Supporting Information

Mima and Wickner 10.1073/pnas.0908694106

SI Materials and Methods

Protein Isolation. Vam3p, Vti1p, and Nyv1p, were produced in *E. coli* Rosetta2 (Novagen) and purified (1). Full-length, untagged Vam7p was purified from inclusion bodies in *E. coli* Rosetta2(DE3)pLys (Novagen). The GST and linker sequences upstream of VAM7 were deleted from the expression vector for GST-Vam7p by QuikChange mutagenesis kit (Stratagene). After the induction at 37 °C for 3 h by 1 mM IPTG, cells from a 2-L culture were harvested, suspended in 80 mL of buffer A [20 mM Hepes-NaOH (pH 8.0), 0.5 M NaCl] containing 1 mM PMSF and 1 μg/mL pepstatin, lysed by French press at 4 °C, and centrifuged (60Ti [Beckman], 50,000 rpm, 30 min, 4 °C). Pellets were washed with 40 mL of buffer A containing 50 mM β-octyl glucoside (β-OG) followed by 80 mL of buffer A, resuspended with 20 mL of buffer A containing 50 mM DTT, 1 mM EDTA, and 6 M GdnHCl, and incubated at 37 °C for 1 h with shaking. The suspension was centrifuged (60Ti [Beckman], 20,000 rpm, 30 min, 4 °C) and the supernatant was dialyzed against 2-L portions of buffer A at 4 °C for 12 h, then 3 h, and centrifuged again (60Ti [Beckman], 50,000 rpm, 30 min, 4 °C), yielding refolded Vam7p in the supernatant. TEV protease (2), affinity-purified antibodies against vacuolar SNAREs, Sec17p, Sec18p, and Vps33p (3), Sec17p (4), Sec18p (4), and HOPS (5) were purified as described.

Preparation of SNARE Proteoliposomes. RPLs were prepared with purified vacuolar SNAREs and POPC [44 or 46% (mol/mol) for donor or acceptor, respectively], POPE (18%), soyPI (18%), POPS (4.4%), POPA (2.0%), CL (1.6%), ERG (8.0%), DAG (1.0%), and fluorescent lipids (1.5% each of NBD-PE/Rh-PE or 1.0% dansyl-PE for donor or acceptor RPLs, respectively) (1). DiC8- or diC4-phosphoinositides (Echelon) in RB150 [20 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 10% (vol/vol) glycerol] were added to fusion reactions.

Proteoliposome Lipid Mixing Assay. The assay of lipid mixing between SNARE-RPLs (1) was modified. Reaction mixtures (RB150, 1 mM ATP, 2 mM $MgCl_2$, 50 μM donor RPLs, 400 μM acceptor RPLs, and 90 μM diC8- or diC4-phosphoinositides) were prepared in black 384-well plates (no. 3676, Corning) on ice and incubated at 27 °C for 10 min in a SpectraMAX Gemini XPS plate reader (Molecular Devices) preequilibrated at 27 °C. After 10 min, premixed Sec17p (620 nM or 1.2 μM), Sec18p (510 nM or 1.0 μM), HOPS (28 or 55 nM), and Vam7p (40 nM−4 μM) were added as indicated and NBD fluorescence was monitored. For calculating the ratios of NBD fluorescence, F/F_0, the fluorescence before the Sec17p/Sec18p/HOPS addition (at 0 min) was used for F_0, except for the reactions with the Qabc-SNARE RPLs and R-SNARE RPLs (Figs. 3*C*, 5, and 6), where F_0 was the lowest values during the initial 10 min incubation. Topology of lipid mixing (Fig. S2 *C* and *D*) was analyzed by adding sodium dithionite (Sigma) while monitoring fluorescence (1).

Flotation Assays of Liposome Association. SNARE RPL reactions (100 μL each) in RB150, with 1 mM ATP, 2 mM $MgCl_2$, 90 μM diC8- or diC4-phosphoinositides, 620 nM Sec17p, 510 nM Sec18p, 28 nM HOPS, and 400 nM or 4 μM Vam7p, where indicated, were incubated at 27 °C for 30 min, placed on ice for 10 min, and mixed with 400 μL 50% Histodenz (Sigma) in RB150 to a final Histodenz concentration of 40%, transferred to 11 × 60 mm tubes, overlaid with 1.6 mL 35% Histodenz in RB150, 2 mL 30% Hisotodenz in RB150, and 200 μL RB150, and centrifuged (SW60Ti, 55,000 rpm, 3 h, 4 °C). RPLs were harvested from the top and analyzed by SDS/PAGE and immunoblotting.

1. Mima J, Hickey CM, Xu H, Jun Y, Wickner W (2008) Reconstituted membrane fusion requires regulatory lipids, SNAREs and synergistic SNARE chaperones. *EMBO J* 27:2031–2042.
2. Jun Y, Xu H, Thorngren N., Wickner W (2007) Sec18p and Vam7p remodel trans-SNARE complexes to permit a lipid-anchored R-SNARE to support yeast vacuole fusion. *EMBO J* 26:4935–4945.
3. Collins KM, Thorngren NL, Fratti RA, Wickner WT (2005) Sec17p and HOPS, in distinct SNARE complexes, mediate SNARE complex disruption or assembly for fusion. *EMBO J* 24:1775–1786.
4. Haas A, Wickner W (1996) Homotypic vacuole fusion requires Sec17p (yeast α-SNAP) and Sec18p (yeast NSF). *EMBO J* 15:3296–3305.
5. Starai VJ, Hickey CM, Wickner W (2008) HOPS proofreads the trans-SNARE complex for yeast vacuole fusion. *Mol Biol Cell* 19:2500–2508.

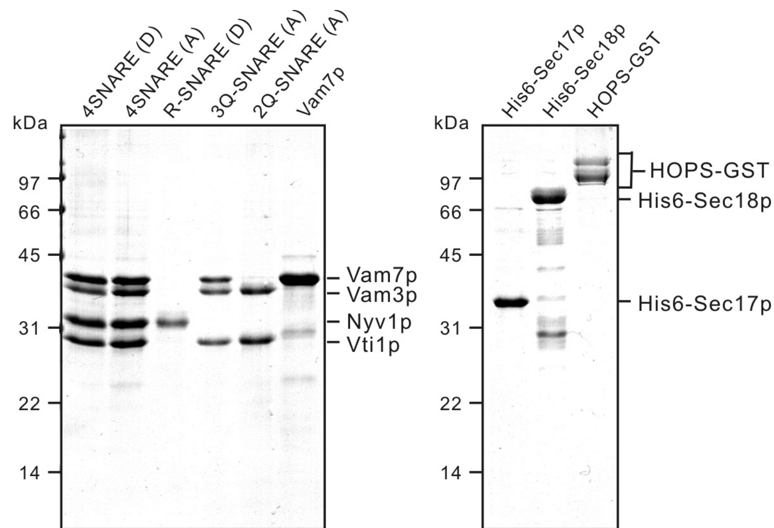


Fig. S1. Coomassie-stained gels of reconstituted proteoliposomes (RPLs), soluble SNARE (Vam7p), and SNARE chaperones (His_6-Sec17p, His_6-Sec18p, and HOPS-GST). RPLs bear 4SNAREs, 3Q-SNAREs (Vam3p, Vti1p, and Vam7p), 2Q-SNAREs (Vam3p and Vti1p), or 1R-SNARE (Nyv1p). Donor and acceptor RPLs are indicated as (D) and (A). Vam7p is the soluble Qc-SNARE, which does not have a transmembrane domain. HOPS-GST is the heterohexameric complex, containing Vps11p, Vps16p, Vps18p, Vps33p, Vps39p, and Vps41p, in which one of the subunits, Vps33p, is tagged with a GST moiety at its C terminus.

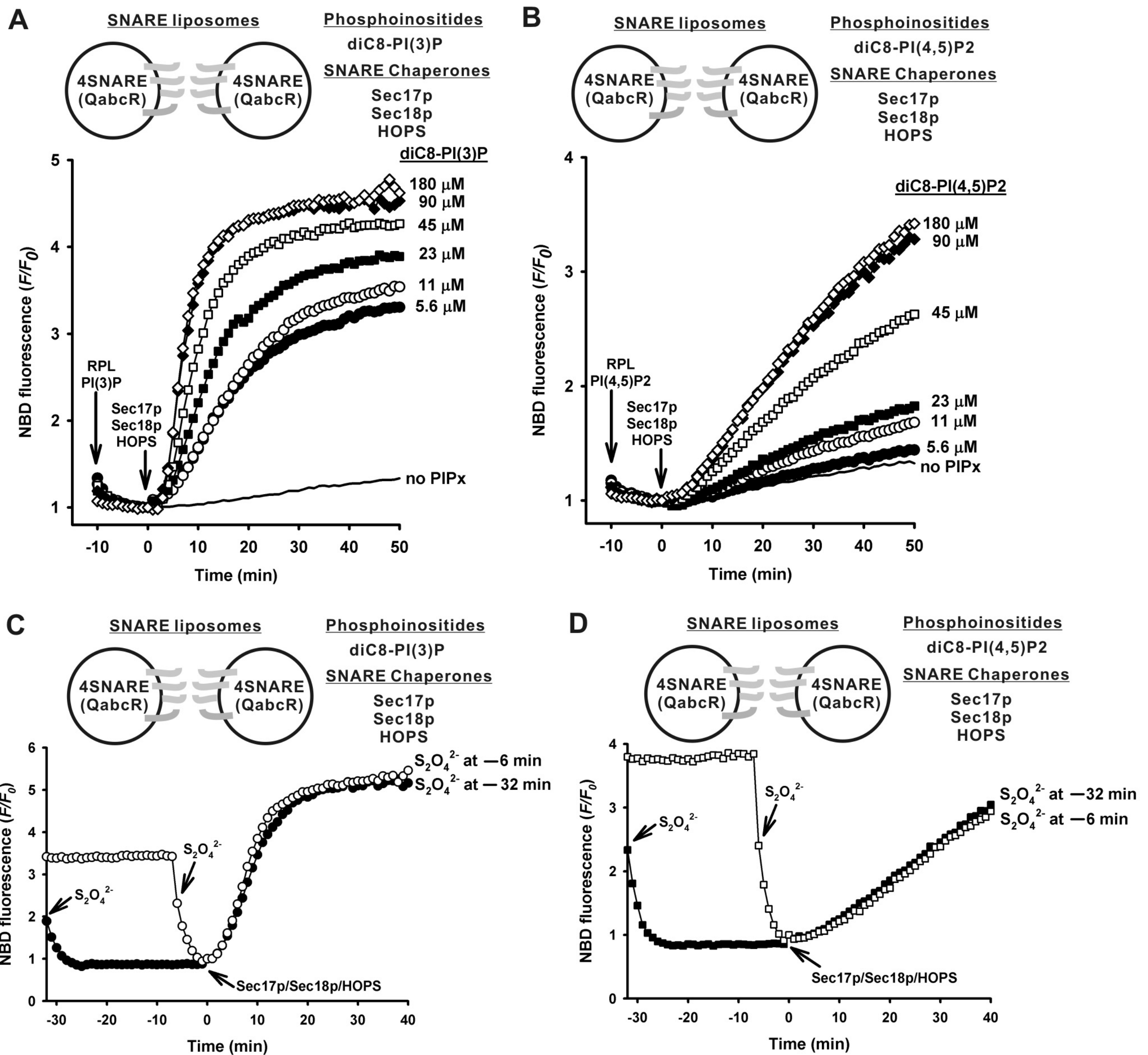


Fig. S2. Either PI(3)P or PI(4,5)P_2 strongly stimulates membrane fusion of RPLs bearing vacuolar SNAREs and SNARE chaperones. Lipid mixing was assayed in RB150 with the 4SNARE-RPLs (450 μM total lipids, 300–750 nM of each SNARE), ATP (1 mM), $MgCl_2$ (2 mM), His_6-Sec17p (620 nM), His_6-Sec18p (510 nM), HOPS-GST (28 nM), and either diC8-PI(3)P or diC8-PI(4,5)P_2 (5.6–180 μM each). Each panel shows representative data from at least 3 experiments. (*A* and *B*) The stimulation of lipid mixing by diC8-PI(3)P (*A*) or diC8-PI(4,5)P_2 (*B*). (*C* and *D*) The reconstituted lipid mixing in the presence of 90 μM diC8-PI(3)P (*C*) or diC8-PI(4,5)P_2 (*D*) shows complete membrane fusion with little accompanying lysis. Topology analysis of the lipid-mixing reactions was performed with sodium dithionite ($S_2O_4^{2-}$) (14, 50). $S_2O_4^{2-}$ (4 mM in final) was added either 32 min or 6 min before Sec17p/Sec18p/HOPS. The exposed NBD groups on donor RPLs were reduced, inactivating their fluorescence. After the Sec17p/Sec18p/HOPS addition, the $S_2O_4^{2-}$, which had been added 6 min before, was still active for reduction of the exposed NBD groups whereas the $S_2O_4^{2-}$, which had been added 32 min before the chaperones, was no longer active (14).

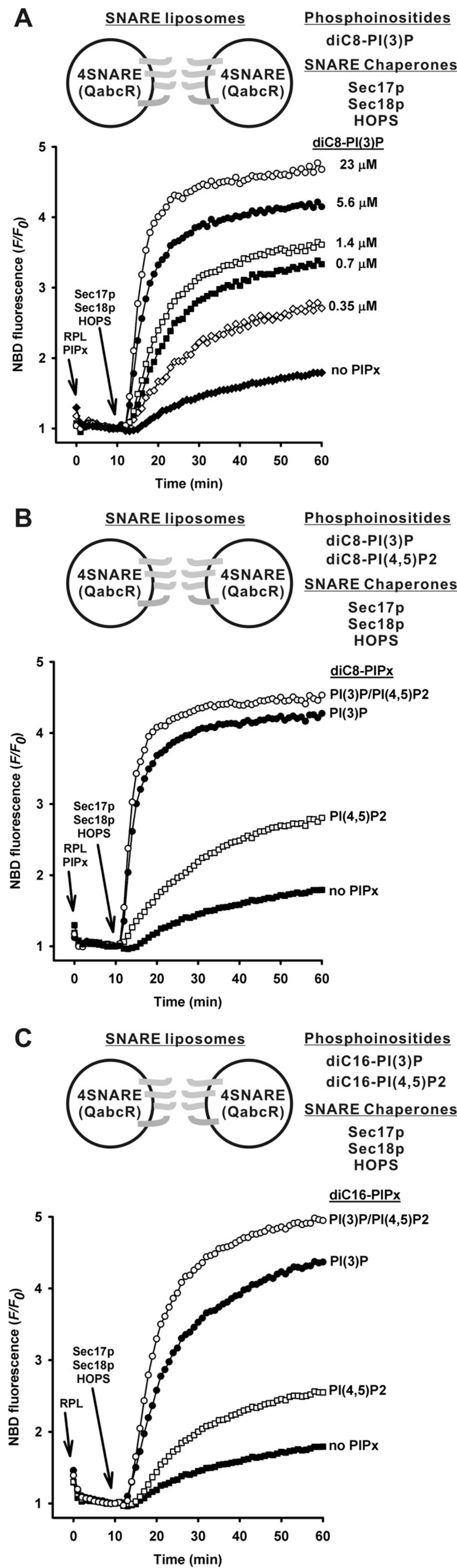


Fig. S3. SNARE-RPL lipid-mixing assays in the presence of either diC8- or diC16-phosphoinositides. (*A*) A low concentration of diC8-PI(3)P can robustly stimulate fusion between 4 SNARE-RPLs in the presence of SNARE chaperones, Sec17p/Sec18p/HOPS. Lipid mixing between 4SNARE-RPLs was assayed as in Fig. S2*A* in the presence of 0.35–23 μM diC8-PI(3)P. (*B*) Lipid mixing between 4SNARE-RPLs was assayed as in Fig. S2*A* but in the presence of either diC8-PI(3)P (23 μM, solid circles) or diC8-PI(4,5)P_2 (23 μM, open squares), or both diC8-phosphoinositides (23 μM each, open circles). (*C*) Either diC16-PI(3)P or diC16-PI(4,5)P_2 strongly stimulates fusion between 4 SNARE-RPLs in the presence of SNARE chaperones. Lipid mixing between 4SNARE-RPLs was assayed as in Fig. S2*A* but in the presence of either diC16-PI(3)P (solid circles) or diC16-PI(4,5)P_2 (open squares), or both diC16-phosphoinositides (open circles). Each diC16-phosphoinositide (1% of total lipids, mol/mol) had been reconstituted into RPLs during the preparation.

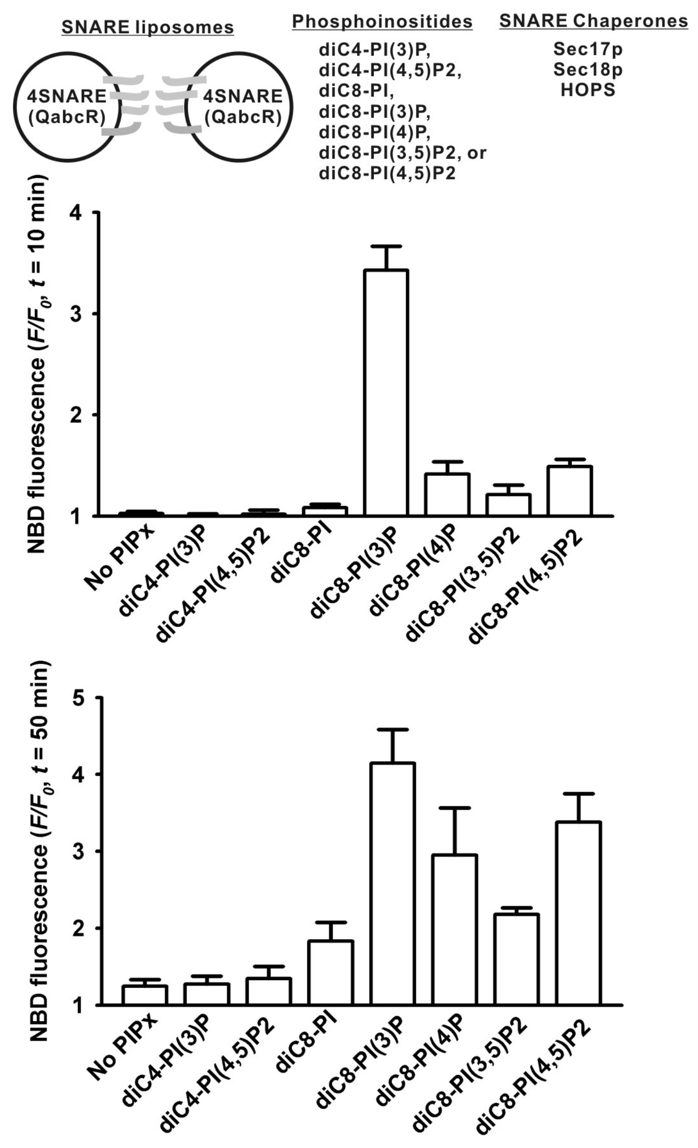


Fig. S4. Phosphoinositide specificity of fusion. The mean values and standard deviations of the NBD fluorescence signals of the lipid-mixing assay in Fig. 2*A* (the relative fluorescence, F/F_0) at 10 (*Upper* panel) and 50 (*Lower* panel) mins.

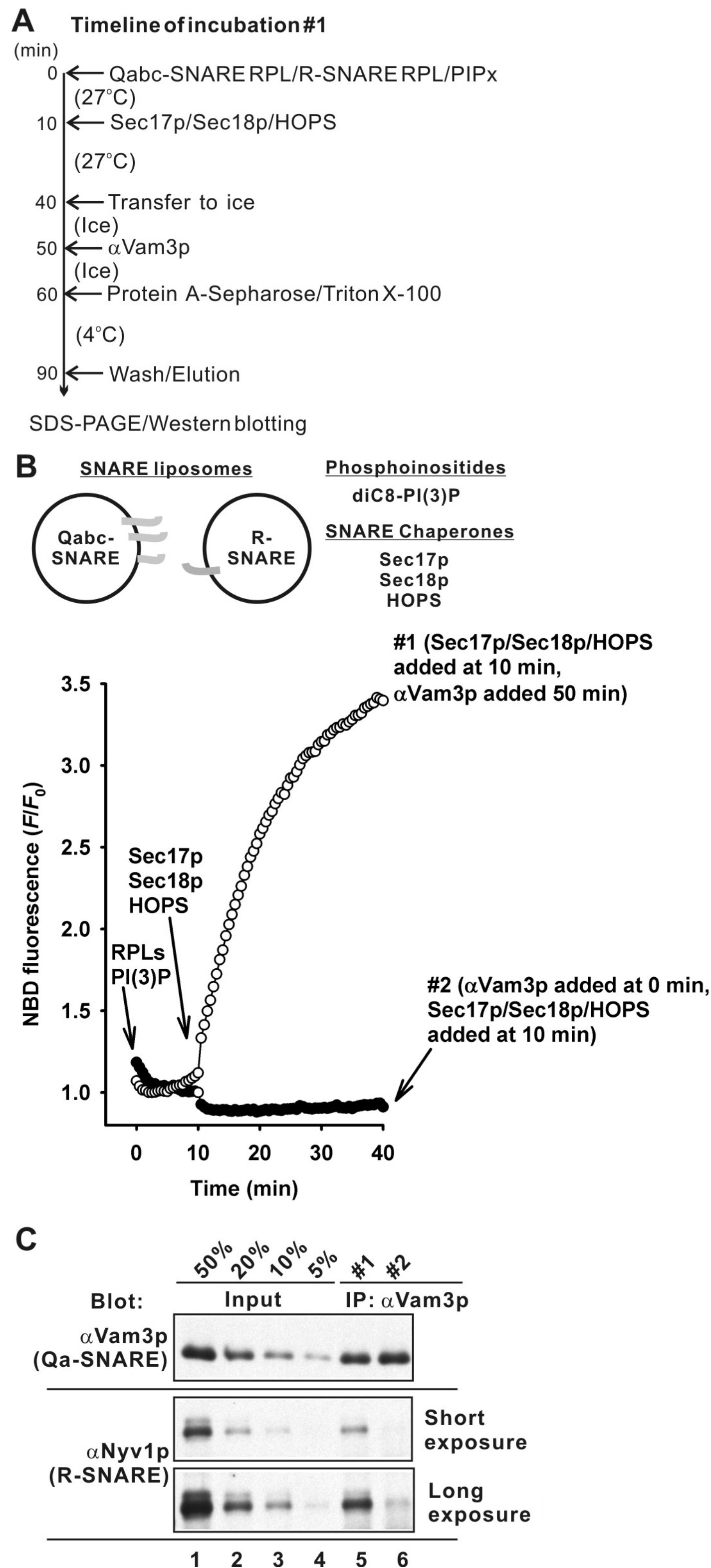
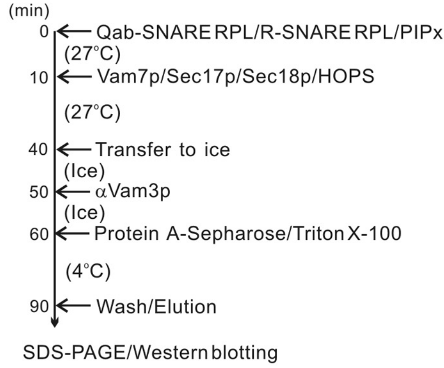
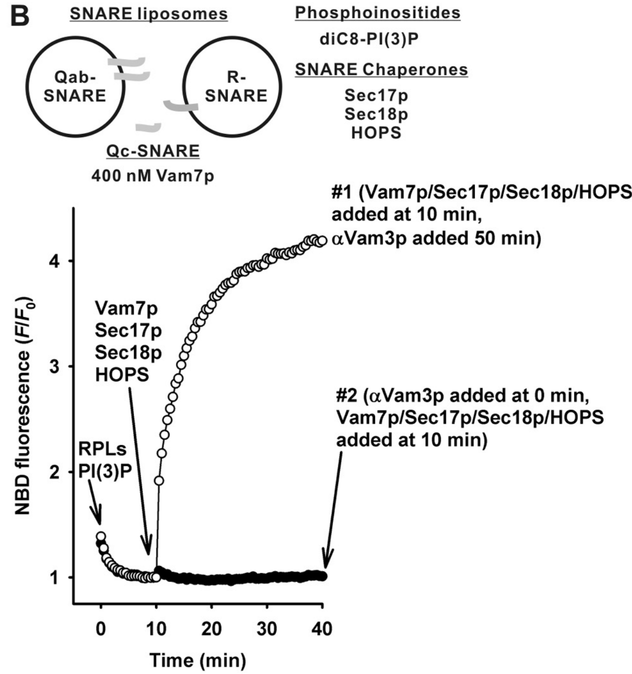
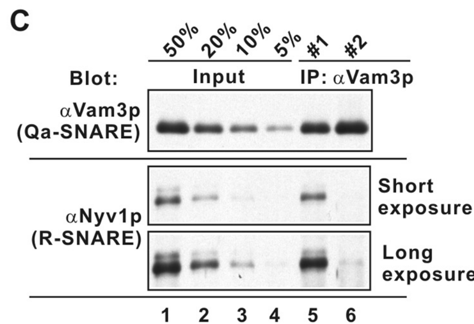
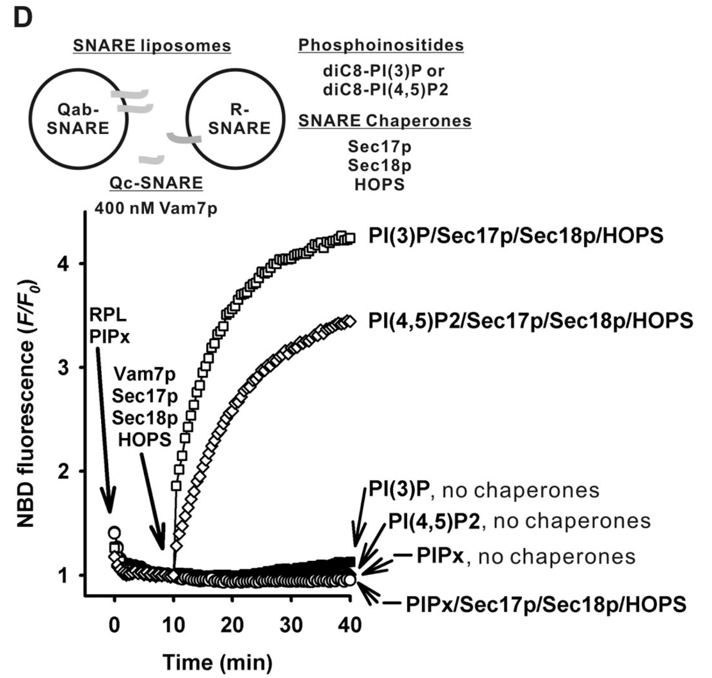


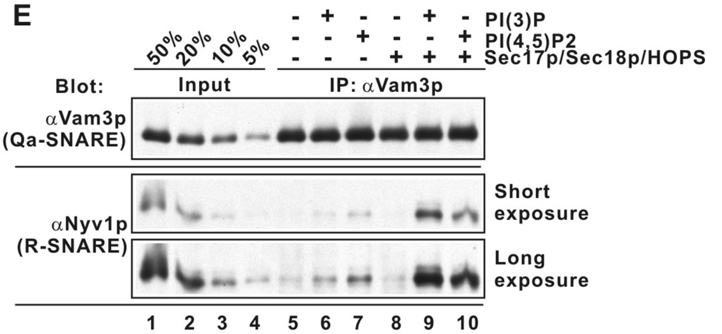
Fig. S5. Control experiments for the SNARE-complex assembly assay with Qabc-and R-SNARE RPLs. (*A*) Design of the experiment. (*B* and *C*) Nyv1p specifically associates with Vam3p during fusion. (*B*) Lipid mixing between the Qabc-SNARE RPLs and R-SNARE RPLs was assayed with 90 μM diC8-PI(3)P, as in Fig. 5*B*, but αVam3p was added at 50 min (no. 1) or 0 min (no. 2) and Sec17p/Sec18p/HOPS were added at 10 min (nos. 1 and 2). (*C*) Nyv1p bound to Vam3p was analyzed by SDS/PAGE and immunoblotting. Sample no. 1 showed rapid fusion in *B*, open circles, with substantial Nyv1p association with Vam3p (*C*, lane 5), whereas neither detectable fusion (*B*, solid circles) nor Nyv1p:Vam3p association was obtained with sample no. 2 (*C*, lane 6)











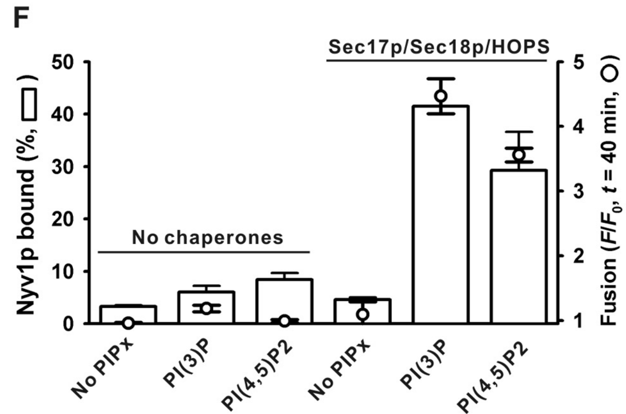


Fig. S6. SNARE chaperones and phosphoinositides cooperate for SNARE-complex assembly. (*A*) Design of the experiment. (*B* and *C*) Nyv1p specifically associates with Vam3p during fusion reactions. (*B*) Lipid mixing with Qab-SNARE RPLs, R-SNARE RPLs, and exogenous Vam7p was assayed with 90 μM diC8-PI(3)P, as in Fig. 4*B*, but αVam3p was added at 50 min (no. 1) or 0 min (no. 2) and Vam7p/Sec17p/Sec18p/HOPS were added at 10 min (nos.1 and 2). (*C*) Nyv1p bound to Vam3p in reactions nos.1 and 2 were analyzed by SDS/PAGE and immunoblotting. (*D–F*) Either PI(3)P or PI(4,5)P_2 and SNARE chaperones synergistically promote the association of Nyv1p with Vam3p during fusion. (*D*) Lipid mixing was assayed in the absence of phosphoinositides or in the presence of either diC8-PI(3)P or diC8-PI(4,5)P_2, as in Fig. 4 *A–C*, with SNARE chaperones Sec17p/Sec18p and HOPS. (*E*) Nyv1p bound to Vam3p in the reactions in *D*. (*F*) Means and standard deviations of the fusion signals (F/F_0, open circles) in *D* and Nyv1p bound (%, open bars) in *E*.