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HOPS Initiates Vacuole Docking by Tethering Membranes before trans-SNARE Complex Assembly

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Vacuole homotypic fusion has been reconstituted with all purified components: vacuolar lipids, four soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, Sec17p, Sec18p, the Rab Ypt7p, and the hexameric homotypic fusion and vacuole protein sorting complex (HOPS). HOPS is a Rab-effector with direct affinity for SNAREs (presumably via its Sec1-Munc18 homologous subunit Vps33p) and for certain vacuolar lipids. Each of these pure vacuolar proteins was required for optimal proteoliposome clustering, raising the question of which was most directly involved. We now present model subreactions of clustering and fusion that reveal that HOPS is the direct agent of tethering. The Rab and vacuole lipids contribute to tethering by supporting the membrane association of HOPS. HOPS indirectly facilitates trans-SNARE complex formation by tethering membranes, because the synthetic liposome tethering factor polyethylene glycol can also stimulate trans-SNARE complex formation and fusion. SNAREs further stabilize the associations of HOPS-tethered membranes. HOPS then protects newly formed trans-SNARE complexes from disassembly by Sec17p/Sec18p.

INTRODUCTION

Membrane fusion is essential for cell growth, hormone secretion, and neurotransmission (Wickner and Schekman, 2008). Each fusion event in the secretory pathway involves Rab GTPases, Rab effectors (proteins that bind Rabs), tethering factors (which are often Rab effectors), soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs), SNARE-associated proteins (including Sec1-Munc 18 [SM] proteins and chaperones that mediate SNARE complex disassembly), and specific lipids. Fusion proceeds through sequential stages of tethering, docking, outer leaflet lipid mixing (hemifusion), and finally inner leaflet lipid mixing and contents mixing. Tethering, the initial contact between membranes, requires the Rab GTPases and tethering factors (Grosshans *et al*., 2006). Tethered membranes can form trans-SNARE complexes (anchored to apposed membranes) and are then referred to as docked membranes. Localized bilayer destabilization leads to fusion. The resulting cis-SNARE complexes (bound to one membrane) are recycled to individual SNAREs by the SNARE disassembly machinery (soluble NSF attachment protein or Sec17p and NSF or Sec18p), allowing subsequent rounds of fusion. Several factors may increase the formation of trans-SNARE complexes and promote fusion (Collins and Wickner, 2007; Shen *et al*., 2007; Mima *et al*., 2008; James *et al*., 2009). However, it has been unclear how most of these factors, and particularly SM proteins, accomplish this stimulation (Sudhof and Rothman, 2009). Here, we address this issue by studying the docking and fusion of proteoliposomes that bear purified yeast vacuolar fusion factors, including the heterohexameric homotypic fusion and vacuole protein sorting complex (HOPS), a tethering factor that also has direct affinity for SNAREs.

We study membrane fusion using isolated yeast vacuoles (Ostrowicz *et al*., 2008) and proteoliposomes reconstituted with purified vacuolar proteins and lipids (Mima *et al*., 2008; Hickey *et al*., 2009; Stroupe *et al*., 2009). Vacuole fusion requires the Rab Ypt7p, HOPS, Sec17p, Sec18p, chemically minor yet functionally essential lipids, termed "regulatory" lipids, and four vacuolar SNAREs. Three of the SNAREs, Vam3p, Vti1p, and Vam7p, have a conserved glutamine at the core of their heptad-repeat SNARE domain and are therefore termed Q-SNAREs. One of these Q-SNAREs, Vam7p, lacks a typical transmembrane anchor, but contains a phox homology domain that binds phosphatidylinositol 3-phosphate [PI(3)P]. Nyv1p is the R-SNARE for vacuole fusion, with a conserved arginyl residue at the center of its SNARE domain. These SNAREs are represented schematically in Figure 1A.

HOPS has affinity for Ypt7p (Seals *et al*., 2000), acidic phospholipids (including phosphoinositides; Stroupe *et al*., 2006), and multiple vacuolar SNAREs (Dulubova *et al*., 2001; Collins *et al*., 2005; Stroupe *et al*., 2006), and each of these affinities contribute to HOPS association with the organelle or reconstituted liposome. The affinity of HOPS for SNAREs is presumably via its SM protein subunit Vps33p. Rapid proteoliposome fusion requires HOPS, vacuolar SNAREs, and vacuolar lipids (Mima *et al*., 2008). Sec17p and Sec18p are also essential if all four SNAREs are present on each membrane (i.e., if cis-SNARE complexes need to be disassembled). Fusion is possible in the presence of Sec17p/Sec18p, the SNARE complex disassembly chaperones, because HOPS specifically protects trans-SNARE complexes

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Abbreviations used: HOPS, homotypic fusion and vacuole protein sorting complex; PEG, polyethylene glycol; Q-SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor with glutamine at the zero layer of the SNARE motif; R-SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor with arginine at the zero layer of the SNARE motif; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

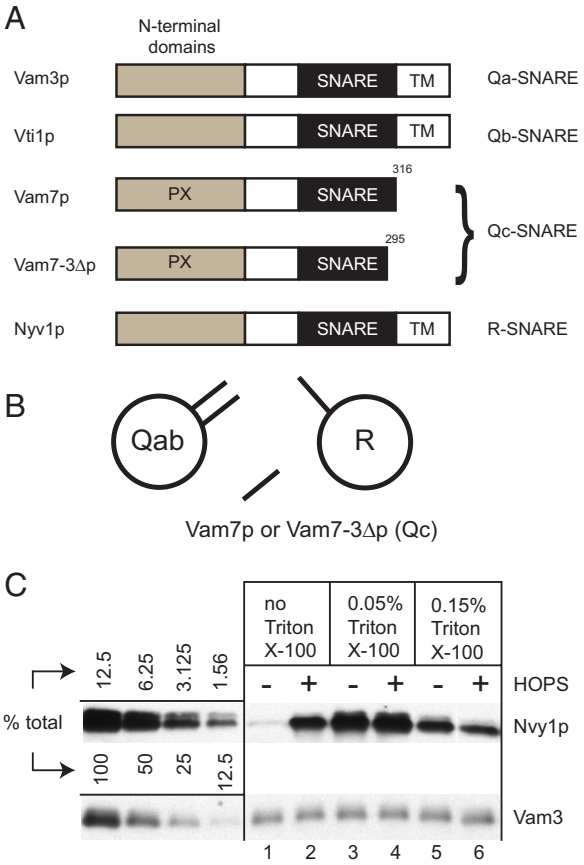

Figure 1. HOPS stimulates trans-SNARE complex assembly between proteoliposomes but does not affect the assembly of SNAREs in detergent. (A) Schematic representation of the SNAREs used in this study. (B) Schematic representation for the topology of the SNAREs used to drive trans-SNARE complex formation in the absence of cis-SNARE complex disassembly. (C) Proteoliposomes bearing Qab-SNAREs (acceptor) and R-SNARE (donor) were incubated with HOPS (90 nM) or HOPS buffer but without Vam7p for 10 min at 27°C. All reactions contained 0.5 mM $MgCl_2$. Triton X-100 (in RB150) or RB150 (no Triton X-100) was added where indicated, immediately followed by Vam7-3Δp (200 nM) addition. After 15 min, RIPA buffer was added, Vam3p was immunoprecipitated, and Vam3p and Nyv1p were detected by immunoblot (see *Materials and Methods*). % total refers to the indicated percentage of the starting proteoliposome mixture, to allow evaluation of the efficiency of SNARE coisolation. Data shown are representative of three independent experiments. See Supplemental Figure S2 for mean ± SD of three experiments.

from Sec17p/Sec18p action (Xu *et al*., 2010). Ypt7p is essential for reconstituted fusion under conditions where HOPS cannot bind directly to lipids, such as when the Vps41p subunit of HOPS is phosphorylated by the vacuolar kinase Yck3p (Hickey *et al*., 2009). Thus, the major function of Ypt7p is to bind HOPS to the membrane.

HOPS promotes trans-SNARE complex formation (Collins and Wickner, 2007). However, it has been unclear whether HOPS promotes trans-SNARE complex formation directly, by catalyzing the assembly of SNARE domains into a complex, indirectly, by promoting the tethering of SNARE-liposomes, or both, as these are not mutually exclusive. Vps33p, the vacuolar SM protein, is a subunit of HOPS, and SM proteins are proposed to catalyze trans-SNARE complex assembly (Rizo and Sudhof, 2002). We now report that we find no evidence for a direct catalysis of SNARE complex assembly by HOPS, but that HOPS does indirectly catalyze the assembly of trans-SNARE complexes via tethering membranes. HOPS tethers liposomes of vacuolar lipids, and this tethering is strongly stimulated by Ypt7p, which stimulates HOPS binding to the membrane, consistent with HOPS membrane association being the major role of Ypt7p (Hickey *et al*., 2009; Stroupe *et al*., 2009). This is the first demonstration of a direct tethering activity of HOPS without SNAREs. Trans-SNARE pairing then captures tethered membranes. HOPS also blocks Sec17p/Sec18p-mediated disassembly of trans-SNARE complexes (Xu *et al*., 2010) and proofreads their structures (Starai *et al*., 2008) and thus has functions beyond tethering.

MATERIALS AND METHODS

Reagents and Strains

Polyethylene glycol (PEG 8000) was from Sigma-Aldrich (St. Louis, MO; P-5413). SNAREs (Mima *et al*., 2008), Ypt7p (Hickey *et al*., 2009), Sec17p (Schwartz and Merz, 2009), and Sec18p (Haas and Wickner, 1996) were purified as described previously.

The HOPS overproducing yeast strain CHY61 was constructed like CHY31 (Starai *et al*., 2008) except that the *VPS33* gene under control of the GAL1 promoter was from p403GAL1-VPS33-LINKER-TEVSITE-GST (Stroupe *et al*., 2009), which allows tobacco etch virus (TEV) protease-mediated cleavage of glutathione transferase (GST) from the Vps33p-GST in purified HOPS. HOPS used in this study was isolated from CHY61 vacuolar membranes (purified in 8% Ficoll) as reported for CHY31 (Starai *et al*., 2008) followed by TEV cleavage, gel filtration, and storage as reported for HOPS from CHY69 (Stroupe *et al*., 2009), with the exception of the HOPS used for Supplemental Figure S1, which was HOPS-GST from CHY31.

Liposome Production and Lipid Mixing Assays

Liposomes were produced as described previously (Mima *et al*., 2008), with vacuole-mimic plus regulatory lipids, hereafter termed vacuolar lipids, unless indicated otherwise. All liposomes had fluorescent lipids (Mima *et al*., 2008). SNAREs, where added, were each at protein to lipid molar ratios of 1:1000. Ypt7p, where added, was at protein to lipid molar ratios of 1:2000 (1×) to 1:333 (6×). Ypt7p concentrations in liposomes were determined using a combination of a Sypro Ruby-stained (Invitrogen, Carlsbad, CA) gel with a standard curve of bovine serum albumin (Sigma-Aldrich) and immunoblotting for Ypt7p. Lipid mixing assays in 384-well plates consisted of donor (50 μM lipids) and acceptor (400 μM lipids) liposomes mixed in RB150, with the specified amounts of $MgCl_2$ and ATP.

Liposome Clustering Assays

After incubation, each reaction was mixed by pipetting, diluted where indicated, and 4 μl was placed on a microscope slide (Gold Seal no. 3051; Thermo Scientific, Portsmouth, NH) and covered with a 22-mm coverslip (2810-22; Corning Life Sciences, Lowell, MA). Images were collected using a TCS SP UV confocal microscope (Leica Microsystems, Deerfield, IL) with a krypton (568 nm) laser, a 63× objective, and confocal software (Leica Microsystems). Random fields were obtained as follows: the appropriate plane of focus (z coordinate) was determined with the x and y coordinates near the lower left corner of the coverslip; this field was the first image collected. Subsequent images were collected as the slide was moved in the y plane until the objective was close to the edge of the coverslip, at which point the slide was moved in the x plane. This procedure was continued until several images were obtained (the number of fields used per reaction is indicated in the figure legends). Occasional images were removed from analyses due to the obvious presence of large, highly fluorescent foreign material. Otherwise, successive images were used for analyses. Particle sizes were measured using ImageJ (National Institutes of Health, Bethesda, MD) after setting the lower threshold level to 50 and the upper threshold level to 255. Each pixel equals 0.096 μm^2, as determined using a micrometer slide. Pixels are used in the probability plots because the actual size of the particles (liposomes or liposome clusters) is unknown; its assay depends on the power of the laser, which was higher for imaging POPC/POPS (no proteins) liposomes.

Trans-SNARE Complex Assembly Assay

Immobilized αVam3p was prepared as described (Xu *et al*., 2010). Liposome solubilization, Vam3p immunoprecipitation, Western blotting, and quantification were performed as described, except that each reaction received only 400 μl of radioimmunoprecipitation assay (RIPA) buffer (with EDTA and protease inhibitor cocktail, but without phenylmethylsulfonyl fluoride) and the αVam3p resin was resuspended in only 400 μl of RIPA buffer during each of the washes.

RESULTS

HOPS promotes SNARE complex assembly among intact, isolated vacuoles (Collins and Wickner, 2007) and is required for organelle fusion (Stroupe *et al*., 2006). To test whether HOPS directly catalyzes SNARE complex assembly, we prepared recombinant vacuolar SNAREs without their transmembrane segments (Vam7p naturally has no transmembrane domain), an approach used previously to study the relationship between the SM protein Sly1p and the SNAREs involved in transport from the endoplasmic reticulum to the Golgi (Peng and Gallwitz, 2002). The pure vacuolar SNAREs showed slow complex assembly, assayed as the association of the other SNAREs with MBP-Vti1p (Supplemental Figure S1). Even stoichiometric amounts of added HOPS had no effect on the rate of SNARE assembly, consistent with a previous study of Sly1p (Peng and Gallwitz, 2002).

Because the failure of HOPS to promote complex formation between the soluble domains of SNAREs is only a negative result, we turned to mixtures of proteoliposomes bearing Nyv1p (the vacuolar R-SNARE) or the Q-SNAREs Vti1p and Vam3p (Figure 1B). We added a C-terminally truncated form of Vam7p, Vam7-3Δp (Schwartz and Merz, 2009), which supports trans-SNARE complex assembly without fusion. Use of Vam7-3Δp ensures that newly formed SNARE complexes are of the trans configuration and not cis-SNARE complexes that might otherwise have formed after the bilayers had merged (i.e., after fusion). In this proteoliposome incubation, HOPS strongly increased SNARE complex assembly (Figure 1C, lanes 1 and 2; and Supplemental Figure S2). The addition of detergent to the liposomes before the addition of Vam7-3Δp allowed us to assay SNARE complex assembly in detergent micelles. HOPS had no effect on the assembly of SNAREs using two different Triton X-100 concentrations (Figure 1C, lanes 3–6). Higher detergent concentrations yielded slower kinetics of SNARE complex assembly because SNARE assembly is probably best when the interacting SNAREs occupy the same micelle and more micelles reduce the likelihood of this copopulation (Carman *et al*., 1995).

How might HOPS promote SNARE complex assembly on these liposomes, which have no other proteins? HOPS associates with protein-free liposomes of vacuolar lipids far more stably than with liposomes of the simpler POPC/POPS (82%:15%) lipid composition (Hickey *et al*., 2009). We tested the ability of HOPS to cluster each of these types of liposomes. HOPS clustered vacuolar lipid liposomes (Figure 2, A and B; and Supplemental Table S1) but not POPC/POPS liposomes (Figure 2C and Supplemental Table S1).

Prenylated Ypt7p binds HOPS to liposomes (Stroupe *et al*., 2009), including those made of POPC/POPS lipids (Hickey *et al*., 2009). Because Ypt7p is essential for physiological vacuole clustering (Mayer and Wickner, 1997), we assayed the effects of Ypt7p on HOPS-mediated liposome clustering. HOPS mediates striking vacuolar-lipid liposome clustering in the presence of Ypt7p (Figure 3, A and B; and Supplemental Table S1). At the concentrations of Ypt7p used in previous reports (Hickey *et al*., 2009; Stroupe *et al*., 2009), the addition of Ypt7p to POPC/POPS liposomes gave far less HOPS-mediated clustering (Figure 3C and Supplemental Table S1). However, the presence of a sixfold higher concentration of Ypt7p on the liposomes supported robust HOPS-mediated clustering (Figure 3D and Supplemental Table S1). The formation of large, stable clusters requires higher concentrations of tethering factors than are needed for fusion (Mima *et al*., 2008; Hickey *et al*., 2009; Stroupe *et al*., 2009),

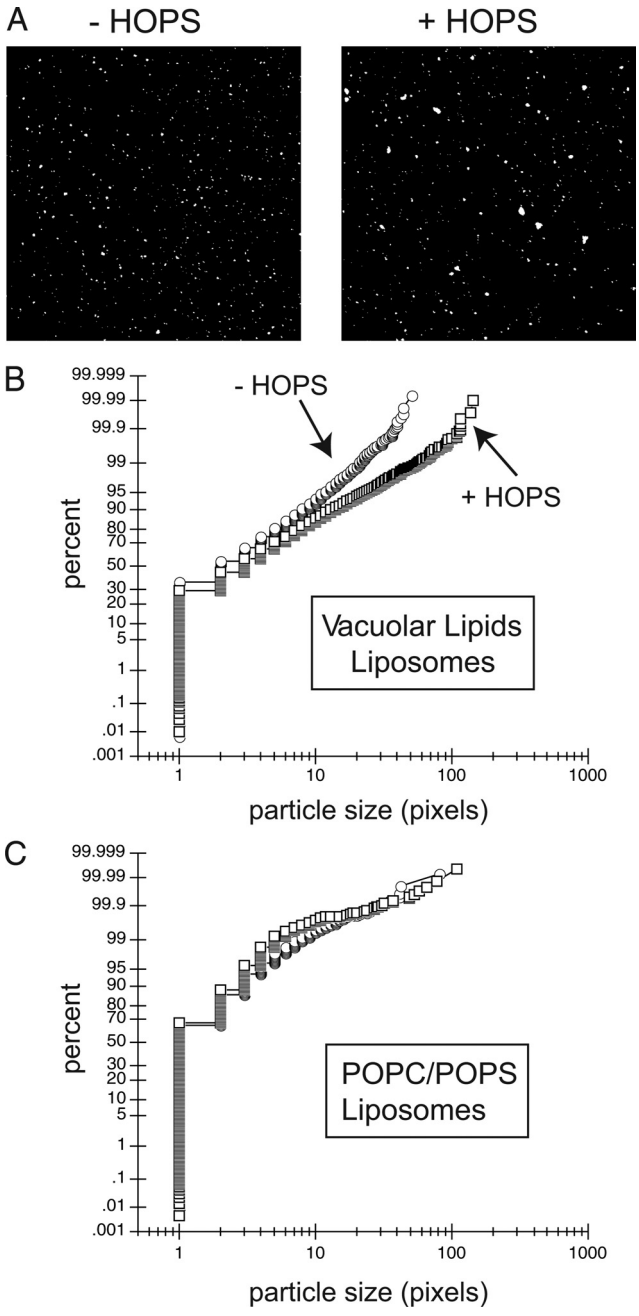

Figure 2. HOPS directly tethers liposomes of vacuolar lipid composition. (A) Protein-free liposomes (450 μM lipids) of vacuolar lipid composition were incubated as in lipid mixing assays except that all liposomes bore fluorescent NBD-PE and RH-PE ("donors"), the final volume was 10 μl, the reactions were in microcentrifuge tubes, and the incubations were performed in a water bath. All reactions contained 0.5 mM $MgCl_2$. After 25 min at 27°C, each reaction was diluted sixfold in RB150 + 0.5 mM $MgCl_2$ and assayed by fluorescence microscopy. Images were thresholded at 50 by using Photoshop (Adobe Systems, Mountain View, CA). (B) Particle sizes for the reactions in A were measured with ImageJ (see *Materials and Methods*) using 10 fields per reaction. HOPS (90 nM; squares) or HOPS buffer (circles) were added as indicated. (C) POPC/POPS liposomes were incubated as described in A, and particle sizes were measured as described in B. B and C are each representative of three independent experiments. See Supplemental Table S1 for the reproducibility and statistical significance of the clustering reactions.

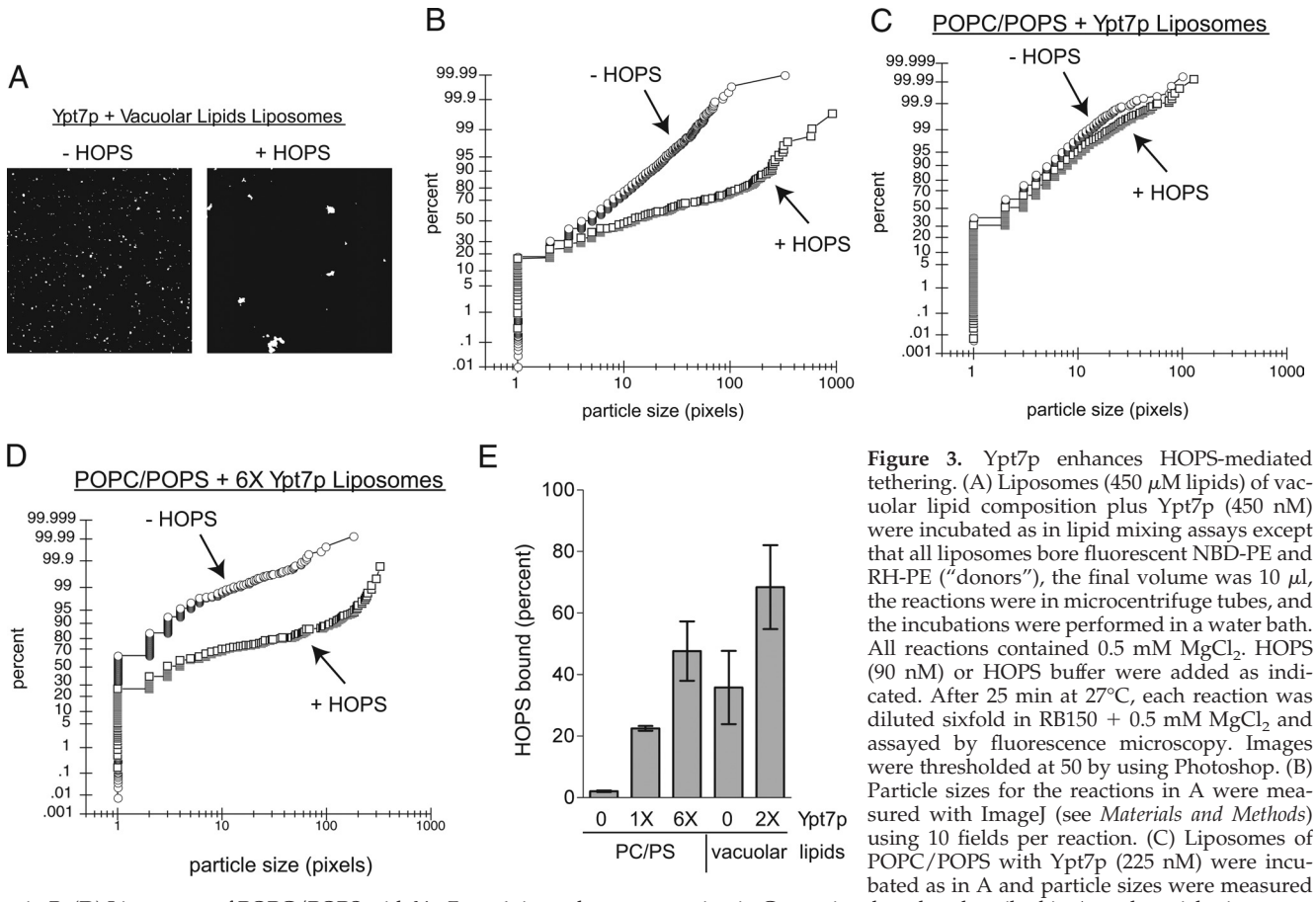

Figure 3. Ypt7p enhances HOPS-mediated tethering. (A) Liposomes (450 μM lipids) of vacuolar lipid composition plus Ypt7p (450 nM) were incubated as in lipid mixing assays except that all liposomes bore fluorescent NBD-PE and RH-PE ("donors"), the final volume was 10 μl, the reactions were in microcentrifuge tubes, and the incubations were performed in a water bath. All reactions contained 0.5 mM $MgCl_2$. HOPS (90 nM) or HOPS buffer were added as indicated. After 25 min at 27°C, each reaction was diluted sixfold in RB150 + 0.5 mM $MgCl_2$ and assayed by fluorescence microscopy. Images were thresholded at 50 by using Photoshop. (B) Particle sizes for the reactions in A were measured with ImageJ (see *Materials and Methods*) using 10 fields per reaction. (C) Liposomes of POPC/POPS with Ypt7p (225 nM) were incubated as in A and particle sizes were measured as in B. (D) Liposomes of POPC/POPS with Ypt7p at 6 times the concentration in C were incubated as described in A, and particle sizes were measured as described in B. B–D are each representative of three independent experiments. See Supplemental Table S1 for the reproducibility and statistical significance of the clustering reactions. (E) The association of HOPS (90 nM) with liposomes of the indicated compositions was assessed by liposome floatation as described previously (Hickey *et al*., 2009). Results are displayed as the mean ± SD of triplicates.

suggesting that even transient tethering events may be captured for fusion. HOPS only mediated the clustering of liposomes to which it could bind, whether via vacuolar lipids, Ypt7p, or both (Figure 3E). Because Ypt7p can contribute to tethering, we tested whether our HOPS preparation contained significant Ypt7p. Through immunoblot for Ypt7p, we determined that HOPS at the concentration used in Figures 2 and 3 (90 nM) contributed <65 pM Ypt7p (Supplemental Figure S3), less than one thousandth the concentration of Ypt7p used in Figure 3C, where HOPS-mediated clustering was barely detectable. Therefore, the stronger HOPS-mediated clustering of vacuolar lipids liposomes (Figure 2, A and B) is Ypt7p independent.

HOPS has affinity for multiple vacuolar SNAREs (Dulubova *et al*., 2001; Collins *et al*., 2005; Stroupe *et al*., 2006), and SNAREs can enhance the binding of HOPS to liposomes (Stroupe *et al*., 2009). However, vacuolar lipid liposomes bearing the four vacuolar SNAREs did not exhibit enhanced HOPS-mediated tethering compared with liposomes of vacuolar lipids alone (Figure 4A). These data suggest that any role of the SNAREs in docking would only be seen after cis-SNARE complexes are disassembled by Sec17p and Sec18p; indeed, vacuole docking requires prior Sec17p/Sec18p activity (Mayer and Wickner, 1997) and reconstituted proteoliposome clustering can require Sec17p/Sec18p (Stroupe *et al*., 2009). We therefore tested the effects of adding Sec17p/Sec18p and HOPS to vacuolar lipids liposomes with and without the four vacuolar SNAREs. Although Sec17p/Sec18p addition had little effect on the HOPS-mediated clustering of liposomes without SNAREs (Figure 4A, filled squares vs. filled triangles; Supplemental Table S1), liposomes bearing SNAREs formed massive clusters when both Sec17p/Sec18p and HOPS were added (Figure 4A, open squares vs. open triangles). To ensure that the Sec17p/Sec18p-dependent increase in cluster size was due to enhanced docking instead of fusion, we prepared liposomes with all four vacuolar SNAREs but with Vam7-3Δp instead of Vam7p. These liposomes also required both HOPS and Sec17p/Sec18p to form giant clusters (Figure 4B and Supplemental Table S1).

To further resolve the roles of HOPS and SNAREs in docking, a mixture of proteoliposomes bearing either the R-SNARE Nyv1p or the two integral membrane Q-SNAREs Vam3p and Vti1p (as represented in Figure 1B) offered several technical advantages: 1. Although SNAREs are present, trans-SNARE pairing and fusion can only occur upon addition of the soluble Q-SNARE Vam7p, providing an additional layer of experimental control. 2. The separation of the SNAREs from the start creates a state that is similar to prior Sec17p/Sec18p-mediated disassembly of cis-SNARE complexes. 3. The Vam7p-dependent association of Vam3p and Nyv1p can be directly assayed. With defined truncation at its C terminus, Vam7-3Δp supports SNARE complex assembly without fusion (Schwartz and Merz, 2009), allowing

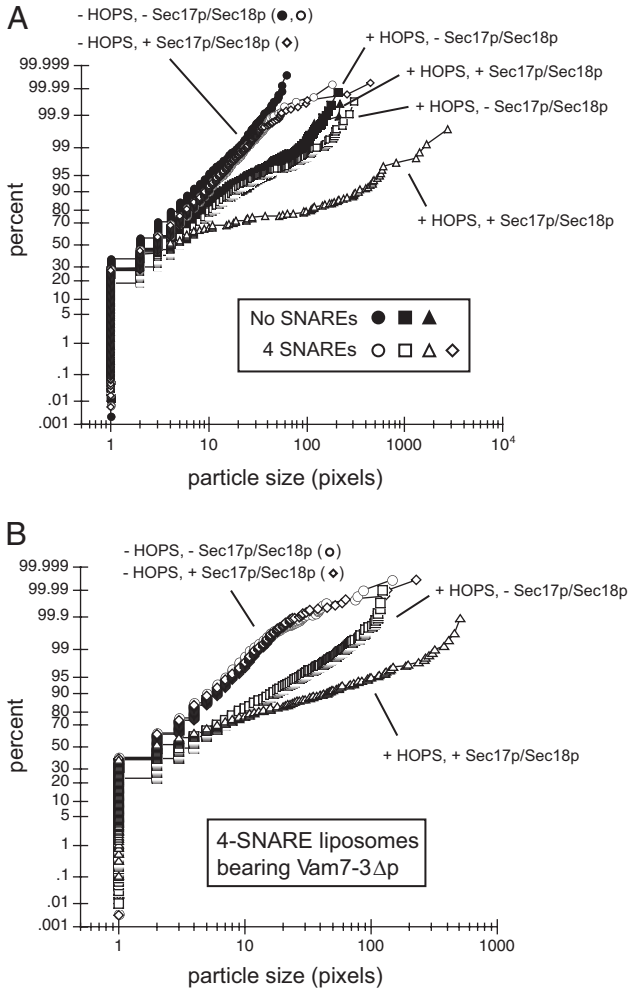

Figure 4. cis-4-SNARE complexes do not increase HOPS-mediated tethering. (A) Liposomes of vacuolar lipid composition which had no proteins or bearing the four vacuolar SNAREs were incubated as in lipid mixing assays except all liposomes bore fluorescent NBD-PE and RH-PE ("donors"), the final volume was 10 μl, the reactions were in microcentrifuge tubes, and the incubations were performed in a water bath. HOPS (90 nM) or HOPS buffer (−HOPS), Sec17p (600 nM), and Sec18p (600 nM) were added as indicated. All reactions contained 1.5 mM $MgCl_2$ and 1 mM ATP. After 25 min at 27°C, each reaction was diluted sixfold in RB150 + 0.5 mM $MgCl_2$ and assayed by fluorescence microscopy. Particle sizes were measured with ImageJ (see *Materials and Methods*) using 10 fields per reaction. (B) Liposomes of vacuolar lipid composition bearing the four vacuolar SNAREs but with Vam7-3Δp were incubated as described in A. Images were taken and particles sizes were measured using 10 fields per reaction. A and B are each representative of three independent experiments. See Supplemental Table S1.

resolution of these events. For these reasons, 2Q-SNARE and 1R-SNARE proteoliposomes (Mima *et al*., 2008) were used in all subsequent experiments.

The clustering of 2Q and 1R proteoliposomes (Figure 5A, open circles) was unaffected by 200 nM Vam7p (Figure 5A, open diamonds) or Vam7-3Δp (Figure 5A, open squares; and Supplemental Table S1). The addition of HOPS alone promoted clustering (Figure 5A, filled circles; and Supplemental Table S1), but there was enhanced clustering upon addition of either Vam7p (Figure 5A, filled diamonds) or Vam7-3Δp (Figure 5A, filled squares; and Supplemental Table S1) in addition to HOPS. Unlike full-length Vam7p, the addition of Vam7-3Δp did not result in fusion (Figure 5B), demonstrating directly that our assays resolve clustering and fusion. We suggest that a low-affinity tethering by HOPS can be followed by stable "capture" of the tethered state through trans-SNARE pairing, yielding stably docked membranes. This model predicts that random proteoliposome collisions that are not tethered by HOPS, although of shorter duration, might be captured by trans-SNARE complexes if SNARE pairing was kinetically favored. Indeed, micromolar concentrations of Vam7p cause fusion of 2Q and 1R proteoliposomes in the absence of HOPS (Mima *et al*., 2008).

Is tethering the principle positive role of HOPS in membrane fusion? To test this, we asked whether the need for HOPS can be bypassed under minimal fusion conditions (without Sec17p/Sec18p) by a nonspecific membrane-clustering agent such as PEG (Dennison *et al*., 2006). PEG aggregates liposomes of POPC/POPS (Figure 6A) or of vacuolar lipids bearing either 2Q- or 1R-SNAREs (Figure 6B) without promoting lipid mixing (data not shown). On addition of 200 nM Vam7p, this PEG-induced aggregation allows SNAREs to form trans-SNARE complexes (Figure 6C, lane 4; and Supplemental Figure S2) and to undergo lipid mixing (Figure 6D). Even concentrations of PEG that did not cause visible clustering led to an increase in lipid mixing (Figure 6, B and D, diamonds), suggesting that transient tethering events can be captured by trans-SNARE pairing. PEG supports trans-SNARE complex formation by Vam7-3Δp (Figure 6C, lane 3; and Supplemental Figure S2) but does not cause fusion (Figure 6E). The ability of Vam7-3Δp to support nearly as much SNARE complex formation as Vam7p (Figure 6C and Supplemental Figure S2) in the absence of fusion shows that the majority of new SNARE complex formed in this time frame is in the trans configuration rather than representing cis-SNARE complex which might have otherwise formed after a fusion event.

Strikingly, although Vam7p-dependent fusion is supported by either PEG or HOPS, the combination of PEG and HOPS did not result in more fusion than PEG alone (Figure 6F). Thus, in the absence of Sec17p and Sec18p, the primary positive function of HOPS is to tether membranes and thereby enhance SNARE complex formation in trans. When that function is fulfilled by PEG, HOPS does not further stimulate fusion.

HOPS is not required for lipid mixing of 2Q-SNARE and 1R-SNARE proteoliposomes if high concentrations of Vam7p are added (Mima *et al*., 2008). However, the addition of Sec17p/Sec18p blocks this lipid mixing unless HOPS is also added (Mima *et al*., 2008). HOPS inhibits the Sec17p/Sec18p-mediated disassembly of trans-SNARE complexes (Xu *et al*., 2010). Although the mechanism by which HOPS achieves this protection is unclear, one possibility is that Sec17p/Sec18p cannot act on trans-SNARE complexes that are part of any large membrane cluster. We therefore tested whether clustering caused by PEG might also allow fusion in the presence of Sec17p/Sec18p. This is not the case (Figure 7, lanes 1–4), because Sec17p/Sec18p inhibited PEG-mediated fusion but enhanced HOPS-mediated fusion. PEG does not prevent HOPS from allowing fusion stimulation by Sec17p/Sec18p (Figure 7, lanes 5 and 6).

DISCUSSION

Although the reconstitution of SNARE-mediated lipid mixing with proteoliposomes has been studied extensively, studies of reconstituted tethering and docking reactions upstream of fusion, typically involving Rabs and tethering factors, have lagged. The establishment of reconstituted fusion reactions that depend not only on SNAREs but also on

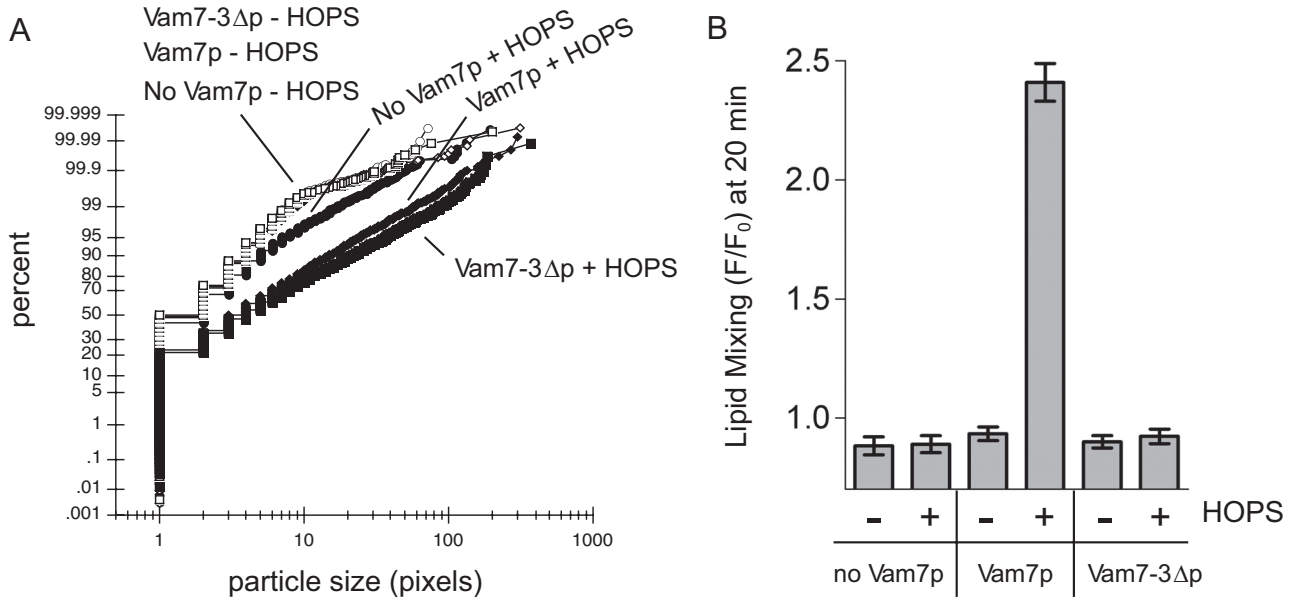

Figure 5. Synergy between HOPS-dependent tethering and SNARE-complex completion of docking. (A) Clustering of Qab-SNAREs (acceptor) and R-SNARE (donor) proteoliposomes by HOPS in absence or presence of Vam7p or Vam7-3Δp. After incubation as in lipid mixing assays (see B), 4 μl of each reaction was used to capture images. Particle sizes were measured with ImageJ (see *Materials and Methods*) using 10 fields per reactions. Data are representative of three independent experiments (see Supplemental Table S1). (B) Lipid mixing between Qab-SNAREs (acceptor) and R-SNARE (donor) proteoliposomes requires HOPS and Vam7p. HOPS (40 nM) was present as indicated. Vam7p (200 nM) or Vam7-3Δp (200 nM) was added as indicated at time 0, which followed a preincubation of the plate at 27°C for 10 min. All reactions contained 0.5 mM $MgCl_2$. Results are displayed as the mean ± SD of three independent experiments.

Rabs and Rab effectors (Hickey *et al*., 2009; Ohya *et al*., 2009; Stroupe *et al*., 2009) has validated the study of these pure factors in proteoliposomes. HOPS stimulation of SNARE-dependent proteoliposome fusion can be either Rab-independent (Mima *et al*., 2008) or Rab-dependent (Hickey *et al*., 2009; Stroupe *et al*., 2009). Rab-independent, HOPS-dependent fusion requires a lipid composition that resembles the vacuole (Mima *et al*., 2008). Vacuolar lipids support the association of HOPS with liposomes in the absence of other proteins, indicating a direct interaction of HOPS with membrane lipids (Stroupe *et al*., 2006; Hickey *et al*., 2009). In the absence of HOPS association with vacuolar lipids, the Rab Ypt7p is required for HOPS membrane association and fusion (Hickey *et al*., 2009; Stroupe *et al*., 2009). Thus, HOPS must bind lipids, Ypt7p, or both to stimulate membrane fusion. How does membrane-bound HOPS stimulate fusion? Our current study shows that membrane-bound HOPS stimulates trans-SNARE complex formation indirectly via tethering the membranes before SNARE interactions (i.e., before docking). Although we do not see direct catalysis of SNARE complex assembly by HOPS (Figure 1 and Supplemental S1), we cannot preclude this occurring under other conditions. Nevertheless, when PEG is added to cause artificial tethering, HOPS remains active (as judged by its continued capacity to allow fusion in the presence of Sec17p/Sec18p; Figure 7) yet does not stimulate fusion in the absence of Sec17p/Sec18p (Figure 6F), suggesting that it is not directly catalyzing trans-SNARE assembly in our assays.

The formation of SNARE complexes between proteoliposomes with relatively physiological SNARE densities (protein/lipid ratios of ~1:1000–1:2000) can be stimulated by the artificial tethering agent PEG (Dennison *et al*., 2006) (Figure 6C), indicating that liposome-liposome interactions are limiting under these conditions. PEG-mediated stimulation of trans-SNARE complexes can lead to enhanced lipid mixing between proteoliposomes (Figure 6E). Although higher SNARE densities can yield significant lipid mixing without HOPS or PEG, high SNARE densities have been shown to compromise membrane integrity (Dennison *et al*., 2006). Isolated vacuoles with elevated SNARE densities also exhibit lysis during in vitro fusion, whereas vacuoles with native SNARE densities do not lyse under these conditions (Starai *et al*., 2007). HOPS-dependent proteoliposome fusion is lysis-free when SNARE protein/lipid ratios of 1:1000–1:2000 are used (Mima *et al*., 2008; Stroupe *et al*., 2009). Is HOPS simply acting like low concentrations of PEG, that is, increasing liposome–liposome interactions? Indeed, HOPS does not further stimulate proteoliposome fusion when PEG is first added to cluster the proteoliposomes (Figure 6F). HOPS can directly stimulate clustering of liposomes that bear either vacuolar lipids (Figure 2) or Ypt7p alone (Figure 3). Unlike HOPS, PEG does not require specific membrane receptors (vacuolar lipids or Ypt7p) to cluster liposomes (Figure 6A) because it operates by dehydration rather than directly cross-linking membranes (Lentz, 2007). By tethering liposomes, HOPS dramatically facilitates SNARE assembly in trans and thus allows low SNARE densities to promote fusion.

Vacuole docking requires Ypt7p and *cis*-SNARE complex disassembly by Sec17p and Sec18p (Mayer and Wickner, 1997), suggesting that robust, stable vacuole clustering depended on trans-SNARE interactions. In the current study, clustering of 2Q-SNARE liposomes and 1R-SNARE liposomes by HOPS was strongly enhanced by the addition of Vam7-3Δp (Figure 5A), which is required for trans-SNARE complex formation (Figure 1C). Clustering was modest, yet detectable, without Vam7-3Δp or Vam7p (Figure 5A). However, fusion upon addition of Vam7p under these conditions was robust; much lower concentrations of HOPS that yield no detectable clustering without Vam7p still yield signifi-

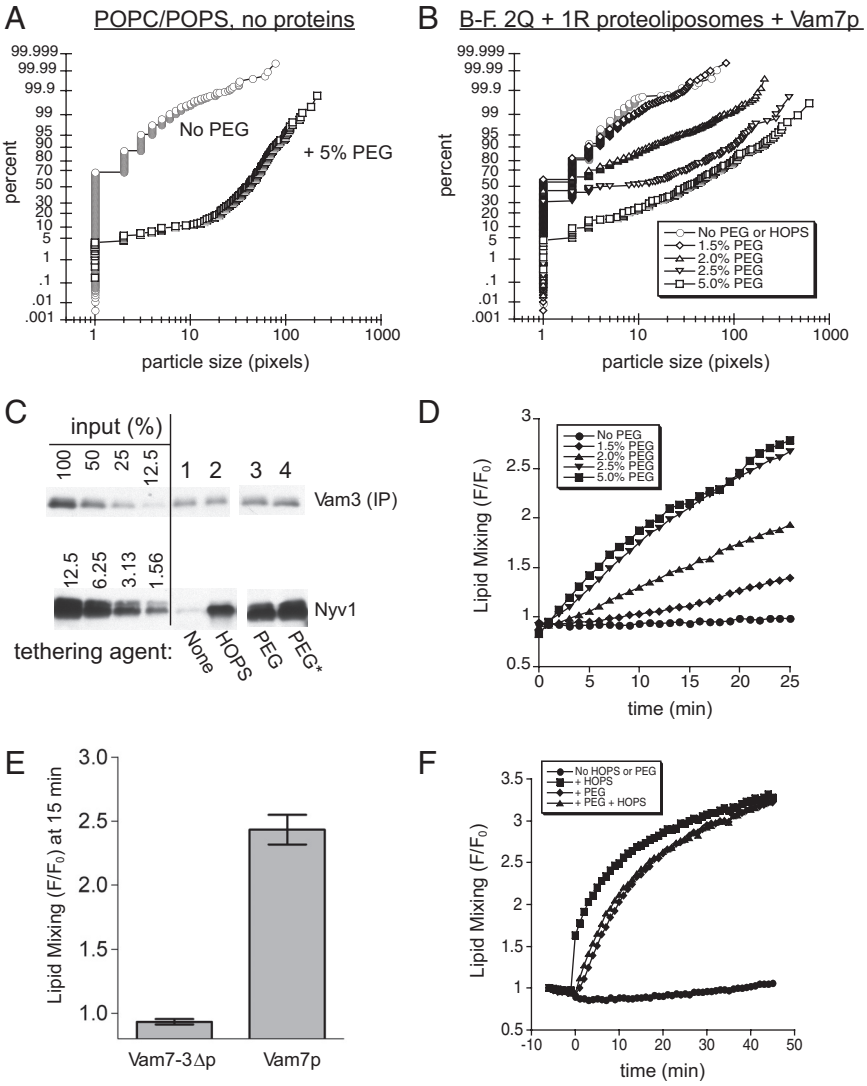

Figure 6. PEG-mediated tethering supports SNARE complex assembly and fusion. (A) PEG alone clusters liposomes. PC/PS liposomes were incubated with or without 5% PEG in 10 μl for 25 min at 27°C. Both reactions contained 0.5 mM $MgCl_2$. The reaction without PEG was diluted sixfold in RB150 + 0.5 mM $MgCl_2$, and the reaction with PEG was diluted sixfold in RB150 + 0.5 mM $MgCl_2$ + 5% PEG. Both reactions were assayed by fluorescence microscopy. Particle sizes were measured with ImageJ (see *Materials and Methods*) using 10 fields per reaction. Results are representative of three independent experiments. (B) PEG-mediated clustering. After lipid mixing (see D), 4 μl of each reaction was used to capture images. Particle sizes were measured with ImageJ (see *Materials and Methods*) using seven fields per reactions. (C) SNARE complex assembly. Reactions and immunoprecipitations were performed as described in Figure 1C, except that 200 nM Vam7p (*; lane 4) or 200 nM Vam7-3Δp (all others) was added at time 0, and PEG (6% final) was added just before Vam7p addition. Input samples and lanes 1 and 2 are from Figure 1C. Lanes 3 and 4 were used to generate lanes 7 and 8 in Supplemental Figure S2. See Supplemental Figure S2 for mean ± SD of three experiments. (D) Lipid mixing between Qab-SNAREs (acceptor) and R-SNARE (donor) proteoliposomes supported by PEG. All reactions contained 0.5 mM $MgCl_2$. PEG was present as indicated. Vam7p (300 nM) was added to all wells at time 0, which followed a pre-incubation of the plate at 27°C for 10 min. (E) Lipid mixing between liposomes bearing Qab-SNAREs (acceptor) and R-SNARE (donor). PEG (6%) was added to both reactions just before Vam7p. Vam7p (200 nM) or Vam7-3Δp (200 nM) was added at time 0, which followed a preincubation of the plate at 27°C for 10 min. These lipid mixing data are from the same reactions used for the trans-SNARE assay in C. Results are displayed as the mean ± SD of three independent experiments. (F) Lipid mixing between liposomes bearing Qab-SNAREs (acceptor) and R-SNARE (donor). All reactions contained 0.5 mM $MgCl_2$. PEG (3% final) was added where indicated 10 min before time 0, and then the plate was incubated at 27°C. HOPS (90 nM) or HOPS buffer were added where indicated 5 min before time 0, and the plate was returned to 27°C. Vam7p (300 nM) was added to all wells at time 0. Results are representative of three independent experiments.

cant fusion upon addition of Vam7p (data not shown). In addition, this experiment was performed in the absence of Sec17p/Sec18p, which strongly stimulate the fusion of these proteoliposomes (Mima *et al*., 2008) (Figure 7). The enhancement of liposome-liposome interactions needed to stimulate fusion is near the lower detection limit of the current microscopic assay, because concentrations of PEG that do not enhance clustering (Figure 6B, diamonds) can stimulate SNARE-mediated fusion (Figure 6D, diamonds).

The robust, SNARE-independent tethering reconstituted in the current study (Figure 3, B and D) was generated with concentrations of HOPS and Ypt7p above those required for proteoliposome fusion. However, these biochemical reactions allow studies of important physiological interactions that have not been possible with previous systems. For example, our current finding that either vacuolar lipids or Ypt7p is sufficient for HOPS-mediated clustering explains why we previously observed both Rab-independent and Rab-dependent fusion conditions.

Although the current study is the first reconstitution of liposome clustering with only Rab and Rab effector, liposome-clustering reactions mediated by pure factors involved in other membrane fusion events have been reported previously (Arac *et al*., 2006; Connell *et al*., 2008; Drin *et al*., 2008). It is noteworthy that these reactions required specific membrane lipids, albeit different lipids than those required for lipid-dependent, HOPS-mediated tethering (Figure 2). An impressive reconstitution of liposome tethering involving the small GTPase Arf and the golgin GMAP-210 required both membrane curvature and a region of the golgin that senses membrane curvature known as an amphipathic lipid-packing sensor (ALPS) motif (Drin *et al*., 2008). Interestingly, the Vps41p subunit of HOPS contains a putative ALPS motif (Drin *et al*., 2007). Although the significance of this domain is unknown, Yck3p, a kinase that negatively regulates HOPS activity by abrogating its binding to vacuolar lipids (Hickey *et al*., 2009), phosphorylates HOPS at serines within this ALPS motif of Vps41p (Cabrera *et al*., 2009).

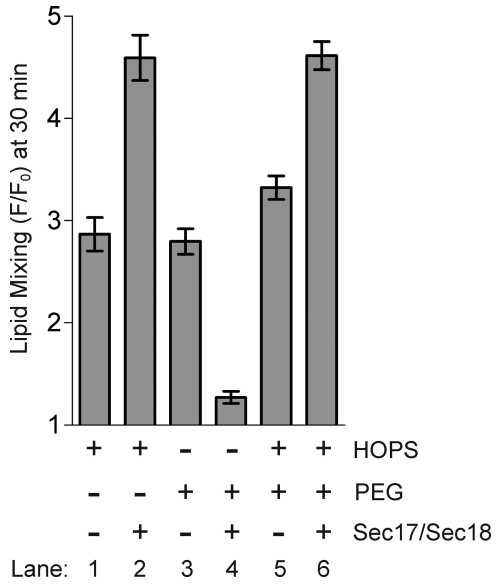

Figure 7. The synergy between HOPS and Sec17/Sec18 is not solely due to HOPS tethering activity. Lipid mixing between liposomes bearing Qab-SNAREs (acceptor) and R-SNARE (donor). All reactions contained 1.5 mM $MgCl_2$ and 1 mM ATP. HOPS (40 nM) followed by PEG (3% final) were added as indicated followed by incubation of the plate at 27°C for 5 min. Sec17p (1.2 μM) and Sec18p (1.4 μM) were added as indicated followed by further incubation of the plate at 27°C for 5 min. Vam7p (300 nM) was added to all reactions at time 0. Results are the mean ± SD of three assays.

Although vacuolar lipids are not absolutely required for tethering liposomes with Ypt7p (Figure 3D), they can suffice for moderate tethering (Figure 2, A and B) and/or work in combination with the Rab for tethering (Figure 3B). This link between Rab effectors and lipids is not limited to the vacuolar fusion reaction and the HOPS complex. The Rab5 effector early endosomal antigen (EEA)1 (Simonsen *et al*., 1998) requires PI(3)P for tethering isolated early endosomes (Lawe *et al*., 2002). EEA1 contains a FYVE domain (Stenmark *et al*., 1996) which mediates PI(3)P binding (Burd and Emr, 1998). The exocyst, which binds the Rab Sec4p on secretory vesicles (TerBush *et al*., 1996), binds phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] and phosphatidylserine at the plasma membrane to dock vesicles (He *et al*., 2007; Liu *et al*., 2007; Zhang *et al*., 2008). Although the exocyst subunits Exo70 and Sec3 have no defined lipid-binding domains, polybasic regions important for PI(4,5)P_2 binding have been identified in both of these proteins. HOPS also has no defined phosphoinositide-binding domain, despite its ability to bind multiple phosphoinositides (Stroupe *et al*., 2006).

We have recently shown that the stable interaction of proteoliposomes requires Ypt7p, HOPS, Sec17p, and Sec18p and the three vacuolar Q-SNAREs (which are unable to pair in trans) (Stroupe *et al*., 2009). However, this study used liposomes that did not bind HOPS in the absence of Ypt7p or SNAREs (i.e., lipids only), which was not the case for the liposomes in our initial reconstitution work (Mima *et al*., 2008; Hickey *et al*., 2009). In the current study, phosphatidylcholine (PC)/ phosphatidylserine (PS) liposomes bind essentially no HOPS without Ypt7p (Figure 3E), and only moderate amounts of HOPS bind to PC/PS liposomes bearing low concentrations of Ypt7p (Figure 3E). Neither of these conditions supports robust HOPS-mediated liposome clustering (Figures 2C and 3C). Robust HOPS-mediated clustering of liposomes made with PC/PS lipids was only observed when Ypt7p concentrations were increased above those used previously (Figure 3D) (Hickey *et al*., 2009; Stroupe *et al*., 2009). HOPS associations with both vacuolar lipids and Ypt7p are regulated in vivo. Lipid binding is negatively regulated by Yck3-mediated phosphorylation (LaGrassa and Ungermann, 2005; Cabrera *et al*., 2009; Hickey *et al*., 2009), and Rab binding is negatively regulated by the RabGAP Gyp7p (Albert *et al*., 1999; Brett *et al*., 2008). The induction of vacuole fragmentation by Gyp7p requires the presence of Yck3p, indicating that both negative regulators are required for complete inhibition of fusion in vivo (Brett *et al*., 2008). Our current in vitro findings suggest that this dual-factor inhibition is required because vacuolar lipids and Ypt7p, separately or in combination, can lead to HOPS-mediated tethering and hence increased trans-SNARE complex assembly and fusion.

A key function of HOPS is to allow fusion in the presence of Sec17p/Sec18p (Mima *et al*., 2008) through protection by HOPS of trans-SNARE complexes from Sec17p/Sec18p-mediated disassembly (Xu *et al*., submitted). Cis-SNARE complexes are not protected from Sec17p/Sec18p action by HOPS (Xu *et al*., unpublished data), consistent with HOPS not affecting the Sec17p/Sec18p-mediated disassembly of a complex of the soluble domains of the vacuolar SNAREs (Mima *et al*., 2008). We propose that HOPS bound to trans-SNARE complexes sterically inhibits the access of Sec17p and/or Sec18p and that the avidity of HOPS for the trans-SNARE complex is greater than that for the cis-SNARE complex because HOPS interacts with SNAREs and each of the two apposed membranes at the sites of trans-SNARE complexes. Before the current study, it was possible that the resistance of trans-SNARE complexes to Sec17p/Sec18p-mediated disassembly in the presence of HOPS was simply due to the liposomes being physically clustered together. However, PEG clustered liposomes do not show resistance to Sec17p/Sec18p action, whereas PEG does increase fusion in the absence of Sec17p/Sec18p (Figure 7). These data show that in the presence of Sec17p/Sec18p, HOPS function extends beyond its role as tethering factor.

HOPS can also distinguish normal vacuolar SNAREs from vacuolar SNAREs with mutations or truncations (Starai *et al*., 2008). This activity also may be separable from tethering, and may require HOPS associations with one or multiple vacuolar SNAREs during trans-SNARE complex formation. HOPS also has a role in membrane microdomain formation (Wang *et al*., 2002, 2003; Fratti *et al*., 2004). Because our current proteoliposomes are far smaller than vacuoles and bear higher concentrations of fusion factors, the microdomain assembly function or putative induction/sensing of membrane curvature function of HOPS may not yet be reflected in our assays. While additional activities of HOPS may require new reconstitution platforms, our current reconstitutions have the same protein and lipid requirements as vacuole fusion in vitro and in vivo, and are thus important chemically defined models of those biological reactions.

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