels of Vam7p, Nyv1p, or HOPS to increase the levels of prefusion trans-SNARE complex suggests that the assembly rate is proportional to their steady-state levels, rather than assembly being limited by the stoichiometry of one constituent.

Discussion

Although trans-SNARE complexes are crucial intermediates in fusion, their direct assay has been elusive and is often not distinguished from the more abundant cis-SNARE complexes. We now show that trans-SNARE assembly is sensitive to inhibitors of tethering, is promoted by elevated SNARE and Rab effector concentrations, and occurs before aqueous compartment mixing. Either elevated concentrations of Sec17p, a co-



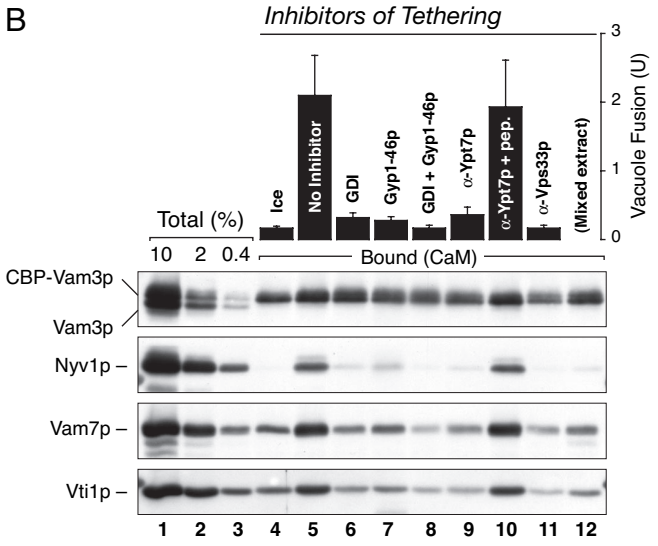


Fig. 3. Control of trans-SNARE complex assembly. (*A*) Testing cis-SNARE disassembly inhibitors and their bypass by Vam7p. Fusion reactions containing trans-SNARE tester vacuoles were incubated with buffer alone (lanes 4 and 8), antibodies to Sec17p (lanes 5 and 9) or Sec18p (lanes 6 and 10), or excess Sec17p (750 nM; lanes 7 and 11). Vam7p was then added where indicated (lanes 8–11). Reactions were incubated (27°C, 45 min) and fusion (black bars) and CBP-Vam3p associations with Nyv1p (gray bars) and other SNAREs were assayed. Data are representative of three independent experiments. Bars represent the average fusion and Nyv1p associations found in three (without Vam7p) or four experiments (with Vam7p). Error bars indicate the standard deviation of the values obtained. (*B*) Role of Ypt7p and HOPS. Fusion reactions with 40 nM Vam7p were incubated with buffer alone (lanes 4 and 5), GDI (lane 6), Gyp1-46p (lane 7), GDI and Gyp1-46p (lane 8), antibodies to Ypt7p (lane 9), antibodies to Ypt7p premixed with cognate peptide (lane 10), or antibodies to Vps33p (lane 11). Reactions were incubated (27°C, 60 min), and fusion (*Top*) and CBP-Vam3p association with other SNAREs assayed. Fusion values are the average of three independent experiments and error bars represent the standard deviation. SNARE associations are representative of three independent experiments.

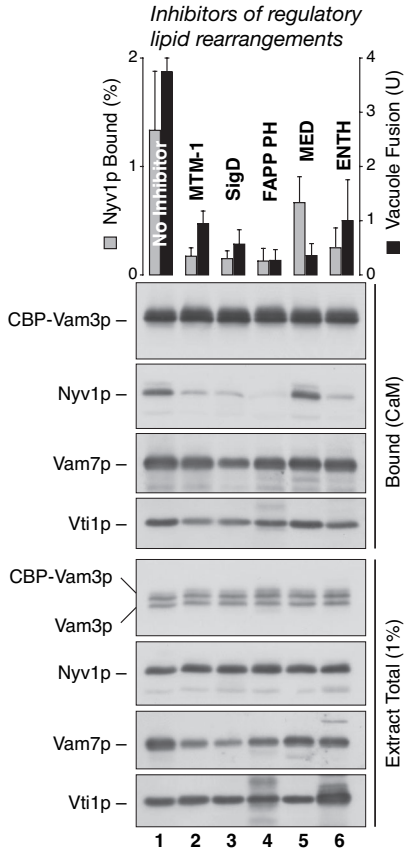


Fig. 4. Regulatory lipid requirements during trans-SNARE complex assembly. Fusion reactions were incubated with Vam7p with no added inhibitors (lane 1) or with the indicated inhibitors (lanes 2–6). After incubation (45 min, 27°C), reactions were placed on ice and fusion (black bars) and CBP-Vam3p associations with Nyv1p (gray bars) and other SNAREs were assayed. Data are representative of four experiments whose results were averaged; error bars represent the standard deviation of the values obtained after densitometric analysis of Nyv1p associations.

chaperone of cis-SNARE complex disassembly, or the lipid ligand MED will allow trans-SNARE complex assembly while preventing fusion.

Sec17p and MED may act through a shared inhibition of HOPS–SNARE interactions. Without added Vam7p, Sec17p prevents the vertex accumulation of HOPS and SNAREs (20, 25), physically disrupts HOPS–SNARE interactions (6), and prevents trans-SNARE complex assembly (Fig. 3). Added Vam7p bypasses the requirement for Sec17/18p function to liberate Vam7p for trans-SNARE complex assembly (24). In the presence of added Vam7p, elevated concentrations of Sec17p permit trans-SNARE complex formation while preventing fusion. Sec17p may block fusion through direct interactions with the trans-SNARE complex. Recent studies of the binding of Sec1/Munc18 proteins, complexin, and synaptotagmin to the synaptic SNARE complex suggest intriguing parallels to our results. Sec1/Munc18 proteins bind to SNARE complexes (28, 29). Complexin interacts with and stabilizes synaptic SNARE complexes next to the membrane proximal region (10). Synaptotagmin, when bound to Ca^{2+}, can displace complexin to facilitate the transition from hemifusion to fusion (9, 30, 31). Like complexin, Sec17p/SNAP binds to the SNARE complex and its binding may also preclude the binding of another factor, such as HOPS.

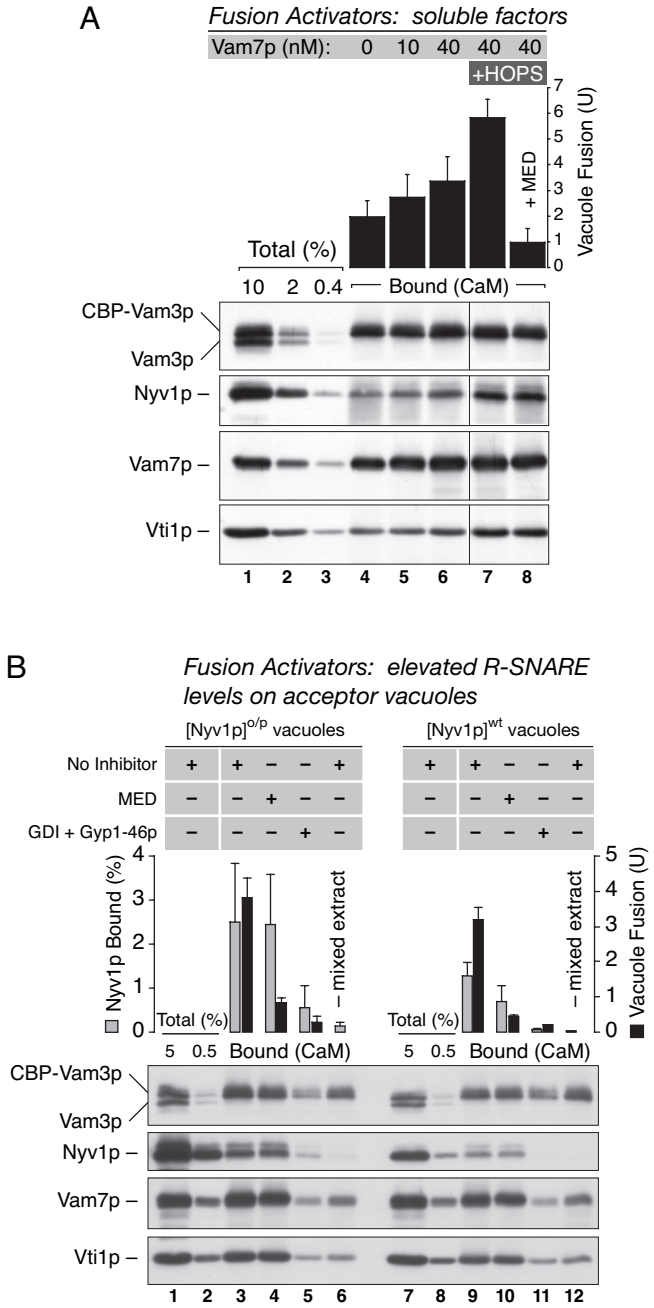


Fig. 5. Promotion of trans-SNARE complex assembly. (*A*) Added Vam7p and HOPS. Fusion reactions contained added Vam7p. Purified HOPS complex (5 μg/ml) was added after Vam7p addition (lanes 7 and 8), and MED (lane 8) was added to one HOPS + Vam7p reaction. Vacuole fusion and CBP-Vam3p associations were assayed after incubation (45 min, 27°C). Data are representative of three independent experiments. Bars represent the average fusion data from the three trials, and error bars represent their standard deviation. (*B*) Elevated Nyv1p on acceptor vacuoles. Fusion reactions were initiated with vacuoles from BJ3505 CBP-Vam3p *nyv1*Δ and a DKY6281 strain which either overproduced Nyv1p from the ADH1 promoter element ([Nyv1p]$^{o/p}$, lanes 1–6; Y. Jun and WTW, unpublished data) or bore wild type levels of Nyv1p ([Nyv1p]^{wt}, lanes 7–12). After incubation (45 min, 27°C), reactions were placed on ice, and fusion (black bars) and associations of CBP-Vam3p with Nyv1p (gray bars) and other SNAREs were assayed. Vacuole detergent extracts from reactions containing a single type of vacuole (CBP-Vam3p *nyv1*Δ, Nyv1p$^{o/p}$, or Nyv1p^{wt}) were also prepared as in Fig. 2*B*. These reaction extracts were mixed to determine the extent of Nyv1p associations that occurred in the extract (lanes 6 and 12). Three independent experiments were averaged, and error bars represent the standard deviation of the fusion values and the Nyv1p associations as determined by densitometry.

HOPS has direct affinity for phosphoinositides and ligands to phosphoinositides cause HOPS release from vacuoles (19). Because HOPS promotes trans-SNARE complex assembly, we suggest that HOPS binding to the SNARE complex is required for HOPS and/or SNARE localization to the vertex for fusion, consistent with a role for SNAREs in HOPS and lipid localization (15, 25). Because Sec17p can block fusion after trans-SNARE assembly, HOPS may stabilize the forward pathway and directly occlude Sec17/18p-mediated trans-SNARE complex disassembly. Just as synaptotagmin displaces complexin during a late step of membrane fusion, Sec17p alone or in combination with Sec18p may similarly be able to associate with the prefusion trans-SNARE complex (4).

trans-SNARE pairing is undoubtedly required for fusion, but it may not be rate limiting. Elevated concentrations of HOPS or the SNAREs Vam7p or Nyv1p stimulate trans-SNARE assembly to a greater extent than they promote fusion (Figs. 3 and 5). Tethered vacuoles that are deprived of Vam7p undergo SNARE pairing within minutes of its addition, yet complete fusion, as assayed by aqueous compartment mixing, occurs much more slowly (27). Lipid bilayer rearrangements are thought to be the terminal events of fusion; our finding that MED blocks fusion after trans-SNARE pairing is in accord with this idea. MED may generally inhibit vacuolar lipid rearrangements or may interfere with essential interactions of SNAREs with lipids (32). There are conserved, basic residues in SNARE proteins between the SNARE motif and the transmembrane domain (33). MED is a basic polypeptide and may interfere with SNARE binding to lipids during the transition from a proposed hemifusion intermediate to aqueous compartment mixing. trans-SNARE pairs may contribute to fusion through the enrichment of other fusion factors (15, 20, 25), by aligning microdomains of the membrane that are poised for fusion and by exerting physical stress on the bilayer (13). There is no quantitative data as to the relative importance of each of these to fusion. In each regard, it is of fundamental importance to be able to directly measure trans-SNARE assembly and fusion in an assay system with normal rates of fusion.

Materials and Methods

Yeast Strains. The *S. cerevisiae* strains for the vacuole fusion assay include BJ3505 [MATα *pep4*::*HIS3 prb1-Δ1.6R his3-Δ200 lys2-801 trp1-Δ101 (gal3) ura3-52 gal2 can1*] and DKY6281 (MATα *pho8*::*TRP1 leu2-3 leu2-112 ura3-52 his3-200 trp1-901 lys2-801 suc2-9*). The CBP coding sequence was inserted within the Vam3p ORF by modifying an existing vector (pFA6a-kanMX6-PGAL1-GST) (34). A DNA fragment containing the VAM3 promoter (300 bases upstream of initiator ATG) and coding sequences for the first 154 amino acids was amplified from yeast genomic DNA by PCR using two oligonucleotides, one with a flanking BglII site (GAAGATCT-CATATAGTTTACCTAGGTGCT) and the other with a flanking PacI site (CGCGTTAATTAAGTTTACTTTTATAGAA-ATATA). The GAL1 promoter was excised from pFA6a-kanMX6-PGAL1-GST by BglII/PacI digestion and replaced with the digested VAM3 promoter and partial coding region. A DNA fragment containing the CBP coding sequence was amplified from pBS1479 (23) by PCR using two oligonucleotides, one containing a flanking PacI site (TCCCCCTTAATTAACAAGAGAAGAT-GGAAAAAGAATTTC) and the other with a flanking AscI site (TGGCGCGCCAAGTGCCCCGGAGGATGAGAT). The GST coding sequence was excised by PacI and AscI digestion and replaced with the digested CBP coding sequence. This vector (pFA6a-kanMX6-PVAM3–2) was used as template for PCR with two oligonucleotides containing flanking homology with the VAM3 locus (TGTACAATAAATTAGGTTGTTTTCCT-CAGGATAAAAGTGATCTATTTGTAAGAATTCGAGCT-CGTTTAAAC and CTGTAATTGGTGTTGTCCTTCGTTA-TGCAGTAAAGGACTCTGCTCGTTCGCGCCAAGTGC-

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CCC). This PCR product was transformed into BJ3505 and DKY6281 yeast strains, and all G418-resistant transformants had recombined via appended VAM3 homology engineered downstream of the CBP sequence. NYV1 was disrupted with the natMX4 cassette (35) using PCR products amplified with homology flanking the NYV1 coding sequence (AGCGACAATTTATTAAGCTGTTAGAGCATTGGACTTTTATATTTTTACCAAAGATTGTACTGAGAGTGCAC and GGAACAAAAGAAATACAACCGTTATTAATGTTATTGTCGTGGGACAGCTCCCTGTGCGGTATTTCCACCG). A DKY6281 strain overexpressing Nyv1p from the ADH1 promoter (Fig. 5*B*) was constructed as described previously (27).

Vacuole Fusion and Reagents. Yeast vacuoles were prepared by flotation through discontinuous Ficoll gradients (22). Standard fusion reactions were performed at 27°C in PIPES/sorbitol buffer (20 mM Pipes/KOH, pH 6.8, 200 mM sorbitol) containing 125 mM KCl, 5 mM $MgCl_2$, 10 μM CoA, 38.6 μg/ml Pbi2p (IB_2), an ATP regeneration system (1 mM ATP, 1 mM $MgCl_2$, 0.5 mg/ml creatine kinase, 3 mM creatine phosphate) and 3 μg of each vacuole type. One unit of vacuole fusion activity yields 1 μmol of *p*-nitrophenol per minute per milligram of BJ3505 vacuole protein. Vacuole fusion inhibitors and activators have been described previously (6, 15, 19, 27, 36) and were used at the following concentrations: His_6-Sec17p (24 μg/ml), anti-Sec17p IgG (225 μg/ml), anti-Vam3p Fab fragments (3 μg/ml), Gdi1p (60 μg/ml), Gyp1–46p (230 μg/ml), anti-Ypt7p peptide antibody (3 μg/ml), Ypt7p peptide (66 μg/ml), anti-Sec18p (7 μg/ml), anti-Vps33p peptide antibody (23 μg/ml), MTM-1 (115 μg/ml), SigD (115 μg/ml), GST-FAPP PH domain (600 μg/ml), MED (10 μM), and GST-ENTH (570 μg/ml). Vam7p was purified as a chitin-binding domain fusion protein, eluted by intein cleavage, and added at 40 nM unless indicated. HOPS complex was purified from a yeast strain coexpressing each subunit from a GAL1 promoter; details of this strain's construction and use in HOPS purification will be published elsewhere. Bovine brain calmodulin (Calbiochem, San Diego, CA) was labeled with EZ-Link Sulfo NHS-LC-LC biotin (Pierce, Rockford, IL).

Assay of trans-SNARE Complexes. Standard trans-SNARE and fusion assays were 11× the scale of fusion assays alone (above) and contained 33-μg vacuoles from BJ3505-CBP-Vam3p *nyv1*Δ and 33-μg vacuoles from DKY6281. After incubation, reactions were placed on ice (5 min), 30 μl was withdrawn to assay Pho8p maturation, and the remainder (300 μl) was centrifuged (11,000 × *g*, 5 min, 4°C). The pellet was overlaid with ice-cold solubilization buffer [200 μl; 20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM imidazole, 0.5% Triton X-100, 20% glycerol, 5 mM β-mercaptoethanol, 1× protease inhibitor mixture (0.46 μg/ml leupeptin, 3.5 μg/ml pepstatin, 2.4 μg/ml pefabloc-SC, 1 mM PMSF)], resuspended, and solubilization buffer was added to a final volume of 400 μl. The extracts were mixed on a nutator rocker and then centrifuged (16,000 × *g*, 20 min, 4°C). Ten percent of the extract was removed for a total sample and the remaining extract was brought to 2 mM $CaCl_2$. The CBP-Vam3p was recovered with biotinylated bovine brain calmodulin (0.3–3 μg) and NeutraAvidin agarose (Pierce, 20–50 μl per 400 μl) with rocking overnight. In some experiments, the biotinylated calmodulin was bound to NeutrAvidin agarose before its addition to extracts (200 μg of protein per milliliter of beads). Beads were collected by centrifugation (3,000 × *g*, 2 min, 4°C) and suspended four times with solubilization buffer containing 0.1% Triton X-100 and 2 mM $CaCl_2$ (600 μl each) followed by bead sedimentation. Bound proteins were eluted with solubilization buffer containing 0.1% Triton X-100 and 5 mM EGTA. The eluates were precipitated with trichloroacetic acid, acetone washed, and heated in sample buffer (94°C, 5 min) for SDS/PAGE and immunoblotting. ECL exposed films were quantitated using a BioRad Molecular Imager GS-800 Densitometer using Quantity One software. All data are representative of experiments performed at least three times.

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