HOPS Proofreads the trans-SNARE Complex for Yeast Vacuole Fusion

Vincent J. Starai, Christopher M. Hickey, and William Wickner

Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755

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The fusion of yeast vacuoles, like other organelles, requires a Rab-family guanosine triphosphatase (Ypt7p), a Rab effector and Sec1/Munc18 (SM) complex termed HOPS (homotypic fusion and vacuole protein sorting), and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). The central 0-layer of the four bundled vacuolar SNAREs requires the wild-type three glutaminyl (Q) and one arginyl (R) residues for optimal fusion. Alterations of this layer dramatically increase the \(K_m\) value for SNAREs to assemble trans-SNARE complexes and to fuse. We now find that added purified HOPS complex strongly suppresses the fusion of vacuoles bearing 0-layer alterations, but it has little effect on the fusion of vacuoles with wild-type SNAREs. HOPS proofreads at two levels, inhibiting the formation of trans-SNARE complexes with altered 0-layers and suppressing the ability of these mismatched 0-layer trans-SNARE complexes to support membrane fusion. HOPS proofreading also extends to other parts of the SNARE complex, because it suppresses the fusion of trans-SNARE complexes formed without the N-terminal Phox homology domain of Vam7p (Qc). Unlike some other SM proteins, HOPS proofreading does not require the Vam3p (Qa) N-terminal domain. HOPS thus proofreads SNARE domain and N-terminal domain structures and regulates the fusion capacity of trans-SNARE complexes, only allowing full function for wild-type SNARE configurations. This is the most direct evidence to date that HOPS is directly involved in the fusion event.

INTRODUCTION

Proteins are routed to their correct intracellular compartment by selective sorting into budding vesicles, targeting of these vesicles to their proper organelle, and regulated membrane fusion. Fusion entails successive steps of tethering; enrichment of key proteins and lipids to form a fusion-competent membrane microdomain (Lang et al., 2001; Wang et al., 2002; Fratti et al., 2004); assembly of proteins from each apposed membrane into fusion complexes; and lipid bilayer rearrangements, often via a hemifusion intermediate. Intracellular membrane fusion requires Rab family guanosine triphosphatases (GTases), multisubunit Rab-effector complexes, Sec1/Munc18 (SM) proteins, and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (Jahn et al., 2003). Rab GTases promote tethering and the enrichment of proteins and lipids in microdomains that support fusion (Lang et al., 2001; Miaczynska and Zerial, 2002; Wang et al., 2002). Rab-effector complexes bind to the active, GTP-bound form of the Rabs and are thereby activated to further the fusion cascade. SM proteins associate with SNAREs and are required for the ensuing membrane fusion (Rizo and Südhof, 2002). Model studies, using SNARE proteins reconstituted into liposomes (Weber et al., 1998) or organelles bearing different levels of SNARE proteins (Starai et al., 2007), have shown that complexes of membrane-anchored SNAREs alone can promote either fusion with preservation of vesicular integrity and mixing of luminal compartments (Nickel et al., 1999), membrane lysis (Dennison et al., 2006; Chen et al., 2006), or both (Starai et al., 2007). In contrast, fusion is accompanied by very little lysis when it occurs with physiological levels of SNAREs, Rab GTPase, and Rab effector (Starai et al., 2007). We are only beginning to see how SNAREs promote fusion or lysis, what regulates their function and directs it toward fusion, and how this is coordinated with Rabs, their effectors, and SM proteins.

SNARE proteins are recognized by their characteristic heptad-repeat “SNARE motifs” which assemble into four-helical coiled coils to form a SNARE complex (Fasshauer et al., 1998). Structural studies (Sutton et al., 1998) have shown that most of the amino acyl residues that face each other on the apposed surfaces of the four helices in a SNARE complex are apolar, with the notable exception of the clustered central residues of each SNARE domain, termed the 0-layer. In virtually all organisms and organelles, the 0-layer consists of three glutaminyl and one arginyl residues, forming an ionic core of the SNARE complex (Fasshauer et al., 1998). Genetic, physiological, and biochemical studies have established the importance of the 0-layer, although it remains unclear why it is required.

SM family proteins are required for the SNARE-dependent fusion of biological membranes (Toonen and Verhage, 2003; Burgoyne and Morgan, 2007). SM proteins interact with SNARE proteins in at least three different modes. Munc18-1 binds to the “closed” conformation of syntaxin, thereby preventing its assembly into SNARE complexes (Dulubova et al., 1999). Other SM proteins can bind to the extreme N terminus of their cognate syntaxins without preventing SNARE complex assembly (Dulubova et al., 2002; Yamaguchi et al., 2002). SM proteins can also bind to a preassembled SNARE complex and not the free syntaxin (Carr et al., 1999; Scott et al., 2004). Elucidating the molecular
mechanisms of SNARE-dependent membrane fusion will require understanding the functional interactions between SNAREs and SM family proteins during fusion.

Vacuoles (lysosomes) from *Saccharomyces cerevisiae* are convenient for the study of SNARE-, SM protein-, and Rab GTPase-dependent fusion. Vacuole homotypic fusion is required for normal organelle structure in vivo (Wada *et al.*, 1992), and it can be monitored conveniently in vitro by colorimetric assays (Haas *et al.*, 1994; Jun *et al.*, 2007). When purified vacuoles are incubated with ATP, their cis-SNARE complexes are disassembled early in the incubation by the combined action of Sec17p and Sec18p (Mayer *et al.*, 1996; Ungerma *et al.*, 1998a; Jun and Wickner, 2007a). Tethering of a vorticam Rab GTPase Ypt7p (Mayer and Wickner, 1997) and its hexameric effector complex, termed HOPS (homotypic fusion and vacuole protein sorting; Stroupe *et al.*, 2006). HOPS consists of a core of four subunits, Vps1, Vps16, Vps33 and 33, associated on the vacuole with two other subunits, Vps39p and Vps41p (Sato *et al.*, 2000; Seals *et al.*, 2000). After tethering, vacuoles are drawn against each other, establishing three microdomains: the disk-shaped boundary domain of tightly apposed membrane from each vacuole; the ring-shaped vertex domain at the edge of the boundary domain; and the outside domain, which does not touch the other vaucole (Wang *et al.*, 2002). Each of the proteins (Wang *et al.*, 2002) and lipids (Fratti *et al.*, 2004) that are required for vacuole fusion become enriched at the vertex ring, and fusion ensues. The vertex ring-enriched proteins (Yptp7p, HOPS, SNAREs, and others) and lipids (phosphoinositides, ergosterol, and diacylglycerol) are interdependent for their vertex enrichment (Wang *et al.*, 2003; Fratti *et al.*, 2004). Studies with reversible inhibitory ligands of these proteins and lipids show that the formation of fusion-competent vertex microdomains is reversible or highly cooperative (Jun *et al.*, 2006).

HOPS has a direct affinity for phosphoinositides and for the Vam7p SNARE (Stroupe *et al.*, 2006). The HOPS subunit Vps39p has nucleotide exchange activity for Ypt7p (Wurmon *et al.*, 2000), and HOPS binds specifically to the GTP-bound form of Ypt7p (Seals *et al.*, 2000). The HOPS subunit Vps33p is a member of the SM protein family. Ypt7p and HOPS are necessary for SNAREs to pair in trans (Collins and Wickner, 2007). Vacuole fusion requires three glutaminyl (Q)-SNAREs (Vti1p, Vam3p, and Vam7p) and one arginyl (R)-SNARE (Nvy1p). Although the wild-type 3Q:1R set of SNAREs gives optimal fusion, fusion can also occur in vitro with 4Q SNAREs or even with 2Q SNAREs and 2R SNAREs when complex assembly is driven by high concentrations of SNAREs (Fratti *et al.*, 2007). Vacuolar SNARE complexes are associated with Sec17p (a-SNAP) or HOPS, but not with both (Collins *et al.*, 2005). Although Sec17p association allows SNARE complexes to be disassembled, the role of HOPS association in SNARE complex function is unknown.

We now show that the addition of purified HOPS to vacuole fusion reactions reduces the levels of noncanonical trans-SNARE complexes formed during fusion, and further reduces the capacity of these mismatched complexes to undergo fusion. With wild-type 3Q:1R SNAREs, additional HOPS stimulates trans-SNARE complex formation (Collins and Wickner, 2007) and fusion. However, with 4Q SNAREs, 2Q:2R SNAREs, or 3Q:1R SNAREs in rotated positions in the transcomplex, the addition of purified HOPS suppresses the level of trans-SNARE complex and even further inhibits the subsequent fusion. Furthermore, exogenous HOPS strongly suppresses vacuole fusion when trans-SNARE complexes include a mutant Vam7p SNARE lacking its phosphoinositide-binding Phox homology (PX) domain. Thus, the HOPS association with SNARE complexes proofreads the wild-type conformations of the trans-SNARE complex and regulates its capacity to lead to membrane fusion.

**MATERIALS AND METHODS**

*Yeast Strains, Vacuole Isolation, and In Vitro Fusion Assay*

Vacuoles were isolated by浮动 on discontinuous Ficol gradients (Haas *et al.*, 1994) from *S. cerevisiae* strains BJS005 (MATa ura3-52 trplΔ101 his3Δ200 lys2-801 gal1 (gal3 can1 prb1Δ1Δ16 pep4Δ1his5)) (Jones, 2002) and DKY6281 (MATα ura3-52 leu2-3112 trplΔ101 his3Δ200 lys2-801Δ1Δ16 pep4Δ1Δ16 TRP1). (Haas *et al.*, 1994), or isogenic strains expressing Nv1101p (RFY1 and RFY2) (Fratti *et al.*, 2007). Unless otherwise stated, the in vitro vacuolar fusion reactions in this study were ATP-free fusion reactions (Thorngren *et al.*, 2004), incubated at 27°C for 90 min. These reactions contained 3 μg of pep4Δ vacuoles (from BJS005 derivatives) and 3 μg of pho8Δ vacuoles (from DKY6281 derivatives) in 20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES)-KOH, pH 6.8, 200 mM sorbitol, 125 mM KCl, 5 mM MgCl2, 10 μM CaCl2, 835 nM purified Pbi2p (2%), and 10 mg/ml bovine serum albumin (BSA). Mature Phox alkylphosphatase activity was assessed as a measure of vacuolar fusion (Haas, 1995). Fusion units (U) are micromoles of p-nitrophenol formed per minute per microgram of pep4Δ vacuole.

**Reagents**

Antibodies were purified as described previously, and they were dialyzed into PS buffer (20 mM PIPES-KOH, pH 6.8, and 200 mM sorbitol) with 125 mM KCl. Anti-Vam3p (Wang *et al.*, 2003) was used at 900 nM, and affinity-purified anti-Sec17p (Haas and Wickner, 1996) was used at 209 nM. Recombinant glutathione transferase (GST)-Vam7p, GST-Vam7Q283Rp, and GST-Vam7p fusion proteins were purified via glutathione-affinity chromatography and dialyzed into PS containing 125 mM KCl (Fratti *et al.*, 2007; Fratti and Wickner, 2007).

**HOPS Overproduction**

A yeast strain producing a Vps33-TEV-GST fusion protein was constructed by transfecting BJ2168 (MATα ura3-52 leu2-3112 trplΔ101 pep4Δ1 pep5-3 prc1-407 gal2) (Zubenko *et al.*, 1980) with a polymerase chain reaction (PCR) product formed from a two-round PCR amplification. A GST-TRP1 PCR product was amplified from pFAna-GST-TRP1 (Longtime *et al.*, 1998) by using primers 1a and 1b (Table 1), introducing a tobacco etch virus (TEV) protease site upstream of the GST coding region. This product was used as a template in a second round of PCR with primers 1b and 1c. This PCR product was transfected into BJ2162, directing the recombination of the -3' end of VPS33. This strain was named CSR3.

The p40GAL1 series of vectors, based on the P8540 vectors (Sikorski and Hieter, 1989), were a generous gift from E. Schielb (ZMBH, Heidelberg, Germany). VPS33-TEV-GST was amplified from C514 bosomosomal DNA by using primer set 2 and 2t cloned into the SpeI/SalI sites of pAG44. VPS18, VPS51, and VPS16 were amplified from FY834 (MATa ura3-52 leu2Δ1 Δ lip2Δ2Δ1 trplΔ63 his3Δ200) (Winost *et al.*, 1995) by using primer sets 3, 4, and 5, respectively. Each of these products was cloned into p40GAL1, p40GAL1, and p40GAL1, respectively, by using the SpeI/Xhol, BamH/Xhol, and BamH/BamH sites

**Construct a strain expressing all six HOPS genes under the control of the GAL1 promoter, FY834 was sequenced to confirm that the following linearized plasmids p40GAL1-VPS33-TEV-GST fused were constructed by Nol digestion. p40GAL1-VPS30 digested with Stul and p40GAL1-VPS11 digested with Baxl and p40GAL1-VPS16 digested with NdeI. The GAL1 promoter was amplified from pMAY2A-GAL1, y5N23 (Janke *et al.*, 2004) with primer set 6, and it was targeted to recombinant upstream of VPS41 through flanking homologous DNA sequence in each direction. The GAL1 promoter was amplified from pMAY2A-GAL1, y5N23 (Janke *et al.*, 2004) with primer set 6, and it was targeted to recombinant upstream of VPS33 by extending the flanking VPS39 homology of the initial PCR product with primer set 8. This created the strain CHY26, which overproduced all of the HOPS subunits upon growth in the presence of galactose.

A cassette from pAG32 (Goldstein and McCusker, 1999) was then used to delete the PEP4 gene from CHY26 by using the PEP4 primer sequences used in the commercial nonessential deletion library (Gaevet *et al.*, 2002), resulting in CHY31 (CHY24 pep4Δ::KanMX4).

**GST-HOPS Purification**

CHY31 from solid medium was grown at 30°C in CSM-bris-leu-trp-ura drop-out medium (MP Biomedicals, Irvine, CA), supplemented with yeast nitrogen base (YNB) and monosodium glutamate, 2% glucose, 100 μg/ml Clomat (WERNER BioAgents, Jena, Germany), and 200 μg/ml G418, adjusted to pH 6.5 with KOH, for 14 h. This culture was used to inoculate 600 ml of the medium, and growth was continued for 8 h, when OD600 reached ~1.3. Four 6-l flasks, each...
with 2.5 l of YP + 2% galactose, were inoculated with 160 ml each of the starter cultures and grown at 30°C for 14 h to OD 600 (proPho8p), and the other has normal vacuole lumenal pro-
two yeast strains. One lacks vacuolar lumenal proteases and

### Table 1. Primer sets used in this study

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<th>Primer set</th>
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<td>1a</td>
<td>GGT CCA GGT GAA AAT TTTG TA TTT TTTA CAG TGG TCC AGG TGCG ATG CTT ACC CCC GTT TAT CTA ATA TTTA AAG TGG TCT GTC TTA AAT ACA</td>
</tr>
<tr>
<td>1b</td>
<td>GCC CAT TTT ATG GAA TTA TTT TTG TTA ACG AAG CAG GCT AAT TTT AAG CTT TTT ACA TCC GCA GAT GGT TAT CCA ATG</td>
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<tr>
<td>1c</td>
<td>GCC GGG GATCCATGTCCCTGAGCTCCTGGAG</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>6</td>
<td>GCC CCT CGA GCT ATA TCC TGC TCA TAG TTT CAT TTT</td>
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</tr>
<tr>
<td>8</td>
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</tr>
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### RESULTS

We assay the fusion of vacuoles that have been purified from two yeast strains. One lacks vacuolar luminal proteases and therefore accumulates inactive proalkaline phosphatase (proPho8p), and the other has normal vacuole luminal pro-
tees but is deleted for the gene encoding Pho8p. Although neither vacuole population has phosphatase activity, bilayer fusion and content mixing allow the proteases to gain access to proPho8p and cleave it to active Pho8p. We measure vacuole fusion and content mixing by a colorimetric assay of this active Pho8p. The homotypic fusion of yeast vacuoles normally depends upon the formation of 3Q:1R transSNARE complexes (Fratti et al., 2007). Although vacuoles with either 4Q or 2Q:2R SNARE complexes can fuse, fusion requires either higher levels of SNAREs or the addition of exogenous compounds that promote lipid rearrangements (Fratti et al., 2007). Because the purified HOPS complex interacts with both the Vam7p SNARE and with certain lipids (Stroupe et al., 2006), we tested whether it might restore full fusion to vacuoles with noncanonical 0-layer residues.

### HOPS Complex Inhibits Fusion of Vacuoles with a Mismatched SNARE 0-Layer

Purified vacuoles have substantial levels of unpaired, membrane-anchored Vam3p, Vti1p, and Nyv1p (Collins et al., 2005). When Vam7p, a soluble SNARE, is provided exogenously, fusion does not require ATP-dependent cis-SNARE complex disassembly (Thorngren et al., 2004; Figure 1A, filled squares). Although HOPS is required for in vitro vacuole fusion (Seals et al., 2000; Stroupe et al., 2006), isolated vacuoles bear sufficient HOPS for fusion (~0.52 nM in the fusion reaction; Stroupe et al., 2006), and supplementation with purified HOPS complex at levels 30-fold over the endogenous vacuolar levels (15.8 nM) has little effect on fusion at any level of added Vam7p (Figure 1A, open squares). High concentrations of recombinant Vam7p263Rα2, mutated to change the 0-layer residue from Q to R, can drive fusion as a component of a 2Q:2R SNARE complex (Figure 1A, filled circles), as reported previously (Fratti et al., 2007). Unlike wild-type 3Q:1R SNARE complex fusion, however, exogenous HOPS addition caused a striking inhibition of the Vam7p263Rα2-mediated 2Q:2R fusion (Figure 1A, open circles). The HOPS SM protein subunit Vps33p, when purified from yeast cytosol, did not have proofreading activity under these conditions, even when added at a higher molar level than HOPS (data not shown). This suggests that the proof-
HOPS Regulates the Assembly, and Function, of trans-SNARE Complexes

In contrast to a previous report from our group (Fratti et al., 2007)—but in agreement with other reports showing that 4Q-SNARE-mediated membrane fusion is well-tolerated to cause massive lysis, detectable lysis can be triggered on wild-type vacuoles by the addition of recombinant Vam7p (Starai et al., 2007). Added HOPS can increase the number of trans-SNARE complexes formed during fusion (Collins and Wickner, 2007), although this increase does not cause additional lysis with wild-type vacuoles (Starai et al., 2007). To test whether 0-layer mismatch promotes lysis, and whether HOPS inhibits the 2Q:2R fusion driven by Vam7Q283Rpb by redirecting docked vacuoles toward lysis, we assayed the release of green fluorescent protein (GFP) from the vacuole lumen during fusion.

Under ATP-free conditions, the addition of Vam7p drives fusion (Figure 3, bar 5 vs. 7) as well as measurable lysis (Figure 3, bars 4 vs. 8). The addition of HOPS complex does not significantly affect this Vam7p-driven fusion or lysis (Figure 3, bars 9 and 10), and a SNARE ligand, α-Vam3p, inhibits fusion and lysis to basal levels (Figure 3, bars 11 and 12). A higher concentration of Vam7p does not drive significantly more fusion or lysis (Figure 3, bars 13–16). Vam7Q283Rpb causes less fusion and lysis than the wild-type protein when used at the same concentrations (Figure 3, bars 19 and 20 and bars 25 and 26), in accordance with the finding that Vam7p must form functional transcomplexes to drive the lysis of vacuoles (Starai et al., 2007). HOPS complex inhibits the fusion caused by Vam7Q283Rpb (Figure 3, bars 19 vs. 21 and 25 vs. 27), and it does not enhance Vam7Q283Rpb-dependent lysis (Figure 3, bars 20 vs. 22 and 26 vs. 28). Therefore, HOPS complex does not inhibit the fusion of mismatched trans-SNARE complexes through increased membrane lysis, but rather through a 0-layer proofreading mechanism.

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Figure 3. HOPS does not promote fusion-dependent lysis with altered 0-layer SNARE complexes. Reactions (90 μl) containing 9 μg each of vacuoles from a BJ3505 derivative bearing luminal soluble GFP (Starai et al., 2007) and from DKY6281 were incubated at 27°C under ATP-free conditions (see Materials and Methods) with the following modifications: BSA was used at 3 mg/ml, and reactions contained 0.1× protease inhibitor cocktail (50× stock: 13 μg/ml leupeptin, 25 mM 1,10-phenanthroline, 25 μg/ml pepstatin A, and 5 mM Pefabloc SC). This concentration of protease inhibitor does not inhibit the proteolytic activation of proPho8p during fusion. Where indicated, HOPS was added to 15.8 nM. After 45 min, reactions were gently mixed and placed on ice; 30 μl was withdrawn to measure fusion (black bars) via Pho8p activity, and 30 μl was assayed for vacular lysis (white bars) via centrifugation (5200 × g, 6 min, 4°C) and assay of GFP fluorescence in pellets and supernatants, as described previously (Starai et al., 2007).

Figure 4. HOPS proofreads after SNARE complex disassembly and before content mixing. Vacuoles were assayed for fusion under ATP-containing conditions (20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol, 150 mM KCl, 6 mM MgCl₂, 10 μM CoA, 1 mM ATP, 1 mg/ml creatine kinase, 29 mM creatine phosphate, and 815 nM indoleacetic acid, HOPS was added to 20.1 nM. Results are mean white bars) were measured for fusion after 90 min at 27°C. Where indicated, HOPS was added to 20.1 nM. Results are mean ± SD. (B) HOPS (30 μl) containing either BJ3505 and DKY6281 (3 μg each, black bars) or RYF1 and RYF2 (3 μg each, white bars) were measured for fusion after 90 min at 27°C. Where indicated, HOPS was added to 20.1 nM. Results are mean ± SD. (B) HOPS (30 μl) containing either BJ3505 and DKY6281 (3 μg each, black bars) or RYF1 and RYF2 (3 μg each, white bars) were incubated at 27°C, and either the fusion inhibitors α-sec17p (209 nM; squares), HOPS complex (201 nM; triangles), α-ypt7p (467 nM; inverted triangles), or α-vam3p (900 nM; diamonds) was added to the fusion reactions at 0-, 10-, 20-, 45-, 60-, and 90-min time points, or the reactions were removed to ice (circles) at the same time intervals. After 90 min, the extent of fusion was measured and compared with the fusion levels of an uninhibited fusion reaction after 90 min (maximal fusion). Results are the mean ± SD of three independent experiments (maximal fusion was 5.32, 5.89, and 5.0 U). The first 45 min of the reaction is plotted for clarity.

(Innhibitor of mismatched 0-layer fusion in a standard in vitro fusion assay.

By taking advantage of an assay designed to test whether vacuoles become resistant to a given fusion inhibitor, we can dissect the vacuole fusion reaction into "priming," and "content mixing" stages (Ungermann et al., 1998b). Using this assay, we can compare the kinetics of HOPS-mediated fusion inhibition to inhibition by reagents that act at specific reaction stages. Vacuoles bearing Nyy1p-R192Qp become resistant to HOPS addition well after resistance to antibodies against Sec17p (Figure 4B, squares vs. triangles). HOPS resistance mirrors the resistance curves of α-ypt7p (inverted triangles) and α-vam3p (diamonds), and HOPS acts before content mixing (ice, circles). These data suggest that the inhibitory HOPS proofreading activity occurs after priming (defined by Sec17p function), during the trans-SNARE pairing/docking stage of vacuole fusion (defined by Ypt7p and Vam3p functions), and before membrane fusion and content mixing.

To test how HOPS may function at the trans-SNARE pairing/docking stage, we have exploited a recently developed assay of trans-SNARE complexes (Collins and Wickner, 2007) to directly measure the effects of added HOPS when the 0-layer of trans-SNARE complexes are mismatched. Under ATP-free conditions, the association of Nyy1p with Vam3p in trans and the subsequent fusion requires added Vam7p (Table 2, lines 2 and 4). Adding HOPS does not significantly change the fusion yield or trans-SNARE complex formation (Table 2, line 5). Vam7pQ283Rp forms fewer 2Q:2R transcomplexes compared with the wild-type 3Q:1R complex, and fusion is similarly reduced (Table 2, line 6). Added HOPS complex strongly reduces the amount of Vam7pQ283Rp-containing trans-SNARE complex formed (line 7). Surprisingly, even the measurable amount of trans-SNARE complex formed with Vam7pQ283Rp in the presence of HOPS is unable to drive proportional fusion (line 7). Together, these data suggest that HOPS is intimately involved in both proofreading the formation of physiological 3Q:1R 0-layer trans-SNARE complexes and in regulating the capacity of those few trans-SNARE complexes which do assemble to support fusion.

(Ossig et al., 2000; Katz and Brennwald, 2000)—vacuoles bearing Nyy1p-R192Qp fuse with ~78% the yield of wild-type vacuoles under our standard, ATP-containing fusion conditions (Figure 4A, bars 1 vs. 2). Adding HOPS to wild-type vacuoles does not significantly change this fusion (Figure 4A, bars 1 vs. 7), but strongly inhibits the 4Q:0R SNARE complex-mediated fusion of Nyy1p-R192Qp vacuoles (Figure 4A, bars 2 vs. 8). These data show that HOPS is a potent

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HOPS Proofreads SNARE Complexes

SM proteins can bind their cognate syntaxins through a conserved N-terminal “H_{ABC}” domain (Toonen and Verhage, 2003; Burgoyne and Morgan, 2007). The N-terminal domain of syntaxin 1 is essential for Munc18-1–mediated SNARE complex activation in a reconstituted system (Shen et al., 2007), and it has been reported that HOPS can associate with the syntaxin orthologue Vam3p at its N terminus (Laage and Ungermann, 2001). Therefore, we tested whether the N-terminal domain of Vam3p is required for HOPS-dependent SNARE complex 0-layer proofreading. Under ATP-free conditions, vacuoles bearing Vam3p lacking its N-terminal H_{ABC} domain (Vam3ΔN, Laage and Ungermann, 2001) can fuse when 200 nM Vam7p is provided (Figure 5, bar 4). Added HOPS slightly inhibits this fusion (bar 5). Vam7^{Q283R} protein also supports the fusion of vacuoles from strains BJ3505 npr1Δ CBP-VAM3 (Collins and Wickner, 2007) and DKY6281 were incubated with the indicated additions at 27°C for 45 min. Reactions were transferred to ice for 5 min, gently mixed, and a 30-μl aliquot was withdrawn to assay alkaline phosphatase activity as a measure of fusion. CBP-Vam3p affinity pull-downs were performed (Collins and Wickner, 2007; Figure 6, compare closed circles with closed squares). Thus, HOPS can proofread the SNARE complex 0-layer in the absence of the N terminus of the Vam3p syntaxin.

HOPS has been shown to associate with Vam7p in solution via the Vam7p phosphoinositide-binding PX domain (Stroupe et al., 2006). To test whether the HOPS proofreading of vacuole SNARE complexes extends to the Vam7p PX domain, we exploited the fact that the purified Vam7p SNARE domain, which lacks the PX domain, can support vacuole fusion in the absence of ATP (Fratti and Wickner, 2007). Vacuole fusion supported by up to 1 μM full-length, wild-type Vam7p is essentially unaffected by the addition of purified HOPS (Figure 6, compare closed squares with open squares). As reported previously, purified Vam7p SNARE domain (Vam7-SD) supports fusion with a much higher K_{on} value compared with wild-type Vam7p (Fratti and Wickner, 2007; Figure 6, compare closed circles with closed squares). Adding HOPS to reactions containing Vam7-SD causes a striking inhibition of fusion (Figure 6, open circles), showing that the HOPS proofreading activity also gauges features of the SNARE complex in addition to the 0-layer and outside the SNARE domain coiled coils.

DISCUSSION

SM family proteins perform two related functions, the proofreading of SNARE-dependent membrane fusion events and

Table 2. HOPS modulation of trans-SNARE complexes and fusion activity

<table>
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<tr>
<th>Reaction condition</th>
<th>Fusion (U)</th>
<th>trans-SNARE complex</th>
<th>Fusion (U) trans-SNARE complex</th>
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<tbody>
<tr>
<td>1. ice</td>
<td>0.17 ± 0.02</td>
<td>0.4 ± 0.13</td>
<td>0.5 ± 0.17</td>
</tr>
<tr>
<td>2. no additions</td>
<td>0.16 ± 0.02</td>
<td>0.9 ± 0.49</td>
<td>0.3 ± 0.19</td>
</tr>
<tr>
<td>3. HOPS</td>
<td>0.16 ± 0.02</td>
<td>0.5 ± 0.08</td>
<td>0.3 ± 0.05</td>
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<tr>
<td>4. 150 nM Vam7p</td>
<td>4.45 ± 0.51</td>
<td>4.1 ± 1.98</td>
<td>1.4 ± 0.81</td>
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<tr>
<td>5. 150 nM Vam7p + HOPS</td>
<td>4.97 ± 0.51</td>
<td>4.3 ± 1.29</td>
<td>1.2 ± 0.32</td>
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<tr>
<td>6. 150 nM Vam7^{Q283R}</td>
<td>2.92 ± 0.96</td>
<td>3.1 ± 1.35</td>
<td>1.0 ± 0.39</td>
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<tr>
<td>7. 150 nM Vam7^{Q283R} + HOPS</td>
<td>0.62 ± 0.06</td>
<td>1.3 ± 0.18</td>
<td>0.5 ± 0.12</td>
</tr>
</tbody>
</table>

ATP-free fusion reactions (11 × scale) with vacuoles from strains BJ3505 npr1Δ CBP-VAM3 (Collins and Wickner, 2007) and DKY6281 were incubated with the indicated additions at 27°C for 45 min. Reactions were transferred to ice for 5 min, gently mixed, and a 30-μl aliquot was withdrawn to assay alkaline phosphatase activity as a measure of fusion. CBP-Vam3p affinity pull-downs were performed (Collins and Wickner, 2007). Vacuoles were sedimented (12,000 × g, 5 min, 4°C) and dissolved in ice-cold 600 μl of solubilization buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 0.5% NP-40 Alternative [Calbiochem, San Diego, CA], 10% glycerol, 0.46 μg/ml leupeptin, 3.5 μg/ml pepstatin, 2.!μg/ml pefabloc-SC, and 1 mM PMSF). After 20 min at 4°C with nutation, insoluble material was removed by centrifugation (16,000 × g, 20 min, 4°C), and 500 μl of each vacuole extract was mixed with 50 μl of calmodulin agarose, which had been pre-equilibrated with solubilization buffer containing 2 mM CaCl2. CaCl2 was added to the vacuolar extract to final concentration of 2 mM, and the suspension was rocked for 16 h at 4°C. Beads were harvested by centrifugation (1400 × g, 2 min, 4°C) and washed five times with 500 μl of ice-cold solubilization buffer, sedimenting beads after each wash as described. Bead-bound proteins were eluted with SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer containing 5 mM EGTA, incubated 5 min at 98°C, separated by SDS-PAGE electrophoresis, and transferred to nitrocellulose. CBP-Vam3p and Nyv1p were detected by immunoblotting. Films were scanned with a computer-controlled PowerLook 1100 scanner (UMAX) and VueScan software version 8.0.4 (Hamrick Software, Phoenix, AZ). Image files were digitized, and the total pixels making up the CBP-Vam3p and Nyv1p bands were quantified using UN-SCAN-IT version 5.1 (Silk Scientific, Orem, UT). Reported values are the mean ± SD of three independent experiments.

a Standard ATP-free reactions (see Materials and Methods): HOPS added to a final concentration of 16.7 μg/ml, where noted.

b Defined as (Nyv1p pixels/CBP − Vam3p pixels) × 10.
the activation of the SNARE complex for fusion (Peng and Gallwitz, 2002; Scott et al., 2004; Shen et al., 2007). The biochemical and genetic data regarding SM proteins clearly point to an essential role, yet the molecular interactions governing SM protein function at each distinct step are only recently being elucidated. Our current study examines the physical and functional relationship during homotypic vacuolar fusion between the HOPS complex, which includes Vps33p, an SM protein, and the SNARE complex.

We find that HOPS plays a significant role in ensuring correct trans-SNARE complex formation and function. Alterations in the buried 0-layer of the SNARE complex that cause a non3Q:1R—or even a rotated 3Q:1R—structure are significant enough to cause HOPS to inhibit assembly (Figure 1 and Table 2). Because these residues are not surface exposed (Fasshauer et al., 1998; Sutton et al., 1998), HOPS may sense a conformational change in the SNAREs resulting from these seemingly slight changes in the 0-layer, due to a dedicated and specific mode of binding to SNAREs, or the assembled SNARE complex. Binding interactions are often regulated by slight conformational differences, which are necessary to ensure interaction specificity above the background of other similar biological structures (Savir and Tilsty, 2007). In other studies (Shen et al., 2007), mutations in residues adjacent to the +1 and +5 layers in the SNARE motif, which are surface-exposed in the SNARE complex, abolish Munc18-1-mediated stimulation of liposome lipid mixing without abolishing the basal SNARE-mediated lipid mixing. In contrast, added HOPS complex inhibits the fusion activity of noncanonical 0-layer SNARE complexes which otherwise have significant fusion activity (Figures 1–6). Although the N terminus of Vam3p promotes HOPS recruitment to the vacuole (Laage and Ungermann, 2001), the 0-layer proofreading activity does not require the N-terminal H_{ABC} domain, SM-protein binding domain of Vam3p (Figure 5), and thus HOPS recognition of the assembled SNARE complex is not simply focused on the Vam3p H_{ABC} domain. HOPS strongly suppresses the fusion capacity of trans-SNARE complexes lacking the Vam7p PX domain (Figure 6), possibly reflecting the direct affinity of this PX domain for HOPS (Stroupe et al., 2006). HOPS may use several modes of binding to the assembled SNARE complex to regulate its assembly (Jun et al., 2007b) and may continue to function as part of the trans-SNARE complex during fusion. These results support the finding that Munc18-1 can bind to synaptic SNARE complexes in the absence of the syntaxin N-terminal H_{ABC} domain (Shen et al., 2007), and they are in accord with another report describing multiple modes of SM protein: SNARE complex binding for yeast Vps45p (Carppp et al., 2006).

We have reported previously (Fratti et al., 2007; Fratti and Wickner, 2007) that the K_{m} value for wild-type Vam7p to support vacuole fusion is far lower than the K_{m} value for either Vam7^{2Q:2R} or Vam7-SD. This may be caused by the proofreading function of the endogenous vacuolar HOPS. Added Vam7^{2Q:2R} or Vam7-SD, which exceeds the HOPS proofreading capacity, may lead to fusion. Fusion, whether supported by wild-type or 2Q:2R trans-SNARE complexes (Fratti et al., 2007), is blocked by antibodies to the HOPS subunit Vps33p. Because HOPS activity is required for the formation of even mismatched trans-SNARE complexes and for the resulting membrane fusion, it is likely that SNARE complex proofreading and the support of fusion are distinct HOPS activities, each essential for physiological vacuole fusion.

SM proteins can bind to the closed conformation of free syntaxin and prevent them from entering SNARE complexes in solution (Pevsner et al., 1994; Dulubova et al., 1999; Yang et al., 2000; Misura et al., 2000). Yeast Sly1p, when prebound to the Golgi syntaxin Sed5p, can also prevent the formation of soluble 2Q:2R SNARE complexes (Peng and Gallwitz, 2002). However, without SNARE transmembrane anchors, this study could not measure the effects of the assembled 2Q:2R SNARE complexes or the SM protein on membrane fusion. Peng and Gallwitz (2004) also found that direct, high-affinity binding of Sly1p to Sed5p was, surprisingly, not required for either protein’s activity in vivo. Furthermore, mutant versions of Sly1p could bind a number of nonsyntaxin SNAREs, suggesting that SM proteins could potentially interact with each of the SNAREs involved in a particular SNARE complex assembly pathway. How, then, do SM proteins prevent non3Q:1R or noncognate SNARE complexes from forming? Simple steric hindrance from a prebound and specific SM:syntaxin complex may be responsible, because each SM protein is well localized to an individual subcellular compartment, and it cannot compensate for the absence of another SM protein at another organelle (Toonen and Verhage, 2003). In contrast, SM proteins may enhance the stability of cognate SNARE complexes, thereby increasing the chances of that SNARE complex causing a fusion event. Vacuole SNAREs, like their neuronal counterparts, may initially form a fusion-incompetent, “open” transcomplex that must be converted to the fusion-competent “closed” SNARE complex. HOPS and its SM subunit Vps33p might prevent noncognate SNARE complexes from progressing from the open to the closed state, whereas SNAREs alone may freely assemble and disassemble without the ability to cause fusion. It has also been proposed that SM proteins can spatially segregate noncognate SNAREs from the active sites of fusion, concentrating the correct SNAREs and increasing the probability that appropriate SNARE complexes will form (Bethani et al., 2007). In addition, the activity of Munc18-1 on the SNARE-dependent lipid mixing of reconstituted proteoliposomes is only seen with the cognate neuronal SNAREs, and not with other SNAREs that can support a background lipid mixing rate in this assay (Shen et al., 2007). This SM protein-mediated proofreading of SNARE identity is distinct from the proof-
reading of the proper 0-layer and N-domain state of the SNARE complex, which we present here.

In addition to proofreading the SNARE complex, HOPS has tethering and Ypt7p/Rab GTPase nucleotide exchange functions, each of which is also required for efficient membrane fusion (Price et al., 2000; Seals et al., 2000; Wurmser et al., 2000; Stroupe et al., 2006). It is unclear whether the RabGTPase nucleotide exchange activity of HOPS is required for its proofreading activity, but neither normal vacuole fusion nor HOPS proofreading activity responds to exogenous additions of GTP (data not shown). However, SM proteins could link Rab GTPase and SNARE functions; a direct interaction between Munc18-1 and Rab5A was recently detected in bovine brain extracts, in accord with this idea (Graham et al., 2008). Therefore, the proofreading or fusion activities of SM proteins could be regulated by cognate Rab GTPase proteins, although SM proteins and HOPS can activate SNARE-dependent lipid mixing in reconstituted systems that lack Rab GTPases (Scott et al., 2004; Shen et al., 2007; Mima and Wickner, unpublished data).

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