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HOPS Proofreads the trans-SNARE Complex for Yeast Vacuole Fusion

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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 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 (Qc) 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 (GTPases), multisubunit Rab-effector complexes, Sec1/Munc18 (SM) proteins, and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (Jahn et al., 2003). Rab GTPases 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 associated with fusion complexes. SNARE 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...
HOPS Proofreads SNARE Complexes

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., 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; Ungermann et al., 1998a; Jun and Wickner, 2007a). Tethering requires the vacuolar 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, Vps 11, 16, 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 vacuole (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 (Ypt7p, 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 (Wurmser 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 (α-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 floatation on discontinuous Ficol gradients (Haas et al., 1994) from S. cerevisiae strains BJ2305 (MATa ura3-52 trpl-Δ101 his3-Δ200 lys2-801 gal2 [gal3] can1 trpl-Δ1.6 rplk::HIS3) (Jones, 2002) and DKy6281 (MATa ura3-52 leu2-3112 trpl-Δ101 his3-Δ200 lys2-801 gal2::Δ9 pho8::TRP1) (Haas et al., 1994), or isogenic strains expressing Nye119 (RFP1 and RFP2) (Fratti et al., 2007). Unless otherwise stated, the in vitro vacuole 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 pepΔ vacuoles (from BJ305 derivatives) and 3 μg of phoΔ 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 CoA, 815 nM purified Pho8p (P2), and 10 mg/ml bovine serum albumin (BSA). Mature Pho8p alkaline phosphatase activity was assayed as a measure of vacuole fusion (Haas, 1995). Fusion units (U) are micromoles of p-nitrophenyl phosphate formed per minute per microgram of pepΔ 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-Vam2p, 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 transducing strain FY834 (MATA leu2-3,112 [Mey1::TEV] pep4-3 [Vps33:: Hyg]) with the yeast artificial chromosome (YAC) YCp50, which contains the yeast GAL1 promoter. The resulting strain FY835 (MATA leu2-3,112 [Mey1::TEV] pep4-3 [Vps33::Hyg]) was amplified with primer set 3, 4, and 6, and it was targeted to the 3′ end of VPS33. This strain was named CHY31 (CHY24 pep4-3::Hisp). To overproduce HOPS, chromosomal disruption of GAL1 was performed by flanking the gene with sequences derived from the TEV promoter.

The p400GAL1 series of vectors, based on the pRS400 vectors (Sikorski and Hieter, 1989), were a generous gift from E. Schielbe (ZMBH, Heidelberg, Germany). VPS33-TEV-GST was amplified from CSY14 chromosomal DNA by using primer set 2 and cloned into the SpeI/Sall sites of p400GAL1 (VP518, VP511, and VP516) were amplified from FY834 (MATa ura3-52 leu2ΔI plp1Δ63 his3Δ200) (Winston et al., 1995) by using primer sets 3, 4, and 5, respectively. Each of these products was cloned into p405GAL1, p406GAL1, and p404GAL1, respectively, by using the SpeI/Xhol, BamH1/Xhol, and BamH1/Xhol sites.

To construct a strain expressing all six HOPS genes under the control of the GAL1 promoter, FY834 was sequentially transformed with the following linearized plasmids p403GAL1-VPS39-TEV-GST digested with SfiI, p405GAL1-VP518 digested with SfiI, p406GAL1-VP511 digested with BaxI, and p404GAL1-VP516 digested with NdeI. The GAL1 promoter from pYM-N23 (Janke et al., 2004) was amplified with primer set 6, and it was targeted to recombine upstream of VP541 through flanking homologous DNA sequence in each plasmid. The GAL1 promoter was amplified from pMXM6-PGAL (Longtine et al., 1998) by using primer set 7, and it was targeted to recombine upstream of VPS39 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 (Gaezert et al., 2002), resulting in CHY31 (CHY24 pep4-3::KanMX4).

GST-HOPS Purification

CHY31 from solid medium was grown at 30°C in CSM-his-leu-trp-ura dropout medium (MP Biomedicals, Irvine, CA), supplemented with yeast nitrogen base (YNB), lysine monosodium glutamate, 2% glucose, 100 μg/ml Clonat (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 culture and grown at 30°C for 2 days before each flask then
received 130 ml of 50% glucose, and cells were grown for an additional 90 min
(final OD_{600} ~ 2.0). Cells were harvested by centrifugation (Beckman JA-10
rotor; 5000 rpm, 5 min, 23°C), resuspended with a glass rod, and mixed by
inversion in 100 mM Tris-Cl, pH 7.8, 10 mM dithiothreitol (50 ml/OD
~ 1.5. Each flask then
incubated with occasional inversion in a 30°C water bath for 10 min, and
centrifuged as described above. Each pellet was resuspended in 15 ml/OD
1.5. Each flask then
centrifuged at 10,000 rpm, 15 min, 4°C. Pellets were suspended in
800 ml of HOPS vacuole lysis buffer (HVLB: 20 mM HEPES-NaOH, pH 7.8,
400 mM NaCl, 10% glycerol, 5 mM 2-mercaptoethanol, and 1.0% Triton X-100)
and incubated on ice for 2 min. Insoluble material was removed by centrif-
fathered (60Ti; 20 min, 50,000 rpm, 4°C), and the supernatant was passed
through a 0.2-
ugation (60Ti; 20 min, 50,000 rpm, 4°C), and the supernatant was passed
through a 0.2-
through a 0.2-
vacuoles were collected, glycerol was added to 10%, phenylmethylsulfonyl fluo-
rine (PMSF) was added to 1 mM, and the suspension was added dropwise
directly to liquid nitrogen. The frozen droplets were stored at ~80°C.
Frozen vacuoles (~300 mg of protein) were thawed, mixed with 1.2 l of cold
20 mM HEPES-NaOH, pH 7.8, 200 mM sorbitol, and 50 mM NaCl, and
centrifuged (JA-14 rotor; 10,000 rpm, 15 min, 4°C). Pellets were suspended in
800 ml of HOPS vacuole lysis buffer (HLVB: 20 mM HEPES-NaOH, pH 7.8,
400 mM NaCl, 10% glycerol, 5 mM 2-mercaptoethanol, and 1.0% Triton X-100)
and incubated on ice for 2 min. Insoluble material was removed by centrif-
igation (60Ti; 20 min, 30,000 rpm, 4°C), and the supernatant was passed
through a 0.2-μm filter (Millipore, Billerica, MA). This was applied to a 25 x
8 cm glutathione-Sepharose 4B column (GE Healthcare, Chalfont St. Giles,
United Kingdom), which had been pre-equilibrated with HLVB at 4°C. The
column was washed with 3 bed volumes of HLVB, and then 3 bed volumes
of low Triton X-100 HOPS buffer (LTHB: 20 mM HEPES-NaOH, pH 7.8,
400 mM NaCl, 10% glycerol, 5 mM 2-mercaptoethanol, and 0.004% Triton X-100). Protein was eluted from the column with LTHB + 10 mM glutathione.
Fractions containing HOPS were pooled, concentrated from ~15 to ~1 ml,
with a final protein concentration of 0.5-1.0 mg/ml in an Amicon Ultra-15
(100,000 NMWL; Millipore) centrifugal filter device, distributed into small
aliquots, and frozen in liquid nitrogen. When calculated, the molar concen-
trations of HOPS considered the molecular mass of HOPS to be 663 kDa,

RESULTS

We assay the fusion of vacuoles that have been purified from
two yeast strains. One lacks vacuolar lumenal proteases and
therefore accumulates inactive proalkaline phosphatase
(proPho8p), and the other has normal vacuole lumenal pro-
teases 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 trans-
SNARE 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, mem-
brane-anchored Vam3p, Vti1p, and Nvy1p (Collins et al.,
2005). When Vam7p, a soluble SNARE, is provided exog-
enoously, 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 vac-
ucle fusion (Seals et al., 2000; Strope et al., 2006), isolated
vacuoles bear sufficient HOPS for fusion (~0.52 nM in the
fusion reaction; Strope et al., 2006), and supplementation
with purified HOPS complex at levels 30-fold over the en-
dogenous vacuolar levels (15.8 nM) has little effect on fusion
at any level of added Vam7p (Figure 1A, open squares).
High concentrations of recombinant Vam7p, 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 Vam7p-mediated 2Q:2R fusion (Figure 1A, open cir-
cles). 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-
Figure 1. HOPS complex inhibits fusion when the SNARE 0-layer is altered. Vacuoles from either BJ3505 and DKY6281 (A) or RY1 and RY2 (bearing Nyv1R192Qp) (B) were assayed for fusion under standard ATP-free conditions (see Materials and Methods). Either wild-type Vam7p (squares) or mutant Vam7Q283Rp (circles) was added at the indicated concentrations, either in the absence (closed symbols) or presence (open symbols) of 15.8 nM purified HOPS complex. Results are the mean of three independent experiments ± SD.

Figure 2. HOPS complex proofreads the 0-layer in the presence of ATP. Vacuoles were assayed for fusion in reactions where the priming block by α-Sec17p was bypassed by added Vam7p (Thorngren et al., 2004). Reactions (30 μl) contained vacuoles from BJ3505 and DKY6281 (3 μg each) in 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, 815 nM purified P2, and 209 nM affinity-purified α-Sec17. After 5 min on ice, HOPS and either Vam7p (A) or Vam7Q283Rp (B) was added at the indicated concentrations. Fusion was measured after 90 min at 27°C. Fusion data are from three independent reactions, standardized as a percentage of the maximum fusion signal obtained with each Vam7p addition, which was 4.78 ± 0.72 U (A) and 1.38 ± 0.33 U (B), mean ± SD.

HOPS Does Not Inhibit Fusion by Promoting Lysis

Whereas wild-type levels of SNAREs, Ypt7p, and HOPS support fusion without triggering lysis, increasing the number of trans-SNARE complexes raises the overall amount of organelle rupture, leading to fusion inhibition (Starai et al., 2007). Although levels of all four SNAREs must be elevated 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 Vam7Q283Rp 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). Vam7Q283Rp 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 Vam7Q283Rp (Figure 3, bars 19 vs. 21 and 25 vs. 27), and it does not enhance Vam7Q283Rp-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.

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

By taking advantage of an assay designed to test when vacuoles become resistant to a given fusion inhibitor, we can dissect the vacuole fusion reaction into “priming,” “docking,” 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 Nyv1R192Qp 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 is mismatched. Under ATP-free conditions, the association of Nyv1p with Vam3p in trans and the subsequent fusion require 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 6). Vam7Q283Rp forms fewer 2Q:2R transcomplexes compared with the wild-type 3Q:1R trans-SNARE complex, and fusion is similarly reduced (Table 2, line 6). Surprisingly, even the measurable amount of trans-SNARE complex formed (line 7). Surprisingly, even the measurable amount of trans-SNARE complex formed (line 7). This concentration of protease inhibitor does not inhibit the proteolytic activation of proHph1p 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 vacuolar 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).
Table 2. HOPS modulation of trans-SNARE complexes and fusion activity

<table>
<thead>
<tr>
<th>Reaction condition</th>
<th>Fusion (U)</th>
<th>trans-SNARE complex[^a]</th>
<th>Fusion (U) trans-SNARE complex[^a]</th>
</tr>
</thead>
<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>
</tr>
<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>
</tr>
<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>
</tr>
<tr>
<td>6. 150 nM Vam7Q283Rp</td>
<td>2.92 ± 0.96</td>
<td>3.1 ± 1.35</td>
<td>1.0 ± 0.39</td>
</tr>
<tr>
<td>7. 150 nM Vam7Q283Rp + HOPS</td>
<td>0.63 ± 0.96</td>
<td>1.3 ± 0.18</td>
<td>0.5 ± 0.12</td>
</tr>
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</table>

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

HOPS Complex Can Proofread Other Features of the SNARE Complex

SM proteins can bind their cognate syntaxins through a conserved N-terminal “HABC” 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 activity. Under ATP-free conditions, vacuoles bearing Vam3p lacking its N-terminal HABC 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). Vam7Q283R protein also supports the fusion of Vam3ΔN vacuoles (Figure 5, bar 6), but this fusion is strongly inhibited by the addition of exogenous HOPS (Figure 5, bar 7). 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 Keq 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...
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 (Fasshawer 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 Tlusty, 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 which lacks the PX domain (Vam7-SD, circles). Assays were performed in the absence (closed symbols) or presence (open symbols) of 20.1 nM purified HOPS complex. Results are the mean ± SD of three independent experiments.

Figure 6. HOPS proofreads at least one domain outside of the SNARE motif. Vacuoles from BJ3505 and DKY6281 were assayed for fusion under standard ATP-free conditions after 90-min incubation with either wild-type Vam7p (squares) or the Vam7p SNARE domain which lacks the PX domain (Vam7-SD, circles). Assays were performed in the absence (closed symbols) or presence (open symbols) of 20.1 nM purified HOPS complex. Results are the mean ± SD of three independent experiments.

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 (Carr 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 Vam7Q283Rp or Vam7-SD. This may be caused by the proofreading function of the endogenous vacuolar HOPS. Added Vam7Q283Rp 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 syntaxins 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 the 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; Wurmsner 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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