Phosphatidylinositol and phosphatidylinositol-3-phosphate activate HOPS to catalyze SNARE assembly, allowing small headgroup lipids to support the terminal steps of membrane fusion

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ABSTRACT **Intracellular membrane fusion requires Rab GTPases, tethers, SNAREs of the R, Qa, Qb, and Qc families, and SNARE chaperones of the Sec17 (SNAP), Sec18 (NSF), and SM (Sec1/Munc18) families. The vacuolar HOPS complex combines the functions of membrane tethering and SM catalysis of SNARE assembly. HOPS is activated for this catalysis by binding to the vacuolar lipids and Rab. Of the eight major vacuolar lipids, we now report that phosphatidylinositol and phosphatidylinositol-3-phosphate are required to activate HOPS for SNARE complex assembly. These lipids plus ergosterol also allow full *trans*-SNARE complex assembly, yet do not support fusion, which is reliant on either phosphatidylethanolamine (PE) or on phosphatidic acid (PA), phosphatidylserine (PS), and diacylglycerol (DAG). Fusion with a synthetic tether and without HOPS, or even without SNAREs, still relies on either PE or on PS, PA, and DAG. These lipids are thus required for the terminal bilayer rearrangement step of fusion, distinct from the lipid requirements for the earlier step of activating HOPS for *trans*-SNARE assembly.**

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INTRODUCTION

Fusion at each stage of the exocytic and endocytic membrane trafficking pathways relies on conserved catalytic proteins (Wickner and Rizo, 2017). These include Rab family GTPases as master regulators, Rab effector tethers, membrane-anchored SNARE proteins, and SNARE chaperones of the SM (Sec1/Munc18), Sec18/NSF, and Sec17/SNAP families. Membranes are first tethered by associations between Rab GTPases and their effector tethers. SM proteins, alone or in complex, then catalyze the assembly of SNAREs anchored to the two tethered membranes. SNARE proteins consist of N-terminal domains, heptad-repeat SNARE domains, short, polar juxtamembrane regions, and often C-terminal membrane anchors. The SNAREs are grouped into subfamilies termed R, Qa, Qb, or Qc, according to whether they have a central arginyl or glutaminyl residue (Fasshauer *et al.*, 1998). SNAREs assemble into four-helical coiled coils of RQaQbQc composition. Heptad-repeat apolar residues face each other at the core of this four-helical structure (Sutton *et al.*, 1998), though polar arginyl or glutaminyl residues at the center of the SNARE coiled coils form a polar center to the otherwise apolar SNARE complex core. Each SNARE domain is thought to be unstructured until the four SNAREs assemble into a coiled coil in an N-to-C direction, a process termed zippering (Fasshauer *et al.*, 1998; Hazzard *et al.*, 1999). Partially zippered SNAREs can bind Sec17, promoting further zippering (Ma *et al.*, 2016) and providing critical membrane-proximal apolar loops of Sec17 (Zhao *et al.*, 2015). These Sec17 apolar loops and the completion of SNARE zippering drive fusion per se (Song *et al.*, 2021). Despite this extensive study of fusion proteins, and although membrane fusion is fundamentally a process of lipid rearrangements between apposed bilayers, less is known of the roles of individual lipids in fusion.

We study membrane fusion with yeast vacuoles (lysosomes). The development of a colorimetric assay for fusion of purified vacuoles

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Abbreviations used: DAG, diacylglycerol; Erg, ergosterol; FRET, fluorescence resonance energy transfer; HOPS, homotypic fusion and vacuole protein sorting; MIN, minimal lipid mixture; NSF, NEM-sensetive factor; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PI3P, phosphatidylinositol-3-phosphate; PS, phosphatidylserine; SM, Sec1/Munc18 homolog; SNAP, soluble NEM-sensitive factor attachment protein; SNARE, soluble NEM-sensitive factor attachment protein receptor; VML, vacuolar mixed lipids.

(Haas *et al.*, 1994) allowed study of the fusion proteins: the Rab Ypt7 (Haas *et al.*, 1995), the R-SNARE Nyv1 (Nichols *et al.*, 1997), Qa-SNARE Vam3, Qb-SNARE Vti1, and Qc-SNARE Vam7 (referred to as simply R, Qa, Qb, and Qc hereafter), the SNARE chaperones Sec17 (SNAP) and Sec18 (NSF; Haas and Wickner, 1996), and the hexameric multifunctional HOPS protein (homotypic fusion and vacuole protein sorting). The HOPS subunits, discovered in the *VAM* and *VPS* (vacuole protein sorting) screens, are stably associated (Nakamura *et al.*, 1997; Seals *et al.*, 2000; Wurmser *et al.*, 2000). Each fusion protein has been purified and reconstituted in functional form, and each is required for reconstituted proteoliposome (RPL) fusion at physiological SNARE concentrations (Mima *et al.*, 2008; Stroupe et al, 2009; Zick and Wickner, 2016).

While there has been substantial study of the proteins of fusion, less is known of the lipid requirements. Even at unphysiologically high SNARE levels where the Rab is not required, simple lipid mixtures of PC and PS supported much less fusion than a vacuolar lipid mix (Fukuda *et al.*, 2000; Mima *et al.*, 2008). Vacuoles have 46 mol% phosphatidylcholine (PC), 18% phosphatidylethanolamine (PE), 18% phosphatidylinositol (PI), 4.4% phosphatidylserine (PS), 2% phosphatidic acid (PA), 8% ergosterol (Erg), 1% diacylglycerol (DAG), and 1% phosphatidylinositol-3-phosphate (PI3P; Zinser and Daum, 1995; Schneiter *et al.*, 1999). Fusion relies on small headgroup, nonbilayer-prone lipids (Zick *et al.*, 2014), including ergosterol (Kato and Wickner, 2001), PA (Sasser *et al.*, 2012), and diacylglycerol (Miner *et al.*, 2017). PI3P contributes to the membrane affinity of Qc (Cheever *et al.*, 2001), and HOPS has direct affinity for acidic lipids such as PI (Orr *et al.*, 2015) and PI3P (Stroupe *et al.*, 2006). At physiological SNARE levels, the fluidity of the fatty acyl chain composition is also important for fusion (Zick and Wickner, 2016). Recent work has revealed that HOPS catalyzes the assembly of vacuolar SNAREs (Baker *et al.*, 2015; Orr *et al.*, 2015; Jiao *et al.*, 2018; Torng *et al.*, 2020). HOPS is activated for this catalysis by association with the Rab Ypt7 when bound to liposomes of complete vacuolar lipid composition (Torng *et al.*, 2020). Ypt7 on liposomes of only PC can bind HOPS (Orr *et al.*, 2015), but this does not suffice for the catalytic activation of the bound HOPS (Torng *et al.*, 2020).

Membrane lipids are not merely a passive bilayer sheet upon which SNARE zippering and Sec17 wedge insertion induce fusion (Song *et al.*, 2021). Fusion of intact, purified vacuoles entails the formation of a ring-shaped microdomain, the "vertex ring," surrounding the apposed regions of each fusion partner (Wang *et al.*, 2002, 2003; Fratti *et al.*, 2004). Specific lipids and proteins, each needed for fusion, become highly enriched in the vertex ring. This enrichment is interdependent (Fratti *et al.*, 2004), in that blocking individual lipids by adding ligands or degrading them enzymatically blocks the enrichment of other critical lipids and of fusion proteins. Conversely, ligands to fusion proteins block lipid enrichment at the vertex (Fratti *et al.*, 2004). The full panoply of lipids and proteins that form the vertex ring is unclear. Vacuolar fusion reconstitution with all purified components has used proteoliposomes that are submicroscopic, precluding visualization of microdomain assembly. A more complete understanding of the roles of individual lipids will be a foundation for understanding the vertex ring assembly and functions.

HOPS catalyzes SNARE assembly on proteoliposomes in a strikingly asymmetric manner (Torng *et al.*, 2020). While HOPS supports modest assembly of the soluble forms of SNAREs on proteoliposomes bearing only R or only Qa, Ypt7 greatly enhances HOPS-dependent SNARE assembly when the R-SNARE, but not the Qa-SNARE, is bound to the same membrane. Proteoliposome fusion also shows a far stronger requirement for activated Ypt7 on the R proteoliposomes than on the Q-SNARE proteoliposomes (Zick and Wickner, 2016). Taken together, these studies show that Ypt7-bound HOPS engages preferentially with the R-SNARE in *cis*, and then with the Q-SNAREs in *trans*. However, this activation appeared to be contingent upon the presence of several lipids present on the vacuole membrane, as proteoliposomes of PC alone did not support HOPS-dependent SNARE assembly (Torng *et al.*, 2020).

We now report that PI and PI3P are required for the activation of HOPS as a catalyst of SNARE assembly, and that the addition of even a single additional vacuolar lipid such as Erg gives substantial HOPS activity for *cis*-SNARE assembly. A minimal lipid composition of PC, PI, PI3P, and Erg supports HOPS-dependent *trans*-SNARE assembly as well as the complete vacuolar lipid composition does, but these minimal lipids do not suffice for membrane fusion. In addition to this minimal set of four lipids, fusion requires either PE or the other three vacuolar lipids, PS, PA, and DAG. When HOPS is bypassed by preassembly of the Q-SNAREs into a 3Q-SNARE complex and the provision of a synthetic tether, PI is no longer required for fusion, suggesting that its role is linked to its affinity for HOPS. Even in the biophysical model reaction of polyethylene glycol-driven fusion in the absence of any fusion proteins, the minimal lipids are not optimal, and PE and the other three vacuolar lipids (PS, PA, and DAG) promote fusion. Thus, two stages of fusion, HOPS activation for *trans*-SNARE assembly and lipid bilayer rearrangement into complete fusion, have distinct lipid requirements.

RESULTS

Because vacuolar lipids activate HOPS (Torng *et al.*, 2020), we asked which specific vacuolar lipids are required for this activation, whether this activation suffices for *trans*-SNARE complex assembly and for fusion, and whether other vacuolar lipids are important for fusion mediated by other tethers and SNAREs or even by a nonproteinaceous dehydrating agent.

Lipid requirements for HOPS activation

Optimal HOPS catalysis of SNARE assembly on proteoliposomes of vacuolar mixed lipids (VMLs) requires the R-SNARE in *cis* to the Rab Ypt7 (Torng *et al.*, 2020). Qc and a cysteine-tagged synthetic SNARE domain of Qb were labeled with distinct fluorophores that undergo FRET when assembled into a four-SNARE complex (Torng *et al.*, 2020). These were incubated with sQa, a soluble form of Qa deprived of its membrane anchor (Song and Wickner, 2017), with Ypt7/R proteoliposomes of complete VML composition or with single lipids omitted, and with HOPS. In all experiments, any omitted lipids were replaced with a compensatory equimolar percent of PC. FRET between the fluorophores bound to Qb-SNARE domain and to Qc was assayed as a measure of SNARE assembly (Torng *et al.*, 2020). SNARE assembly with the complete lipid mixture (Figure 1A, lane 1, and Supplemental Figure 1, red curve) was reduced to nearly the background levels seen without proteoliposomes (lane 2) by omission of either PI (lane 4) or PI3P (lane 9). Because PI constitutes 18% of the lipid mixture and PI3P only 1%, they likely fulfill distinct functions. Each is known to have direct affinity for HOPS (Stroupe *et al.*, 2006; Orr *et al.*, 2015). None of the other lipids are uniquely required. As a complementary approach, we started from a simple lipid composition of only PC, which did not support SNARE assembly (Figure 1B, lane 6; PC only) as reported (Torng *et al.*, 2020). The inclusion of either PI (lane 4) or PI3P (lane 5) had little effect, and even their inclusion together in the proteoliposomal lipid mixture (lane 3) gave little SNARE assembly in comparison to that seen with complete VML (lane 1). The further inclusion of either PE (Figure 1C, lane 4), Erg (lane 5), or other lipids (lanes 6–8) supported additional SNARE assembly.

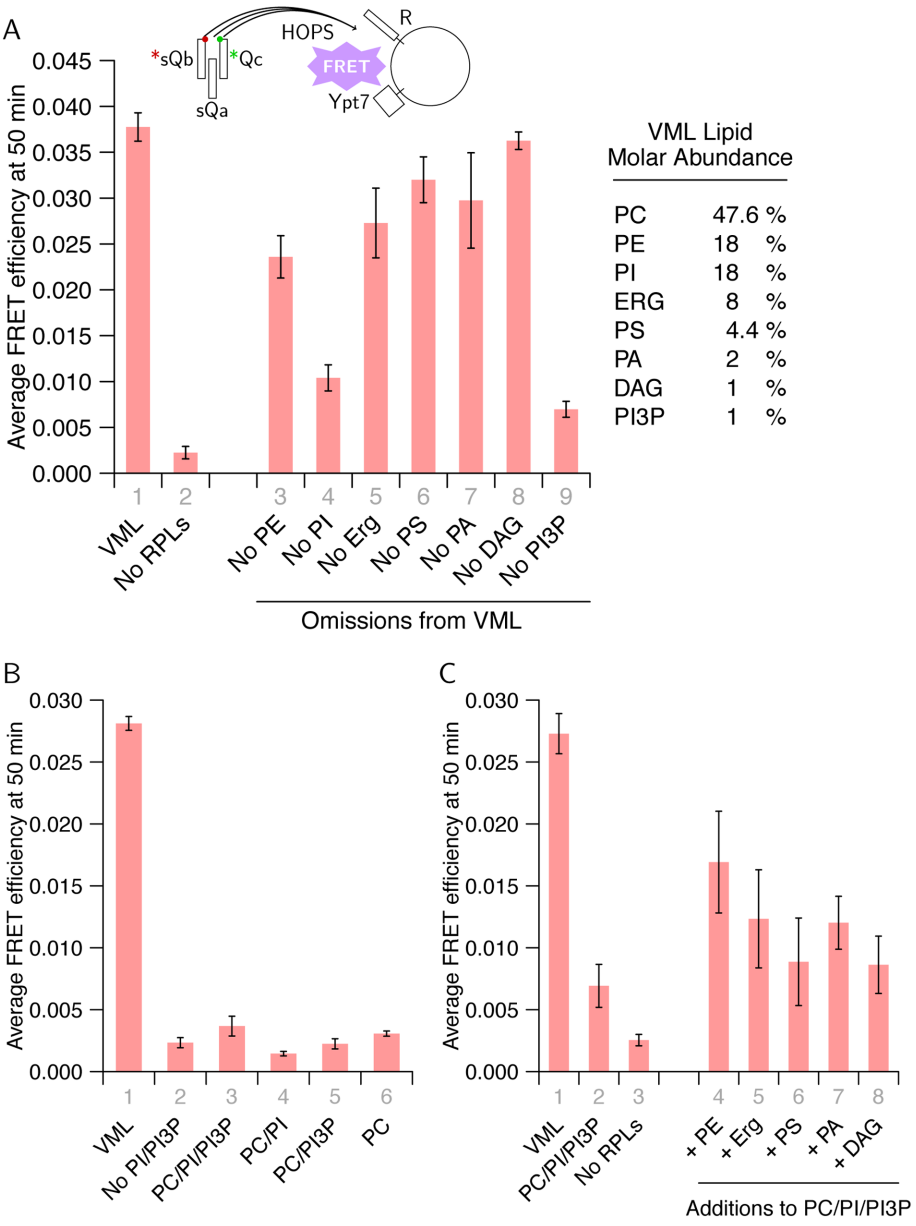

FIGURE 1: PI and PI3P are required for HOPS-catalyzed *cis*-SNARE complex assembly. (A) Proteoliposomes of various lipid compositions were assessed for SNARE complex assembly with the standard reaction as described in *Materials and Methods*. In all figures, omitted lipids were replaced with an equal mole percent of PC. For convenience, the lipid composition is reported on the side. See Supplemental Figure 1 for a representative plot of the kinetic data. (B) Combinations of PI and PI3P without the other lipids were tested for SNARE complex assembly. (C) Single lipid additions to PC/PI/PI3P were tested for SNARE complex assembly. In this figure, two independent proteoliposome preparations were each assayed twice. The mean and standard deviation (SD) are shown. Gray numbers for each reaction are included for ease of reference in the main text.

Subreactions of SNARE complex assembly

While SNAREs can slowly assemble spontaneously, they are catalyzed to assemble by direct association with HOPS bound to vacuolar lipids, proteoliposome-bound R-SNARE, and Ypt7 (Torng *et al.*, 2020). We assayed a possible subreaction in this assembly, the binding of fluorescent Qb-SNARE domain (*Qb) to HOPS:Ypt7/R proteoliposomes, by sedimentation (Figure 2). The complete *Qb binding assay has HOPS, proteoliposomes bearing Ypt7 and R, and the soluble SNAREs sQa, Qc, and *Qb. Under the sedimentation conditions used, more than 85% of proteoliposomes sediment for all reactions tested (Supplemental Figure S2). *Qb does not sediment, alone (Figure 2A, lane 4), with protein-free liposomes (lane 3), or with all the SNAREs but without liposomes (lane 2). Approximately 6–8% of the *Qb sedimented in the complete reaction (Figure 2, A–C, lane 1). Sedimentation was reduced by the omission of PI or PI3P (Figure 2B, lane 1 vs. lanes 7, 12, and 13), but the other lipids contributed as well. Sedimentation was greatly reduced by the omission of either HOPS or of even one other SNARE, either sQa, Qc, or membrane-bound R (Figure 2A), but fell to background levels comparable to those seen without proteoliposomes (Figure 2A, lane 3) upon concurrent omission of both HOPS and sQa (Figure 2C, lane 4). This assay therefore measures both HOPS-dependent binding, which occurs when SNARE assembly is blocked by the absence of sQa (Figure 2C, lane 3 vs. lane 4), and SNARE assembly, which occurs at these high SNARE concentrations in the absence of HOPS (Figure 2C, lane 2 vs. 4), in accord with fusion studies (Mima *et al.*, 2008).

In addition to HOPS having specific lipid requirements, the Rab Ypt7 activates HOPS for SNARE complex assembly (Torng *et al.*, 2020). This activation is seen when Ypt7 has bound GTP (Figure 3A, red curve 1). In contrast, with bound GDP (blue curve 3) or with GDP generated by GTP hydrolysis under the influence of the GTPase activating protein Gyp1-46 (yellow curve 2), HOPS-mediated SNARE assembly is reduced to the level seen in the absence of Ypt7 (purple curve 4). This assay of FRET from the association of *Qb and *Qc reflects full SNARE complex assembly as reported (Torng *et al.*, 2020), as it is 100 times more rapid in the presence of sQa than in its absence (Figure 3A, red curve 1 vs. gray curve 5). In contrast, about 25% of the HOPS-dependent sedimentation of *Qb seen in the complete reaction (Figure 3B, lane 1) persists in the absence of sQa (Figure 3B, lane 5). Thus, *Qb is likely associating with HOPS before assembling into a four-SNARE complex. The interdependent association of the SNAREs with Rab- and lipid-bound HOPS may precede the catalysis of their assembly into a four-SNARE complex that exhibits FRET.

Distinct lipid requirements for *trans*-SNARE assembly and for fusion

To test whether the lipids which support HOPS-dependent SNARE association and *cis*-SNARE complex assembly on Ypt7/R proteoliposomes (Figure 1) will also suffice for *trans*-SNARE assembly and fusion, we prepared proteoliposomes with Ypt7 and with either the

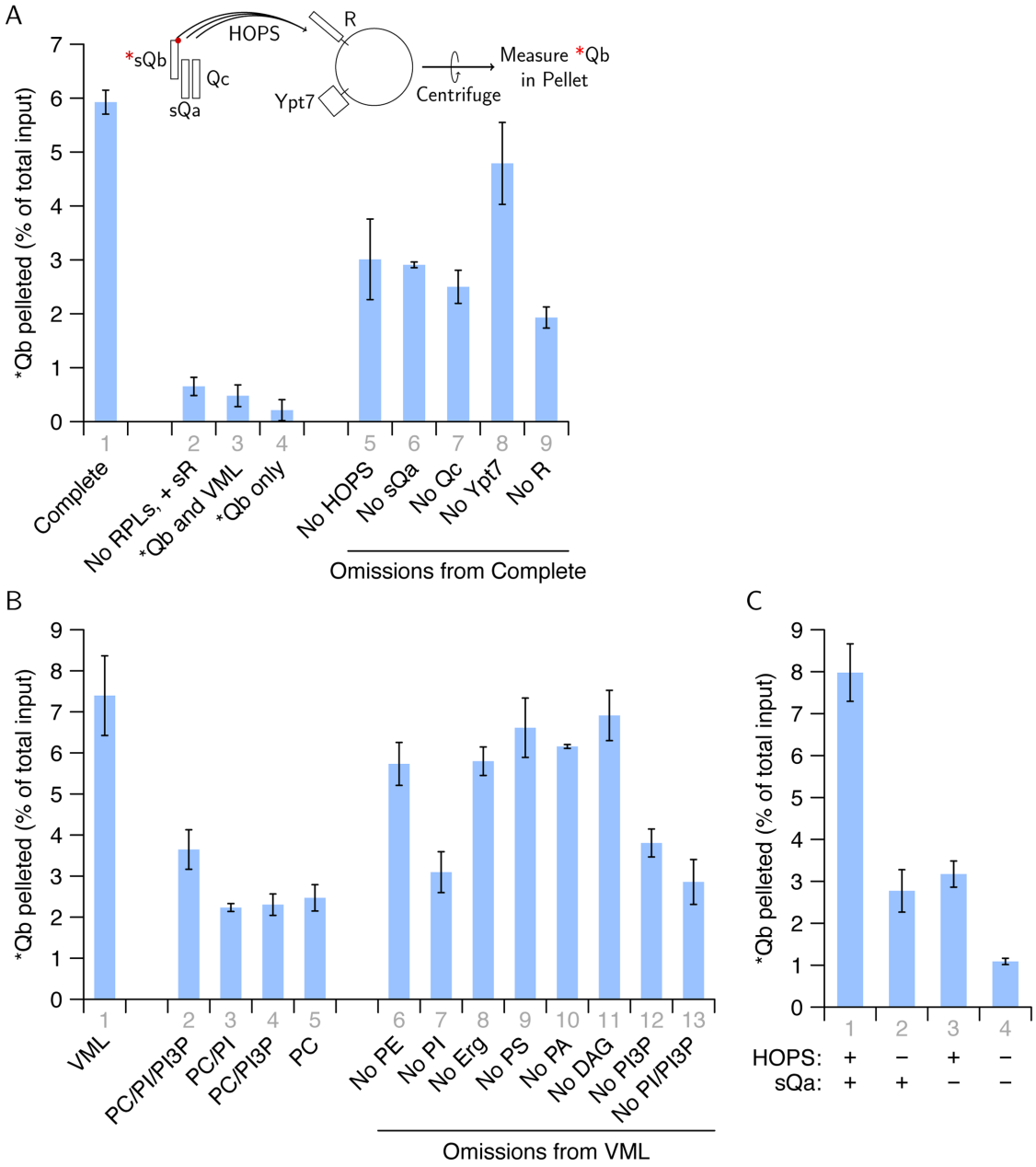

FIGURE 2: Qb association with the membrane requires HOPS or four-SNARE assembly. (A) Proteoliposomes of various lipid compositions were tested for Qb association as described in *Materials and Methods*. (B) Various components were excluded from the standard ("complete") Qb association reaction. Reaction 2 contains sR instead of membrane-bound R, while Ypt7 and membranes are missing. (C) The dependence of Qb association with the membrane on HOPS and sQa was explicitly tested. In this figure and in Figure 3, the mean and SD of triplicate assays are shown.

R-SNARE or the Qa and Qb SNAREs, and with either the complete eight VMLs or with PC, PI, PI3P, and Erg (termed MIN, or minimal, mix; Figure 4). Erg was chosen for its documented role in fusion in vivo (Seeley *et al.*, 2002), with isolated vacuoles (Kato and Wickner, 2001), and with reconstituted proteoliposomes (Zick *et al.*, 2014), and in promoting *cis*-SNARE complex assembly (Figure 1C). Proteoliposomes bore either of two lumenal fluorescent proteins, Cy5-labeled streptavidin or biotinylated phycoerythrin. Upon fusion-induced mixing of the proteoliposome lumenal contents, the tight association of biotin to streptavidin brings the two fluorophores into intimate contact, yielding a strong FRET signal (Zucchi and Zick, 2011). Fusion incubations had proteoliposomes, HOPS, Qc, and either Sec17, Sec18, and Mg:ATPγS, or their buffers. Though the proteoliposomes of either VML or MIN composition bore the same levels of the Ypt7 Rab and the R, Qa, or Qb SNAREs (Figure 4A), only the VML proteoliposomes exhibited HOPS-dependent fusion, stimulated by Sec17 and Sec18 as reported (Song *et al.*, 2017), while the MIN proteoliposomes did not fuse (Figure 4B, red vs. blue). To assay *trans*-SNARE assembly in each case, aliquots were taken from the fusion mixtures after 5- or 30-min incubation and solubilized in a modified RIPA detergent mixture that solubilizes membranes without disrupting SNARE complexes (Zick *et al.*, 2014). The RIPA mixture included a substantial molar excess of GST-Nyv1 to competitively block any SNARE assembly with wild-type R after solubilization. Detergent extracts were mixed with magnetic protein A-beads bearing affinity-purified antibody to the Qa-SNARE,

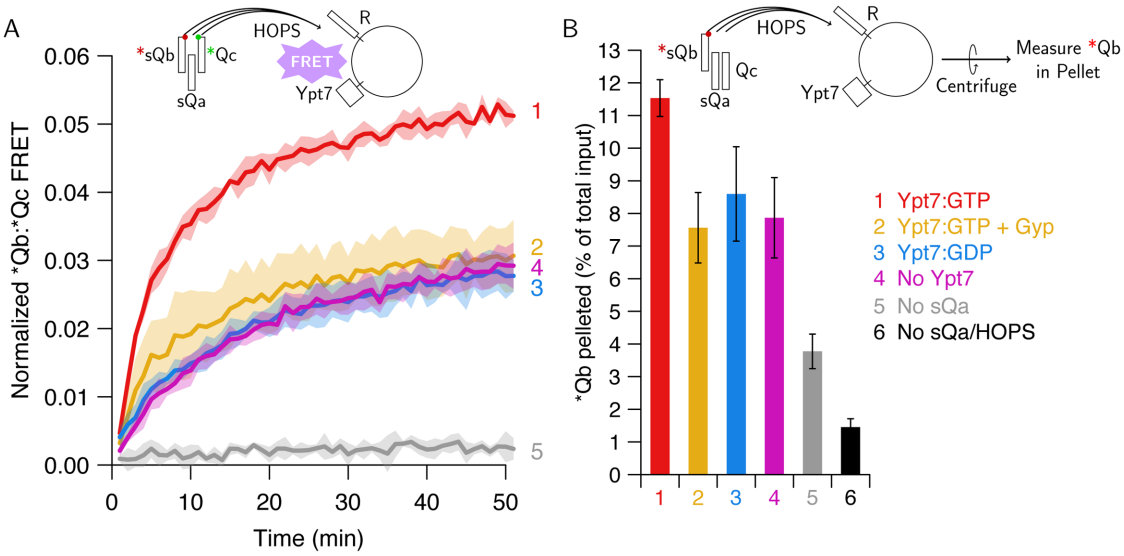

FIGURE 3: Requirements for Qb SNARE binding and SNARE complex assembly. Parallel SNARE assembly and Qb association reactions were performed in panels A and B, respectively. Each reaction is color-matched. Proteoliposomes bearing R and prenyl-Ypt7, each at 1:2000 protein-to-lipid molar ratio, were incubated with GTP or GDP and EDTA for 10 min at 27°C. $MgCl_2$ was then added to complete nucleotide exchange of Ypt7, followed by addition of Gyp where indicated. Prenyl-Ypt7 was used instead of Ypt7-TM, as Ypt7-TM is not GTP-sensitive (Lee *et al.*, 2020). After another 10 min incubation, HOPS, sQa, fluorescent Qb, and (A) fluorescent Qc or (B) nonfluorescent Qc were added to the reaction. SNARE assembly (A) and Qb association (B) were then measured as described in *Materials and Methods*. Final reaction concentrations were 0.75 mM proteoliposomes, 20 μM GTP or GDP, 1 mM EDTA, 0.5 mM free $MgCl_2$, 2 μM Gyp, 150 nM HOPS, 1 μM Qb and Qc, and 0.5 μM sQa. The proteoliposomes were not prepared using the microdialysis procedure, but rather as described previously (Torng *et al.*, 2020).

washed to remove unbound proteins, and assayed by immunoblot for Qa-bound R-SNARE, a measure of *trans*-SNARE complex. Comparable levels of *trans*-SNARE complex assembled with proteoliposomes of VML or MIN lipid compositions, unaffected by the addition of Sec17, Sec18, and ATPγS (Figure 4C). Both VML and MIN proteoliposomes showed comparable *trans*-SNARE complex assembly after 5 min or 30 min (Figure 4D, green bars), but fusion required the VML proteoliposomes (purple bars).

As HOPS-dependent SNARE assembly needs PI and PI3P (Figure 1), we tested the roles of these lipids in fusion. Fusion between Ypt7/R and Ypt7/QaQb proteoliposomes of complete VML composition was strongly reduced by the absence of either PI, PI3P, or both (Figure 5). The requirement for PI and PI3P was also seen for fusion in the absence of Sec17 and Sec18 (Supplemental Figure 3).

Because MIN lipids suffice for *trans*-SNARE assembly but fusion needs VML (Figure 4), we examined which lipids among PE, PS, PA, and DAG, which are in VML but not in MIN, are needed for fusion (Figure 6). Strikingly, the addition of any one of these lipids to MIN restored some fusion. The omission of the neutral lipid PE had only a small effect on fusion (open squares, lane 2), while the further omission of the acidic lipids PS, PA, or both (open triangles or diamonds, filled squares; lanes 3, 4, and 6) caused further diminution of fusion. Fusion without Sec17 or Sec18, though diminished, still required either PE or PS, PA, and DAG (Supplemental Figure 4).

Do these specific lipid requirements for progression from *trans*-SNARE assembly to fusion reflect properties of HOPS, or of the lipid rearrangements that constitute fusion per se? We first tested for the requirement of PI and PI3P. When the three Q-SNAREs are preassembled during proteoliposome preparation, a synthetic tether can support SNARE-dependent fusion instead of HOPS (Song and Wickner, 2019). Dimeric GST-PX effectively tethers membranes, based on its affinity for PI3P in each fusion partner. As reported (Song and Wickner, 2019), Ypt7/R and Ypt7/QaQbQc proteoliposomes of VML lipids will fuse with either HOPS or dimeric GST-PX as tether (Figure 7A, red, filled vs. open circles; Figure 7B, lane 1 vs. lane 2). Fusion supported by HOPS is greatly reduced when PI is omitted (Figure 7A, blue closed circles; Figure 7B, lane 3), but PI is not needed for fusion with the synthetic tether GST-PX (Figure 7A, blue open circles; 7B, lane 4). The requirement for PI, which is known to contribute to HOPS membrane binding (Orr *et al.*, 2015), is thus specific for HOPS function. PI3P is also needed for fusion with HOPS (Figure 7A, green filled circles), but the dependence on PI3P of fusion with GST-PX cannot be assayed as PI3P is already required to allow tethering by dimeric GST-PX (Song and Wickner, 2019).

To test whether the requirement for some combination of PE, PS, PA, and DAG seen for Ypt7/R and Ypt7/QaQb proteoliposomes with HOPS (Figure 6) is specific to HOPS or is a more general requirement for tethered membranes to progress to full fusion, we examined the fusion of Ypt7/R and Ypt7/QaQbQc proteoliposomes of either VML, MIN + PE, MIN + PS/PA/DAG, or MIN lipids with either HOPS or dimeric GST-PX. There was no fusion without a tether (Figure 8, gray symbols), and little or no fusion with MIN lipids and either tether (Figure 8A, diamonds, and Figure 8B, lanes 10–12). Either PE or PS/PA/DAG sufficed to support fusion with either tether (squares and triangles), and optimal fusion was seen with MIN plus all four, i.e., VML (Figure 8, circles). Because PE, and PS/PA/DAG, had comparable effects with either tether, these four lipids are not simply needed for some specific HOPS function that might follow *trans*-SNARE assembly.

Because the lipids PE, PS, PA, and DAG were not needed for HOPS function (Figure 8), we further tested whether they directly affected SNARE-mediated fusion, or membrane fusion in general. We prepared liposomes with entrapped Cy5-streptavidin or biotinylated phycoerythrin but without SNAREs or Rab. These liposomes were

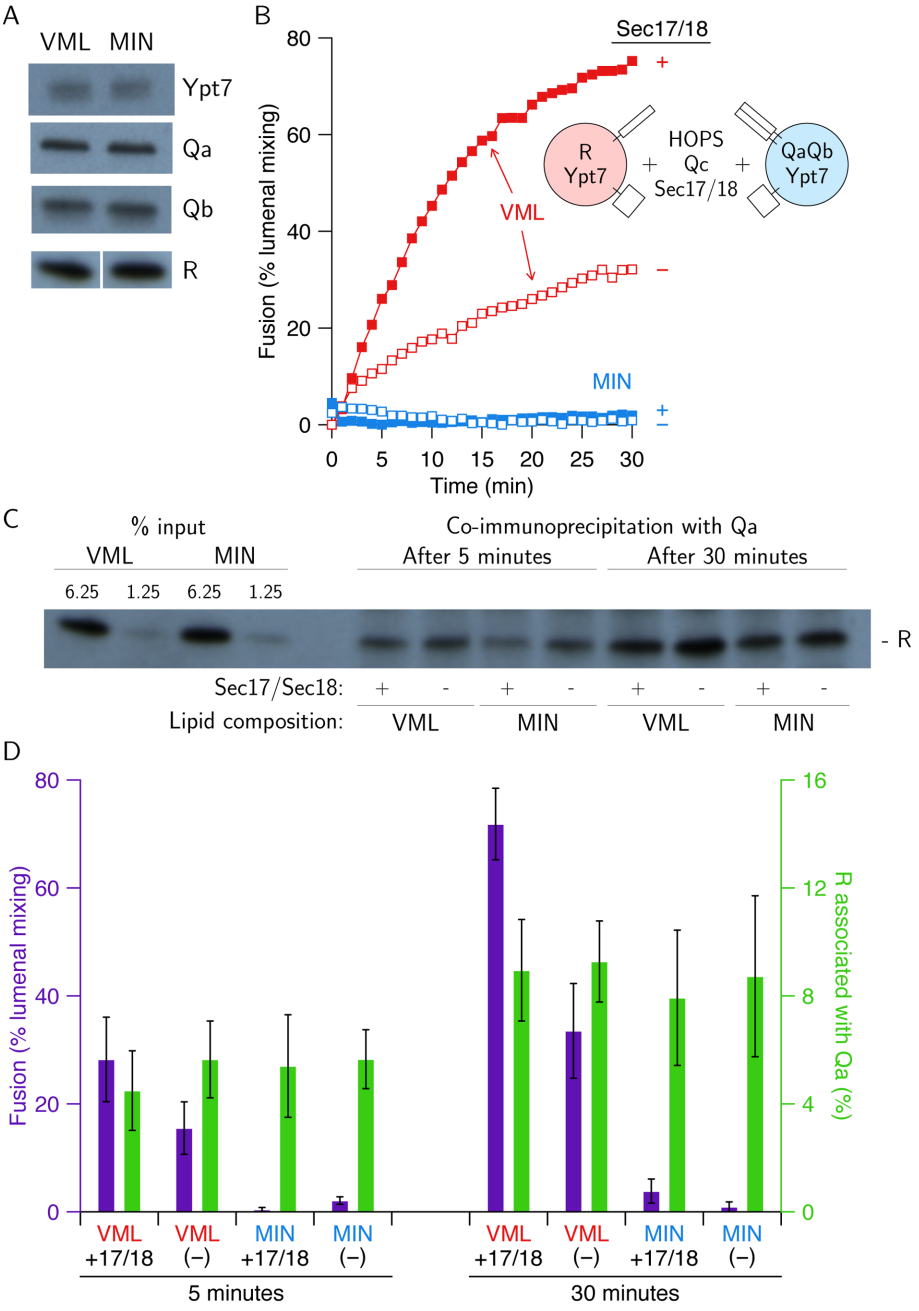

FIGURE 4: Distinct lipid requirements for *trans*-SNARE assembly and fusion. Proteoliposomes were prepared as described (Song *et al.*, 2020) with vacuolar mixed lipids (VMLs) or with a chosen minimal set of lipids (MIN) of PC, PI, PI3P, and Erg. Proteoliposomes had either Ypt7 and R (1:8000 and 1:16,000 M ratios to lipid, respectively), or Ypt7 and QaQb at the same concentrations. (A) Immunoblot assay of MIN and VML proteoliposomes for Ypt7, R, Qa, and Qb. (B) Fusion assays were performed in the presence or absence of 50 nM HOPS, 100 nM Qc, 100 nM Sec17, 300 nM Sec18, and 1 mM Mg:ATPγS as described in *Materials and Methods*. (C) Aliquots of fusion incubations were removed after 5 min and 30 min of incubation and mixed with modified RIPA buffer (Song *et al.*, 2020), a nondenaturing detergent mixture. Qa was recovered from the RIPA extract with affinity-purified antibody bound to protein-A magnetic beads, and the Qa-bound R was assayed by SDS–PAGE and immunoblot as described (Song *et al.*, 2020). (D) Quantification of triplicate assays of parts B and C. In this figure and subsequent bar graphs, error bars represent standard deviations.

mixed in the presence of excess nonfluorescent streptavidin to quench any FRET signal from extralumenal fluorophores. The fusion of such liposomes can be induced by 18% polyethylene glycol (PEG) (Burgess *et al.*, 1992) through membrane apposition and dehydration. Liposomes of MIN composition did not fuse with added PEG, while those of VML composition gave substantial fusion (Figure 9, blue vs red squares). The omission of PE, or of PS, PA, and DAG, strongly reduced the PEG-induced fusion signal (though the signal after fusion drifts due to liposome cluster drift in the plate reader). These lipids thus contribute to bilayer rearrangements during fusion, whether supported by HOPS and SNAREs, by a synthetic tether and SNAREs, or even by PEG, a dehydrating chemical fusogen (Lentz, 2007).

DISCUSSION

HOPS is activated as a catalyst for the assembly of the four vacuolar SNAREs by binding to both Ypt7 and to vacuolar lipids (Torng *et al.*, 2020). This is an allosteric activation of HOPS for catalysis of SNARE assembly rather than simply a colocalization of HOPS with its membrane-bound SNARE substrates, because either Ypt7 or the vacuolar lipids alone suffice for HOPS binding to membranes yet both are required for HOPS activation, and the activation of HOPS for catalysis of SNARE assembly is even seen when the four SNAREs are in aqueous solution without membrane anchors (Torng *et al.*, 2020). We now report that PI and PI3P are the crucial vacuolar lipids for this activation. PI, as the major vacuolar acidic lipid (Zinser and Daum, 1995), has direct affinity for HOPS (Orr *et al.*, 2015) but is not the sole HOPS receptor, as Ypt7 can also fulfill this function (Hickey and Wickner, 2010; Orr *et al.*, 2015). Both HOPS:membrane association and membrane:membrane fusion require PI, but these requirements for PI can be bypassed by elevated levels of another acidic vacuolar lipid, PS (Orr *et al.*, 2015). PI is not needed for the last step of fusion, lipid rearrangements into transient nonbilayer fusion intermediates, because PI is not required for SNARE-dependent fusion supported by a synthetic tether when the Q-SNAREs are preassembled (Figure 7). HOPS also has a direct affinity for phosphoinositides, including PI3P (Stroupe *et al.*, 2006). In contrast, the need for either PE or for PS, PA, and DAG for fusion is seen whether the Q-SNAREs are initially disassembled (Figure 6) or preassembled (Figure 8), whether the tethering is provided by Ypt7:HOPS or by dimeric GST-PX (Figure 8), and even for SNARE-free fusion induced by PEG (Figure 9). These lipids likely contribute directly to bilayer rearrangements.

HOPS has been proposed to have an elongated and somewhat unstructured conformation (Chou et al, 2016), though recent studies have shown that its association with Ypt7 can induce a major

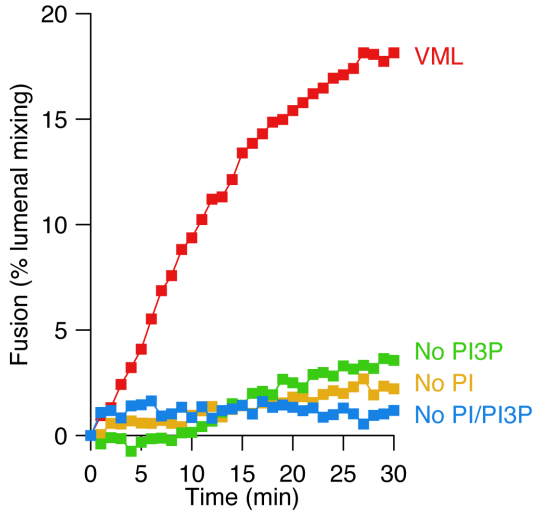

FIGURE 5: HOPS-catalyzed fusion also needs PI and PI3P. (A) Proteoliposomes were prepared with Ypt7 and R, and with Ypt7, Qa, and Qb as described in *Materials and Methods*. Proteoliposomes were separately prepared with either VML lipid composition or with omission of PI, omission of PI3P, or omission of both PI and PI3P. These were incubated with HOPS, Sec17, Sec18, and Qc and assayed for fusion by FRET as described in *Materials and Methods*.

conformational change (Fuellbrunn *et al.*, 2021), which is likely related to the functional activation reported here. Its binding to three coreceptors on the vacuole, PI, PI3P, and Ypt7, may orient and activate it for productive engagement with SNAREs. In accordance with this hypothesis, there is far greater engagement for HOPS-catalyzed SNARE assembly when HOPS is bound to Ypt7 on the same proteoliposomes as R than when on the same proteolipomes as Qa (Torng *et al.*, 2020), and fusion requires Ypt7:GTP on the R proteoliposome far more than on the Qa (Zick and Wickner, 2016).

Synaptic fusion also shows specific lipid requirements. Cholesterol, a major lipid in synaptic vesicles, is important for fusion mediated by synaptic SNAREs (Tong *et al.*, 2009), and acidic lipids promote fusion with synaptotagmin and calcium as well (Lai and Shin, 2012). Fusion reconstituted with neuronal SNAREs, Munc18-1, and Munc 13-1 is stimulated by DAG and phosphoinositides (Liu *et al.*, 2016). While synaptic fusion and vacuole fusion share requirements for SNAREs and an SM protein, synaptic fusion has not required a clear homologue to HOPS subunits other than Vps33, the SM subunit, and vacuolar fusion does not need homologues of Munc13, synaptotagmin, or complexin. Shared mechanistic lipid requirements between synaptic fusion and vacuolar fusion are not yet evident.

Our current results contribute to our working model of vacuole membrane fusion. Vacuoles bear the prenyl-anchored Rab Ypt7, integrally bound R, Qa, and Qb SNAREs, and peripherally bound fusion proteins such as HOPS, Sec17, and Sec18 as well as the Qc SNARE, which cycles between the cytoplasm and membrane (Collins *et al.*, 2005). HOPS binds to vacuoles by its affinities for Ypt7 (Seals *et al.*, 2000), for vacuolar SNAREs (Song *et al.*, 2020), and for acidic lipids such as PI, PA, and PS (Orr *et al.*, 2015) and phosphoinositides including PI3P (Stroupe *et al.*, 2006). HOPS membrane binding can be modulated by its phosphorylation by the Yck3 kinase (Zick and Wickner, 2012), and it may be displaced by Sec17:SNARE association (Collins *et al.*, 2005; Schwartz *et al.*, 2017) or by Ypt7 cycling to its inactive GDP-bound state (Seals *et al.*, 2000). Qc binds by its affinities for the other SNAREs, for PI3P (Cheever *et al.*, 2001), and for HOPS (Stroupe *et al.*, 2006), and is displaced from vacuoles during ATP-dependent SNARE complex disassembly (Ungermann and Wickner, 1998). ATP hydrolysis by Sec18 drives disassembly of postfusion *cis*-SNARE complexes, preparing the organelle for the next round of fusion (Mayer *et al.*, 1996). While HOPS and Ypt7 can tether membranes (Stroupe *et al.*, 2006; Hickey and Wickner, 2010), HOPS is activated by its associations with Ypt7 and the R-SNARE to bind the Q-SNAREs and initiate SNARE zippering (Torng *et al.*, 2020). At some point after zippering has progressed past the SNARE 0-layer, Sec17 and Sec18 associate with the zippering SNARE bundle to both promote further zippering and to allow insertion of the Sec17 N-loop into the apposed bilayers (Song *et al.*, 2021). Specific lipids support distinct stages of this pathway (Figure 10). PI and PI3P are needed to activate HOPS for SNARE assembly (Figures 1 and 4). PE, DAG, PA, and PS are needed for the completion of fusion, and this requirement remains even when HOPS (Figure 8) or SNAREs (Figure 9) are not present,

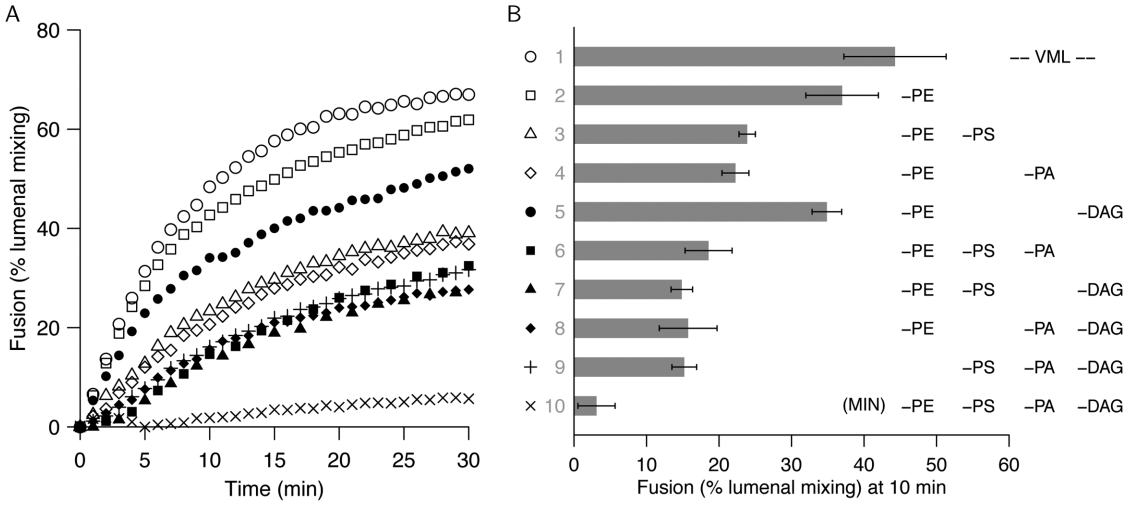

FIGURE 6: Fusion promoted by combinations of PE, PS, PA, and DAG. Proteoliposomes with Ypt7/R and Ypt7/QaQb were prepared with MINimal lipids (PC, PI, PI3P, and Erg), with MIN plus the indicated combinations of PE, PS, PA, and DAG, or with all of these (constituting VML). Proteoliposomes were mixed with HOPS, Sec17, Sec18, ATPγS, and Qc and assayed for fusion. (B) Quantification of triplicate assays of part A.

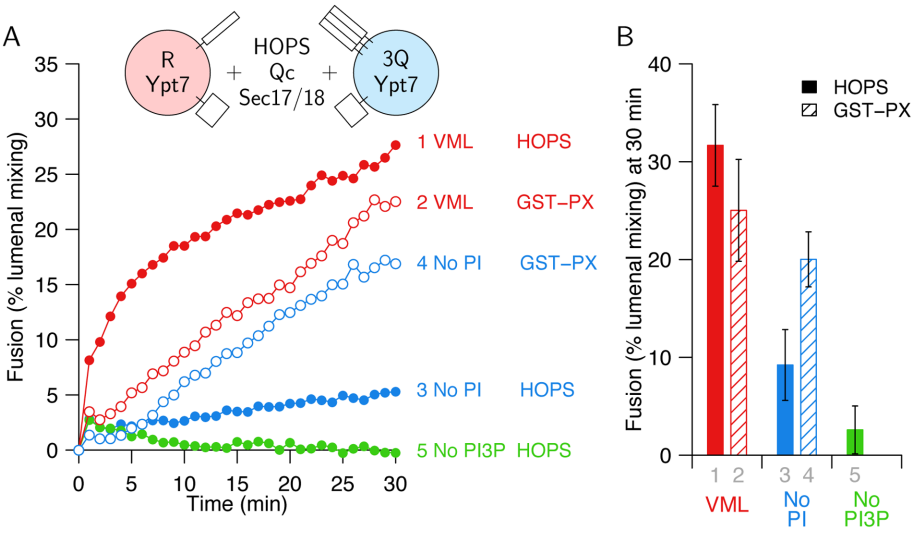

FIGURE 7: The requirement for PI is specific to HOPS. (A) Ypt7/R and Ypt7/3Q proteoliposomes were prepared with VML lipid composition, or with PI, PI3P, or both omitted. These were assayed for fusion with either 50 nM HOPS or 2 μM GST-PX. (B) Quantification of triplicate assays of part A.

suggesting that these lipids act at apposed bilayers to support the lipid rearrangements, which are the essence of fusion, progressing from two tightly apposed bilayers through possible nonbilayer intermediates such as hemifusion to a fully fused bilayer.

MATERIALS AND METHODS

Proteins

HOPS (Zick and Wickner, 2013), the vacuolar SNAREs (Mima *et al.*, 2008; Zucchi and Zick, 2011), soluble R and Qa (Thorngren *et al.*, 2004; Song and Wickner, 2017), the MBP-tagged SNARE domain of Qb (Song and Wickner, 2017), Ypt7-TM bearing an apolar membrane anchor of the same sequence as Qb (Song *et al.*, 2020), prenyl Ypt7 (Zick and Wickner, 2013), Sec17 (Schwartz and Merz, 2009), Sec18 (Mayer *et al.*, 1996), and GST-PX (Fratti and Wickner, 2007) were purified as described.

SNARE assembly assay

Assays were performed as described previously (Torng *et al.*, 2020), and SNARE assembly is reported as Average FRET efficiency (Song *et al.*, 2021). Standard reactions contained proteoliposomes (0.75 mM lipid) bearing R and Ypt7-TM at protein-to-lipid molar ratios of 1:3000 and 1:6000 respectively, fluorescently labeled Qb and Qc (1 μM each), sQa (1.5 μM), and HOPS (150 nM) in a 20 μl reaction of RB150 (20 mM HEPES/NaOH, pH 7.4, 0.15 M NaCl, 10% glycerol) and 0.5 mM $MgCl_2$. Three fluorescence channels were read every minute for 50 min at 27°C in a SpectraMax Gemini XPS (Molecular Devices) plate reader. These channels are reported again here for convenience: Oregon Green 488 (excitation [ex]: 497 nm; emission [em]: 527 nm; cutoff [c/o]: 515 nm), FRET (ex: 490 nm; em: 615 nm; c/o: 590 nm), and Alexa-568 (ex: 568 nm; em: 605 nm; c/o: 590 nm).

Qb association assay

Pelleting reactions were set up with the same reaction concentrations as the SNARE assembly assays, but in 50-μl volumes in PCR tube strips. Reactions were incubated in a water bath set to 27°C for 40 min, then transferred to a 500-μl Eppendorf tube. RB150 (150 μl) was added to each tube, and then the tubes were centrifuged for 40 min at 13,000 RPM at 4°C. The supernatant was removed with a gel-loading pipette tip, and the pellet was resuspended in 20 μl RB150. The Alexa-568 fluorescence of 15 μl aliquots was measured with the SpectraMax plate reader and compared with a mock full reaction to calculate the amount of fluorescent Qb that pelleted. Under these reaction concentrations, the maximum pelleting signal ranges between 12.5 and 25%, assuming that Qb associates with one-to-one stoichiometry with either R, Ypt7, or HOPS.

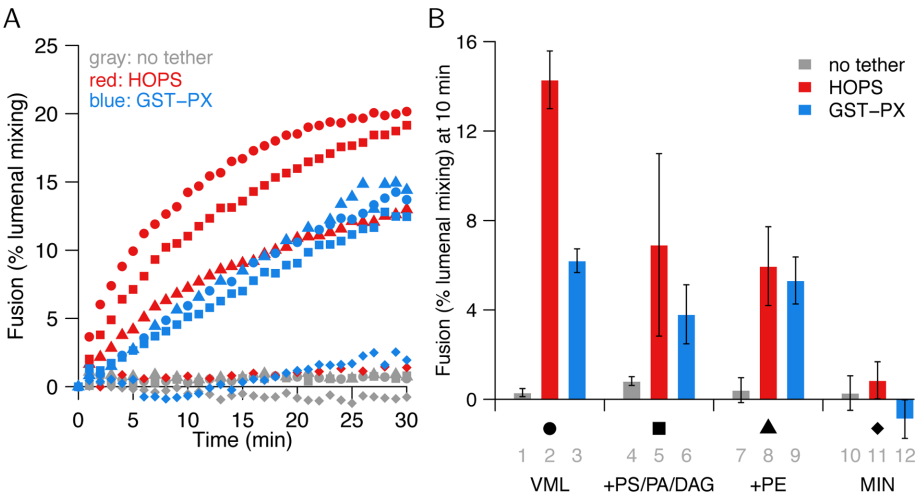

FIGURE 8: Fusion needs either PE or PS, PA, and DAG, whether HOPS or GST-PX is used as tether. (A) Proteoliposomes were prepared with Ypt7/R and Ypt7/3Q and with the indicated lipid compositions. Fusion assays contained either no tether, 2 μM GST-PX, or 50 nM HOPS as indicated; Sec17 and Sec18 were not added, as they block fusion with GST-PX (Song and Wickner, 2019). (B) Quantification of quadruplicate assays of part A.

Microdialysis proteoliposome preparation

Reconstituted proteoliposomes for SNARE assembly and Qb association assays were prepared with a detergent dialysis method (Zick and Wickner, 2013) modified for small volumes (Overall, 1987). Lipids dissolved in chloroform were mixed with β-octylglucoside dissolved in methanol for a final lipid-to-detergent molar ratio of 1:12.5. The mixture was then dried by a stream of nitrogen, followed by spinning for 3 h in a speed-vac. Pellets were dissolved in RB150/$MgCl_2$ buffer and then combined with TEV, then with R and Ypt7 as described in the figure legends to form a mixed micellar solution. The final concentrations of this solution were 5 mM lipid, 62.5 mM β-octylglucoside, R and

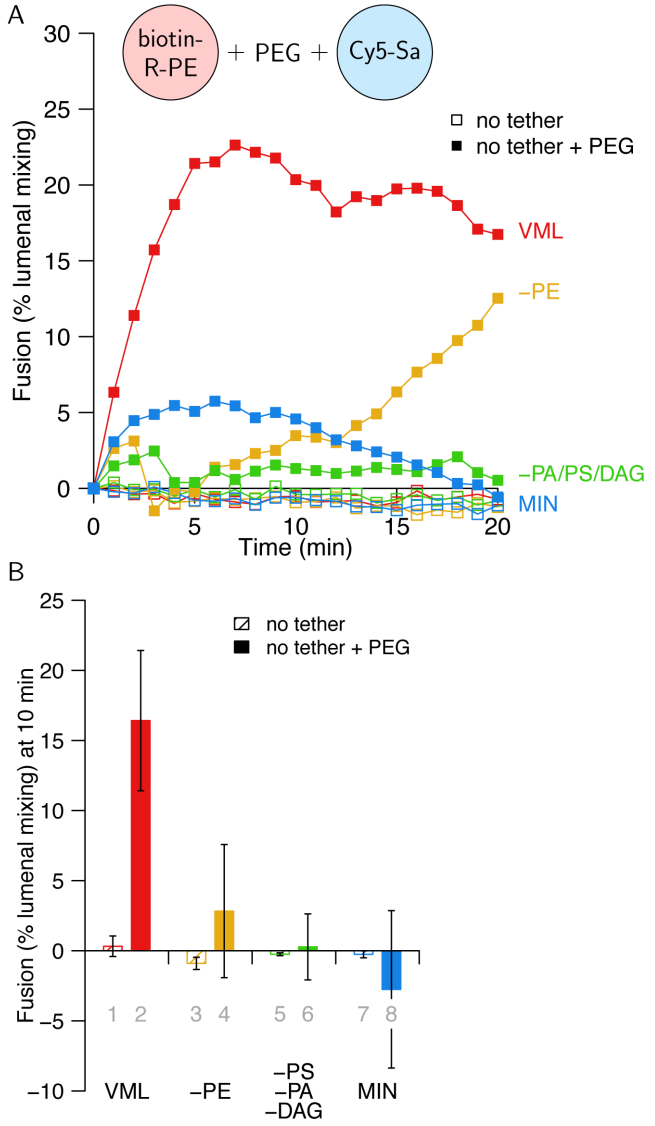

FIGURE 9: PEG-induced fusion with protein-free liposomes also needs PE and PS/PA/DAG. (A) Liposomes of the indicated compositions were prepared with either Cy5-streptavidin or biotinylated phycoerythrin as lumenal markers but without membrane proteins. Fusion was assayed in RB150 after addition of either buffer alone or buffer with polyethylene glycol 8000 (Sigma; 18% final concentration). (B) Quantification of quadruplicate assays of part A.

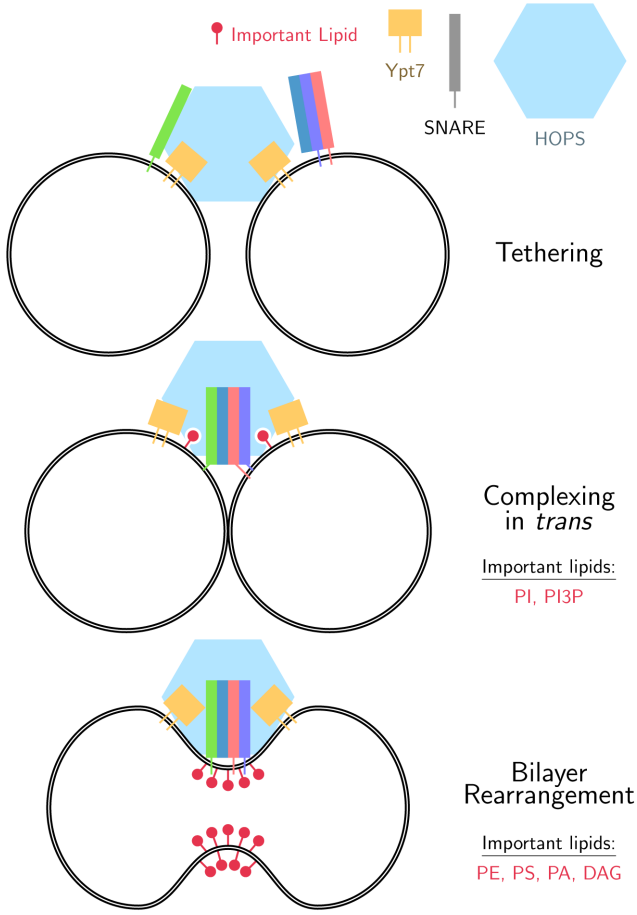

FIGURE 10: Summary schematic of the roles that different lipids play at the distinct stages of fusion. PI and PI3P are important for HOPS catalysis of the *trans*-SNARE complex, while PE, PS, PA, and DAG promote bilayer rearrangements for content mixing. See text for discussion.

Ypt7 at 1:3000 and 1:6000 protein-to-lipid molar ratios, respectively, 1 mM $MgCl_2$, and 1.2 µM TEV in 180 µl. Each proteoliposome prep was added to a 1.5-ml Eppendorf tube with a hole melted through its cap via a Pasteur pipet (Overall, 1987). The opening of the tube was then covered with a piece of dialysis tubing (25 kDa cutoff, 12 mm flat width; Spectrum Labs, Rancho Dominguez, CA), and the cap was shut over the tubing to lock it in place. Pairs of tubes were suspended inverted into a 150-ml beaker containing 120 ml of RB150/1 mM $MgCl_2$ and 0.5 g Biobeads SM-2 (Biorad). Bubbles within the cap of each tube were removed with a pipettor and a loop of tubing. After overnight dialysis, the proteoliposomes were added to a Histodenz gradient in 11 × 34 centrifuge tubes and then centrifuged in a TLA120.2 rotor at 60,000 rpm for 75 min (Zick and Wickner, 2013).

Proteoliposome fusion

Proteoliposomes were prepared and assayed for fusion as described (Song *et al.*, 2021; Song *et al.*, 2017; Song and Wickner, 2019). Briefly, mixed micellar solutions of the indicated lipids in β-octylglucoside were mixed with Ypt7-TM (Song *et al.*, 2020) and SNAREs at molar ratios to lipids of 1:8,000 and 1:16,000, respectively, as well as either biotinylated phycoerythrin or Cy5-labeled streptavidin. After 16 h of dialysis in the dark at 4°C against RB150/1 mM $MgCl_2$ and Biobeads SM2 (1 g/ml mixed micellar solution) to remove the detergent, the resulting proteoliposomes with entrapped fluorescent marker protein were isolated by floatation through a Histodenz step gradient. Fusion assays contained Ypt7/R and either Ypt7/QaQb or Ypt7/QaQbQc proteoliposomes. Fusion was initiated by the addition of 50 nM HOPS, 100 nM Qc (where it was not present on the proteoliposomes), (where indicated) 100 nM Sec17, 300 nM Sec18, and 1 mM Mg:ATPγS. FRET between the Cy5 and phycoerythrin fluorophores was measured in a SpectraMax fluorescence plate reader.

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