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Reconstituted membrane fusion requires regulatory lipids, SNAREs and synergistic SNARE chaperones

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The homotypic fusion of yeast vacuoles, each with 3Q- and 1R-SNARE, requires SNARE chaperones (Sec17p/Sec18p and HOPS) and regulatory lipids (sterol, diacylglycerol and phosphoinositides). Pairs of liposomes of phosphatidylcholine/phosphatidylserine, bearing three vacuolar Q-SNAREs on one and the R-SNARE on the other, undergo slow lipid mixing, but this is unaffected by HOPS and inhibited by Sec17p/Sec18p. To study these essential fusion components, we reconstituted proteoliposomes of a more physiological composition, bearing vacuolar lipids and all four vacuolar SNAREs. Their fusion requires Sec17p/Sec18p and HOPS, and each regulatory lipid is important for rapid fusion. Although SNAREs can cause both fusion and lysis, fusion of these proteoliposomes with Sec17p/Sec18p and HOPS is not accompanied by lysis. Sec17p/Sec18p, which disassemble SNARE complexes, and HOPS, which promotes and proofreads SNARE assembly, act synergistically to form fusion-competent SNARE complexes, and this synergy requires phosphoinositides. This is the first chemically defined model of the physiological interactions of these conserved fusion catalysts.
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Introduction

Membrane fusion is required for protein trafficking, cell surface growth, hormone secretion and neurotransmission. It is catalyzed by conserved proteins and lipids that become enriched in fusion-competent microdomains (Lang *et al*, 2001; Miaczynska and Zerial, 2002; Wang *et al*, 2002; Fratti *et al*, 2004). Rab GTPases in their GTP-bound state cooperate with Rab:GTP-binding proteins ('effectors') to tether organelles, providing an initial layer of specificity (Novick and Zerial, 1997; Grosshans *et al*, 2006). Further specificity is provided by the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins (McNew *et al*, 2000; Parlati *et al*, 2000; Jahn and Scheller, 2006), distinguished by their heptad-repeat SNARE domains that form 4-helical bundles (Poirier *et al*, 1998; Sutton *et al*, 1998), in *cis* (anchored to one membrane) or in *trans* (anchored to apposed membranes). SNARE complex assembly and disassembly is governed by distinct SNARE chaperones. αSNAP (soluble *N*-ethylmaleimide-sensitive factor attachment protein) (Sec17p) and NSF (*N*-ethylmaleimide-sensitive factor) (Sec18p) perform ATP-driven SNARE complex disassembly (Söllner *et al*, 1993). Sec1p/Munc18-1p family (SM) proteins associate with SNAREs and SNARE complexes to permit *trans*-SNARE complex assembly (Rizo and Südhof, 2002; Dulubova *et al*, 2007; Shen *et al*, 2007). Membrane fusion also requires specific lipids. Diacylglycerol (DAG), which can trigger the fusion of liposomes (Siegel *et al*, 1989), is required for the fusion of isolated organelles (Fratti *et al*, 2004; Jun *et al*, 2004). Sterols support fusion (Kato and Wickner, 2001; Fratti *et al*, 2004), perhaps by stabilizing fusion-competent microdomains (Valdez-Taubas and Pelham, 2003). Phosphoinositides can bind fusion proteins (Cheever *et al*, 2001; Fratti *et al*, 2004; Stroupe *et al*, 2006) in a common microdomain (Miaczynska and Zerial, 2002; Fratti *et al*, 2004).

SNARE proteins, discovered in the neuronal synapse, are vital for membrane fusion in all eukaryotic cells (Jahn and Scheller, 2006). They associate through their conserved SNARE domains into SNARE complexes. The buried, conserved 0-layer at the centre of the 4-helical SNARE complex has three glutamyl residues and one arginyl residue. It is the basis for classifying SNAREs as Q- or R-SNAREs (Fasshauer *et al*, 1998). Most SNAREs have a C-terminal *trans*-membrane anchor.

SNARE proteins that function together in the cell can mediate lipid mixing between proteoliposomes (Weber *et al*, 1998; McNew *et al*, 2000), suggesting that SNAREs are the engine of fusion and determinants of fusion specificity and that other factors only regulate SNARE-driven fusion. Whereas SNAREs alone can drive fusion (Nickel *et al*, 1999), they can also cause substantial lysis of reconstituted proteoliposomes, 'RPLs,' or yeast vacuoles (Dennison *et al*, 2006; Starai *et al*, 2007). Synaptotagmin can associate with neuronal SNAREs in RPLs to enhance fusion and provide Ca^{2+} regulation (Tucker *et al*, 2004; Bhalla *et al*, 2006). However, most intracellular fusion does not use Ca^{2+} or synaptotagmin. The inclusion of Sec1p during the formation of RPLs with yeast exocytic SNAREs enhances their rate of lipid mixing (Scott *et al*, 2004), and stronger stimulation is seen with Munc18-1p and neuronal SNARE liposomes (Shen *et al*, 2007). Although there has been progress in reconstituting regulated neuronal SNARE-driven lipid mixing (Tucker *et al*, 2004; Shen *et al*, 2007), there has been less progress with RPL reactions with non-neuronal SNAREs.

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We study membrane fusion with yeast vacuoles. Vacuole fusion (Ostrowicz *et al*, 2008) requires the Rab-GTPase Ypt7p, the heterohexameric HOPS (homotypic fusion and vacuole protein sorting)/Vps Class C complex (Seals *et al*, 2000; Wurmser *et al*, 2000), four vacuolar SNAREs (the Q-SNAREs Vam3p, Vti1p and Vam7p and the R-SNARE Nyv1p), SNARE disassembly chaperones (Sec17p/Sec18p) and several chemically minor but functionally vital regulatory lipids (ergosterol (ERG), DAG and phosphoinositides). The HOPS complex, which includes an SM protein (Vps33p) and acts downstream of Ypt7p (Starai *et al*, 2007), has been isolated in active form (Stroupe *et al*, 2006) and can bind SNAREs such as Vam7p (Stroupe *et al*, 2006), Vam3p (Sato *et al*, 2000; Dulubova *et al*, 2001) and the SNARE complex (Collins *et al*, 2005; CMH and WW, unpublished data) as well as phosphoinositides (Stroupe *et al*, 2006). HOPS and the regulatory lipids are continuously needed until fusion (Jun *et al*, 2006) rather than only regulating *trans*-SNARE complex formation. *Trans*-SNARE complex can undergo continuous remodelling by Sec17p/Sec18p (Jun *et al*, 2007), whereas HOPS permits SNARE complex assembly and proofreads its structure (Starai *et al*, 2008). Nevertheless, liposomes bearing only vacuolar SNAREs can undergo lipid mixing (Fukuda *et al*, 2000), much like liposomes with their neuronal counterparts (Weber *et al*, 1998). Understanding the physiological fusion machinery will require chemically defined *in vitro* reactions that need more than SNARE proteins.

Our current studies began by reproducing the capacity of PC/PS (phosphatidylcholine/phosphatidylserine) liposomes with vacuolar SNAREs to undergo lipid mixing (Fukuda *et al*, 2000). This lipid mixing is insensitive to HOPS and is blocked by Sec17p/Sec18p. To seek reconstitution conditions that require these physiological factors, we varied the lipid composition, the presence of the SNAREs on one or the other fusion-partner liposome and the addition of Sec17p/Sec18p, HOPS and regulatory lipids. With vacuolar lipid composition, rapid lipid mixing requires the four SNAREs, Sec17p/Sec18p, HOPS and regulatory lipids. Our studies of this reconstituted system reveal clear requirements for specific lipids and a novel, phosphoinositide-dependent interplay of the HOPS complex, which promotes and proofreads SNARE complex assembly (Starai *et al*, 2008), and Sec17p/18p, which promote disassembly, to facilitate productive SNARE pairing and fusion. This reconstituted system drives complete membrane fusion without lysis, fulfilling the definition of physiological fusion.

Results

To reconstitute yeast vacuole membrane fusion, we purified recombinant GST-Vam3p, Vti1p, Vam7p and Nyv1p (Supplementary Figure S1A) and removed the GST moiety of GST-Vam3p by TEV protease. Proteoliposomes were reconstituted by dialysis from detergent micellar solutions of defined lipid mixtures and untagged SNAREs (Supplementary Figures S1B–I and Table SI). In addition to liposome pairs bearing 3Q or 1R SNARE (Fukuda *et al*, 2000), we prepared proteoliposomes bearing all four SNAREs, as in the organelle. We employed three lipid compositions (Supplementary Figure S1 and Table SI): (1) PC/PS, (2) a vacuolar lipid composition (Zinser *et al*, 1991; Schneiter *et al*, 1999) that contained PC, PE (phosphatidylethanolamine), PI (phosphatidylinositol), PS, PA (phosphatidic acid), CL (cardiolipin) and ERG and (3) vacuole lipids plus the 'regulatory' lipids DAG, PI(3)P (phosphatidylinositol 3-phosphate) and PI(4,5)P_2 (phosphatidylinositol 4,5-bisphosphate), which are required for *in vitro* vacuole fusion (Fratti *et al*, 2004). Analysis of isolated proteoliposomes showed successful reconstitution in each case (Supplementary Figure S1). We employed molar ratios of lipid/SNARE from 500:1 to 2000:1 (Supplementary Table SI), as proteoliposomes with higher SNARE densities, for example, less than 100:1 lipid/SNARE, may lose membrane integrity and leak lumenal contents (Dennison *et al*, 2006).

Lipid mixing with PC/PS proteoliposomes requires separate reconstitutions of 3Q-SNAREs and 1R-SNARE

To study fusion, we employed a lipid-mixing assay using NBD-PE (*N*-(7-nitro-2,1,3-benzoxadiazole-4-yl)-phosphatidylethanolamine) and Rh-PE (*N*-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine) (Struck *et al*, 1981; Weber *et al*, 1998). As reported (Fukuda *et al*, 2000), PC/PS liposome pairs where one partner has three vacuolar Q-SNAREs and the other partner has the R-SNARE undergo lipid mixing (Figures 1A and Supplementary Figure S2A; the

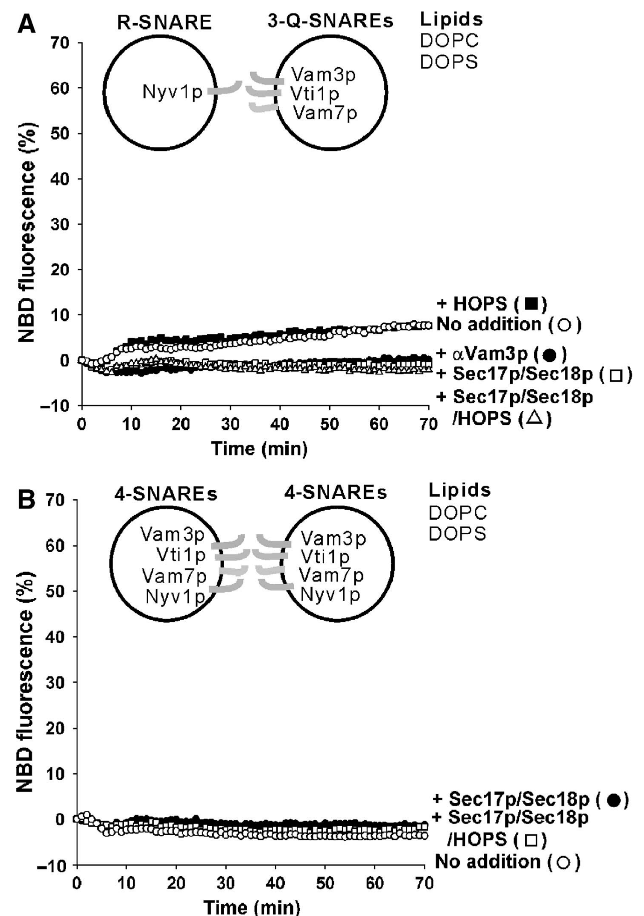

Figure 1 SNARE-mediated lipid mixing of PC/PS liposomes. (**A**) Lipid mixing between PC/PS liposomes with vacuolar 3Q-SNAREs (270–560 nM) or R-SNARE (94 nM). (**B**) Lipid mixing between PC/PS liposomes bearing all the four SNAREs (480–700 nM). Sec17p (400 nM), Sec18p (400 nM) and HOPS (10 nM) were added where indicated. All data were from one experiment and are representative of five independent experiments.

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Supplementary data presents statistics for the total dequenching) after a short lag for the components to warm to temperature (this lag is not seen when the system is warmed before an essential missing component is added, as in Figures 2D, 5 and 6). This does not require Sec17p, Sec18p and HOPS, which are needed for fusion of the organelle. Lipid mixing was inhibited by antibody to Vam3p (Figure 1A, αVam3p) and by antibodies to the other SNAREs but not by pre-immune IgG proteins (data not shown), indicating that all four SNAREs are involved in lipid mixing. Lipid mixing was blocked by Sec17p/Sec18p (Figure 1A and Supplementary Figure S2A), suggesting that they disassemble either *trans*-SNARE complexes or the 3Q-SNARE complexes on the acceptor liposomes. As the HOPS complex is required for SNARE complex assembly on isolated vacuoles (Collins *et al*, 2005), we expected that HOPS addition would enhance the lipid mixing. However, it had little effect on the PC/PS liposome pairs and did not restore lipid mixing in the presence of Sec17p and Sec18p (Figure 1A and Supplementary Figure S2A). In contrast to these PC/PS proteoliposomes, vacuoles bear all four SNAREs, which are disassembled from *cis*-SNARE complexes by Sec17p, Sec18p and ATP (Ungermann *et al*, 1998). PC/PS proteoliposomes bearing all four SNAREs did not undergo lipid mixing, even when incubated with Sec17p, Sec18p, ATP and HOPS (Figure 1B and Supplementary Figure S2B). Thus PC/PS proteoliposomes are not suitable for studying the functions of the HOPS, Sec17p and Sec18p SNARE chaperones.

Vacuolar lipids

As lipid mixing between liposomes bearing 3Q-SNAREs and those with 1R-SNARE was promoted by vacuolar lipids (Fukuda *et al*, 2000), we asked whether this lipid composition might promote a more physiological response to HOPS, Sec17p and Sec18p. Although lipid mixing between these liposomes bearing the 3Q- or 1R- SNAREs was still inhibited by Sec17p/Sec18p/ATP, it was stimulated by HOPS, which overcame the Sec17/Sec18p/ATP inhibition (Figure 2A and Supplementary Figures S2C and S3A). Earlier studies of the effects of SM proteins on lipid mixing with SNARE liposomes (Scott *et al*, 2004; Shen *et al*, 2007) used only PC/PS and required lengthy preincubation at 4°C and amounts of SM proteins which were comparable with the SNARE levels to stimulate lipid mixing. The effects of HOPS in our current studies require no preincubation, and HOPS was present at 1/50 the molar concentration of the Qa-SNARE Vam3p.

Liposomes with four vacuolar SNAREs require Sec17p, Sec18p, HOPS and vacuolar lipids for lipid mixing

Sec17p/Sec18p can disassemble 4-SNARE complexes in *cis*. Vacuolar lipid proteoliposomes bearing the four SNAREs exhibited lipid mixing which required Sec17p/Sec18p, ATP and HOPS and was blocked by αVam3p (Figure 2B and Supplementary Figure S2D). Lipid mixing was proportional to the concentrations of Sec17p/Sec18p and HOPS (Supplementary Figures S3B and C). Thus the synergistic actions of Sec17p, Sec18p, HOPS and vacuolar lipid composition are necessary for lipid mixing between 4-SNARE proteoliposomes.

Stimulation by vacuole fusion regulatory lipids

We have reconstituted vacuole fusion with defined components: the cognate SNAREs, SNARE chaperones, HOPS and bulk vacuolar lipids. However, these lipids lack the regulatory lipids DAG and the phosphoinositides PI(3)P and PI(4,5)P_2 (Fratti *et al*, 2004). ERG is also a regulatory lipid (Fratti *et al*, 2004), but was reported (Zinser *et al*, 1991; Schneiter *et al*, 1999) as part of the vacuole membrane bulk lipid composition and thus was included in our bulk vacuolar mixture. We prepared two sets of vacuolar lipid donor and acceptor proteoliposomes with the four SNAREs, one without further supplement and one with 1% of each of DAG, PI(3)P and PI(4,5)P_2 (Figure 2C and Supplementary Figure S2E). Regulatory lipids strongly enhanced the initial rate of lipid mixing when compared with vacuolar lipids alone (Figure 2C, open circles versus diamonds). This stimulated lipid mixing was blocked by omission of either Sec17p/Sec18p or HOPS (Figure 2C and Supplementary Figure S2E). These findings establish that the regulatory lipids are important components of the fusion machinery.

For comparison with earlier studies (Fukuda *et al*, 2000), we employed dioleoyl (DO) lipids in these liposomes, yet a 1-palmitoyl 2-oleoyl (PO) fatty acyl chain composition is more physiological and may yield more stable bilayers. Liposomes with all four SNAREs and with PO- lipids exhibit similar lipid mixing to their DO-lipid counterparts (Figure 2D), and this lipid mixing still requires HOPS, Sec17p/Sec18p, ATP and the SNAREs. Vps33p, the SM subunit of HOPS, will not substitute for HOPS (Figure 2D). We therefore employed PO lipids for the rest of the study. Dynamic light scattering shows that these proteoliposomes are 200–400 nm in diameter (Figure 2E).

Topology of lipid mixing

Liposomes can undergo fusion, lysis/reannealing and hemifusion (Düzgünes *et al*, 1987; Meers *et al*, 2000; Dennison *et al*, 2006). To distinguish these, we employed dithionite ($S_2O_4^{2-}$), a membrane-impermeable reducing agent that inactivates the fluorescence of exposed NBD (McIntyre and Sleight, 1991) but not Rh (Meers *et al*, 2000). The fluorescence of the NBD-PE in our liposomes is quenched by Rh-PE, as both fluorophores are present in the lumenal and outer monolayers. Added β-octylglucoside (β-OG) dilutes the fluorophores and thereby relieves quenching (Figure 3A). Dithionite concentrations of 3 mM or higher cause complete inactivation of the fluorescence of the fully accessible NBD-PE (Figure 3A). Dithionite itself is labile, and it loses potency after 30 min under the conditions of our lipid-mixing assay (Figure 3B). When intact SNARE proteoliposomes bearing NBD-PE and Rh-PE were treated with dithionite, the fluorescence of accessible NBD-PE was inactivated, whereas dithionite-inaccessible NBD-PE remained quenched by Rh-PE (Figure 3C). After 30 min of further incubation, when the dithionite had lost its potency, β-OG was added to assay the fluorescence of the remaining NBD-PE that had faced the lumen. Approximately 30–40% of the NBD-PE had been inaccessible to dithionite, showing that it had been on the lumenal monolayer of intact, sealed proteoliposomes (Figure 3C).

We tested whether SNARE, HOPS and Sec17p/Sec18p-mediated fusion was accompanied by lysis. Donor and acceptor proteoliposomes, each with four SNAREs and vacuolar

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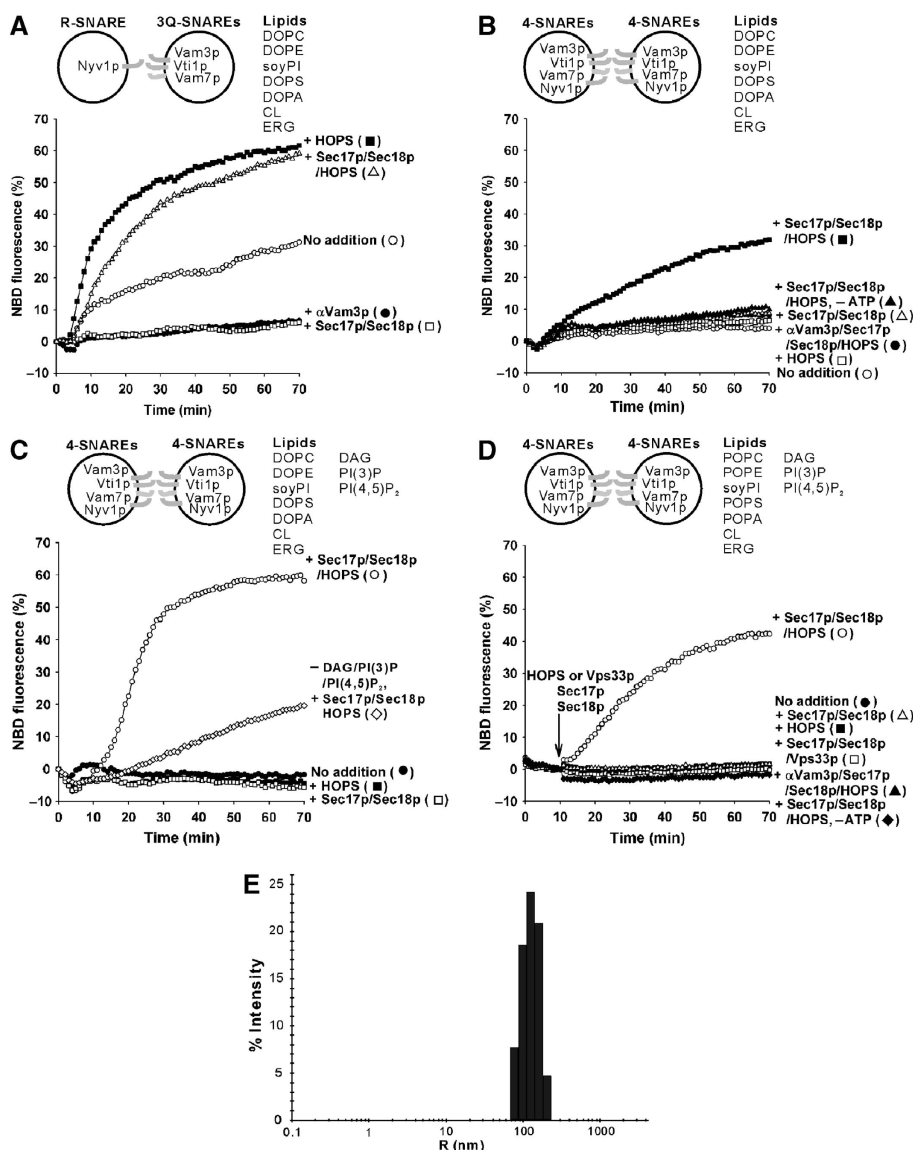

Figure 2 Lipid mixing between SNARE proteoliposomes with vacuolar lipids, regulatory lipids, Sec17p/Sec18p and HOPS. (**A**) Effect of Sec17p (400 nM), Sec18p (400 nM) and HOPS (10 nM) on lipid mixing between vacuolar-lipid liposomes bearing either the 3Q-SNAREs (220–580 nM) or the R-SNARE (85 nM). (**B**) Lipid mixing with vacuolar-lipid liposomes bearing all four SNAREs (380–630 nM) in the presence of Sec17p (400 nM), Sec18p (400 nM) and HOPS (10 nM). (**C**) Regulatory lipids (DAG, PI(3)P and $PI(4,5)P_2$) stimulated the lipid mixing of liposomes bearing the four SNAREs (450–550 nM) in the presence of Sec17p (400 nM), Sec18p (400 nM) and HOPS (10 nM). All liposomes, except those depicted with open diamonds, had regulatory lipids. (**D**) More physiological PO-lipids. Assays of lipid mixing used liposomes bearing vacuolar lipids including PO lipids, regulatory lipids and all the four SNAREs (310–450 nM), in the presence of Sec17p (1 μM), Sec18p (1 μM) and either HOPS (50 nM) or Vps33p (250 nM). Soluble components were added to the reactions after 10 min at 27 °C. All data (A–D) was from one experiment and is typical of more than three independent experiments. (**E**) Size distribution of SNARE liposomes. Dynamic light scattering experiments were performed (Araç *et al*, 2006), with the SNARE liposomes used in (D) (450 μM lipids in RB150 with 1 mM ATP and 6 mM $MgCl_2$) and 50% laser intensity.

and regulatory lipids, were incubated in portions that received 4 mM dithionite after 10 min (Figure 3D, filled circles) or after 35 min (open circles). At 40 min, the dithionite that had been added at 10 min would be inactive (Figure 3B), whereas the dithionite added at 35 min largely retains activity. At 40 min, Sec17p, Sec18p and HOPS were added, inducing rapid dequenching. Had this dequenching been due to, or even accompanied by, lysis/reannealing, the lumenally oriented NBD would have been reduced by dithionite added at 35 min and inactivated as a fluorophore. However, comparable dequenching was seen in each sample (Figure 3D, open and filled circles), establishing that Sec17p, Sec18p and HOPS promote fusion but do not promote lysis/reannealing. The addition of β-OG at 100 min showed that the same amount of NBD had remained inaccessible to dithionite added at 35 min regardless of Sec17p, Sec18p and HOPS being added or not (125 and 122 fluorescence units, respectively).

To test whether the dequenching of lumenally oriented NBD-PE reflected full fusion or only the dilution of quenching Rh-PE in the outer monolayer upon hemifusion, we employed 4-SNARE proteoliposomes bearing NBD-PS, which is less prone to translocation than NBD-PE (Meers *et al*, 2000) and a quenching DiD lipid, dil(5)C18ds (1,1′-diocradecyl-3,3,3′3′-tetramethylindodicarbocyanine-5′,5′-disulfonic acid), which

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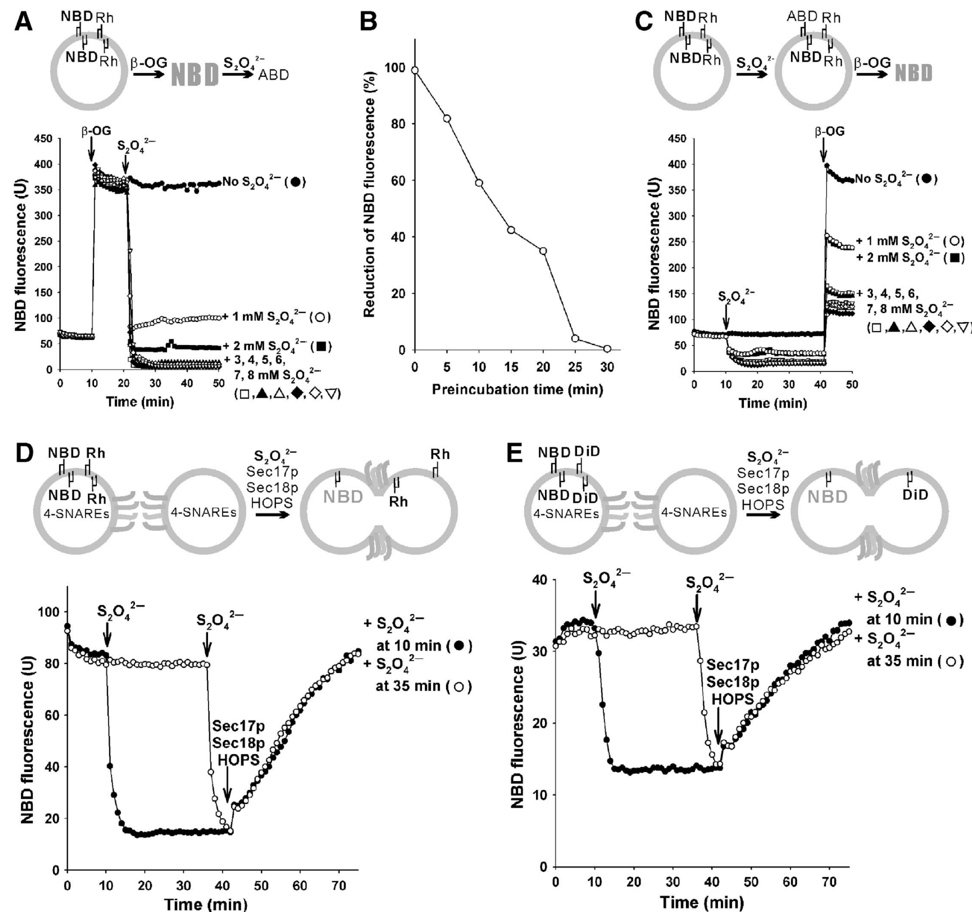

Figure 3 Reconstituted membrane fusion without lysis. Topology of lipid mixing was analysed with dithionite ($S_2O_4^{2-}$), a membrane-impermeable reductant that inactivates the fluorescence of accessible NBD. All liposomes had four SNAREs (310–450 nM), vacuolar lipids (PO-lipids) and regulatory lipids, as shown in Figure 2D. (**A**) Dithionite reduction of NBD. 4-SNARE liposomes (450 μM lipids) were incubated in RB150 with 1 mM ATP and 6 mM $MgCl_2$ at 27 °C, with addition of 100 mM β-OG at 10 min and 0–8 mM sodium dithionite at 20 min. (**B**) Lability of dithionite at 27 °C. Dithionite (40 mM in RB150 with 1 mM ATP, 6 mM $MgCl_2$) was preincubated at 27 °C for 0–30 min, then added (4 mM final concentration) to mixtures of the SNARE liposomes and β-OG, as in (A), followed by further incubation at 27 °C while monitoring NBD fluorescence. The plateau fluorescence value was compared with the value before dithionite addition to derive the percent reduction. (**C**) Liposomes bear dithionite-inaccessible NBD-PE. 4-SNARE liposomes, as in (A), were incubated at 27 °C, and dithionite was added to 4 mM at 10 min. After 30 min further incubation, when dithionite had lost its potency (B), 100 mM β-OG was added to relieve Rh-PE quenching and assay the NBD-PE that had remained dithionite inaccessible. (**D**) Sec17p (1 μM), Sec18p (1 μM) and HOPS (50 nM) trigger lipid mixing among the four SNARE liposomes without causing lysis. Dithionite (4 mM) was added to the four SNARE liposomes at either 10 min (closed circles) or 35 min (open circles), before adding Sec17p/Sec18p and HOPS at 40 min. (**E**) Lysis-free inner monolayer mixing of four SNARE liposomes was triggered by Sec17p, Sec18p and HOPS. Lipid mixing was assayed with dithionite as in (D), except that donor liposomes bore NBD-PS and a quencher, diI(5)C18ds, which is rendered nonfluorescent by dithionite. The fluorescent moiety of diI(5)C18ds is indicated as DiD in the scheme. All data (A–E) were from one experiment and are representative of data from more than three independent experiments.

is reduced and inactivated by dithionite (Meers *et al*, 2000). Fluorescent and nonfluorescent liposomes were mixed and incubated, then dithionite was added to inactivate any accessible NBD-PS or dil(5)C18ds either 30 or 5 min before the addition of Sec17p, Sec18p and HOPS. Dequenching was the same whether the dithionite remained active or had become inactivated (Figure 3E, filled and open circles) and thus reflects full fusion with its accompanying inner monolayer mixing, but not lysis.

Roles of SNARE chaperones

SNARE complexes on isolated vacuoles are associated with Sec17p or HOPS but not both (Collins *et al*, 2005). With pure chaperones and SNAREs, Sec17p allows Sec18p to disassemble SNARE complexes but HOPS does not (Figure 4A). The role of HOPS is not simply to stabilize SNARE complexes in the presence of Sec17p/Sec18p, as HOPS stimulates lipid mixing in the absence of Sec17p/Sec18p (Figure 2A) and does not prevent Sec17p/Sec18p-dependent disassembly of SNARE complexes (Figure 4B), as Sec17p can displace HOPS (Collins *et al*, 2005). To further explore the interplay of these SNARE chaperones, we first asked which SNARE topologies were functional. Proteoliposomes will fuse when the soluble SNARE Vam7p is added with Sec17p, Sec18p and HOPS rather than during proteoliposome assembly from detergent mixed micelles (Figure 5A). Proteoliposomes were also prepared with each single, membrane anchored SNARE in one liposome and the two complementary integral membrane SNAREs in its fusion partner (Figure 5B–D). The only combination that fused was with the R-SNARE Nyv1p on one fusion partner and the Qab-SNAREs Vam3p and Vti1p on the other, in accord with earlier studies of yeast ER to Golgi transport (Parlati *et al*, 2000). With the SNAREs thus artificially separated, high levels (6 μM, approximately 10-times higher

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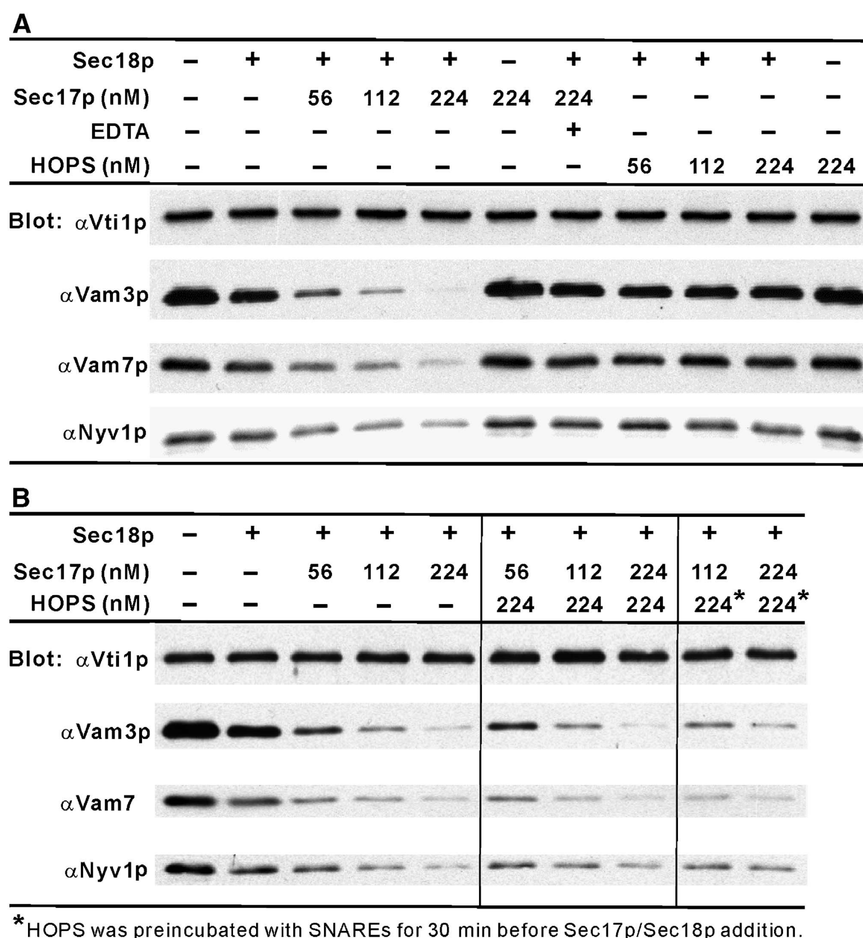

Figure 4 Sec18p and Sec17p, but not HOPS, disassemble a complex of four vacuolar SNARE soluble domains (**A**), and HOPS does not prevent a disassembly of the 4-SNARE complex by Sec18p/Sec17p (**B**). The four soluble SNAREs, lacking a transmembrane domain, were purified as described (Jun *et al*, 2006). They were mixed at 10 μM each on ice in SSB (20 mM HEPES–NaOH, pH 7.4, 10% glycerol, 125 mM NaCl, 5 mM $MgCl_2$, 0.008% Triton X-100), incubated at 4 °C overnight, diluted to 0.5 μM each in SSB, and mixed for 1 h with amylose beads (NEB) equilibrated in SSB. Beads were isolated by centrifugation (8000 r.p.m., 2 min, 4 °C) and washed four times in 450 μl SSB. Sec18p (2.3 μM), Sec17p (56–224 nM), HOPS (56–224 nM) and EDTA (10 mM) were mixed as indicated in SSB with 1 mM $MgCl_2$:ATP on ice in separate tubes, then mixed with the washed beads. After incubation at 30 °C with rotation for 45 min, the beads were further washed four times as above, then bound proteins were eluted (90 °C, 0.4% SDS) and assayed by SDS–PAGE/immunoblot.

concentrations than the Qab-SNAREs) of Vam7p promote a lipid mixing (Figure 5B) which is sensitive to Sec17p/Sec18p, indicating their disassembly of productive SNARE complexes that could otherwise lead to fusion. HOPS stimulates lipid mixing, and the combination of HOPS and Sec17p/Sec18p stimulates further. The synergy of SNARE chaperones was even more evident at lower levels of Vam7p (600 nM, comparable concentrations with the Qab-SNAREs), which alone could not support lipid mixing (Figure 5Ea, filled circles). In this condition, HOPS gave a modest fusion signal (filled squares), which was dramatically stimulated by the further addition of Sec17p and Sec18p (open triangles). This stimulation required each of Sec17p, Sec18p and Mg/ATP (data not shown), and thus likely reflects their concerted action of disassembling SNARE complexes. To determine whether these SNARE chaperones were regulating the formation of new SNARE complex containing both Nyv1p and Vam3p, the same samples (Figure 5Ea) were mixed with EDTA and soluble GST-Nyv1p (to block any further binding of Nyv1p to Vam3p), solubilized by detergent, and incubated with bead-bound antibody to Vam3p to assay the amount of Nyv1p that had become associated with Vam3p (Figure 5Eb; S samples are from the fusion incubations of part a; in C, donor and acceptor proteoliposomes were incubated separately and their detergent extracts mixed). Sec17p/Sec18p increased the Vam3p–Nyv1p association in the presence of HOPS (Figure 5Eb, lane 4 versus 6), consistent with its disassembly of non-productive SNARE *cis*-complexes to allow the formation of more productive SNARE complexes, which function with HOPS for *trans*-SNARE complex assembly. Sec17p/Sec18p may disassemble all SNARE complexes, whether productive or non-productive for later fusion, whereas HOPS confers selectivity for productive SNARE complex formation. This is consistent with earlier findings that Sec17p/Sec18p can disassemble *cis*- or *trans*-complexes of vacuolar SNAREs (Ungermann *et al*, 1998; Jun *et al*, 2007), whereas HOPS is selective for SNARE 0-layer composition and other structural features (Starai *et al*, 2008).

Regulatory lipid requirement

Each regulatory lipid has an important role in fusion. When compared with the fusion of 4-SNARE liposomes of vacuolar

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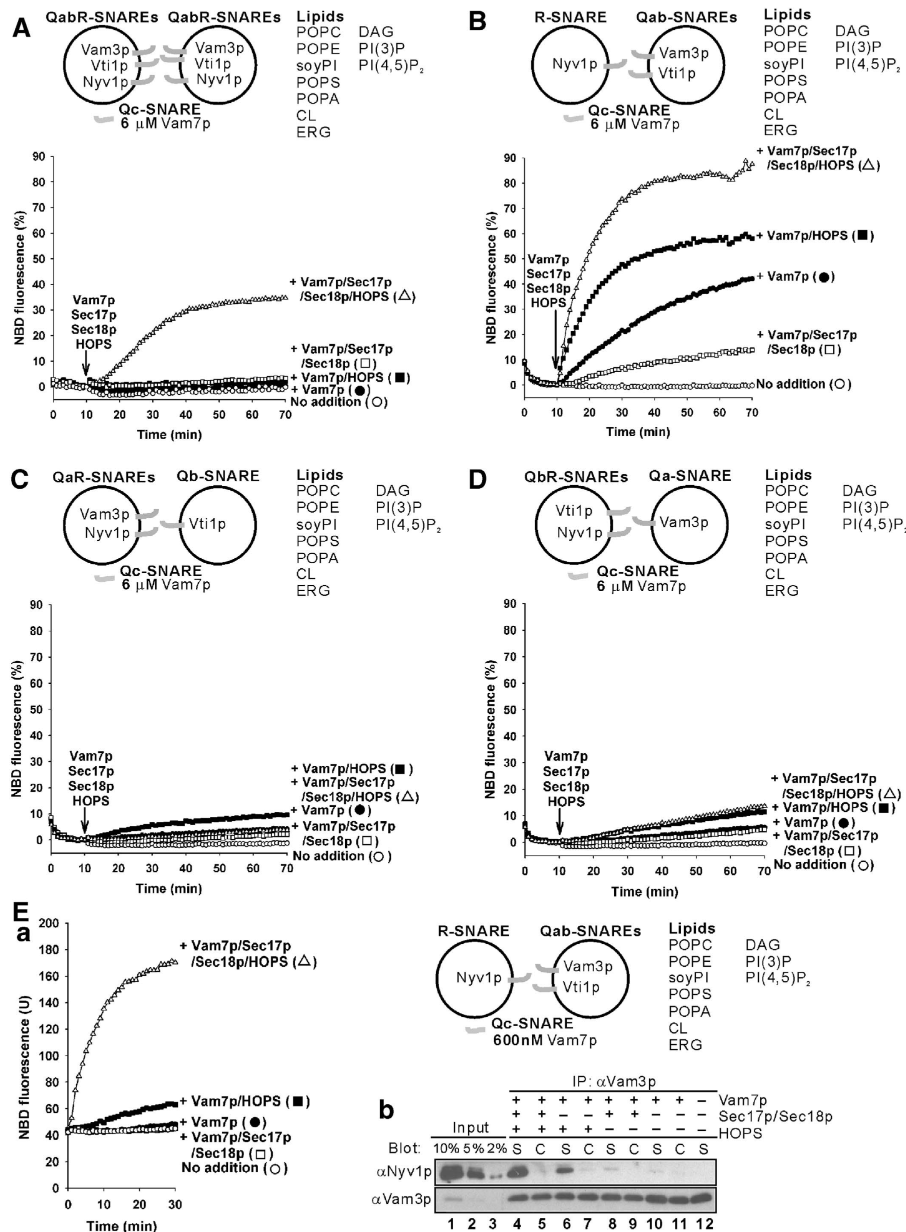

Figure 5 Synergistic actions of Sec17p/Sec18p and HOPS. All liposomes bore vacuolar PO lipids and regulatory lipids. Vam7p (6 μM), Sec17p (1 μM), Sec18p (1 μM) and HOPS (50 nM) were added to the reactions after 10 min preincubation of liposomes at 27 °C. (**A**) Lipid mixing between liposomes bearing vacuolar QabR-SNAREs (850–920 nM). (**B–D**) Lipid mixing between liposomes bearing (B) Qab-SNAREs (580–800 nM) and R-SNARE (70 nM), (C) Qb-SNARE (820 nM) and the QaR-SNAREs (25–100 nM), and (D) Qa-SNARE (700 nM) and the QbR-SNAREs (100–120 nM). (**E**) Synergy of Sec17p/Sec18p and HOPS in promoting *trans*-SNARE pairing. (a) Lipid mixing assays had liposomes bearing either vacuolar Qab-SNAREs (580–800 nM) or the R-SNARE (70 nM), as in (B) but with 600 nM Vam7p. (b) Assay of Nyv1p bound to Vam3p. After 30 min, reaction mixtures from (a) were mixed with 560 ng of soluble GST-Nyv1p, 400 μl of ice-cold solubilization buffer (20 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 150 mM NaCl, 10% glycerol, 0.5% NP-40, 0.46 μg/ml leupeptin, 3.5 μg/ml pepstatin, 2.4 μg/ml pefabloc-SC and 1 mM PMSF) and 10 mM EDTA and mixed at 4 °C for 5 min. After centrifugation (2 min, 16 000 *g*, 4 °C), supernatants (350 μl) were mixed with protein A agarose beads with covalently bound anti-Vam3p N-domain antibodies and incubated (4 °C, 1 h). Beads were washed with 600 μl of solubilization buffer four times. Bound proteins were eluted at 90 °C with 0.4% SDS, followed by SDS–PAGE and immunoblot. As a control, Qab-SNARE liposomes and R-SNARE liposomes were incubated and solubilized separately, mixed and assayed for Nyv1p:Vam3p association as above. The samples and their controls are labelled 'S' and 'C', respectively. The data in A-Ea were from one experiment and are representative of more than three independent experiments.

PO lipids and all regulatory lipids (Figure 6A), omission of DAG halved the rate of fusion, whereas omission of ERG, $PI(4,5)P_2$ or PI(3)P caused far greater reduction. The dependence on regulatory lipids was stricter in these experiments with PO lipids than in studies with DO lipids (Figure 2C). None of the regulatory lipids was simply required for the proteoliposomal association of soluble fusion proteins, as there was similar recovery of HOPS, Sec17p, Sec18p and Vam7p with the proteoliposomes (Figure 6B). Vam7p has direct affinities for other SNAREs (Collins *et al*, 2005), HOPS (Stroupe *et al*, 2006) and PI(3)P (Cheever *et al*, 2001); the strict requirement for PI(3)P for liposomal fusion (Figure 6A) does not simply reflect the role of this lipid in facilitating Vam7p binding to the membrane (Figure 6B).

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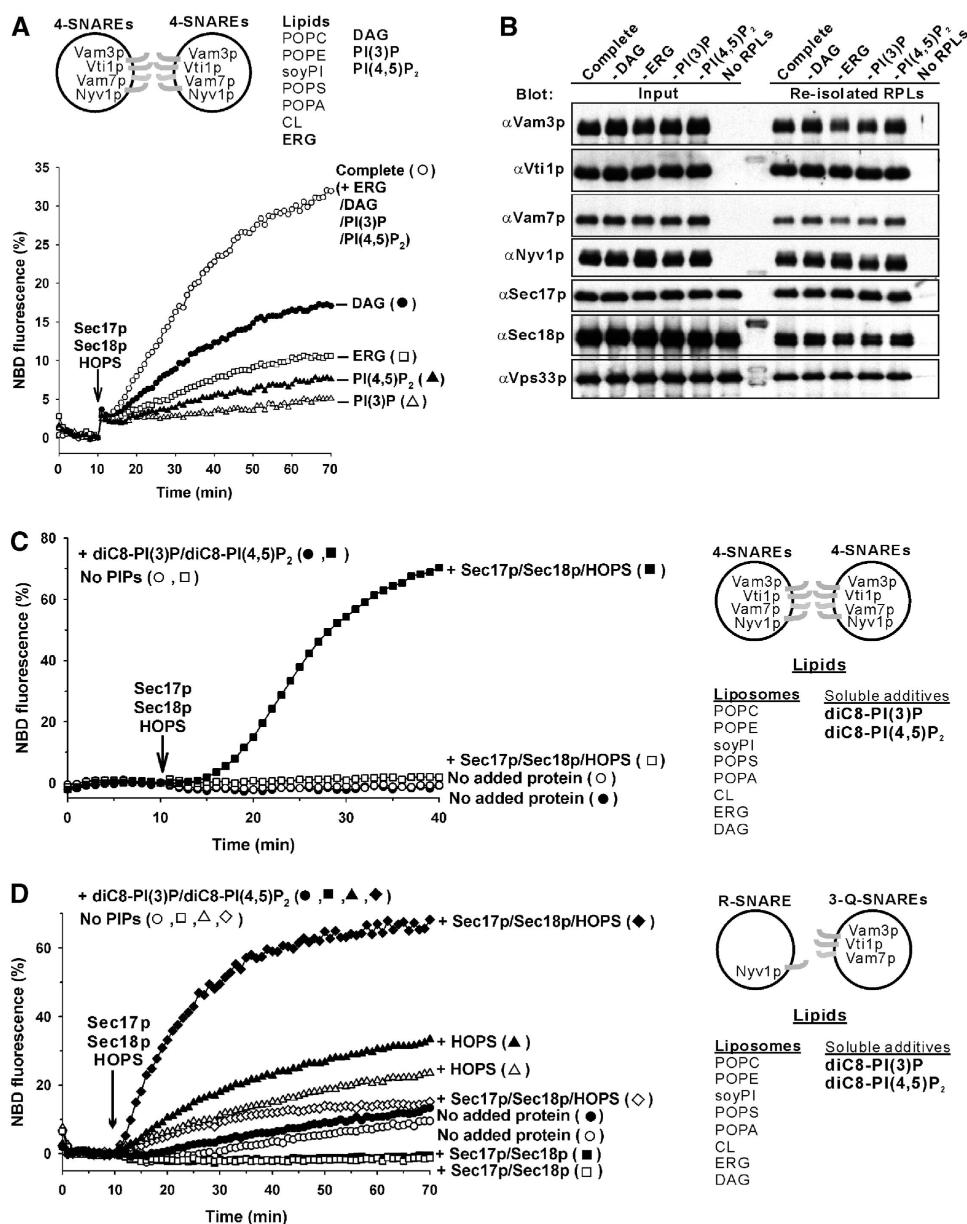

Figure 6 Regulatory lipids support membrane fusion. (**A**) Liposomes bearing the four SNAREs (320–880 nM) with the complete lipid composition (vacuolar lipids (PO lipids) and regulatory lipids) or lacking DAG, ERG, PI(3)P or PI(4,5)P_2 were assayed for lipid mixing. Sec17p (1 μM), Sec18p (1 μM) and HOPS (50 nM) were added at 10 min. (**B**) The binding of Sec17p, Sec18p, and HOPS to each set of liposomes was analysed by flotation (Tucker *et al*, 2004). Donor liposomes (450 μM) bearing the four SNAREs (200–650 nM) with the indicated lipid compositions were incubated with Sec17p, Sec18p and HOPS at 27 °C for 1 h, as shown in (A). Samples (80 μl) were mixed with 320 μl of 50% Histodenz in RB150, transferred to a 11 × 60 mm tube, then overlaid with 1.6 ml of 35% Histodenz in RB150, 2.0 ml of 30% Histodenz in RB150, and 200 μl of RB150. After centrifugation (SW60Ti (Beckman), 55 000 r.p.m., 3 h, 4 °C), liposomes were harvested and analysed by SDS–PAGE/immunoblot. (**C**) Liposomes lacking PI(3)P and PI(4,5)P_2 and bearing four SNAREs (390–680 nM) were assayed for lipid mixing as in (A) with lower concentrations of ATP (0.5 mM) and $MgCl_2$ (3 mM) and with Sec17p (500 nM), Sec18p (500 nM), HOPS (28 nM) and diC8-PI(3)P/diC8-PI(4,5)P_2 (45 μM each) added at 0 min where indicated. (**D**) Liposomes lacking PI(3)P and PI(4,5)P_2 and bearing 3Q-SNAREs (430–650 nM) and R-SNARE (82 nM) were assayed for lipid mixing as in (C) but with lower concentrations of diC8-PI(3)P/diC8-PI(4,5)P_2 (23 μM each). The data in A, C and D were from one experiment and are representative of more than three independent experiments.

A mixture of soluble diC8-PI(3)P and diC8-PI(4,5)P_2 restores Sec17p/Sec18p/HOPS-dependent full fusion to 4-SNARE proteoliposomes, which had been prepared and isolated without phosphoinositides (Figure 6C). With 4-SNARE RPLs, Sec17p/Sec18p will always be required for *cis*-SNARE complex disassembly. To obviate this need and facilitate study of whether phosphoinositides support a synergistic cooperation of Sec17p/Sec18p with HOPS, RPLs with 3Q-SNAREs or with R-SNARE were prepared without phosphoinositides and were assayed for fusion. Without added proteins, the low level of fusion was only slightly stimulated by phosphoinositides (Figure 6D, open versus filled circles). Sec17p/Sec18p blocked fusion, whereas the addition of HOPS without Sec17p/Sec18p stimulated fusion, with only modest enhancement by phosphoinositides. In the absence of phosphoinositides, HOPS relieved much of the inhibition by Sec17p/Sec18p (open diamonds), but there was no synergistic stimulation of fusion. Strikingly, added phosphoinositides

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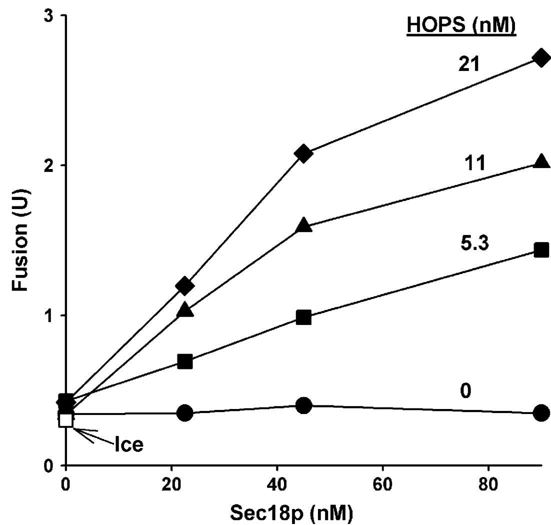

Figure 7 Fusion of ypt7Δ vacuoles requires Sec18p and HOPS. Standard fusion reactions (Jun *et al*, 2007) employed vacuoles from BJ3505 4SNARE^{++} ypt7Δ and DKY6281 4SNARE^{++} ypt7Δ (Starai *et al*, 2007). Vacuoles were incubated on ice (open square) or at 27°C with recombinant Sec18p, HOPS or both. After 90 min, Pho8p phosphatase activity was assayed to measure vacuole fusion (Haas, 1995). Fusion units are μmol of *P*-nitrophenolate formed per min per μg of BJ3505 4SNARE^{++} ypt7Δ vacuoles.

supported a strong, synergistic stimulation of fusion by HOPS and Sec17p/Sec18p (filled diamonds). Thus phosphoinositides are required for the synergy between these SNARE chaperones, and both chaperones are essential for stimulation by phosphoinositides. This may in part reflect the affinity of these lipids for both HOPS and the Vam7p SNARE.

Similarity to vacuolar Rab-bypass fusion

Is HOPS, a Rab-effector complex, ever seen to function without its Rab, Ypt7p, in the context of a biological membrane? To address this, we returned to assays of vacuole fusion. Vacuoles from strains that overexpress each of the four vacuolar SNAREs (termed 4SNARE^{++}) bypass the normal requirement for Ypt7p for fusion (Starai *et al*, 2007). The fusion of 4SNARE^{++} ypt7Δ vacuoles require both Sec18p and HOPS (Figure 7). Thus, for both reconstituted 4-SNARE proteoliposomes and for vacuoles with elevated levels of SNAREs, Sec18p and HOPS synergistically promote fusion in the absence of Ypt7p.

Discussion

There have been three approaches to studying membrane fusion. (1) Liposome fusion can be triggered by bilayer perturbants such as calcium, polyethylene glycol, viral fusion proteins, DAG or phospholipase C (which generates DAG). These studies show how lipids rearrange for fusion, but do not reveal the roles of physiological fusion proteins. (2) Fusion is also studied with isolated organelles. These *in vitro* reactions rely on physiological fusion factors such as Rab GTPases, Rab effectors, SNAREs, SNARE chaperones, SM proteins and specific lipids. (3) Fusion is also studied through the lipid mixing of proteoliposomes bearing v- or t-SNAREs. This approach reveals important SNARE properties, but often employs high SNARE densities, is accompanied by substantial SNARE-dependent lysis (Dennison *et al*, 2006) and does not depend on other established physiological fusion factors. To connect these approaches, we began with assays of lipid mixing of v- and t-SNARE PC/PS proteoliposomes (Weber *et al*, 1998; Fukuda *et al*, 2000), but moved stepwise to more physiological conditions of lipid composition, disposition of SNAREs on fusion partners and addition of other fusion proteins.

Our current reconstitution faithfully reflects many proteins and lipids needed for vacuole fusion and shows that they cooperate to promote true fusion and not lysis. Lipid choice is crucial, as specific lipids form functional membrane microdomains (Lang *et al*, 2001; Fratti *et al*, 2004) and support the association of peripheral membrane proteins such as Vam7p (Cheever *et al*, 2001) or HOPS (Stroupe *et al*, 2006) at the vertex ring domain of yeast vacuole fusion (Wang *et al*, 2002). The reconstitution presented here does not require Ypt7p, yet we have shown that HOPS and Sec17p/Sec18p can bypass the need for Ypt7p, either on the intact vacuole or in proteoliposomes. Studies of this reconstituted system yield four lessons: (1) SNARE chaperones cooperate, with Sec17p/Sec18p disassembling non-productive SNARE complexes and HOPS capturing functional complexes for rapid fusion. (2) Fusion without lysis does not rely on additional protein factors, but is inherent to the complete fusion machinery. (3) The regulatory lipids (ERG, phosphoinositides and DAG), implicated in fusion from genetic studies *in vivo* and biochemical studies of the isolated organelle (Mayer *et al*, 2000; Cheever *et al*, 2001; Kato and Wickner, 2001; Seeley *et al*, 2002; Fratti *et al*, 2004; Jun *et al*, 2004), are an integral part of the core fusion pathway. (4) Phosphoinositides stimulate fusion only when both SNARE chaperone systems are present, and these act synergistically only in the presence of phosphoinositides (Figure 6D).

Both Sec17p/Sec18p, which disassemble SNARE complexes (Söllner *et al*, 1993; Ungermann *et al*, 1998; Jun *et al*, 2007), and HOPS, which promotes the assembly of vacuolar SNARE complexes (Collins and Wickner, 2007), are needed to fuse 4-SNARE liposomes of vacuolar lipids. Unlike Sec17p, HOPS does not function with Sec18p to promote SNARE complex disassembly (Figure 4). However, this does not explain the full HOPS function, as HOPS also promotes fusion of 3Q-SNARE and R-SNARE proteoliposomes in the absence of Sec17p/Sec18p (Figures 2A and 5B). Thus HOPS must catalyze SNARE complex assembly and/or enhance the capacity of assembled *trans*-SNARE complexes for fusion. This is in accord with other studies that found stimulation by Sec1p of lipid mixing between liposomes bearing yeast plasma membrane SNAREs (Scott *et al*, 2004) or by Munc18-1p for liposomes with neuronal SNAREs (Shen *et al*, 2007). In our current reconstitution, nanomolar levels of HOPS, substoichiometric with the SNAREs, support rapid and efficient fusion. Our data reveal new aspects of how Sec17p/Sec18p and HOPS cooperate to promote fusion. In the presence of phosphoinositides, Sec17p/Sec18p inhibits fusion in the absence of HOPS but stimulates fusion in its presence. The strong inhibition of fusion by Sec17p/Sec18p (Figure 5B) likely reflects the ATP-driven disassembly of the 3-Q-SNARE complex or the 4-SNARE *trans*-complex, intermediates on the path to fusion. Proteoliposomes bearing Vam3p and Vti1p will fuse with those with Nyv1p when given Vam7p and HOPS, now stimulated by Sec17p/Sec18p instead of being

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inhibited (Figure 5B and E). How is the role of Sec17/Sec18p switched, from inhibitor to activator, by HOPS and phosphoinositides (Figure 6D)? SNAREs form non-productive as well as productive complexes, and SM proteins can improve the specificity of pairing (Tsui *et al*, 2001; Peng and Gallwitz, 2002; Brandhorst *et al*, 2006). We suggest that Sec17p/Sec18p can disassemble SNARE complexes, whether they are productive or not, allowing a fresh chance for these SNAREs to form complexes that can be captured by HOPS, which proofreads SNARE complex composition and structure (Starai *et al*, 2008), and to thereby form more fusogenic complexes. In accord with this working model, added Sec17p and Sec18p only enhance the total level of SNARE complex between Vam3p from one fusion partner and Nyv1p from the other when HOPS is also present (Figure 5Eb). Phosphoinositides may support this synergy through binding both Vam7p and HOPS.

Fusion in model systems has often been accompanied by lysis (Kendall and MacDonald, 1982; Burgess *et al*, 1992; Lau *et al*, 2004; Dennison *et al*, 2006). Our reconstitution of fusion with physiological lipids, SNAREs and SNARE chaperones has little or no accompanying lysis, suggesting that other proteins of the vacuole are not required to avert SNARE-driven lysis. Vacuoles can be driven to lyse by excess SNAREs (Starai *et al*, 2007); further studies are required to understand whether chaperones and vacuole lipids guide the bilayer destabilization by SNAREs towards fusion and away from lysis.

Regulatory lipids are required for vacuole fusion, whether *in vivo*, *in vitro* with the isolated organelle or with purified proteins and lipids in proteoliposomes (Figure 6). Vacuole fragmentation, a hallmark of defective fusion, is seen in strains with deletions in either ERG biosynthetic genes, in *PLC1*, a phospholipase C that converts PI(4,5)P_2 to DAG, or in *VPS34*, a PI 3-kinase (Seeley *et al*, 2002). The fusion of isolated vacuoles is blocked by sterol ligands such as nystatin, aphotericin B or filipin or by sterol extraction by β-methycyclodextrin (Kato and Wickner, 2001; Fratti *et al*, 2004), by phosphoinositide ligands such as monoclonal antibodies, neomycin or recombinant FYVE or ENTH domains or by phosphoinositide phosphatases (Mayer *et al*, 2000; Fratti *et al*, 2004), or by DAG ligands such as recombinant C1b domain or inhibitors of phospholipase C that generate DAG such as U73122 or 3-nitrocoumarin (Jun *et al*, 2004). Biochemical studies have tied the regulatory lipids to specific subreactions, and catalysts, of fusion. ERG supports Sec17p/Sec18p-mediated priming (Kato and Wickner, 2001) and hence the ensuing steps: 4-phosphoinositides regulate priming and docking (Mayer *et al*, 2000), phosphoinositides contribute to the vacuole association of Vam7p (Cheever *et al*, 2001) and HOPS (Stroupe *et al*, 2006) and support the synergy of the two SNARE chaperone systems (Figure 6D) and DAG is needed for *trans*-SNARE pairing and fusion (Jun *et al*, 2004). These lipids become enriched at the vertex ring fusion microdomain, they are required for each other's vertex ring enrichment, their vertex enrichment is regulated by SNAREs and in turn they regulate the SNAREs spatial enrichment (Fratti *et al*, 2004). Nevertheless, it had remained possible that these lipid requirements were only indirect; for example, they might have regulated a vacuolar ion transport system that itself indirectly regulated more direct fusion catalysts. With our current finding that reconstituted fusion is also governed by each of the regulatory lipids (Figure 6), which are integral components of the fusion pathway, it is clear that the bilayer is not simply a passive substrate for protein action, but has lipids that regulate each stage of fusion.

The current reconstitution is a platform for exploring fusion mechanisms. Other proteins such as Ypt7p might further enhance or regulate the fusion rate. Components can be omitted singly or in groups, and the effects on fusion subreactions are studied in detail, avoiding indirect effects such as the alterations of vacuole content of fusion proteins when genes encoding trafficking proteins or lipid metabolism are deleted from the cell. The ability to control proteoliposome composition is an invaluable aid to studies of mechanism.

Materials and methods

Protein isolation

Protein expression and purification is described in Supplementary data.

Reconstitution of SNARE proteoliposomes

Proteoliposomes with vacuolar SNAREs were prepared as described (Weber *et al*, 1998; Scott *et al*, 2003) with modifications. Non-fluorescent lipids, except for ERG (Sigma) and phosphoinositides (Echelon), were from Avanti Polar Lipids. Fluorescent lipids (NBD-PE, Rh-PE and dansyl-PE) were from Molecular Probes. Donor lipid mixes contain 1.5% (mol/mol) NBD-PE and 1.5% Rh-PE, whereas acceptor lipid mixes contain 1.0% dansyl-PE. For PC/PS liposomes, the lipid mixes are DOPC (82 or 84% for donor or acceptor liposomes, respectively), DOPS (15%) and fluorescent lipids. Vacuolar lipid liposomes contain DOPC or POPC (45 or 47% for donor or acceptor, respectively), DOPE or POPE (18%), soyPI (18%), DOPS or POPS (4.4%), DOPA or POPA (2.0%), CL (1.6%), ERG (8.0%) and fluorescent lipids. For liposomes with vacuolar and regulatory lipids, DAG (1.0%), PI(3)P (1.0%) and PI(4,5)P_2 (1.0%) are included, and DOPC or POPC is reduced to 42 or 44% for donor or acceptor, respectively. Dried films with these compositions were dissolved (final 2 mM lipids) in RB500 (20 mM HEPES–NaOH, pH 7.4, 500 mM NaCl, 10% glycerol) with 40 mM CHAPS, purified vacuolar SNAREs (GST-Vam3p, Vti1p, Vam7p, and Nyv1p) (final 2 μM each), and TEV protease (final 2 μM, only added when GST-Vam3p is present). The detergent-lipid-SNARE mixed micellar solutions were incubated (4 °C, 1 h, gentle agitation), then dialyzed against RB500 in a Slide-A-Lyzer 20 kDa cutoff dialysis cassette (Pierce) at 4 °C to remove detergent. SNARE proteoliposomes were purified by flotation through steps of 40, 30 and 0% Histodenz (Sigma) in RB150 (20 mM HEPES–NaOH, pH 7.4, 150 mM NaCl, 10% glycerol). Each dialysate (1–1.5 ml) was mixed with an equal volume of 80% Histodenz in RB150 and transferred to an 11 × 60 mm tube. Samples were overlaid with 30% Histodenz in RB150 to a total volume of 4 ml, then 200 μl RB150. After centrifugation (SW60Ti [Beckman], 55 000 r.p.m., 3 h, 4 °C), liposomes were harvested from the 0/30% Histodenz interface. Lipid concentrations were determined from the fluorescence of NBD-PE ($\lambda_{ex} = 460$ nm, $\lambda_{em} = 538$ nm, emission cutoff = 515 nm) for donor liposomes and dansyl-PE ($\lambda_{ex} = 336$ nm, $\lambda_{em} = 517$ nm, emission cutoff = 495 nm) for acceptor liposomes, in the presence of 100 mM β-OG. Liposomes were diluted with RB150 to 2 mM lipids and stored at −80 °C without loss of activity. Protein concentrations of each SNARE were determined from Coomassie-stained gels using UN-SCAN-IT gel version 5.1 (Silk Scientific Corporation) and bovine serum albumin as standard.

Lipid mixing assay

Lipid mixing assays were performed as described (Weber *et al*, 1998; Scott *et al*, 2003) with modifications. Typically, reaction mixtures in RB150 were prepared in black 96-well plates (for 100 μl reactions) or 384-well plates (for 20 μl reactions) (Corning) on ice and were composed of donor (50 μM lipids) and acceptor (400 μM lipids) SNARE proteoliposomes, 1 mM ATP, 6 mM $MgCl_2$, and Sec17p (0.4–1.0 μM), Sec18p (0.4–1.0 μM), HOPS (10–50 nM),

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Vps33p (250 nM), Vam7p (0.6–6.0 μM) and αVam3p (1.0 μM) where indicated. Without preincubation, plates were placed in a SpectraMAX Gemini XPS plate reader (Molecular Devices) equilibrated at 27 °C. NBD fluorescence ($\lambda_{ex} = 460$ nm, $\lambda_{em} = 538$ nm, emission cutoff = 515 nm) was monitored at 1 min intervals, 30 reads per well on the 'high' PMT setting (arbitrary units). In some experiments, Sec17p, Sec18p, HOPS, Vps33p and Vam7p were added to liposome reactions that had been preincubated at 27 °C for 10 min in a plate reader. After 1–2 h at 27 °C, 100 mM β-OG was added to fully dequench the NBD. For calculating maximal NBD fluorescence, the signals at 0 min were set to 0% and the signals after β-OG addition were 100%.

Topology analysis of lipid mixing

All experiments with sodium dithionite (Sigma) employed 4-SNARE liposomes with vacuolar and regulatory lipids including POPC, POPE, POPS and POPA, prepared as above, except that, in Figure 3E, NBD-PS (Avanti) and diI(5)C18ds (Molecular Probes) were present in donor liposomes instead of NBD-PE and Rh-PE. For lipid mixing assays with dithionite, reactions (24 μl) containing donor (50 μM lipids) and acceptor (400 μM lipids) liposomes, 1 mM ATP and 6 mM $MgCl_2$ in RB150 were incubated at 27 °C in a black 384-well plate, with addition of dithionite (4 mM), Sec17p (1 μM), Sec18p (1 μM), HOPS (50 nM) and β-OG (100 mM), as described, monitoring NBD fluorescence.

Vacuole isolation and in vitro vacuole fusion assay

Yeast vacuoles were isolated from BJ3505 4SNARE^{++} ypt7Δ and DKY6281 4SNARE^{++} ypt7Δ and fusion was assayed as described (Haas, 1995; Starai *et al*, 2007).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

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