Sec17 (α-SNAP) and Sec18 (NSF) restrict membrane fusion to R-SNAREs, Q-SNAREs, and SM proteins from identical compartments

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Membrane fusion at each organelle requires conserved proteins: Rab-GTPases, effector tethering complexes, Sec1/Munc18 (SM)-family SNARE chaperones, SNAREs of the R, Qa, Qb, and Qc families, and the Sec17/α-SNAP and ATP-dependent Sec18/NSF SNARE chaperone system. The basis of organelle-specific fusion, which is essential for accurate protein compartmentation, has been elusive. Rab family GTPases, SM proteins, and R- and Q-SNAREs may contribute to this specificity. We now report that the fusion supported by SNAREs alone is both inefficient and promiscuous with respect to organelle identity and to stimulation by SM family proteins or complexes. SNARE-only fusion is abolished by the disassembly chaperones Sec17 and Sec18. Efficient fusion in the presence of Sec17 and Sec18 requires a tripartite match between the organellar identities of the R-SNARE, the Q-SNAREs, and the SM protein or complex. The functions of Sec17 and Sec18 are not simply negative regulation; they stimulate fusion with either vacuolar SNAREs and their SM protein complex HOPS or endoplasmic reticulum/*cis*-Golgi SNAREs and their SM protein Sly1. The fusion complex of each organelle is assembled from its own functionally matching pieces to engage Sec17/Sec18 for fusion stimulation rather than inhibition.

membrane fusion | SNARE | SM protein | NSF | α-SNAP

Intracellular membrane fusion mechanisms on the exocytic and endocytic pathways are conserved from yeast to humans and among the organelles (1). Each organelle is marked by 1 or more Rab-family GTPase which binds tethering factors to bring membranes into apposition (2). SNARE proteins are required on each membrane for fusion (3). SNAREs have N-domains, heptad-repeat SNARE domains, and often a C-terminal *trans*-membrane anchor. Their SNARE domains assemble into quaternary complexes, with the polar residues of each helical SNARE domain exposed on the surface of the coiled-coil 4-SNARE structure. Apolar residues of the SNARE domains are buried in the coiled coil, although 3 central internally oriented glutamines and an arginine form the "zero layer" of each SNARE complex. SNAREs are in 4 conserved families, R, Qa, Qb, and Qc, and SNARE complexes are composed of 1 member of each family (4). SNARE complexes are in *cis* if anchored to 1 membrane or in *trans* if anchored to apposed membranes. Sec1/Munc18 family (SM) proteins are required for fusion and may catalyze SNARE assembly (5–9). *Trans*-SNARE complexes become *cis*-complexes as a result of fusion and are then disassembled by Sec17/α-SNAP and Sec18/NSF. Sec17 and Sec18 also act earlier to promote fusion (10–13) and in certain model reactions can disassemble *trans*-SNARE complexes and thereby block fusion (11).

There have been conflicting data as to whether SNAREs alone provide the organelle specificity of membrane fusion. Yeast have a defined repertoire of SNAREs (14). Early studies of lipid mixing among proteoliposomes bearing yeast SNAREs suggested that they sufficed to specify organelle identity through regulated fusion (15). In contrast, studies with mammalian endosomal SNAREs indicated substantial promiscuity of function (16–18). Recent studies have demonstrated additional layers of fusion specificity conferred by tethering factors (19) or SM family SNARE-binding proteins (20).

We study membrane fusion with 2 model systems from yeast, the homotypic fusion of the vacuole/lysosome (21) and the fusion of endoplasmic reticulum (ER)-derived COPII vesicles to the *cis*-Golgi (22). Vacuoles undergo fission and fusion in the cell; mutants which block fusion allow continued fission and thus accrue multiple small vacuoles. These *VAM* (vacuole morphology) mutations were identified in genes encoding the vacuolar Rab Ypt7, each subunit of the hexameric HOPS (homotypic fusion and vacuole protein sorting) complex, and the Qa-SNARE Vam3 and Qc-SNARE Vam7 (23), referred to hereafter as Qa and Qc. The vacuolar R-SNARE Nyv1 was discovered independently (24). The Qb-SNARE Vti1, Sec17/α-SNAP, and Sec18/NSF are required for both vacuole fusion and fusion on the essential exocytic pathway and thus were not detected in the original *VAM* screen. Two of the six HOPS subunits have direct affinity for Ypt7, conferring membrane tethering (25–27). A third subunit, Vps33, is the vacuolar SM protein, with direct affinity for the SNARE domains of the R- and Qa-SNAREs (6). HOPS also has direct affinity for the Qb and Qc SNAREs (28) and directly promotes SNARE complex assembly (8, 9).

Membrane fusion between ER-derived vesicles and *cis*-Golgi requires the Rab Ypt1 and its effectors for tethering, including Uso1 (29) and the large TRAPP complexes (30), as well as the SM protein Sly1, the R-SNARE Sec22, and the Q-SNAREs Sed5, Bos1, and Bet1. The relative roles of Uso1 and TRAPP in tethering

Significance

Although each intracellular fusion event is catalyzed by similar proteins, organelle-specific fusion is essential for accurate protein compartmentation. Prior studies have suggested that specificity resides in the SNARE proteins or in SNAREs and their Sec1/Munc18 (SM) chaperones. The current work, with combinations of SM proteins, R-SNAREs, and Q-SNAREs from yeast vacuole homotypic fusion and from endoplasmic reticulum/*cis*-Golgi fusion, finds that some noncognate combinations support fusion. However, in the presence of the SNARE disassembly chaperones Sec17/α-SNAP and Sec18/NSF, fusion only occurs when the R-SNARE, Q-SNAREs, and SM protein are derived from the same organelle.

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are unclear. Fusion of ER-derived vesicles with the *cis*-Golgi differs from vacuolar fusion in that Sly1 is not part of a large, stable complex as seen for the vacuolar SM protein Vps33.

Prior reconstitutions of fusion with SNAREs alone have suggested either that SNAREs act selectively to confer organelle specificity or that they assemble and function promiscuously. We now compare fusion specificity between proteoliposomes bearing R- or Q-SNAREs from yeast vacuoles or ER/*cis*-Golgi. High concentrations of vacuolar Q-SNAREs will mediate fusion with any tested R-SNARE, but Sec17/Sec18 prevents these simple *trans*-SNARE complexes from supporting fusion. SNARE-only fusion can be stimulated by either the vacuolar tethering/SM complex HOPS or the ER/*cis*-Golgi SM protein Sly1. Strikingly, fusion in the presence of Sec17 and Sec18 requires reconstitutions with a 3-fold match of the organelle of origin between the R-SNARE, the Q-SNAREs, and the SM protein or protein complex. In addition to this regulatory role, Sec17, Sec18, and ATP can stimulate fusion of membranes with either vacuolar SNAREs with the vacuolar SM-protein complex HOPS or with ER/*cis*-Golgi SNAREs and their SM protein Sly1.

Results

Early studies of reconstituted proteoliposome fusion employed an assay of dequenching of pairs of fluorescent lipids incorporated in 1 of the fusion partners. As well as measuring fusion, this assay can report lysis and the attendant membrane reannealing and other forms of lipid exchange (31). A more rigorous assay (32), used throughout the current study, employs a mixture of proteoliposomes with lumenal fluorescent proteins, either Cy5-streptavidin or biotinylated phycoerythrin, mixed with a large excess of external nonfluorescent streptavidin to block any signal from lysis. The Cy5 and phycoerythrin fluorophores, trapped within different proteoliposomes, are initially separated by at least the thickness of 2 bilayers and thus do not exhibit FRET (fluorescence resonance energy transfer). Upon fusion, the mixing of lumenal contents allows tight binding of biotin to streptavidin, bringing their bound Cy5 and phycoerythrin fluorophores into intimate contact and creating a strong FRET signal. Lumenally marked proteoliposomes with high surface concentrations of the 3 vacuolar Q-SNAREs were mixed with proteoliposomes with complementary lumenal marking and bearing either Nyv1, an R-SNARE from the vacuole (Fig. 1 *A* and *B*), Sec22, an R-SNARE

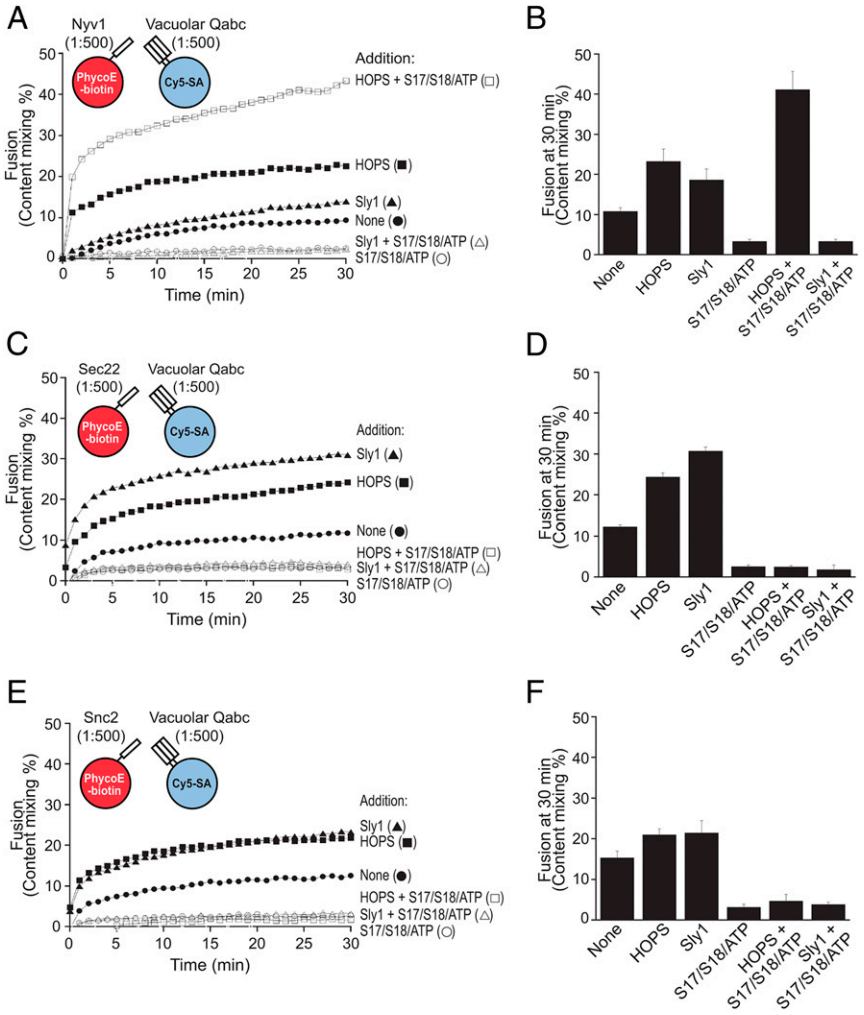

Fig. 1. SNARE-only fusion at high SNARE levels is promiscuous for R-SNARE but suppressed by Sec17/Sec18. (*A*–*F*) Fusion reactions contained proteoliposomes bearing vacuolar Qabc SNAREs at 1:500 SNARE:lipid molar ratios and proteoliposomes carrying the vacuolar R-SNARE Nyv1 (*A* and *B*), the ER/*cis*-Golgi R-SNARE Sec22 (*C* and *D*), or the endocytic R-SNARE Snc2 (*E* and *F*) at 1:500 SNARE:lipid molar ratios. Mixed proteoliposomes were incubated as described (34) for 30 min at 27 °C in the absence or presence of indicated components: HOPS (100 nM), Sly1 (4.5 μM), Sec17 (0.4 μM), Sec18 (0.6 μM), and ATP (1 mM). Content mixing was assayed by FRET between the lumenal markers Cy5 and phycoerythrin. Kinetic curves in this figure are representative of $n \geq 3$ experiments; representative experiments and means and SDs for triplicate repeats are shown.

of the endoplasmic reticulum (Fig. 1 *C* and *D*), or Snc2, an R-SNARE from the plasma membrane (Fig. 1 *E* and *F*). These proteoliposomes had very high SNARE:lipid molar ratios, ~60-fold higher than the organelle itself (33), and lacked a Rab, but allowed direct comparisons with early studies (34). Without further addition, each gave comparable fusion (Fig. 1 *A*, *C*, and *E*, filled circles). Fusion with each R-SNARE was stimulated by either Sly1 (filled triangles), the SM protein of the endoplasmic reticulum, or by HOPS (filled squares), a hexameric complex with a subunit (Vps33) which is the vacuolar SM protein. Without HOPS or Sly1, fusion was blocked by the addition of Sec17, Sec18, and ATP (open circles), which disassemble *trans*-SNARE complexes (11). When HOPS is added to proteoliposomes with entirely vacuolar SNAREs, it not only overcomes the Sec17/Sec18 inhibition, but gives fusion at a higher rate and extent (Fig. 1*A*, open squares) than seen without Sec17/Sec18, as reported (34). This synergy between HOPS and Sec17/18 was completely absent when proteoliposomes bore the ER or PM R-SNAREs (Fig. 1 *C* and *E*; open squares). In these fusion assays, which employed vacuolar Q-SNAREs, the ER/*cis*-Golgi SM protein Sly1 did not show fusion synergy with Sec17/Sec18 or even block their inhibition (Fig. 1 *A*, *C*, and *E*, open triangles). Thus SNAREs at high concentrations promote promiscuous membrane fusion, stimulated by SM proteins from the same or different organelle, but fusion is blocked by Sec17, Sec18, and ATP unless the SNAREs and SM protein or complex originate from the same organelle.

Tethering is a prerequisite for SNAREs to assemble in *trans* in a productive conformation (35). Tethering is normally performed by a Rab and its effector (33). Proteoliposomes were therefore prepared at lower SNARE concentrations (36) with the vacuolar Rab Ypt7 and the 3 vacuolar Q-SNAREs or with Ypt7 and the vacuolar R-SNARE Nyv1 (Fig. 2*A*). These lower concentrations of Q- and R-SNAREs alone did not support membrane fusion (Fig. 2*A*, open circles). HOPS supported fusion (filled circles), and this fusion was further stimulated by Sec17, Sec18, and either ATP or ATPγS (triangles). ATPγS does not support Sec18-mediated SNARE complex disassembly, and thus disassembly is not needed for the stimulation by Sec17 and Sec18. In contrast to HOPS, the ER SM protein Sly1 did not promote fusion with these vacuolar SNAREs, alone or with Sec17 and Sec18 (Fig. 2*C*, diamonds). With HOPS, fusion was strongly suppressed by substitution of the vacuolar Qa SNARE Vam3 with Pep12 (Fig. 2*E*) or substitution of the vacuolar R-SNARE Nyv1 by the ER/*cis*-Golgi R-SNARE Sec22 (Fig. 2*G*). Pep12 is an endosomal Qa-SNARE; its overexpression will restore some level of vacuole fusion to a *vam3*Δ strain (37). Interestingly, when proteoliposomes with far higher concentrations of the Pep12 and the 2 vacuolar Q-SNAREs Vti1 and Vam7 were mixed with proteoliposomes bearing high concentration of the vacuolar R-SNARE Nyv1, fusion was seen in the presence of HOPS, although this fusion was blocked by Sec17 and Sec18 (*SI Appendix*, Fig. S1). HOPS did support limited fusion between proteoliposomes bearing vacuolar Q-SNAREs and those with Snc2, a plasma membrane R-SNARE (Fig. 2*I*, filled circles), although this fusion is blocked by Sec17, Sec18, and ATP or ATPγS (triangles). The ER/*cis*-Golgi SM protein Sly1 failed to stimulate fusion with each R-SNARE (Fig. 2, open diamonds). With each R-SNARE, there was no fusion in the presence of Sec17 and Sec18 without an SM protein (filled squares). Each of these assays employed vacuolar Q-SNAREs; fusion in the presence of Sec17 and Sec18 was only seen with the vacuolar R-SNARE and vacuolar HOPS (Fig. 2*A*). The tripartite match of the organelle of origin of the R-SNARE, Q-SNAREs, and SM-containing protein complex HOPS was crucial for fusion in the presence of Sec17 and Sec18.

HOPS has multiple affinities, for vacuolar SNAREs (6, 28), Rab (38), phosphoinositides (28), and acidic lipids (39). HOPS can tether membranes (26), catalyze SNARE complex assembly (6, 9), and protect against *trans*-SNARE disassembly by Sec17 and Sec18 (11). To determine which of these affinities and activities underlies the specific requirement for an organelle match between the R-SNARE and the vacuolar Q-SNAREs, we compared fusion supported by HOPS to that seen with a synthetic tether, a dimeric fusion protein of GST fused to a Phox domain (PX) which specifically binds phosphatidylinositol 3-phosphate (PI[3]P). Tethering is due to each PX domain of the GST-PX dimer binding to PI(3)P from separate proteoliposomes (35). With 1 fusion partner bearing the 3 preassembled vacuolar Q-SNAREs, GST-PX supports their fusion to vacuolar R-SNARE proteoliposomes (Fig. 3*A*, gray circles), albeit not as well as HOPS (black circles). GST-PX also supports their fusion to proteoliposomes with the plasma membrane R-SNARE Snc2 (Fig. 3*A*, gray triangles). Without Sec17 and Sec18, HOPS largely employs its tethering function to stimulate fusion, since it can be replaced by GST-PX. However, GST-PX lacks SM function and cannot support fusion in the presence of Sec17 and Sec18 (35). Thus HOPS is mainly supplying a tethering function under these conditions; it also protects against fusion inhibition by Sec17/Sec18 when these are present (Fig. 1 and ref. 34).

Might the limited capacity of nonvacuolar R-SNARE proteoliposomes for fusion with vacuolar 3Q-SNARE proteoliposomes reflect an inability to assemble into SNARE complexes? Proteoliposomes were prepared with the 3 vacuolar Q-SNAREs and either no R-SNARE or the vacuolar, ER, or plasma membrane R-SNARE, and these were each assayed for fusion with vacuolar R-SNARE proteoliposomes in the presence of HOPS. The 3Q-SNARE proteoliposomes without R-SNARE were active without Sec17/Sec18 (Fig. 4*A*, open circles), while the presence of any tested R-SNARE on the same membrane as the vacuolar Q-SNAREs suppressed fusion (open squares, triangles, and diamonds), suggesting that any of the 3 R-SNAREs suppressed fusion by forming a *cis*-complex with the 3 Q-SNAREs. In the presence of Sec17, Sec18, and ATP, each of the R + 3Q-SNARE proteoliposomes (Fig. 4*A*, filled symbols) was as active as the 3Q-SNARE proteoliposomes which lacked R-SNARE (filled circles), indicating that each of the R-SNAREs had engaged the 3Q-SNAREs in a *cis*-SNARE complex which was recognized for disassembly by Sec17 and Sec18.

Reconstituted ER/*cis*-Golgi Fusion Is Also Stimulated by Sec17/Sec18/ATP, Requiring a Tripartite Match of SM Protein with R- and Qa-SNAREs. Recombinant ER/*cis*-Golgi SNAREs Sec22 (R), Sed5 (Qa), Bos1 (Qb), and Bet1 (Qc) were purified and incorporated into ER/*cis*-Golgi R-SNARE proteoliposomes and ER/*cis*-Golgi 3Q-SNARE proteoliposomes. Vacuolar Nyv1 R-SNARE proteoliposomes did not fuse with these ER/*cis*-Golgi Q-SNARE proteoliposomes, even when supplemented with other fusion factors such as HOPS, Sly1 and PEG, or Sec17/Sec18 (Fig. 5 *A* and *B*). The fusion of proteoliposomes with the ER/*cis*-Golgi R-SNARE Sec22 to those with the 3 ER/*cis*-Golgi Q-SNAREs was supported by Sly1 and Sec17/Sec18/ATP (Fig. 5*C*, filled diamonds with all 3 proteins vs. open circles with Sly1 alone or filled triangles with Sec17/Sec18/ATP alone; also Fig. 5*D*). ATP hydrolysis was required, as fusion was not seen with ATPγS instead of ATP (open diamonds). As reported (20), Sly1 and the synthetic tethering agent PEG (1%) could also support fusion (Fig. 5 *C* and *D*). There was no fusion with PEG, Sec17/Se18/ATP, or Sly1 alone (Fig. 5 *C* and *D*). Sec17 and Sec18 also stimulated fusion with Sly1 and PEG (Fig. 5 *C* and *D*), as Sec17 and Sec18 stimulated HOPS-dependent fusion with all vacuolar SNAREs (Figs. 2 and 4). Since PEG not only tethers membranes, but is a dehydrating agent which can directly trigger fusion at higher concentrations (40) and is an agent of macromolecular crowding (41), we also tested a tethering agent of more defined mechanism. As above (Fig. 3), we chose the inherently dimeric protein of GST fused to a Phox homology domain (PX), which has direct affinity for PI(3)P on each proteoliposomal membrane. The

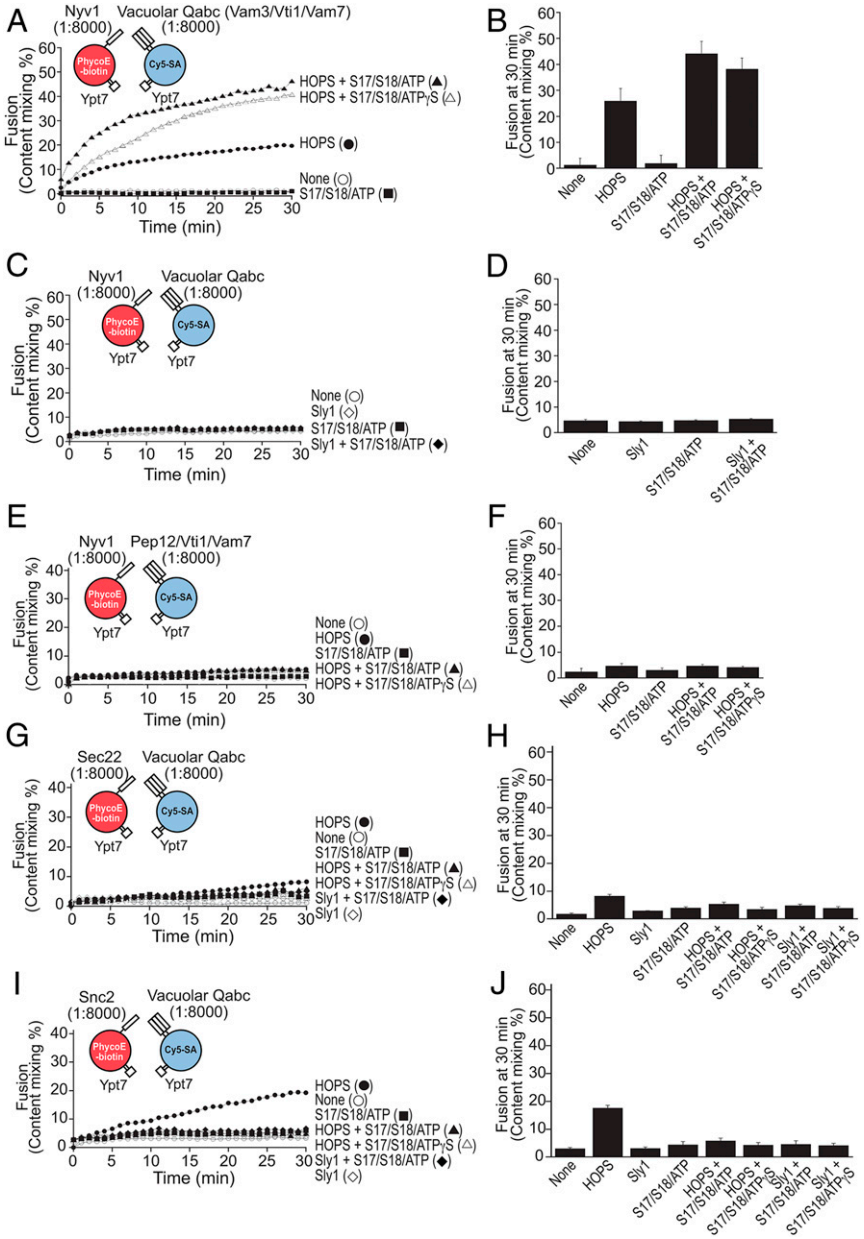

Fig. 2. In the presence of Sec17 and Sec18, fusion to vacuolar Q-SNARE proteoliposomes requires vacuolar R-SNARE and the vacuolar SM protein complex. (*A*–*D*) Proteoliposomes bearing vacuolar Qabc SNAREs and the vacuolar Rab Ypt7-TM, each at 1:8,000 protein:lipid molar ratios, were incubated with proteoliposomes carrying Ypt7-TM and the vacuolar R-SNARE Nyv1 at the same concentrations. Fusion incubations contained the indicated combinations of additional components: HOPS (100 nM), Sly1 (4.5 μM), Sec17 (0.4 μM), Sec18 (0.6 μM), ATP (1 mM), and ATPγS (1 mM). (*E*–*F*) Fusion assays were performed as in *A*, but with proteoliposomes bearing Pep12 instead of Vam3. (*G*–*J*) Fusion incubations contained proteoliposomes with the ER/*cis*-Golgi R-SNARE Sec22 (*G* and *H*), or the endocytic R-SNARE Snc2 (*I* and *J*) at 1:8,000 protein:lipid molar ratios for 30 min at 27 °C in the absence or presence of additional components, as above. Kinetic curves in this figure are representative of $n \geq 3$ experiments; representative experiments and means and SDs for triplicate repeats are shown.

dimeric GST-PX synthetic tether cooperated with Sly1 for fusion of proteoliposomes bearing PI(3)P and the R- and 3Q- ER/*cis*-Golgi SNAREs (Fig. 5*E*, gray diamonds, and Fig. 5*F*), and this fusion was further stimulated by Sec17, Sec18, and ATP (Fig. 5*E*, black triangles, and Fig. 5*F*). The specific resistance of fusion to Sec17/Sec18/ATP when the R- and Q-SNAREs and SM protein are matched, and even stimulation by these chaperones, is thus seen for a second organelle.

Discussion

Reconstitution of fusion with purified components enables combinatorial study of the requirements for fusion efficiency and organelle specificity. Rapid and accurate fusion has several requirements: SNAREs in a *trans* complex, tethering to allow these *trans* complexes to assemble in a functional configuration (34), an SM protein or SM complex to catalyze SNARE complex assembly and protect from Sec17/18 inhibition, Sec17/18 to proofread the SNARE and SM organellar match and promote bilayer mixing, and nonbilayer prone lipids which favor the nonbilayer intermediate states of fusion (42). SNAREs alone, even at high concentrations, support only slow fusion which tolerates substantial R-SNARE or SM protein mismatch (seen with vacuolar Q-SNAREs, Fig. 1) or may support no fusion at all (with ER/*cis*-Golgi Q-SNAREs, Fig. 5). Sec17 and Sec18 prevent SNARE- or SM-mismatched fusions

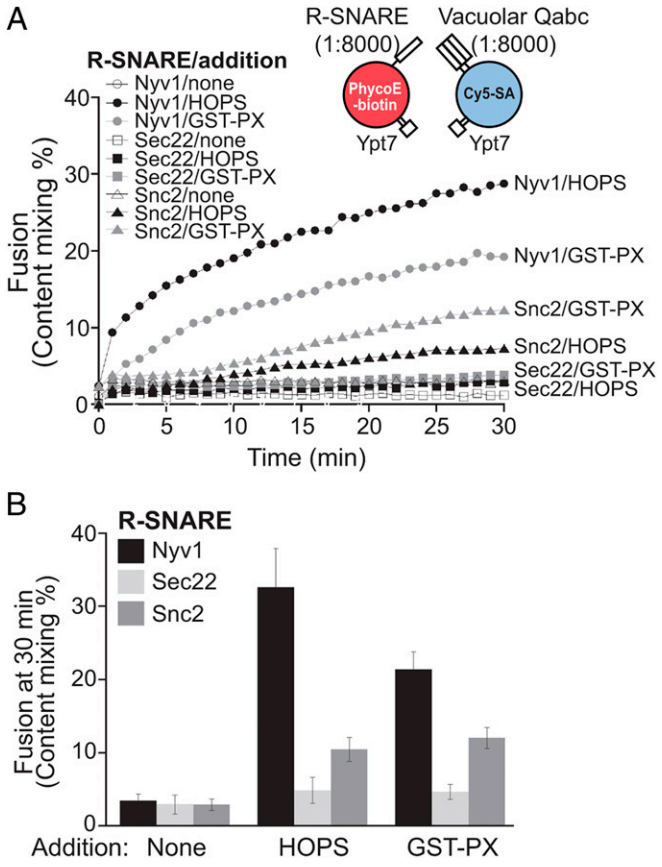

Fig. 3. With preassembled Q-SNAREs and without Sec17/Sec18, HOPS largely drives fusion by its tethering function. Fusion incubations contained proteoliposomes bearing vacuolar Qabc SNAREs and the vacuolar Rab Ypt7-TM at 1:8,000 protein:lipid molar ratios and proteoliposomes with Ypt7-TM and the vacuolar R-SNARE Nyv1 (circles), the ER/*cis*-Golgi R-SNARE Sec22 (squares), or the endocytic R-SNARE Snc2 (triangles) at 1:8,000 protein:lipid molar ratios. Incubations were performed with 100 nM HOPS (black symbols) or 1 μM GST-PX (gray symbols) for 30 min at 27 °C. Kinetic curves in this figure are representative of $n \geq 3$ experiments; representative experiments (*A*) and means and SDs for triplicate repeats (*B*) are shown.

entirely (Figs. 1, 2, and 5). At low, more physiological SNARE levels, tethering is crucial for the assembly of cognate SNARE subassemblies, R- and 3Q complexes, into *trans* 4-SNARE complexes (Fig. 2). When SNAREs are initially even more disassembled, SM proteins catalyze their assembly (6, 8) as well as conferring resistance to disassembly by Sec17/Sec18. Each of these factors cooperates to provide rapid, efficient, and organelle-specific fusion.

Tethering. Vacuolar HOPS combines tethering and SM functions. Two HOPS subunits have direct affinity for the vacuolar Rab, while a third subunit is the SM protein of that organelle. Vacuolar SNAREs on otherwise untethered membranes will readily form spontaneous *trans*-associations in conformations that are largely inactive for fusion (35). Tethering concentrates these SNAREs, resulting in fusion-competent *trans*-complexes. Since vacuolar SNAREs will form abundant inactive *trans*-assemblies without prior tethering (35), we suggest that ER/*cis*-Golgi SNAREs may do so as well. Neither the Sly1 SM protein nor tethering by either PEG or the membrane-binding dimeric GST-PX suffices for fusion with ER/*cis*-Golgi SNAREs, but together Sly1 and either tether will support fusion. This fusion is stimulated, not blocked, by Sec17/Sec18/ATP, just as with vacuolar SNAREs and HOPS, indicating (as studies with the multisubunit HOPS alone could not) that the SM subunit itself confers protection from Sec17/Sec18 inhibition. Strikingly though, Sec17/Sec18/ATP and Sly1 alone support fusion without an obvious tether. We hypothesize that improper ER/*cis*-Golgi *trans*-SNARE pairs may be selectively disassembled by Sec17/Sec18/ATP, while Sly1 association suffices to protect the active *trans*-SNARE complexes. Inactive *trans*-SNARE pairing, though transient, may provide a tether to enhance the proportion of correct SNARE pairings. SM proteins thus have at least 2 functions, catalysis of SNARE assembly and conferring resistance of cognate SNARE assemblies to inhibition by Sec17 and Sec18.

Roles of Sec17 and Sec18. Sec17 binds along the length of the 4-helical SNARE bundle (43), inserting its apolar N-domain loop into the bilayer to aid lipid rearrangements and promote

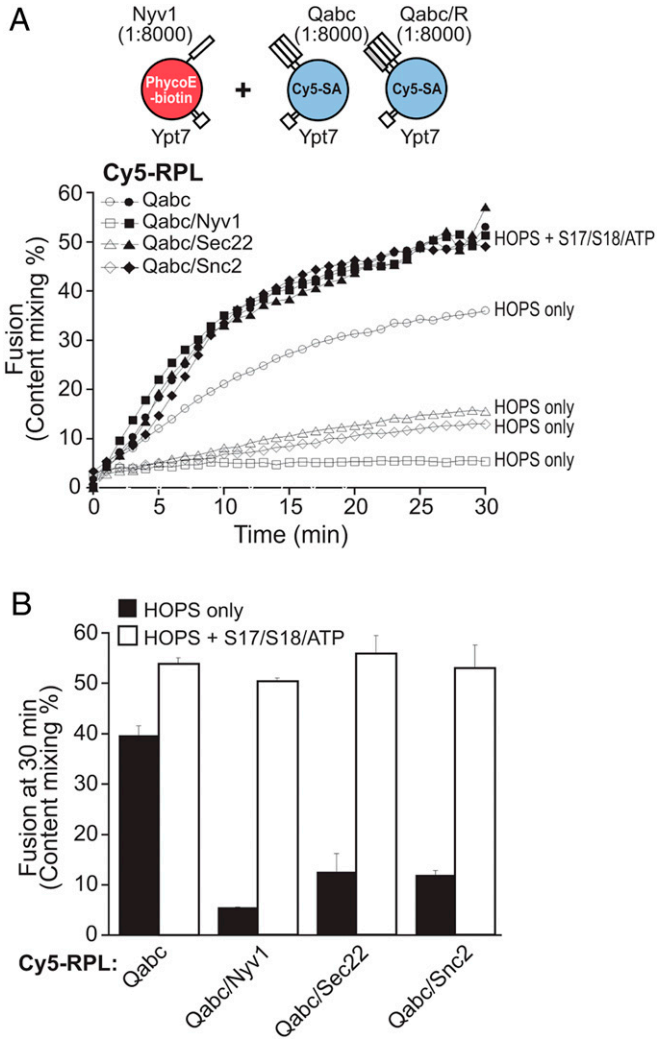

Fig. 4. Noncognate R-SNAREs associate with vacuolar Qabc SNAREs to form fusion-suppressed *cis*-SNARE complexes which can be disassembled by Sec17/Sec18. Fusion reactions had proteoliposomes bearing Ypt7-TM and the vacuolar R-SNARE Nyv1 at 1:8,000 protein:lipid molar ratios and proteoliposomes containing Ypt7-TM and vacuolar Qabc SNAREs only (circles), Qabc and Nyv1 (squares), Qabc and Sec22 (triangles), or Qabc and Snc2 (diamonds) at 1:8,000 protein:lipid molar ratios. Fusion reactions were incubated with HOPS (100 nM) only (open symbols) or HOPS and Sec17/Sec18/ATP (filled symbols) for 30 min at 27 °C. Kinetic curves in this figure are representative of $n \geq 3$ experiments; representative experiments (*A*) and means and SDs for triplicate repeats (*B*) are shown.

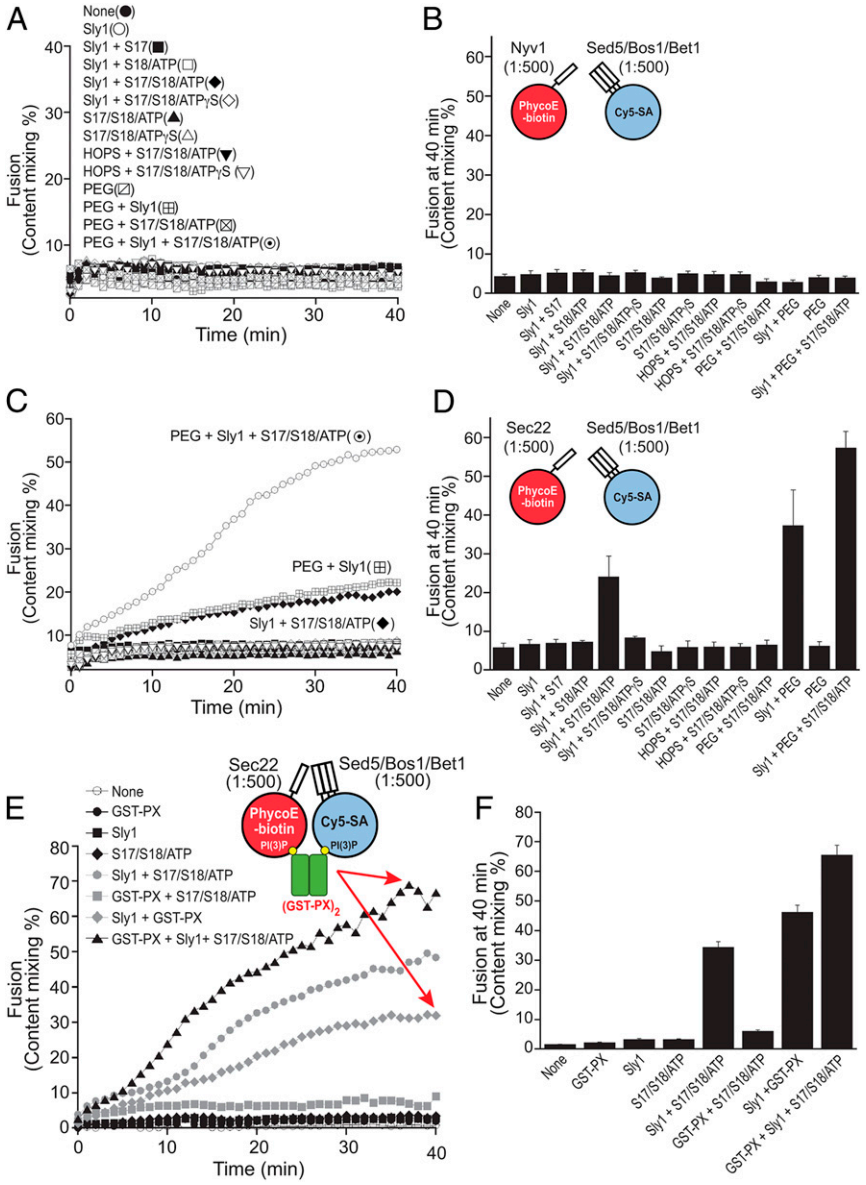

Fig. 5. In the presence of Sec17/Sec18, proteoliposomes with ER/*cis*-Golgi Qabc-SNAREs require Sly1 for fusion with proteoliposomes bearing the ER/*cis*-Golgi R-SNARE Sec22. Proteoliposomes bearing the ER/*cis*-Golgi Qabc-SNAREs Sed5/Bos1/Bet1 at 1:500 SNARE:lipid molar ratios were incubated with proteoliposomes containing either the vacuolar R-SNARE Nyv1 (*A* and *B*) or the ER/*cis*-Golgi R-SNARE Sec22 (*C*–*F*) at 1:500 SNARE:lipid molar ratios for 40 min at 27 °C in the absence or presence of indicated components: HOPS (100 nM), Sly1 (4.5 μM), GST-PX (1 μM), PEG (1%), Sec17 (0.4 μM), Sec18 (1.2 μM), and ATP (1 mM) or ATPγS (1 mM). For *E* and *F*, proteoliposomes were prepared with 1% PI(3)P, represented by yellow circles in the illustration. Kinetic curves in this figure are representative of $n \geq 3$ experiments; representative experiments and means and SDs for triplicate repeats are shown.

the completion of SNARE zippering (12). Sec18 can use the energy of ATP hydrolysis to disassemble SNAREs or may provide localized bulk to aid bilayer bending (41, 44). Based on their distinct needs for ATP hydrolysis, Sec17 and Sec18 may have different functions for vacuolar and ER/*cis*-Golgi fusion. Without Sec17/Sec18/ATP, fusion between R- and vacuolar 3Q-proteoliposomes proceeds almost as well with tethering via GST-PX dimer as via HOPS (Fig. 3). With GST-PX, fusion with vacuolar SNAREs is inhibited by Sec17/18/ATP (35). With HOPS however, there is equivalent stimulation of the initial rate of HOPS-dependent fusion by Sec17 and Sec18 with hydrolyzable or nonhydrolyzable ATP (Fig. 2*A*), indicating that the Sec17/18 stimulation is not via SNARE remodeling. In striking contrast to reconstituted vacuolar fusion, which is supported by either ATPγS or ATP (Fig. 2*A*), fusion reconstituted with SNAREs and SM protein of the ER/*cis*-Golgi (Fig. 5) requires Sec17, Sec18, and ATP and is not supported by ATPγS (Fig. 5 *C* and *D*). Without added tether, a requirement for Sec17, Sec18, and hydrolyzable ATP for ER/*cis*-Golgi fusion shows a need for SNARE complex disassembly (Fig. 5 *C* and *D*), perhaps to provide a tether, as discussed above, or to enforce correct conformations of assembled SNAREs, as demonstrated for a complete neuronal fusion reconstitution (45).

Combinatorial Fusion Specificity. While limited specificity is apparent in the match of R- and Q-SNAREs (Figs. 2 and 5), fusion by SNAREs alone (Fig. 1) is blocked by Sec17, Sec18, and ATP which can disassemble SNARE complexes. Without Sec17/Sec18, SM proteins and SM complexes may stimulate the fusion of proteoliposomes with organelle-matched or mismatched SNAREs (Figs. 1 and 2). HOPS can catalyze vacuolar SNARE assembly (8, 9)

and protect these *trans*-SNARE complexes against Sec17/Sec18 inhibition. While Sec17 and Sec18 and ATP can mediate disassembly of *trans*-SNARE complexes (11), they also stimulate fusion when the SNAREs and SM protein complex are from the same organelle (Figs. 2*A* and 5) as well as inhibiting fusion when they are not (Figs. 1 *C* and *E* and 2*I*). Thus, Sec17 and Sec18 act through more than just SNARE complex disassembly. Sec17 and Sec18 can act prior to fusion in conjunction with HOPS to stimulate fusion per se (10, 12, 13), and ATPγS can support this fusion (12). The synergistic action of the vacuolar SM complex HOPS with Sec17 and Sec18 is only seen with vacuolar SNAREs (Figs. 1, 2, and 5*A*). Although Sly1 only resembles the Vps33 SM subunit of HOPS rather than the entire complex, we now report that Sly1 also cooperates with Sec17 and Sec18 for fusion and that this is only seen with SNAREs from the ER/*cis*-Golgi, the same compartment as Sly1 (Fig. 5 *C*–*F*). An earlier reconstitution of fusion between ER-derived vesicles and *cis*-Golgi relied on the synthetic tethering agent polyethylene glycol (20); we confirm this finding (Fig. 5 *C* and *D*) and find that Sly1 and Sec17/Sec18/ATP (Fig. 5 *C* and *D*) or Sly1 and the synthetic tether of dimeric GST-PX (Fig. 5 *E* and *F*) support fusion in the absence of PEG. By imposing the rigorous fusion assay of protected lumenal compartment mixing and introducing the physiologically ubiquitous SNARE chaperones Sec17 and Sec18, tethering-dependent fusion is seen to be specific for the match between the organelle of origin of the R-SNARE, the Q-SNAREs, and the SM protein or complex. Further studies to explore the roles of Sec17, Sec18, and ATP in ER/*cis*-Golgi fusion may require functional reconstitutions which include purified ER/*cis*-Golgi tethering proteins Ypt1, TRAPP, and Uso1 as well. The full test of the generality of this model of fusion specificity will require the isolation of complete and functional sets of tethering and fusion factors for each stage of the endocytic and exocytic pathways.

Integrated Fusion Complexes. Current studies contribute to 2 conceptual models of fusion (Fig. 6). In 1 (Fig. 6*A*), factors such as SM proteins or SM protein complexes promote *trans*-SNARE complex assembly while Sec17 and Sec18 oppose functional assembly or mediate disassembly. These other proteins would no longer have a role once *trans*-SNARE complexes are correctly assembled, and the rate of fusion would simply be proportional to the steady-state level of *trans*-SNARE complexes. An alternative is an integrated fusion complex model (Fig. 6*B*). If each protein is considered as a piece of the integrated fusion complex jigsaw puzzle (Fig. 6 *B*, *Left*), mutual affinities may guide smooth assembly, and yet, like a puzzle, less efficient assembly or fusion might be seen when 1 component is missing. As an example, a current model of vacuole fusion (ref. 13; Fig. 6 *B*, *Right*) summarizes the multiplicity of known interactions of the vacuolar SNAREs, HOPS, Sec17, Sec18, Rab, and membrane lipids. Each of the components needed for fusion has multiple mutual affinities; for example, HOPS has individual affinities for each SNARE (6, 28), dual affinities for the Rab Ypt7 (27, 38), and binds phosphoinositides (28) and acidic lipids (39). Even a small protein such as Sec17 has affinities for itself, for Sec18, for each SNARE, and for the lipid bilayer (46). Fusion proteins and lipids are interdependent for assembly into fusion microdomains on the organelle (43). Functional reconstitution studies have shown that subcomplexes can assemble which lack either an individual SNARE (42), Sec17 (13), HOPS (31), or a key lipid (47) and which require just the missing protein or lipid to proceed to rapid fusion. In accord with this model, Sec17 is present on all *trans*-SNARE complexes which form during the fusion of purified vacuoles (11). *trans*-SNARE complexes may accumulate which are limited for fusion by their need to bind Sec17 (13). Sec18 is ubiquitous in the cytosol, and the presence of Sec17 on *trans*-SNARE complexes makes Sec18 a likely member of the assembly

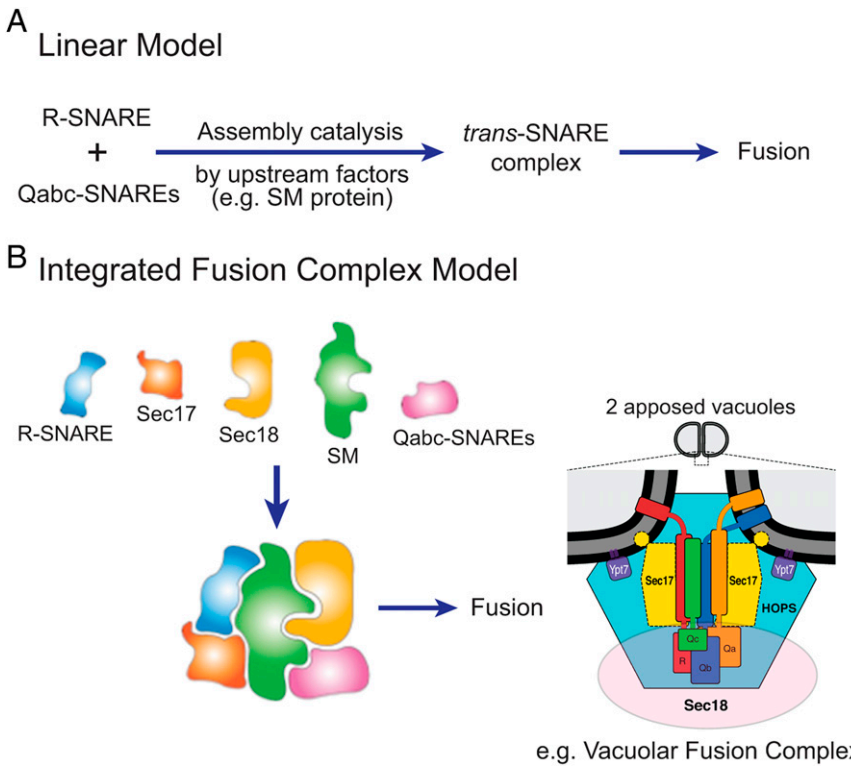

Fig. 6. Two models of membrane fusion. (*A*) In a "linear model," the rate of fusion is proportional to the steady-state concentration of *trans*-SNARE pairs. Other proteins only govern the rate of their assembly and disassembly. (*B*) In an "integrated fusion complex" model, the myriad affinities among fusion proteins and lipids guide their interdependent assembly. The correctly assembled complex is so stable as to resist disassembly by Sec17 and Sec18. See text for further details.

too. HOPS would then be continuously required to prevent the Sec17 and Sec18 from disassembling the *trans*-SNARE complex. This model is in accord with our current study, revealing the need for an organelle-specific match between components when challenged by Sec17 and Sec18.

Materials and Methods

Most lipids were from Avanti. Ergosterol was purchased from Sigma, and phosphatidylinositol 3-monophosphate from Echelon. Cy5-streptavidin and biotinylated R-phycoerythrin were obtained from SeraCare and from Life Technologies. Nonfluorescent streptavidin was obtained from Thermo Fisher.

Protein Expression and Purification. HOPS (26), vacuolar SNAREs (34), MBP-Ypt7-TM, and GST-PX (35) were isolated as described. MBP-tagged yeast SNAREs (Sec22, Snc2, Sed5, Bet1, Bos1, and Pep12) were constructed by In-Fusion cloning (Clontech). Briefly, the coding region of each yeast SNARE was PCR-amplified and cloned into pMBP-Parallel1 vector, and clones were confirmed by sequencing. MBP-tagged yeast SNAREs were produced in E.coli Rosetta(DE3)pLysS (Novagen). Transformants were grown in 2 L of TB media plus ampicillin and chloramphenicol and cultures shaken at 37 °C to A600 nm of 0.7, IPTG added to 1 mM, and shaken overnight at 16 °C. Cells were sedimented and resuspended in buffer A (40 mL, 20 mM Tris·HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, and protease inhibitor mixture; ref. 48), lysed by French Press at 4 °C, and centrifuged (Beckman 60Ti, 50,000 rpm, 30 min, 4 °C). Pellets were resuspended in buffer A plus 1% Triton X-100 (30 mL) with a Dounce homogenizer, then gently agitated (2 h, 4 °C) for solubilization. After centrifugation (60Ti, 50,000 rpm, 60 min, 4 °C), supernatant was mixed with amylose resin (NEB) (7.5 mL) in buffer B (20 mM Hepes-NaOH, pH 7.4, 200 mM NaCl, 10% glycerol, 1% β-octyl glucoside) and nutated (2 h, 4 °C). The suspended resin was poured into a 1.5-cm-diameter column at 4 °C and drained. Unadsorbed proteins were removed with 100 mL of buffer B, and tagged proteins eluted with 20 mM maltose in buffer B. Aliquots of each protein were snap-frozen in liquid nitrogen and transferred to −80 °C. Tobacco Etch Virus (TEV) protease was used to remove the MBP tag during proteoliposome preparation.

Proteoliposome Preparation. Vacuolar mixed lipids (VML; ref. 33) proteoliposomes were prepared from solutions with 50 mM β-octyl glucoside (28), with Ypt7-TM where indicated (1:8,000 molar ratio to lipid), and SNAREs at indicated protein:lipid molar ratios. Lipids in chloroform were mixed with β-OG in the following proportions: for VML (18:2) compositions, 47.6 mol% DOPC (1,2-Dioleoyl-sn-glycero-3-phosphocholine), 18 mol% DOPE (1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine), 18 mol% soy PI (L-α-phosphatidylinositol), 4.4 mol% DOPS (1,2-Dioleoyl-sn-glycero-3-phospho-L-serine), 2 mol% DOPA (1,2-Dioleoyl-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]). Liposomes bearing 3 Q-SNAREs at 1:500 SNARE:lipid molar ratios lacked PI(3)P, except for Fig. 6 *B*, *Right*. Chloroform was removed from samples in glass vials under a stream of nitrogen for 30 min, followed by 3 h speedvac, and the lipid/detergent pellet was solubilized in 400 μL of 2.5×-concentrated Rb150 (Rb150 is 20 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 10% [vol/vol] glycerol) by nutation for 3 h at room temperature, then detergent/lipid mixed micelle portions were stored under argon at −80 °C. After thawing and nutation for 30 min at room temperature, they were mixed with the indicated SNARE and Rab proteins and with Cy5-streptavidin and biotinylated phycoerythrin, dialyzed for at least 16 h at 4 °C in the dark against 250 mL of Rb150 + 1 mM $MgCl_2$ and 1g Biobeads SM2 with rapid stirring and isolated by flotation (12).

Membrane Fusion Assay. Fusion reactions (20 μL) contained pairs of proteoliposomes (each 250 μM lipid) in RB150 which were preincubated for 10 min at 27 °C with 5 μM streptavidin, 1 mM EDTA, and 10 μM GTP before addition of 1.25 mM $MgCl_2$ to complete the guanine nucleotide exchange of Ypt7. An aliquot (10 μL) was moved to a plate with 384 wells. Mixed proteins (10 μL; e.g., HOPS, Sly1, PEG, GST-PX, Sec17, and Sec18) or their buffers were added to start fusion. During 30 min at 27 °C in a fluorescence plate reader, fluorophore FRET (PhycoE:Cy5 FRET: excitation [ex]: 565 nm; emission [em]: 670 nm; cutoff: 630 nm) was measured each minute with a SpectraMax Gemini XPS (Molecular Devices) fluorescence plate reader. Complete content mixing levels were assayed by adding 1% (wt/vol) Thesit to samples lacking streptavidin.

Data Availability. All data discussed in the paper are presented herein.

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