Phosphoinositides Function Asymmetrically for Membrane Fusion, Promoting Tethering and 3Q-SNARE Subcomplex Assembly

Hao Xu and William Wickner

From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755-3844

Phosphatidylinositol 3-phosphate (PI(3)P) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) are essential for rapid SNARE-dependent fusion of yeast vacuoles and other organelles. These phosphoinositides also regulate the fusion of reconstituted proteoliposomes. The reconstituted reaction allows separate analysis of phosphoinositide-responsive subreactions: fusion with SNAREs alone, with the addition of the HOPS tethering factor, and with the further addition of the SNARE complex disassembly chaperones Sec17p and Sec18p. Using assays of membrane tethering, trans-SNARE pairing, and lipid mixing, we found that PI(3)P and PI(4,5)P2 have distinct functions that are asymmetric with respect to R-SNARE (Nyv1p) and the 3Q-SNAREs (Vam3p, Vti1p, and Vam7p). Fusion reactions with the Q-SNAREs and R-SNARE on separate membranes showed that PI(3)P has two distinct functions. PI(3)P on Q-SNARE proteoliposomes promoted Vam7p binding and association with the other two Q-SNAREs. PI(3)P on R-SNARE proteoliposomes was recognized by the PX domain of Vam7p on Q-SNARE proteoliposomes to promote tethering, although this function could be supplanted by the tethering activity of HOPS. PI(4,5)P2 stimulated fusion when it was on R-SNARE proteoliposomes, opposed to Q-SNARE proteoliposomes bearing PI(3)P. These functions are essential for the phosphoinositide-dependent synergy between HOPS and Sec17p/Sec18p in promoting rapid fusion.

Membrane fusion is the final step of vesicular traffic, delivering cargo within donor vesicles to the proper acceptor compartment at the right time. Fusion at each organelle requires a specific set of SNAREs, a Rab GTPase, a tethering factor, and an SM protein. In addition to these proteins, specific lipid molecules are required, including phosphoinositides. Phosphatidylinositol 4-phosphate is required for endoplasmic reticulum-to-Golgi traffic at a step after the tethering of COPII vesicles to the Golgi membrane, accompanying trans-SNARE complex formation (1). In exocytosis, PI(4,5)P2 facilitates the recruitment of the exocytic tethering complex (2, 3), regulates the clustering of syntaxin-1A (4), and is required for the functions of synaptotagmin and CAPS (5, 6). In endocytosis, PI(3)P regulates early endosomal fusion by recruiting the tethering factor EEA1 (7, 8) and controls the fusion of phagosomes with late endosomes (9). Homotypic vacuole fusion (10) also requires PI(3)P and PI(4,5)P2 (11–15).

Like other fusion events, vacuole fusion requires cognate SNARE proteins that form coiled-coil four-helix bundles either on the same membrane or on apposed membranes. Three vacuolar SNAREs, Vam3p, Vti1p, and Vam7p, have a glutamyl residue at the center of their SNARE domain and are therefore termed Q-SNAREs, and the fourth, Nyv1p, has an arginyl residue and is called an R-SNARE. trans-SNARE complexes between the 3Q-SNAREs on one vacuole and the R-SNARE on an apposed vacuole lead to fusion. Vam7p does not have a hydrophobic transmembrane domain, but its N-terminal PX domain binds PI(3)P (16). Ypt7p, the Rab GTpase of vacuoles fusion (17), recruits HOPS (18), a heterohexameric complex with direct affinity for phosphoinositides, SNAREs, and Ypt7p (19). Vacuole fusion has been reconstituted from purified proteins and lipids (14, 20). Although the requirement for Ypt7p can be bypassed under certain reconstitution conditions (14, 18), HOPS supports rapid fusion by directly tethering apposed membranes (21) and protecting trans-SNARE complexes from disassembly by the Sec17p and Sec18p SNARE chaperones (22).

Although PI(3)P and PI(4,5)P2 are minor chemical constituents of the membrane, they regulate the fusion of vacuoles (both in vivo and in vitro) and of proteoliposomes composed of purified vacuolar components. The deletion of nonessential genes of phosphoinositide metabolism blocks vacuole fusion in the cell (12). In vitro fusion of the organelle is inhibited by a wide variety of phosphoinositide ligands or phosphoinositide phosphatases (11, 13), blocking specific stages of priming and tethering. PI(3)P and PI(4,5)P2 are enriched at the “vertex ring” microdomain, where docked vacuoles fuse, and this enrichment is interdependent with other fusion proteins and lipids (13). The optimal fusion of reconstituted proteoliposomes needs both PI(3)P and PI(4,5)P2 (14), although some subreactions can proceed in their absence, and either phosphoinositide can support some level of fusion (15). The striking synergy between the HOPS complex, which directly or indirectly promotes trans-SNARE complex assembly, and the Sec17p and Sec18p chaperones, which mediate SNARE com-

---

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–8.
plex disassembly, is absolutely dependent on phosphoinositides (15). Despite the importance of phosphoinositides in vacuole fusion, the mechanisms they use to regulate each stage of the fusion reaction have been elusive. This is due in part to the homotypic nature of vacuole fusion. With all SNAREs present on both fusion partners and in the presence of HOPS, Sec17p, and Sec18p, it is difficult to distinguish the individual functions of each phosphoinositide and whether each acts in cis or in trans to the 3Q-SNAREs and R-SNARE, which engage to form functional trans-SNARE complexes (23).

The power of the reconstitution approach, in which each relevant protein or lipid can be present or absent on each fusion partner, allows exploration of the roles and asymmetries of PI(3)P and PI(4,5)P2 functions. Are both phosphoinositides needed on each fusion partner, and is this requirement asymmetric with respect to R-SNARE and the 3Q-SNAREs that combine in trans to form SNARE complexes? What are the relationships between the HOPS and Sec17p/Sec18p chaperones and phosphoinositides? We now report studies that began with SNARE liposomes in the absence of HOPS, Sec17p, or Sec18p. Under these conditions, high concentrations of Vam7p are needed to drive 3Q-SNARE complex assembly, and tethering requires a trans-interaction between the PX domain of the Q-SNARE Vam7p on one membrane and PI(3)P on the apposed membrane bearing R-SNARE. At lower, more physiological concentrations of Vam7p, PI(3)P is needed on the same membrane as Q-SNAREs to promote Vam7p binding and assembly into the 3Q-SNARE complex; HOPS is then strictly required for tethering. HOPS also protects trans-SNARE complexes from the disassembly chaperones Sec17p and Sec18p (22). In contrast, PI(4,5)P2 stimulates when it is on the same membrane as R-SNAREs, but only when PI(3)P is simultaneously on the apposed Q-SNARE membranes. Thus, phosphoinositides fulfill crucial but mechanistically distinct and asymmetric roles under different fusion conditions.

**EXPERIMENTAL PROCEDURES**

**Proteins and Antibody Preparation**—Recombinant Vam3p, Vti1p, and Nyv1p were expressed in Rosetta 2 cells (Novagen) (14), GST-Vam7p in Rosetta 2(DE3) pLysS cells (24), and untagged Vam7p and Vam7p(Y42A) in Rosetta 2(DE3) cells (25). Vam7p-Δ3A was isolated from Rosetta 2(DE3) cells as described by Schwartz and Merz (26) with modifications (22). The HOPS complex containing Vps33p-GST was purified from vacuole membranes of yeast strain CHY61 (21), followed by tobacco etch virus protease cleavage, gel filtration, and storage as described (20). Sec17p (22) and His6-Sec18p (27) were prepared as described. Antibodies to Vam3p were purified using immobilized GST-Vam3p-ΔTM and cross-linked to protein A-Sepharose CL-4B (GE Healthcare) (22). Antibodies to Nyv1p and Vam7p were generated as described (28, 29).

**Proteoliposome Reconstitution and Lipid Mixing Assay**—SNARE proteoliposomes were prepared with PI(3)P (1%), PI(4,5)P2 (1%), neither phosphoinositide, or both (14). SDS-PAGE and Coomassie Blue staining were performed to ensure that phosphoinositides did not affect protein incorporation into RPLs. Standard fusion reactions (20 μl) contained RB150 (20 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, and 10% (v/v) glycerol), 1 mM MgCl2, 1 mM ATP, an ATP-regenerating system (1 mg/ml creatine kinase and 29 mM creatine phosphate), donor proteoliposomes (200 μM lipids), and acceptor proteoliposomes (200 μM lipids). Vam7p (0.22–6 μM) or Vam7p-Δ3A (0.22–6 μM), HOPS (35 nM), Sec17p (0.68 μM), and Sec18p (0.24 μM) were present where indicated. Reaction mixtures were prepared on ice and transferred to a 396-well plate (preincubated on ice), and the NBD fluorescence signal was measured (λex = 460 nm, λem = 538 nm, λcutoff = 515 nm) in a SpectraMax Gemini XPS plate reader (Molecular Devices) at 27 °C (14). To monitor the fusion between R- and 3Q-SNARE RPLs, they were preincubated separately at 27 °C for 10 min (each in 10 μl of reaction buffer) before mixing together in a 396-well plate prewarmed to 27 °C. Fusion was calculated as the increase in the fluorescence due to lipid mixing at any time divided by the fluorescence at the first minute (ΔF/F0). An increase of 1 in this parameter is defined as one unit of fusion. The maximal rate of fusion was calculated to compare fusion reactions. Under certain conditions of rapid fusion (supplemental Figs. 2 and 4), the F1 value was obtained from a control reaction in which only the respective R-SNARE RPLs (but not the Q-SNARE RPLs) were present. Error bars are standard deviations from three or more experiments.

**Liposome Clustering Assay**—Following incubation at 27 °C for 5 min, each reaction mixture was diluted 40-fold in RB150. Four microliters were placed on a microscope slide and covered with a 22-mm coverslip. Images were collected using a fluorescence microscope (19). Particle sizes were measured in ImageJ (National Institutes of Health) as described (21).

**Assays for 3Q- and trans-SNARE Complex Formation**—The trans-SNARE complex was assayed as described (22). To examine 3Q-SNARE complex formation, 2Q-SNARE RPLs (200 μM lipids) were incubated with untagged Vam7p at 27 °C for 30 min in a reaction mixture including RB150, 1 mM MgCl2, 1 mM ATP, and an ATP-regenerating system. After 5 min on ice, GST-Vam7p (at 5-fold molar excess to Vam7p in the reaction) was added to the sample. Following 10 min of further incubation on ice, membranes were solubilized, Vam3p was immunoprecipitated with immobilized anti-Vam3p antibody (22, 28), and coprecipitating untagged Vam7p and Vti1p were assayed by immunoblotting.

**RESULTS**

**Phosphoinositides Are Not Needed on Both Fusion Partners**—Rapid homotypic fusion of yeast vacuoles depends on both PI(3)P and PI(4,5)P2 (11, 13), a requirement that has been recapitulated in reconstitution assays (14, 15). To address the specific functions of these phosphoinositides, we first asked if they act from one or both membranes during the fusion reaction. Proteoliposomes with four SNAREs (Vam3p, Vti1p, Vam7p, and Nyv1p) were prepared either with or without the two phosphoinositides. Proteoliposome fusion was monitored by a FRET-based lipid mixing assay that employs “donor” proteoliposomes labeled with lipid-anchored NBD and rhodamine (at concentrations at which the NBD fluorescence signal
is effectively quenched by rhodamine), whereas acceptor proteoliposomes have neither fluorophore. Upon fusion, the mixture of the donor and acceptor membranes relieves this quenching effect via dilution, leading to increased NBD fluorescence (30). Fusion of 4-SNARE proteoliposomes requires the synergistic actions of the tethering factor HOPS and the SNARE complex disassembly chaperones Sec17p and Sec18p (14). Phosphoinositides were essential for fusion but were needed only on one fusion partner, either donor or acceptor (Fig. 1 and supplemental Fig. 1). SNAREs are required on both membranes for fusion (23). How do phosphoinositides fulfill their roles on just one membrane?

**Recognition of PI(3)P in trans by the PX Domain of Vam7p for Tethering**—To determine whether each phosphoinositide has specific functions that are asymmetric with respect to SNAREs, we prepared proteoliposomes with Nyv1p only (R-SNARE RPLs) or with Vam3p, Vti1p, and Vam7p (3Q-SNARE RPLs). Because R- and 3Q-SNARE RPLs can fuse without HOPS or Sec17p/Sec18p (14, 23), we are able to address the specific requirements for phosphoinositides that are in cis or in trans to the R- or Q-SNAREs. In contrast to HOPS- and Sec17p/Sec18p-dependent homotypic fusion of 4-SNARE RPLs (Fig. 1), heterotypic fusion between R- and 3Q-SNARE proteoliposomes could occur without phosphoinositides (Fig. 2, bar 5 versus bar 1; and supplemental Fig. 2, A–C, open triangles). However, there was a significant stimulation of fusion by phosphoinositides on R-SNARE proteoliposomes (Fig. 2, bar 7 versus bar 5). To distinguish the roles of PI(3)P and PI(4,5)P2, we performed SNARE-only fusion reactions using proteoliposomes bearing either PI(3)P or PI(4,5)P2. PI(3)P contributed to this stimulation much more than PI(4,5)P2 (Fig. 2, bar 13 versus 10; and supplemental Fig. 2, B and C). The stimulation by PI(3)P was ablated when wild-type Vam7p was replaced by Vam7p(Y42A) (Fig. 2, bars 15–18; and supplemental Fig. 2D), which has a defective PI(3)P-binding pocket in its PX domain (16). Therefore, the trans-interaction between the PX domain of Vam7p and PI(3)P greatly enhances the rate of fusion.

PI(3)P on the R-SNARE RPL might facilitate the tethering of proteoliposomes, leading to more trans-SNARE complexes and faster fusion, or it might stimulate fusion at a step after the formation of trans-SNARE complexes. To distinguish between these possibilities, we compared the levels of the trans-SNARE complex at an early reaction time when the rate of fusion was still linear. A trans-SNARE complex assay was performed with R-SNARE RPLs plus Q-SNARE RPLs bearing Vam7p-3Δ, a C-terminal deletion mutant that supports trans-SNARE complex formation but not fusion (26). The use of Vam7p-3Δ prevents the post-fusion formation of any cis-SNARE complexes (which had never been trans), allowing an authentic measurement of only the trans-SNARE complex. The level of the trans-SNARE complex was determined by the amount of Nyv1p (from R-SNARE RPLs) co-immunoprecipitated with Vam3p (from Q-SNARE RPLs) 5 min after the proteoliposomes were mixed together at reaction temperature. Under these conditions, PI(3)P dramatically increased the level of the trans-SNARE complex when it was on the R-SNARE RPL (Fig. 3A, lane 8), in accord with the fusion data in Fig. 2. Furthermore, tethering assays showed that PI(3)P in
trans to Vam7p greatly facilitated the formation of large clusters and that the functional interaction between PI(3)P and the PX domain of Vam7p was important for the cluster size increase (Fig. 3B). Fusion was inhibited when PI(3)P was also in cis with Vam7p on Q-SNARE RPLs (Fig. 2, bar 13 versus bar 14), perhaps due to 3Q-SNARE RPLs clustering with each other instead of with R-SNARE RPLs. Thus, PI(3)P can promote fusion by facilitating tethering via a trans-interaction with Vam7p, although an additional post-trans-SNARE complex role remains possible.

HOPS-dependent Tethering Does Not Require Phosphoinositides—Because HOPS tethers vesicles (21) and has affinity for phosphoinositides (19), we asked whether the HOPS-tethering activity, which should promote the fusion of R- and 3Q-SNARE RPLs, is in any way influenced by the topological distribution of phosphoinositides. HOPS stimulated fusion in the absence of phosphoinositides (Fig. 4, bars 5 and 6; and supplemental Fig. 3C), suggesting that HOPS does not need PI(3)P or PI(4,5)P2 to tether SNARE-bearing proteoliposomes. This was confirmed directly by clustering assays using protein-free liposomes (supplemental Fig. 4). When PI(3)P was present on the R-SNARE RPLs in trans to Vam7p, HOPS no longer stimulated fusion (Fig. 4, bars 1 and 2; and supplemental Fig. 3A), suggesting that the trans-interaction between PI(3)P and Vam7p is so efficient at tethering proteoliposomes that the need for HOPS-tethering activity is bypassed under these conditions.

PI(3)P Facilitates 3Q-SNARE Complex Formation When Vam7p Must Be Recruited to the Membrane—Our studies thus far examined the asymmetric requirement for phosphoinositides in the fusion of R- and 3Q-SNARE proteoliposomes in the absence of Sec17p and Sec18p. During vacuole fusion, cis-SNARE complexes are disassembled by Sec17p and Sec18p, and the Qc-SNARE Vam7p is constantly released to the cytosol and recruited to the membrane (31). To reflect Vam7p recruitment in the fusion reaction, we employed an RPL fusion system in which the Q-SNARE proteoliposomes initially bore only Vam3p and Vti1p, and Vam7p was added to the reaction mixture at controlled concentrations. High levels of Vam7p (i.e., 6 μM) drove 3Q-SNARE complex formation by mass action, in a fashion that was insensitive to phosphoinositides. Under these conditions, fusion was greatly stimulated by PI(3)P on the R-SNARE RPLs (Fig. 5, bar 4 versus bar 10).
In accord with findings using 3Q-SNARE RPLs (Fig. 2), this asymmetric stimulation by PI(3)P was also abolished by the Y42A single amino acid change in the PI(3)P-binding pocket of the Vam7p PX domain (supplemental Fig. 5, bar 4 versus bar 12). At low levels of Vam7p (i.e., 0.22 μM), very little fusion was detected regardless of the position of PI(3)P (Fig. 5, bars 1, 5, 9, and 13), suggesting that too little Vam7p was recruited to drive tethering or fusion. We therefore added HOPS, whose tethering activity does not require phosphoinositides (Fig. 4 and supplemental Fig. 4). When HOPS was present to fulfill the tethering function, and Vam7p had to be recruited into SNARE complexes, PI(3)P was needed on the 2Q-SNARE RPLs (Fig. 6, compares bars 5–8 with bars 1–4; and supplemental Fig. 6), in accord with studies showing that PI(3)P is a primary receptor for Vam7p on vacuoles (31). PI(4,5)P2 on either R- or 2Q-SNARE RPLs had little effect by itself on fusion (Fig. 6, bar 7 versus bar 5 and bar 15 versus bar 13). However, with PI(3)P on 2Q-SNARE RPLs, PI(4,5)P2 on R-SNARE RPLs doubled the maximal rate of fusion (Fig. 6, bar 7 versus bar 5 and bar 15 versus bar 13).

To examine the roles of PI(3)P in 2Q-SNARE RPLs, we asked if it enhanced the formation of the 3Q-SNARE complex. 2Q-SNARE RPLs (with or without PI(3)P) were incubated with low levels of Vam7p before membranes were solubilized in detergent. To block Vam3p-Vam7p association in detergent after membrane solubilization, excess GST-Vam7p was added to compete with Vam7p. The 3Q-SNARE complex was assayed as the amount of untagged Vam7p that co-immunoprecipitated with Vam3p (Fig. 7). PI(3)P dramatically enhanced the 3Q-SNARE complex formation (Fig. 7, compare lanes 5 and 9 with bar 1). However, with PI(3)P on 2Q-SNARE RPLs, PI(4,5)P2 on R-SNARE RPLs doubled the maximal rate of fusion (Fig. 6, bar 7 versus bar 5 and bar 15 versus bar 13).

To examine the roles of PI(3)P in 2Q-SNARE RPLs, we asked if it enhanced the formation of the 3Q-SNARE complex. 2Q-SNARE RPLs (with or without PI(3)P) were incubated with low levels of Vam7p before membranes were solubilized in detergent. To block Vam3p-Vam7p association in detergent after membrane solubilization, excess GST-Vam7p was added to compete with Vam7p. The 3Q-SNARE complex was assayed as the amount of untagged Vam7p that co-immunoprecipitated with Vam3p (Fig. 7). PI(3)P dramatically enhanced the 3Q-SNARE complex formation (Fig. 7, compare lanes 5 and 9 with bar 1). However, with PI(3)P on 2Q-SNARE RPLs, PI(4,5)P2 on R-SNARE RPLs doubled the maximal rate of fusion (Fig. 6, bar 7 versus bar 5 and bar 15 versus bar 13).

To examine the roles of PI(3)P in 2Q-SNARE RPLs, we asked if it enhanced the formation of the 3Q-SNARE complex. 2Q-SNARE RPLs (with or without PI(3)P) were incubated with low levels of Vam7p before membranes were solubilized in detergent. To block Vam3p-Vam7p association in detergent after membrane solubilization, excess GST-Vam7p was added to compete with Vam7p. The 3Q-SNARE complex was assayed as the amount of untagged Vam7p that co-immunoprecipitated with Vam3p (Fig. 7). PI(3)P dramatically enhanced the 3Q-SNARE complex formation (Fig. 7, compare lanes 5 and 9 with bar 1). However, with PI(3)P on 2Q-SNARE RPLs, PI(4,5)P2 on R-SNARE RPLs doubled the maximal rate of fusion (Fig. 6, bar 7 versus bar 5 and bar 15 versus bar 13).

To examine the roles of PI(3)P in 2Q-SNARE RPLs, we asked if it enhanced the formation of the 3Q-SNARE complex. 2Q-SNARE RPLs (with or without PI(3)P) were incubated with low levels of Vam7p before membranes were solubilized in detergent. To block Vam3p-Vam7p association in detergent after membrane solubilization, excess GST-Vam7p was added to compete with Vam7p. The 3Q-SNARE complex was assayed as the amount of untagged Vam7p that co-immunoprecipitated with Vam3p (Fig. 7). PI(3)P dramatically enhanced the 3Q-SNARE complex formation (Fig. 7, compare lanes 5 and 9 with bar 1). However, with PI(3)P on 2Q-SNARE RPLs, PI(4,5)P2 on R-SNARE RPLs doubled the maximal rate of fusion (Fig. 6, bar 7 versus bar 5 and bar 15 versus bar 13). To examine the roles of PI(3)P in 2Q-SNARE RPLs, we asked if it enhanced the formation of the 3Q-SNARE complex. 2Q-SNARE RPLs (with or without PI(3)P) were incubated with low levels of Vam7p before membranes were solubilized in detergent. To block Vam3p-Vam7p association in detergent after membrane solubilization, excess GST-Vam7p was added to compete with Vam7p. The 3Q-SNARE complex was assayed as the amount of untagged Vam7p that co-immunoprecipitated with Vam3p (Fig. 7). PI(3)P dramatically enhanced the 3Q-SNARE complex formation (Fig. 7, compare lanes 5 and 9 with bar 1). However, with PI(3)P on 2Q-SNARE RPLs, PI(4,5)P2 on R-SNARE RPLs doubled the maximal rate of fusion (Fig. 6, bar 7 versus bar 5 and bar 15 versus bar 13).

Sec17p and Sec18p Confer HOPS Dependence and a Rigorous Requirement for PI(3)P on Q-SNARE RPLs—PI(3)P fulfills distinct functions in different fusion subreactions, supporting tethering when it is on R-SNARE proteoliposomes and 3Q-SNARE complex formation when it is on Q-SNARE proteoliposomes. What happens when fusion is performed in the presence of Sec17p/Sec18p as well as HOPS, mimicking vacuole fusion in intact cells? Instead of basing these assays on 4-SNARE RPLs as in Fig. 1, which obscures the asymmetry of phosphoinositide requirements with respect to R- or Q-SNAREs, we used R- and 2Q-SNARE RPLs and a low level of soluble full-length Vam7p. As reported (14), this fusion was absolutely dependent on HOPS, Sec17p/Sec18p, and phosphoinositides (Figs. 1 and 8 and supplemental Figs. 1 and 7). PI(3)P on Q-SNARE RPLs was essential for robust fusion, and PI(4,5)P2 on R-SNARE proteoliposomes provided further stimulation (Fig. 8, bars 1, 5, and 7; and supplemental Fig. 7). Thus, in the presence of HOPS, which facilitates tethering.
and Sec17p and Sec18p, which constantly disassemble SNARE complexes and release Vam7p, PI(3)P is required on Q-SNARE RPLs to facilitate 3Q-SNARE complex assembly. Vam7p(Y42A) did not support this fusion regardless of the position of phosphoinositides (Fig. 8, bars 17–20; and supplemental Fig. 7E), in contrast to the observation that Vam7p(Y42A) did support fusion when it was already assembled into the 3Q-SNARE complex during proteoliposome preparation (Fig. 2 and supplemental Fig. 2D). Thus, a functional PI(3)P-binding site appears to be essential for Vam7p membrane rebinding and ongoing 3Q-SNARE formation in the presence of SNARE disassembly chaperones, in accord with studies using yeast vacuoles (31). Taken together, Sec17p and Sec18p confer rigorous HOPS dependence and a requirement for PI(3)P on Q-SNARE RPLs. PI(3)P was also required on Q-SNARE proteoliposomes when both fusion partners bore Ypt7p (supplemental Fig. 8, compare bars 5–8 with bars 1–4, respectively), and PI(4,5)P2 stimulated more when on the R-SNARE RPLs (compare bars 5, 7, and 13), suggesting that Ypt7p, whose major function is to help recruit HOPS to the membrane (18), does not alter the asymmetry of phosphoinositide function.

Although mechanistic insights can be gleaned from experiments with phosphoinositides and R- and Q-SNAREs on distinct fusion partners, the physiological reaction is one of homotypic vacuole fusion where the same phosphoinositides are on both fusion partners. It is therefore of particular interest to note that little or no fusion was seen when both the R- or Q-SNARE RPLs bore no phosphoinositides (Fig. 8, bar 1) or bore PI(4,5)P2 alone (bar 11). Although some fusion was seen when both bore PI(3)P (Fig. 8, bar 6), fusion proceeded with a much faster initial rate when both fusion partners bore both PI(3)P and PI(4,5)P2 (bar 16) and to almost four times the extent (supplemental Fig. 7, B–D, compare filled symbols). This striking effect of PI(4,5)P2 was only observed in the presence of Sec17p/Sec18p (Fig. 8, compare bars 6, 11, and 16 in the presence of Sec17p/Sec18p with those in Fig. 6 in their absence).

**DISCUSSION**

Yeast vacuole fusion is homotypic and complex, with each fusion partner bearing all four SNAREs, an array of important lipids (including phosphatidylethanolamine, phosphatidic acid, 3- and 4-phosphoinositides, ergosterol, and diacylglycerol), HOPS, Sec17p/Sec18p, and the Rab GTPase Ypt7p. The multiplicity of these components and the homotypic nature of the fusion obscure the chemical definition of individual subreactions, requiring a reductionist biochemical approach. To explore the roles of PI(3)P and PI(4,5)P2 in vacuole fusion, we employed defined subreactions in which the membrane-anchored vacuolar R- and Q-SNAREs were artificially segregated onto separate proteoliposomes, allowing tethering, SNARE pairing, and fusion to be examined as we reintroduced peripheral membrane proteins such as the soluble SNARE Vam7p, the SNARE disassembly chaperones Sec17p and Sec18p, and the multifunctional HOPS complex. This approach revealed that the phosphoinositide functions are asymmetric with respect to the Q- and R-SNAREs and are exquisitely dependent on the presence or absence of HOPS and Sec17p/Sec18p.

Our studies led to a working model of asymmetric phosphoinositide function (Fig. 9). Vam3p and Vti1p, which form a stable 2-SNARE complex (23), associate with Vam7p, which binds to the membrane through the affinity of its PX domain for PI(3)P (Fig. 9, A and B) to form a 3Q-SNARE complex (Fig. 9C). Tethering can be promoted by the interaction of the Vam7p PX domain with PI(3)P in trans (Fig. 3) or by HOPS (21), bringing the membranes into close apposition to allow R- and 3Q-SNAREs to pair in trans and form 4-SNARE trans-complexes that lead to fusion (Fig. 9E). However, a significant proportion of the trans-SNARE complexes may assemble in inactive conformations of unknown structure (Fig. 9F, fig leaf). Such nonfunctional conformations were suggested by earlier structural studies of neuronal SNAREs (32) and are inferred for vacuole SNAREs from the need for Sec18p-mediated trans-SNARE complex recycling for fusion of intact vacuoles (33) and the requirement for
Sec17p/Sec18p for optimal rates of fusion of proteoliposomes that bear separated Q- and R-SNAREs (14). HOPS has been shown to proofread several aspects of SNARE structure (34) and to protect functional trans-SNARE complexes from disassembly (22). Our working model postulates that Sec17p and Sec18p disassemble and recycle nonfunctional trans-SNARE complexes that are not protected by HOPS, but as shown (22), they do not efficiently disassemble HOPS-protected functional trans-SNARE complexes.

In contrast to the clear asymmetric functions of PI(3)P, PI(4,5)P2 (without PI(3)P) confers limited stimulation in simple reconstitution systems with SNAREs, with SNAREs and HOPS alone, or with SNAREs, HOPS, and Sec17p/Sec18p. However, in the presence of PI(3)P, it has an important and asymmetric role (Figs. 6 and 8, compare bars 5 and 7). Earlier reconstitution studies have shown that phosphoinositides have no effect on the membrane association of HOPS, Sec17p, or Sec18p (Fig. 4E in Ref. 15). Strikingly, the fusion seen in the presence of HOPS and Sec17p/Sec18p with either PI(3)P or PI(4,5)P2 present on both Q- and R-SNARE RPLs (Fig. 8, bars 6 and 11) is far less than when both phosphoinositides are on both fusion partners (bar 16). Further studies will be needed to define the molecular role(s) of PI(4,5)P2 in this system.

HOPS-tethering activity does not require phosphoinositides (supplemental Fig. 4), even though HOPS has affinity for these acidic lipids (19). The importance of HOPS-lipid interaction is strongly supported by the finding that phosphorylated HOPS, which has diminished binding to lipids, no longer supports fusion unless the HOPS-Ypt7p interaction is present (18). We suggest that acidic lipids other than phosphoinositides may participate in binding HOPS (15).

Asymmetric requirements for specific lipids in membrane fusion are not limited to vacuoles. PI(4,5)P2 on the plasma membrane is essential for vesicle exocytosis. Using an elegantly reconstituted proteoliposome fusion system, it was discovered that PI(4,5)P2 regulates dense core vesicle exocytosis from the Q-SNARE membranes via interaction with CAPS (a Munc13-like protein), which bears a pleckstrin homology domain (5). Thus, one reason for the asymmetric requirement for phosphoinositides in fusion is that their respective protein partners (with a specific lipid-binding motif in their cytosolic domain) regulate fusion from only Q- or R-SNARE membranes. Studying the asymmetric requirement for phosphoinositides will inevitably lead to insights into how these proteins regulate various fusion subreactions. Phosphoinositides and their derivatives might also regulate fusion in an asymmetric fashion because of their capacities to generate membrane curvature. For example, asymmetric but reciprocal distribution of phosphatidic acid and PI(4,5)P2 triggers the in vitro progression of GLUT4 vesicle exocytosis from the hemifused state to full fusion (35). It will be important to determine whether phosphatidic acid and PI(4,5)P2 play similar roles in vacuole fusion.

Acknowledgments—We thank Dr. Joji Mima for sharing reagents at the beginning phase of this project and Amy Orr and Holly Jakubowski for excellent technical support.

References

1. Lorente-Rodriguez, A. (2010) Investigation of the Influence of Lipids in ER to Golgi Transport. Ph.D. thesis, Dartmouth College, Hanover, NH

2. He, B., Xi, F., Zhang, X., Zhang, J., and Guo, W. (2007) EMBO J. 26, 4053–4065

3. Zhang, X., Orlando, K., He, B., Xi, F., Zhang, J., Zajac, A., and Guo, W. (2008) J. Cell Biol. 180, 145–158

4. Murray, D. H., and Tamm, L. K. (2009) Biochemistry 48, 4617–4625

5. James, D. J., Khodzhong, K., Kowalchyk, J. A., and Martin, T. F. (2008) J. Cell Biol. 182, 355–366

6. Bai, J., Tucker, W. C., and Chapman, E. R. (2004) Nat. Struct. Mol. Biol. 11, 36–44

7. Simonsen, A., Lippé, R., Christoforidis, S., Gaultier, J. M., Brech, A., Cal-

8. Burd, C. G., and Emr, S. D. (1998) Mol. Cell 2, 157–162

9. Vieira, O. V., Bucci, C., Harrison, R. E., Trimble, W. S., Lanzetti, L., Gru-

10. Mima, J., Hickey, C. M., Xu, H., Jun, Y., and Wickner, W. (2008) EMBO J. 27, 2031–2042

11. Mayer, A., Schegmann, D., Dove, S., Glatz, A., Wickner, W., and Haas, A. (2000) Mol. Biol. Cell 11, 807–817

12. Seeley, E. S., Kato, M., Margolis, N., Wickner, W., and Etizen, G. (2002) Mol. Biol. Cell 13, 782–794

13. Fratti, R. A., Jun, Y., Merz, A. J., Margolis, N., and Wickner, W. (2004) J. Cell Biol. 167, 1087–1098

14. Mima, J., Hickey, C. M., Xu, H., Jun, Y., and Wickner, W. (2008) EMBO J. 27, 2031–2042

15. Mima, J., and Wickner, W. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 16191–16196

16. Cheever, M. L., Sato, T. K., de Beer, T., Kutateladze, T. G., Enn, S. D., and Overduin, M. (2001) Nat. Cell Biol. 3, 613–618

17. Haas, A., Schegmann, D., Lazar, T., Gallwitz, D., and Wickner, W. (1995) EMBO J. 14, 5258–5270

18. Hickey, C. M., Strome, C., and Wickner, W. (2007) J. Biol. Chem. 284, 16118–16125

19. Strome, C., Collins, K. M., Fratti, R. A., and Wickner, W. (2006) EMBO J. 25, 1579–1589

20. Fratti, R. A., Collins, K. M., Hickey, C. M., and Wickner, W. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 7907–817

21. Haas, A., and Wickner, W. (1996) J. Cell Biol. 131, 1948–1960

22. Fukuda, R., McNew, J. A., Weber, T., Parlati, F., Engel, T., Nickel, W., Rothman, J. E., and Söllner, T. H. (2000) Nature 407, 198–202

23. Fratti, R. A., Collins, K. M., Hickey, C. M., and Wickner, W. (2007) J. Biol. Chem. 282, 14861–14867

24. Mima, J., and Wickner, W. (2009) J. Biol. Chem. 284, 27114–27122

25. Schwartz, M. L., and Merz, A. J. (2009) J. Cell Biol. 185, 535–549

26. Boeddinghaus, C., Merz, A. J., Laage, R., and Unger mann, C. (2002) J. Cell Biol. 157, 79–89

27. Weninger, K., Bowen, M. E., Chu, S., and Brunger, A. T. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 14800–14805

28. Jun, Y., Xu, H., Thorngren, N., and Wickner, W. (2007) EMBO J. 26, 4935–4945

29. Starai, V. J., Hickey, C. M., and Wickner, W. (2008) Mol. Biol. Cell 19, 2500–2508

30. Vicogne, J., Vollenweider, D., Smith, J. R., Huang, P., Frohman, M. A., and Pessin, E. J. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 14761–14766