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Sec17 can trigger fusion of *trans*-SNARE paired membranes without Sec18

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Sec17 [soluble *N*-ethylmaleimide–sensitive factor (NSF) attachment protein; α-SNAP] and Sec18 (NSF) perform ATP-dependent disassembly of *cis*-SNARE complexes, liberating SNAREs for subsequent assembly of *trans*-complexes for fusion. A mutant of Sec17, with limited ability to stimulate Sec18, still strongly enhanced fusion when ample Sec18 was supplied, suggesting that Sec17 has additional functions. We used fusion reactions where the four SNAREs were initially separate, thus requiring no disassembly by Sec18. With proteoliposomes bearing asymmetrically disposed SNAREs, tethering and *trans*-SNARE pairing allowed slow fusion. Addition of Sec17 did not affect the levels of *trans*-SNARE complex but triggered sudden fusion of *trans*-SNARE paired proteoliposomes. Sec18 did not substitute for Sec17 in triggering fusion, but ADP- or ATPγS-bound Sec18 enhanced this Sec17 function. The extent of the Sec17 effect varied with the lipid headgroup and fatty acyl composition of the proteoliposomes. Two mutants further distinguished the two Sec17 functions: Sec17^{L291A,L292A} did not stimulate Sec18 to disassemble *cis*-SNARE complex but triggered the fusion of *trans*-SNARE paired membranes. Sec17^{F21S,M22S}, with diminished apolar character to its hydrophobic loop, fully supported Sec18-mediated SNARE complex disassembly but had lost the capacity to stimulate the fusion of *trans*-SNARE paired membranes. To model the interactions of SNARE-bound Sec17 with membranes, we show that Sec17, but not Sec17^{F21S,M22S}, interacted synergistically with the soluble SNARE domains to enable their stable association with liposomes. We propose a model in which Sec17 binds to *trans*-SNARE complexes, oligomerizes, and inserts apolar loops into the apposed membranes, locally disturbing the lipid bilayer and thereby lowering the energy barrier for fusion.

membrane fusion | SNAREs | α-SNAP | NSF

Intracellular vesicular traffic between organelles is the basis of cell growth, hormone secretion, and neurotransmission. At each step of exocytic and endocytic trafficking, membranes dock and fuse, mixing their lipids and luminal contents while keeping them separate from the cytosol. Families of proteins, conserved from yeast to humans, mediate docking and fusion. Fusion requires Rab family GTPases and "effector" proteins that bind to a Rab in its active, GTP-bound state (1). Among the effectors are large, organelle-specific tethering complexes. Fusion requires SNARE proteins and their chaperones. SNAREs (2) are proteins that can "snare" (bind to) each other, in *cis* (when anchored to the same membrane) or in *trans* (when anchored to apposed, tethered membranes). SNAREs have a conserved "SNARE domain" with a characteristic heptad repeat. SNAREs are categorized as R-SNAREs if they have a central arginyl residue, or Qa-, Qb-, or Qc-SNAREs with a central glutamyl residue (3). SNAREs form RQaQbQc quaternary *cis*- or *trans*-SNARE complexes, which bind SNARE chaperones, including the Sec1/Munc18 family of SNARE binding proteins, and Sec18/NSF (*N*-ethylmaleimide–sensitive factor), an AAA family ATPase that drives SNARE complex disassembly (4). Sec17/α-SNAP (soluble NSF attachment protein) is a cochaperone to Sec18 that enhances its rate of SNARE complex disassembly (5).

We study fusion with yeast vacuoles. The homotypic fusion of vacuoles has been studied extensively through genetic identification of vacuole morphology (*vam*) mutants (6) and vacuole protein sorting (*vps*) mutants (7), through a colorimetric assay of the fusion of isolated vacuoles (8), and more recently through the fusion of proteoliposomes reconstituted with defined, purified proteins and lipids (9–11). Sec17, Sec18, and ATP catalyze the first stage of vacuole fusion, in which *cis*-SNARE complexes are disassembled (12). Tethering is then supported by the Rab Ypt7 and the large, multisubunit tethering complex termed HOPS (13). Vps33, one of the HOPS subunits, is the vacuolar SM (Sec1/mUNC-18 family) protein. HOPS has direct affinity for vacuolar SNAREs (14–16), and helps to catalyze SNARE complex assembly and the subsequent fusion (17).

During in vitro fusion incubations, most Sec17 is released from vacuoles during *cis*-SNARE complex disassembly (12). However, a few percent of the vacuolar SNAREs form *trans*-SNARE complexes (18), and Sec17 is a major constituent of these complexes (19). Furthermore, although Sec17 and Sec18 can disassemble *trans*-SNARE complexes (19) and will block fusion events in which tethering is supplied by an unphysiological agent such as polyethylene glycol (13), Sec17 and Sec18 work synergistically with HOPS to promote fusion (9, 20). This synergy is even seen when the SNAREs are initially disposed with the R-SNARE on one set of proteoliposomes and the three Q-SNAREs on the others (9), a condition that per se does not require *cis*-SNARE complex disassembly by Sec18. Finally, added Sec17 restores fusion to vacuoles where fusion is blocked by a defined C-terminal truncation in the SNARE domain of the Qc-SNARE Vam7, in the apparent absence of ATP or Sec18 activity (21). These observations prompted us to reevaluate the roles of Sec17 and Sec18 in the fusion pathway.

Significance

Intracellular membrane trafficking relies on SNARE proteins from apposed membranes to form *trans*-complexes. Sec18 (*N*-ethylmaleimide–sensitive factor; NSF) and its cochaperone Sec17 (soluble NSF attachment protein; α-SNAP) disassemble *cis*-SNARE complexes, liberating SNAREs for *trans*-complex assembly. We now describe an additional function of Sec17, its ability to trigger the fusion of *trans*-SNARE paired membranes. We propose a model in which Sec17 oligomerizes on *trans*-SNARE complexes, inserting apolar loops into the adjacent membranes. This precisely localized membrane interaction may disturb the lipid bilayer, lowering the energy barrier that prevents the two membranes from merging, and thereby facilitate fusion.

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We now exploit proteoliposomes bearing purified vacuolar Rab and SNAREs to reinvestigate the roles of Sec17 and Sec18. Generous amounts of Sec18 alone can disassemble *cis*-SNARE complexes, allowing proteoliposomes bearing all four vacuolar SNAREs to fuse at a moderate rate. The rate of fusion can be stimulated by wild-type Sec17, as expected, but also by a Sec17 mutant that has greatly diminished capacity to stimulate Sec18. This suggested that Sec17 could act in ways other than through Sec18 stimulation. We therefore examined fusion incubations where the SNAREs are disposed on complementary proteoliposomes such that Sec18 is not required at all. We find that Sec17 can trigger rapid fusion of proteoliposomes that are already joined by *trans*-SNARE associations.

Results

Sec17 and Sec18 disassemble *cis*-SNARE complexes so that SNAREs can associate in *trans* (12, 22). We used our reconstituted reaction of proteoliposome fusion with defined proteins and lipids (9–11) to examine whether Sec17 and Sec18 have separable activities. Sec18, at high levels, promoted a slow fusion reaction without Sec17 (Fig. 1*A* and Fig. 1*B*, first column) whereas, as expected, Sec17 alone did not support fusion when the SNARES are in *cis*-complex (Fig. 1*B*, row *a*). With 10 nM Sec18, fusion strictly required Sec17. Sec17^{L291A,L292A}, modeled on an α-SNAP mutation that does not stimulate NSF (5, 21), did not suffice (Fig. 1*B*, row *b*). Higher levels of Sec17 inhibited fusion, as noted in earlier studies of fusion of the intact organelle (23). With higher Sec18 concentrations (Fig. 1*B*, rows *c* and *d*), Sec17^{L291A,L292A} stimulated Sec18-dependent fusion. With 100 nM Sec18, the fusion seen with 64 nM Sec17^{L291A,L292A} was comparable to that seen with 2 nM wild-type Sec17 (Fig. 1*B*, *c3* and *c8*), suggesting that Sec17^{L291A,L292A} is about 1/32 as active as wild-type Sec17. However, with 10 nM Sec18, there was far more fusion with, for example, 8 nM Sec17 than with 256 nM Sec17^{L291A,L292A} (Fig. 1*B*, *b5* and *b10*). It was thus difficult to fully explain the observed pattern by considering Sec17 only as a cofactor to Sec18. An alternative explanation was that Sec17 fulfills a second function. To seek this function, we sought fusion conditions where Sec17 could be tested in the absence of Sec18.

Sec17 Can Function Without Sec18. To remove the requirement for Sec18, we sought conditions where the SNAREs in a fusion incubation were not initially in complex with each other. Although the Qb-SNARE Vti1 has a C-terminal transmembrane anchor, we have reported (17) that fusion does not depend on this anchor. When vacuolar mixed lipid (VML) proteoliposomes with 18:2 fatty acyl composition and bearing Ypt7–GTP and the R-SNARE Nyv1 were mixed with proteoliposomes with Ypt7–GTP and the Qa-SNARE Vam3, fusion could be triggered by the addition of a mixture of HOPS, soluble Vti1 lacking its membrane anchor (termed sVti1), and the inherently soluble Qc-SNARE Vam7 (Fig. 2*A*). The four SNAREs were separate from each other when they were introduced into these fusion incubations, that is, there were no *cis*-SNARE complexes to disassemble and therefore the reaction did not require Sec18 or ATP. This fusion reaction (Fig. 2*A*) showed a modest but consistent stimulation by Sec17, until by 6.4 μM the stimulation was overcome by the inhibitory effect of Sec17, as noted before. To optimize the stimulation by Sec17, we varied the lipid composition of the proteoliposomes. We have recently reported that acidic lipids (24) and small-headgroup lipids (25) have specific, important roles in the fusion reaction.

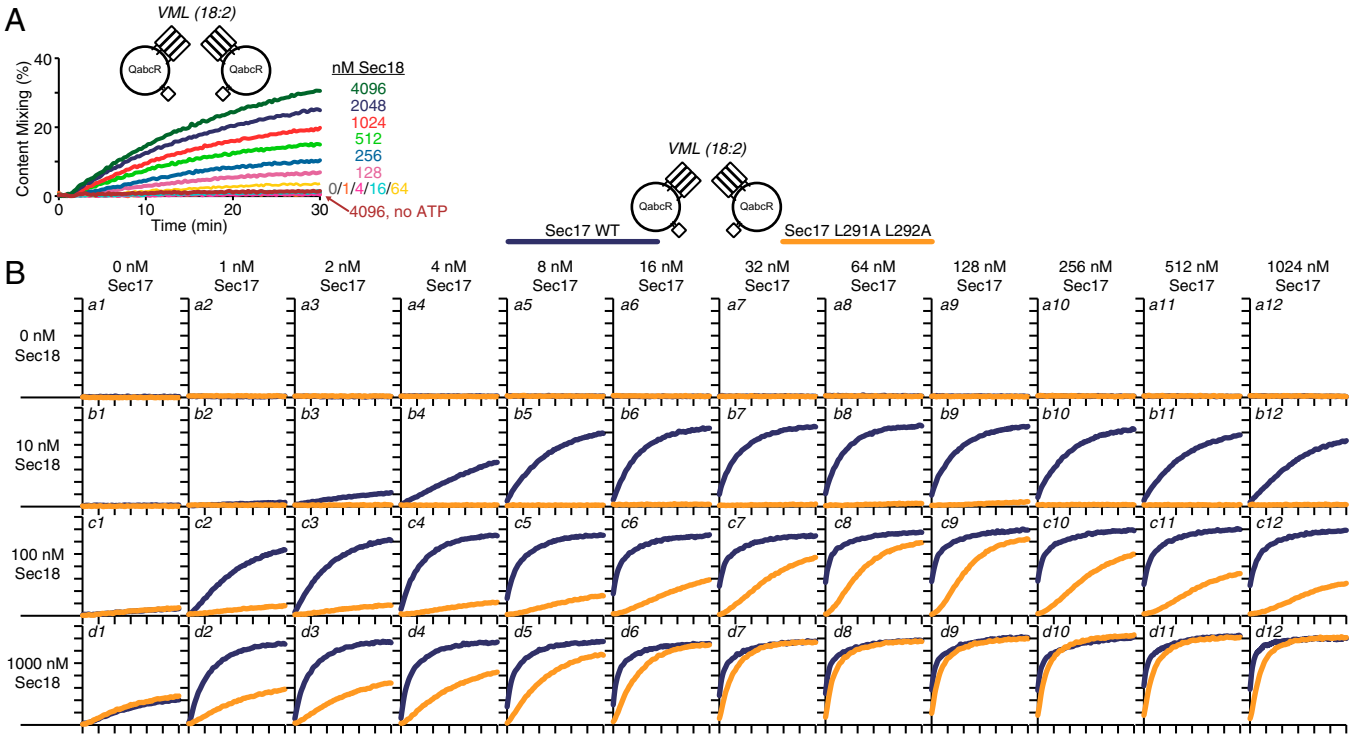

Fig. 1. Mutant Sec17 that is defective in stimulating Sec18 ATPase activity is still able to stimulate four-SNARE–four-SNARE fusion reactions. (*A*) Fusion reactions contained RPLs of vacuolar mixed lipids (18:2) and the four vacuolar SNAREs at a 1:1,000 protein:lipid ratio. Reactions were initiated by addition of 50 nM HOPS, 1 mM ATP (unless specified otherwise), and increasing concentrations of Sec18. (*B*) Four-SNARE fusion reactions as in *A* were initiated with 50 nM HOPS, 1 mM ATP, increasing concentrations of Sec18 (*Top* to *Bottom* rows), and increasing concentrations of Sec17 (*Left* to *Right* columns). The added Sec17 was either the wild-type version (WT; blue curves) or a version that carries mutations that impair Sec17 stimulation of Sec18 (L291A and L292A; orange curves). The *x* axis represents 0–30 min, and the *y* axis represents 0–80% content mixing. Kinetic curves of content mixing assays in this and subsequent figures are representative of $n \geq 3$ experiments.

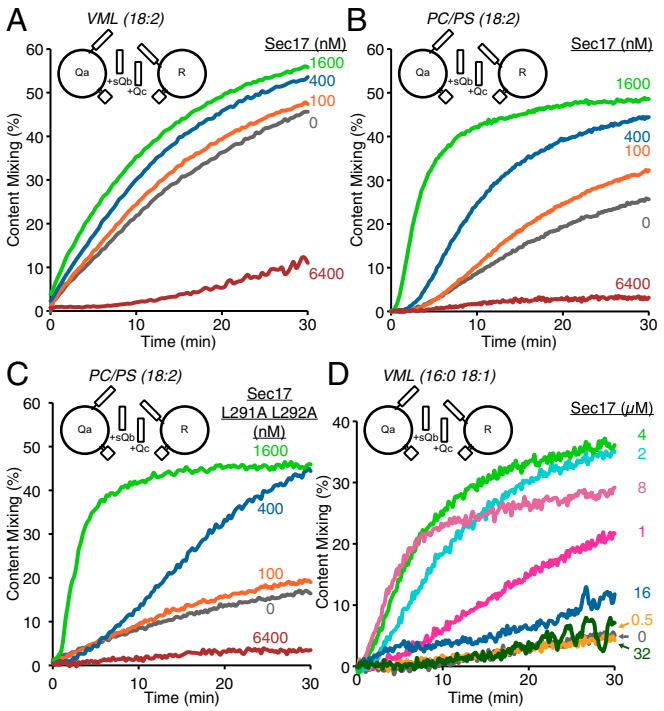

Fig. 2. Sec17 can stimulate fusion reactions without Sec18. (*A*) Fusion reactions had a mixture of RPLs of VMLs (18:2) bearing either the R-SNARE Nyv1 (1:10,000 protein:lipid ratio) or the Qa-SNARE Vam3 (1:2,000 protein:lipid ratio). The Qb-SNARE Vti1 without its transmembrane domain (500 nM) and the Qc-SNARE Vam7 (50 nM) were added, and fusion was initiated by adding 50 nM HOPS and increasing amounts of Sec17. (*B*) Reactions as in *A*, but with RPLs composed of PC/PS (18:2) lipids. (*C*) Reactions as in *B*, but with a mutant version of Sec17 (L291A and L292A). (*D*) Reactions as in *A*, but with RPLs composed of VMLs (16:0 18:1), and with 1 μM sQb- and 1 μM Qc-SNAREs.

Proteoliposomes of only PC and PS were less active than those of vacuolar lipid composition but were strongly stimulated by the addition of Sec17 (Fig. 2*B*) in the absence of Sec18 or ATP. $Sec17^{L291A,L292A}$ was just as active as wild-type Sec17 in stimulating this Sec18-independent fusion (Fig. 2*C*), in marked contrast to its diminished capacity to support Sec18-dependent fusion (Fig. 1*B*, row *b*). Fusion of similar proteoliposomes with a vacuolar lipid composition but with fatty acyl chains 16:0 and 18:1 also required added Sec17 (Fig. 2*D*). Thus, both lipid headgroup and fatty acyl compositions strongly affected the degree to which fusion required added Sec17.

Sec17 Stimulates Fusion After *trans*-SNARE Pairing. It has been suggested that fusion is largely regulated through controlling *trans*-SNARE pairing (26). However, tethering factors and nonbilayer-prone lipids are essential for fusion beyond modulating the level of *trans*-SNARE pairing (20, 25). We therefore assayed both fusion and *trans*-SNARE pairing between proteoliposomes bearing Ypt7–GTP and the R-SNARE Nyv1 and those with Ypt7–GTP and the Qa-SNARE Vam3. Fusion was initiated in duplicate incubations by the addition of soluble protein mixtures, either sVti1, Vam7, HOPS, and Sec17 (Fig. 3*A*, sample b) or this mixture with each of these proteins singly omitted (samples a and c–f). Fusion required each of these soluble proteins, including Sec17 (Fig. 3*A*, *Left*). After 2 min of incubation, one of each of the duplicate incubations was solubilized with detergent and assayed for Nyv1 that was associated with Vam3 by immunoprecipitation with immobilized antibody to Vam3 and immunoblot for Nyv1 (Fig. 3*A*, *Right*). For one sample, which initially did not have Vam7, Vam7 was added with the detergent (sample e). Although Sec17 was essential for fusion (Fig. 3*A*, *Left*), the presence or absence of Sec17 had little effect on the level of *trans*-SNARE complex (Fig. 3*A*, *Right*, a vs. b). The *trans*-SNARE complex that had formed in the absence of Sec17 was not somehow defective, because addition of Sec17 after 10 min triggered a dramatic burst of fusion at a rate that increased with Sec17 concentration (Fig. 3*B*). High concentrations of Sec17 that were present from the start of the incubation inhibited fusion (Fig. 3*C*), as noted (Figs. 1*B* and 2), but the burst of fusion based on

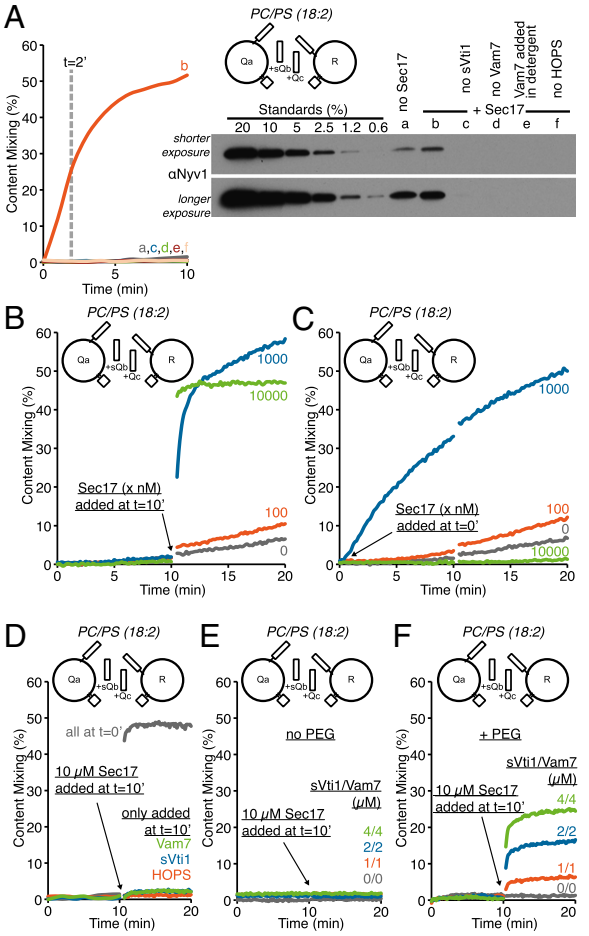

Fig. 3. Sec17 triggers fusion of *trans*-SNARE paired membranes. (*A*) Fusion reactions had RPLs of PC/PS (18:2) lipids bearing either the R-SNARE Nyv1 (1:2,500 protein:lipid ratio) or the Qa-SNARE Vam3 (1:2,500 protein:lipid ratio). The reactions (a–f) were started by adding 636 nM soluble version of the Qb-SNARE Vti1, 304 nM Qc-SNARE Vam7, 66 nM HOPS, and 1 μM Sec17, or their respective buffers. After 2 min, parallel incubations were lysed in detergent and subjected to immunoprecipitation with anti-Vam3 antibodies. For sample e, Vam7 was added to the detergent lysate before immunoprecipitation. All samples were analyzed by SDS/PAGE and Western blotting with anti-Nyv1 antibodies, reflecting the amount of Vam3–Nyv1 *trans*-interactions. (*B*) Fusion reactions had RPLs composed of PC/PS (18:2) lipids bearing either the R-SNARE Nyv1 (1:10,000 protein:lipid ratio) or the Qa-SNARE Vam3 (1:2,000 protein:lipid ratio). A soluble version of the Qb-SNARE Vti1 (500 nM) and the Qc-SNARE Vam7 (50 nM) were added, and the reactions were initiated by addition of 50 nM HOPS. After 10 min, the indicated concentrations of Sec17 were added. (*C*) Fusion reactions as in *B*, but the indicated concentrations of Sec17 were added with HOPS at $t = 0$ min. (*D*) Similar fusion reactions as in *B*, but sVti1 (500 nM), Qc (50 nM), and HOPS (50 nM) were either all added at $t = 0$ min or withheld individually and only added at $t = 10$ min. Sec17 (10 μM) was added at $t = 10$ min. (*E* and *F*) Fusion reactions as in *B*, but without HOPS and with higher concentrations of the soluble SNAREs sVti1 (0–4 μM) and Vam7 (0–4 μM). The soluble SNAREs were added at $t = 0$ min either without a tethering agent (*E*) or with 2% (wt/vol) PEG 8000 (*F*) as a chemical tether. Sec17 (10 μM) was added at $t = 10$ min.

Sec17 addition to preformed SNARE complexes showed no such inhibition (Fig. 3*B*). The burst of fusion seen upon addition of Sec17 after 10 min of incubation relied on preformed *trans*-SNARE complex, as shown by its dependence on the presence of Vam7, sVti1, and HOPS during the initial incubation period (Fig. 3*D*). Although HOPS and Ypt7 were optimal for tethering, the artificial tethering agent polyethylene glycol (PEG) could also support the generation of a fusion-competent state. This reaction was also dependent on the soluble SNAREs, although at higher concentration, and was triggered by Sec17 addition (Fig. 3 *E* and *F*).

Might the capacity of Sec17 to trigger the fusion of proteoliposomes with *trans*-SNARE complexes be limited to reactions with sVti1, lacking its physiological transmembrane domain? Proteoliposomes that bore Ypt7–GTP and either the R-SNARE Nyv1 or the Qa- and Qb-SNAREs Vam3 and full-length Vti1 were mixed with HOPS and Vam7. As reported (9, 17), fusion did not require Sec17 or Sec18. However, the addition of Sec17 after 10 min triggered a concentration-dependent burst of fusion (Fig. 4*A*). Thus, although the C-terminal truncation of Vti1 may enhance the effect, Sec17 also triggered fusion when wild-type SNAREs were disposed such that SNARE complex disassembly was not required. Under these conditions, rapid SNARE complex assembly was followed by slow fusion without Sec17. Sec17 also triggered rapid fusion between proteoliposomes bearing all three Q-SNAREs and those bearing the R-SNARE (Fig. 4*B*). Whereas 8 or 32 μM Sec17 was able to trigger a rapid burst of fusion, the inhibition at intermediate levels of Sec17 (2 μM) was presumably due to the inhibition of further *trans*-SNARE complex formation (as in Fig. 3 *B–D*) without triggering a substantial burst of fusion first. These findings were similar to observations with intact vacuoles, where docking occurs ~20 min before fusion (12, 27).

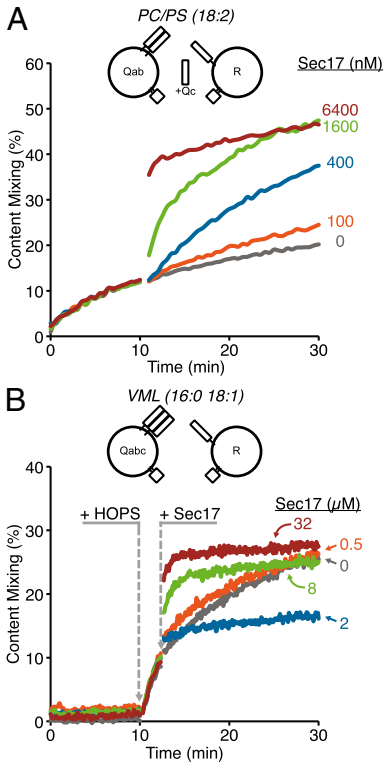

Fig. 4. Sec17 can stimulate fusion reactions with all wild-type SNAREs. (*A*) Fusion reactions had RPLs composed of PC/PS (18:2) lipids bearing either the R-SNARE Nyv1 (1:10,000 protein:lipid ratio) or both the Qa-SNARE Vam3 and the Qb-SNARE Vti1 (1:10,000 protein:lipid ratio). The Qc-SNARE Vam7 (50 nM) was added, and tethering was initiated by addition of 50 nM HOPS. After 10 min the indicated amounts of Sec17 were added. (*B*) Fusion reactions had RPLs composed of VMLs (16:0 18:1) bearing either the R-SNARE Nyv1 (1:1,000 protein:lipid ratio) or the three Q-SNAREs Vam3, Vti1, and Vam7 (1:1,000 protein:lipid ratio). The RPLs were mixed and incubated for 10 min before 50 nM HOPS was added. After 2 min, the indicated amounts of Sec17 were added.

Without ATP Hydrolysis, Sec18 Enhances the Sec17 Fusion Trigger. Can Sec18 affect the capacity of Sec17 to trigger fusion of *trans*-SNARE paired proteoliposomes? Mixed proteoliposomes bearing Ypt7–GTP and either Nyv1 or Vam3 were incubated with sVti1, Vam7, and HOPS for 10 min and then mixed with 50 nM Sec17, a concentration where it has little effect on the rate of fusion (Figs. 3*B* and 5*A*). Supplementation of the Sec17 with up to 640 nM Sec18 and ATP had little effect (Fig. 5*A*). However, supplementation with Sec18 + ADP (Fig. 5*B*) or Sec18 + ATPγS (Fig. 5*C*) gave a strong stimulation that remained fully dependent on Sec17 (Fig. 5*D*). Sec18 cannot disassemble SNARE complexes with ADP or ATPγS, but the affinities of Sec18 for both SNAREs and Sec17 may facilitate their association at low Sec17 levels.

Importance of the Apolar Loop of Sec17. The N-terminal apolar loop of Sec17 (28), residues 19–30, might stimulate fusion through bilayer penetration. A recent structure of the 20S supercomplex (29), which was determined by single-particle electron cryomicroscopy, has shown that this loop is poised next to the SNARE transmembrane segments when Sec17 oligomers are in complex with a four-helical SNARE bundle, which might be envisioned as a *cis*-SNARE complex (29) or as a *trans*-SNARE complex (Fig. 6*A*). Proteoliposomes of full vacuolar lipid mix, Ypt7–GTP, and the four SNAREs did not fuse when given only 10 nM Sec18 (Fig. 1*B*, row *b* and Fig. 6*B*, *b1*) unless they were also supplied with Sec17 (*b2–b6*, blue curves). Substitution of polar residues for hydrophobic residues within the loop by mutations F21S and M22S had almost no effect under Sec18-dependent fusion conditions (Fig. 6*B*, orange curves). However, in incubations with PC/PS proteoliposomes bearing Ypt7–GTP and either the R-SNARE Nyv1 or the Qa-SNARE Vam3, supplemented with sVti1, Vam7, and HOPS, $Sec17^{F21SM22S}$ could not trigger a burst of fusion (Fig. 6*C*, orange curves). The two activities of Sec17 were thus distinguished in three manners: by assays that exclusively require the SNARE complex disassembly function or the fusion-triggering function; through Sec17 mutants that are selectively disabled for each of these functions; and by their exclusive cooperation with either Sec18–ATP or Sec18–ADP.

For another model of these interactions, we incubated liposomes of PC/PS with Sec17 or $Sec17^{F21S,M22S}$ and the mixed recombinant vacuole SNAREs lacking their transmembrane domains, termed sSNAREs (soluble SNAREs). After incubation, liposomes were reisolated by flotation and assayed for bound proteins (Fig. 7). When the sSNAREs and Sec17 were coincubated with liposomes, they each floated (Fig. 7*A*, lane b), whereas Sec17 alone or sSNAREs alone showed much less association with liposomes (lanes a and c). Sec17 is known to oligomerize upon association with SNAREs (29–31), which may multiply its affinity for lipids. Incubations of soluble SNARE domains and $Sec17^{F21S,M22S}$, deficient in the apolar character of its loop domain, showed little association of either the sSNAREs or mutated Sec17 with the liposomes beyond the backgrounds seen in separate incubations (Fig. 7*A*, lane d vs. lanes a and e).

Discussion

Sec17/α-SNAP, which stimulates SNARE complex disassembly by Sec18/NSF, can drive rapid fusion of proteoliposomes that have already formed *trans*-SNARE pairs. These two activities of Sec17 are distinguished in four ways. (*i*) Four-SNARE proteoliposomes rely on Sec18 for *cis*-SNARE complex disassembly (Fig. 1), whereas fusion reactions without *cis*-SNARE complexes

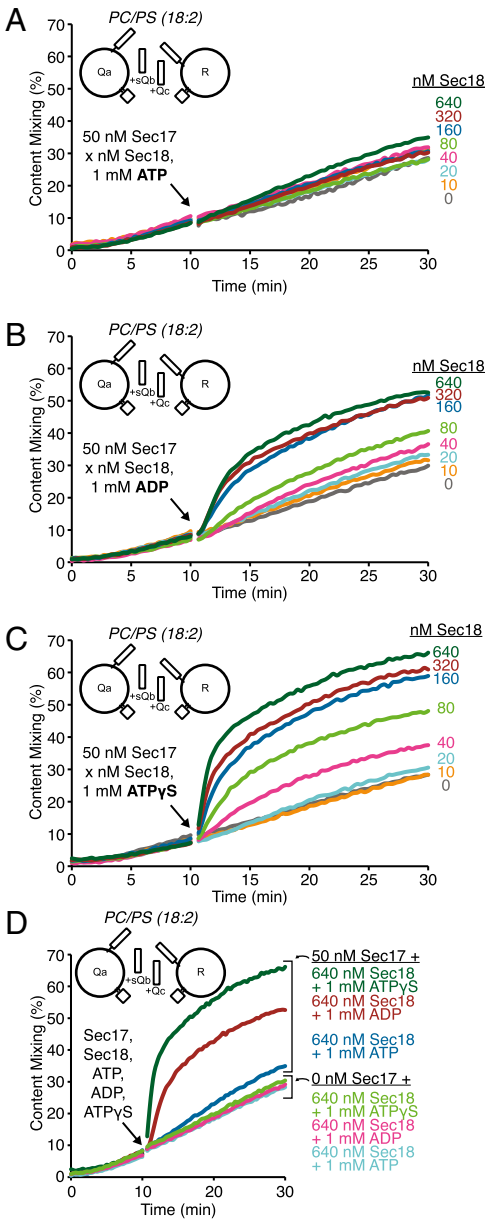

Fig. 5. ADP- or ATPγS-bound Sec18 enhances Sec17-triggered fusion. (*A*–*C*) Fusion reactions contained RPLs of PC/PS (18:2) lipids bearing either the R-SNARE Nyv1 (1:10,000 protein:lipid ratio) or the Qa-SNARE Vam3 (1:2,000 protein:lipid ratio). A soluble version of the Qb-SNARE Vti1 (500 nM) and the Qc-SNARE Vam7 (50 nM) were added, and the reaction was initiated by addition of 50 nM HOPS. After 10 min, 50 nM Sec17, the indicated amounts of Sec18, and either 1 mM ATP (*A*), 1 mM ADP (*B*), or 1 mM ATPγS (*C*) were added. (*D*) The same reactions as in *A*–*C* with 640 nM Sec18 are shown for comparison with corresponding control conditions that lacked Sec17.

are stimulated by Sec17 without Sec18 being required (Fig. 2). (*ii*) Sec17- and Sec18-mediated disassembly of *cis*-SNARE complexes requires ATP, whereas Sec18 only stimulates Sec17 to trigger fusion when it is complexed with ADP or ATPγS (Fig. 5). (*iii*) The fatty acyl composition has a marked effect on the dependence of fusion on Sec17 (Fig. 2). (*iv*) Mutations in the apolar loop of Sec17 near its N terminus prevent it from triggering fusion while allowing it to cooperate with Sec18 for disassembly of *cis*-SNARE complexes (Fig. 6), whereas mutations in a C-terminal region have the opposite effect, blocking stimulation of Sec18 (Fig. 1*B*) while still triggering *trans*-SNARE paired proteoliposomes to fuse (Fig. 2*C*). In sum, it is clear that Sec17 can have two very distinct roles in fusion.

These and other recent findings suggest a revised working model of vacuole homotypic fusion. *cis*-SNARE complexes are disassembled by the Sec18 ATPase, stimulated by its cochaperone

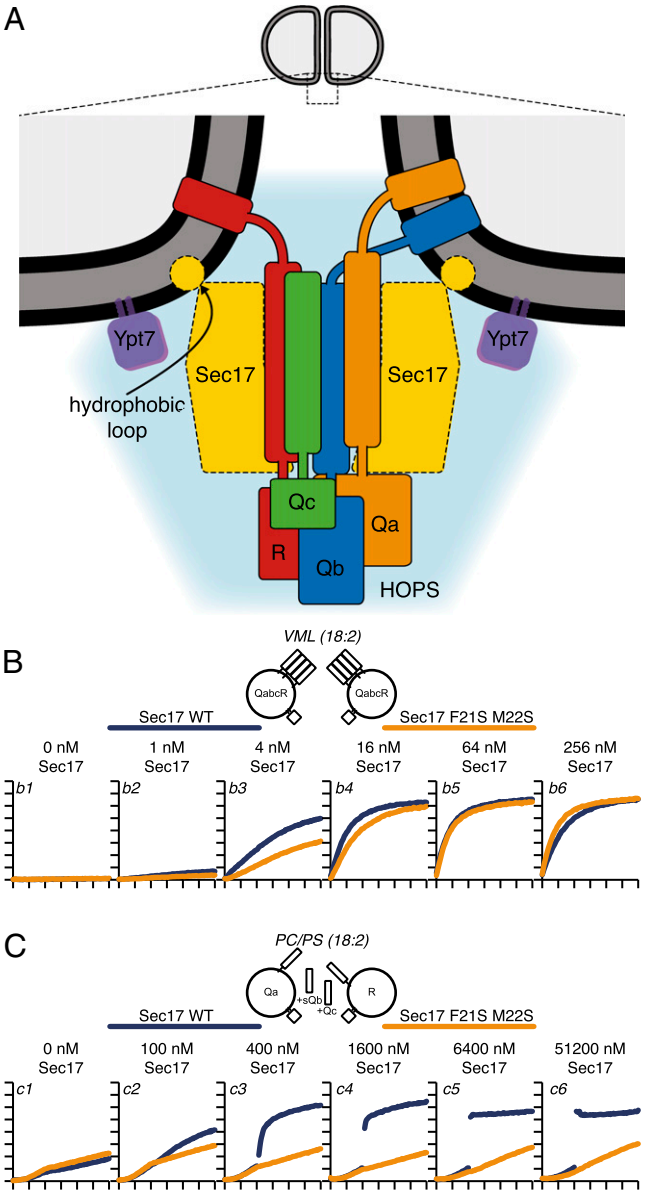

Fig. 6. Hydrophobic loop of Sec17 is critical for its fusion trigger function. (*A*) Model of Sec17 fusion trigger action, based on the structure of the 20S complex (29) modeled to *trans*-SNARE interactions. (*B*) Fusion reactions contained RPLs of VMLs (18:2) and the four vacuolar SNAREs at a 1:1,000 protein:lipid ratio. Reactions were initiated by addition of 50 nM HOPS, 1 mM ATP, 10 nM Sec18, and Sec17. The added Sec17 was either the wild-type version (blue curves) or a version that carries mutations in an N-terminal hydrophobic loop of Sec17 (F21S and M22S; orange curves). The *x* axis represents 0–30 min, and the *y* axis represents 0–80% content mixing. (*C*) Fusion reactions contained RPLs of PC/PS (18:2) lipids bearing either the R-SNARE Nyv1 (1:10,000 protein:lipid ratio) or the Qa-SNARE Vam3 (1:2,000 protein:lipid ratio), a soluble version of the Qb-SNARE Vti1 (500 nM), and the Qc-SNARE Vam7 (50 nM). Reactions were initiated by addition of 50 nM HOPS. After 10 min, either wild-type Sec17 (blue curves) or a version that carries mutations in an N-terminal hydrophobic loop (F21S and M22S; orange curves) was added. The *x* axis represents 0–30 min, and the *y* axis represents 0–80% content mixing.

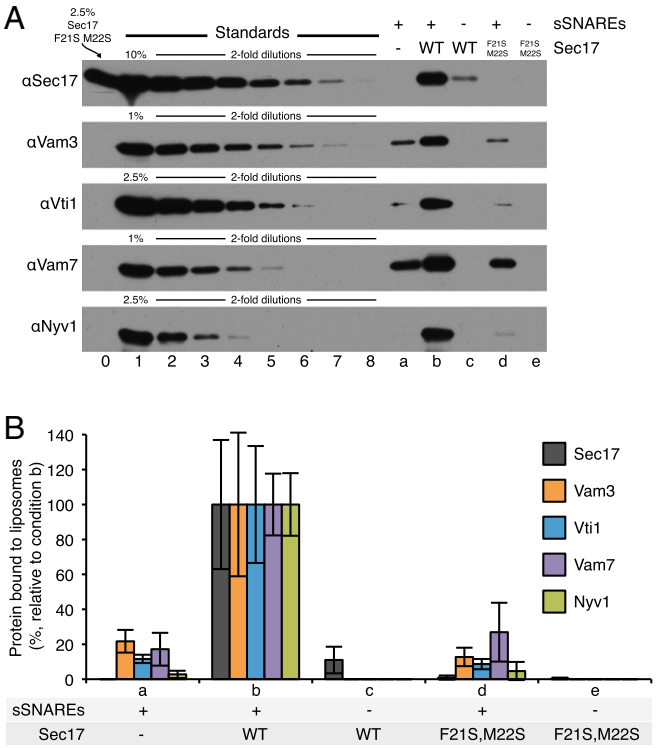

Fig. 7. Mutation of the hydrophobic loop of Sec17 results in reduced membrane association. Liposomes were incubated with a mixture of sSNAREs and Sec17, WT, or bearing mutations F21S and M22S, as indicated, for 1 h and subjected to flotation on a density gradient (see *Methods* for details). The recovered liposome samples were analyzed by SDS/PAGE and Western blot. (*A*) Representative Western blots of proteins that floated with liposomes. (*B*) Quantification of Western blot analysis, as in *A*, from three independent experiments. Different exposures, most suitable to relate individual signals to the corresponding range of the standard curves, were used. The signal intensities of condition b (with sSNAREs and Sec17 WT) were normalized to 100%. The primary quantification data (averages and SDs) are listed in Table S1.

Sec17. HOPS then tethers the membranes (13) through its affinities for Ypt7–GTP on each membrane (32) and for acidic lipid (24). Tethered membranes become drawn against each other, and the proteins and lipids needed for fusion become enriched in an interdependent manner in a ring-shaped microdomain surrounding the two apposed membranes (33, 34). HOPS catalyzes the entry of the soluble Vam7 Qc-SNARE into *trans*-SNARE complexes (17), and may protect the *trans*-SNARE complex from Sec18-mediated disassembly (19) in a manner that requires its Vps33 subunit (35).

Recent studies have clarified the elements that allow docked membranes with SNAREs paired in *trans* to proceed to fuse. At unphysiologically high SNARE levels, multiple *trans*-SNARE complexes alone drive a very slow fusion (20). Even with *trans*-SNARE pairing linking apposed membranes, the rate of fusion is strongly enhanced by the presence of a tether, whether the physiological Ypt7–HOPS tether or a synthetic tether such as polyethylene glycol (17). In either case, small-headgroup lipids, which lower the energy for membrane transitions through nonbilayer intermediates, are required for meaningful fusion rates (25). We now report that Sec17 can dramatically accelerate fusion once *trans*-SNARE pairs have assembled. The concentrations at which these effects were observed were in the range of the cytosolic concentration of Sec17, which can be estimated as 0.5–1.5 μM, based on proteome quantification studies indicating Sec17 abundance at 65–205 parts per million (36, 37) and a cellular protein concentration of 280 mg/mL (38). Vacuole fusion experiments with $Vam7^{3\Delta}$, a truncation mutant of Vam7 (21), support this interpretation. In these studies, $Vam7^{3\Delta}$ formed *trans*-SNARE complexes as efficiently as wild-type Vam7. The partially zipped complexes did not drive fusion but were rescued by Sec17 in the absence of ATP. Enhanced proteoliposome fusion due to Sec17 is lost in the $Sec17^{F21S,M22S}$ mutant, and thus likely depends on the hydrophobicity of the Sec17 19–30 loop region. The structure of the 20S Sec18–Sec17–SNARE complex (29) shows that Sec17 oligomerizes when bound to SNAREs and that each of the several Sec17 apolar loops is poised near the SNARE membrane anchors, where it may insert into a bilayer. In accord with this concept, Sec17 and the mixed soluble domains of the vacuolar SNAREs are interdependent for a lipid association that can withstand flotation (Fig. 7). Sec18–ADP may enhance binding of Sec17 to the *trans*-SNARE complex but only exchange the ADP for ATP and catalyze SNARE complex disassembly after fusion has converted the *trans* complex to *cis*. Alternatively, Sec18–ATP may not be able to bind to a HOPS–Sec17–*trans*-SNARE complex at all. It is also unclear whether Sec17 displaces HOPS from SNARE complexes (39) before fusion.

Fusion, and its dependence on Sec17, is regulated by membrane lipid composition. With vacuolar lipid mix and 18:2 fatty acyl chains there is only minimal effect of Sec17 in enhancing fusion, whereas with PC/PS and 18:2 the effect is striking (Fig. 2 *A* and *B*). However, with 16:0 18:1 fatty acyl chains, fusion of even VML proteoliposomes is strongly stimulated by added Sec17 (Fig. 2*D*). In the context of the complex physiological fatty acyl and headgroup composition of yeast vacuolar lipids (40, 41), further studies will be needed to define the common denominator for the lipid role in this reaction.

Several factors can contribute to lowering the energy barrier for two apposed membranes to rearrange their bilayers for fusion: (*i*) the physical force of membrane deformation deriving from *trans*-SNARE complexes forming continuous α-helices between their SNARE and transmembrane domains (42) [the initial concept that the energy for fusion derives entirely from forming a continuous α-helix between SNARE and transmembrane domains has been questioned by observations that transmembrane domains can be replaced by lipidic anchors for yeast vacuole fusion (43) or neuronal fusion (44)]; (*ii*) the docking of membranes in close proximity (45, 46); (*iii*) the membrane bending that surrounds an extended region of membrane contact, driven by tethers (47); (*iv*) the enrichment of small headgroup lipids that can more readily fit into the nonbilayer lipidic structures of hemifusion and fusion intermediate states (25, 34); and (*v*) localized (e.g., *trans*-SNARE–associated) additional proteins or other factors that can bind to lipids or insert into membranes to promote nonbilayer transitions. The latter includes Ca^{2+}-triggered insertion of synaptotagmin at the neuronal synapse (48) and Sec17 for intracellular fusion. In model systems, transition from apposed bilayers to fusion has even been promoted by interaction of calcium with lipids (49). The relative contribution of each to achieving the activation state for transition to fusion remains for future biophysical studies; important progress has been made in this regard with SNAREs (50).

We have reported (12) that Sec17 is released from vacuoles by the ATP-dependent action of Sec18, and that subsequent fusion becomes insensitive to antibodies to Sec17. How can this be rationalized with our current findings? Only a few percent of SNAREs become engaged in *trans* (18), and only a correspondingly small fraction of the initially bound Sec17 may be needed to trigger fusion. Because the fusion of docked vacuoles is slow in vitro, occurring with a half-time of 20 min or more (27), these kinetics may not depend on Sec17. It is possible that the stimulation we report here may or may not be necessary in all physiological conditions. Our observations reveal more about what can potentially contribute to fusion rather than providing evidence of whether Sec17 physiologically fulfills this function. Nonetheless, the finding that fusion arrested by a C-terminal deletion in the Vam7 SNARE domain (21)

can be rescued by added Sec17 shows that this effect can occur on the native organelle.

The triggering of a burst of fusion by Sec17 may share important mechanistic features with synaptotagmin and Ca^{2+}-triggered fusion. In each case, a protein associates with SNAREs and inserts a domain into the bilayer, providing the impetus for rapid transition to fusion. In neither case is there an absolute fusion dependence on the SNARE-bound, membrane-inserting protein. Synaptotagmin/Ca^{2+}-independent fusion events are seen at the synapse (51), as fusion in the reconstituted yeast vacuolar system can occur independent of Sec17 (9, 17, 52). Because synaptic vesicle fusion also involves SNAPs, the neuronal homologs of Sec17, it is even conceivable that synaptotagmin and SNAPs could cooperate to trigger fast fusion in this system.

Methods

Proteins and Reagents. Lipids were obtained from Avanti Polar Lipids, with the exception that ergosterol was from Sigma-Aldrich, PI(3)P was from Echelon Biosciences, and the fluorescent lipids [Marina Blue-DHPE (1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine), NBD (nitrobenzoxadiazole)-DHPE] were from Life Technologies. Biotinylated R-phycoerythrin was purchased from Life Technologies, Cy5-derivatized streptavidin was from KPL, and unlabeled streptavidin was from Thermo Scientific. Sec18 (53), Sec17 and Sec17^{L291A,L292A} (21), Ypt7 (17), HOPS (17), and vacuolar SNARE proteins (9, 11, 21) were purified as described. The Sec17^{F21S,M22S} construct was created by site-directed mutagenesis and purified as for the other Sec17 constructs. Soluble Vti1 (sQb) was purified as MBP-TEV-Vti1(1–194) as described (17).

Reconstitution of Vacuolar Rab/SNARE Proteoliposomes. Proteoliposomes bearing Cy5-streptavidin or biotinylated R-phycoerythrin were prepared from mixed micellar solutions (containing 50 mM β-octyl-glucoside) by detergent dialysis (20-kDa cutoff membrane) in RB150/Mg^{2+} [20 mM Hepes·NaOH, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 10% glycerol (vol/vol)] as described (17), with modifications. Lipids dissolved in chloroform were mixed at the following proportions: For vacuolar mixed lipids (16:0 18:1), 44.6–47.6 mol% POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), 18 mol% POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine), 18 mol% soy PI (L-α-phosphatidylinositol), 4.4 mol% POPS (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine), 2 mol% POPA (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate), 1 mol% 16:0 DAG (1,2-dipalmitoyl-*sn*-glycerol), 8 mol% ergosterol, and 1 mol% PI(3)P [1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-myo-inositol-3′-phosphate)]. For VMLs (18:2), lipids were used in the same proportions as above, but with the dilinoleoyl forms of PC, PE, PS, and PA. For PC/PS (18:2), 66–68.8 mol% DLPC (1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine), 30 mol% DLPS (1,2-dilinoleoyl-*sn*-glycero-3-phospho-L-serine), and 1 mol% PI(3)P. Fluorescent lipids (0.2 mol% Marina Blue-DHPE, 3 mol% NBD-DHPE) were included to allow assays of lipid dequenching. Molar protein:lipid ratios were 1:1,000–1:10,000 for SNAREs and 1:2,000–1:10,000 for Ypt7, which was present on all proteoliposomes used in this study. Isolation after reconstitution was achieved by floatation on a three-step Histodenz gradient [35%, 25% Histodenz (wt/vol), and RB150/Mg^{2+}].

Reconstituted Proteoliposome Fusion Assays. Fusion reactions were assembled in 20 μL. Fusion assay pairs of reconstituted proteoliposomes (RPLs) (each 250 μM lipid) in RB150, 5 μM streptavidin, 1 mM EDTA, and 10 μM GTP were preincubated for 10 min at 27 °C before addition of 1.25 mM $MgCl_2$ to exchange Ypt7 to its GTP-bound form, and then 10 μL was transferred to wells of 384-well plates. Soluble components (10 μL; e.g., HOPS, Sec17, Sec18, ATP, sVti1, Vam7) or their respective buffers were added to initiate the reactions, either as one addition or two consecutive 5-μL additions. All reactions contained 0.5% (wt/vol) defatted BSA, 5 mM reduced glutathione, and 1 mM DTT. Plates were incubated at 27 °C in a fluorescence plate reader for 30 min, and content mixing signals [PhycoE–Cy5-FRET: excitation (ex): 565 nm; emission (em): 670 nm; cutoff: 630 nm] were recorded at intervals of 10–30 s in a SpectraMax Gemini XPS (Molecular Devices) fluorescence plate reader. Maximal values were determined after addition of 0.1% (wt/vol) Thesit to samples that had not received streptavidin.

Determination of *trans*-SNARE Associations by Coimmunoprecipitation. To estimate the amount of *trans*-SNARE association that formed during a reaction, the amount of Nyv1 that coimmunoprecipitated with Vam3 was determined. A 40-μL fusion reaction was incubated for 2 min at 27 °C, placed on ice, and diluted 10-fold in ice-cold RIPA buffer [20 mM Hepes·NaOH, pH 7.4, 150 mM NaCl, 0.2% (wt/vol) BSA, 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium cholate, 0.1% (wt/vol) SDS] containing 50 μg/mL affinity-purified anti-Vam3 antibody and 10 μM GST-Nyv1. After 20 μL RIPA buffer-washed protein A magnetic beads (Thermo Scientific) was added, the mix was nutated at room temperature for 2 h. After the beads were washed three times with 1 mL of RIPA buffer, samples were eluted in 50 μL of reducing SDS sample buffer at 95 °C for 5 min. Aliquots (20 μL) of each sample were subjected to SDS/PAGE and immunoblotting with anti-Nyv1 antibody.

Sec17 and Soluble SNARE Membrane Binding Assay. Liposomes composed of 83.5 mol% DLPC, 15 mol% DLPS, and 1.5 mol% NBD-DHPE were prepared by detergent dialysis. Liposomes in RB150/Mg^{2+} were mixed with BSA, a mixture of either the four soluble SNAREs or their buffers, and Sec17. Final concentrations were 0.5 mM lipid, 0.2% (wt/vol) BSA, 2 μM each SNARE, and 1 μM Sec17 in 32 μL per reaction. Reactions were gently vortexed, incubated at 30 °C for 1 h, and placed on ice for 5 min. Histodenz [96 μL of a 54% (wt/vol) solution in isoosmolar RB150/Mg^{2+} containing just 2% (vol/vol) glycerol] was added. Tubes were vortexed gently. Samples (80 μL) were transferred to 7- × 20-mm polycarbonate centrifuge tubes (Beckman Coulter), overlaid with 80 μL of 35% (wt/vol) and then 80 μL of 30% (wt/vol) isoosmolar Histodenz in RB150/Mg^{2+}, and finally with 50 μL of RB150/Mg^{2+}. The remaining sample was solubilized in 0.1% (vol/vol) Thesit for lipid recovery determination. Tubes were centrifuged in a TLS55 rotor (Beckman Coulter) for 1.5 h at 55,000 rpm and 4 °C. Floated liposomes (80 μL) were harvested from the top of the tube and solubilized in 0.1% (vol/vol) Thesit. Lipid recovery was determined by measuring NBD fluorescence (ex: 460 nm; em: 538 nm; cutoff: 515 nm) of the starting samples vs. the floated samples. Floated samples and starting samples were incubated in SDS sample buffer for 5 min at 90 °C and then analyzed by SDS/PAGE, followed by immunoblotting. The sample volumes that were loaded were adjusted according to lipid recovery. Serial twofold dilutions of the starting sample provided a standard curve for quantifying the amount of bound protein using UN-SCAN-IT software (Silk Scientific).

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