The tethering complex HOPS catalyzes assembly of the soluble SNARE Vam7 into fusogenic *trans*-SNARE complexes

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ABSTRACT **The fusion of yeast vacuolar membranes depends on the disassembly of *cis*–soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complexes and the subsequent reassembly of new SNARE complexes in *trans*. The disassembly of *cis*-SNARE complexes by Sec17/Sec18p releases the soluble SNARE Vam7p from vacuolar membranes. Consequently, Vam7p needs to be recruited to the membrane at future sites of fusion to allow the formation of *trans*-SNARE complexes. The multisubunit tethering homotypic fusion and vacuole protein sorting (HOPS) complex, which is essential for the fusion of vacuolar membranes, was previously shown to have direct affinity for Vam7p. The functional significance of this interaction, however, has been unclear. Using a fully reconstituted in vitro fusion reaction, we now show that HOPS facilitates membrane fusion by recruiting Vam7p for fusion. In the presence of HOPS, unlike with other tethering agents, very low levels of added Vam7p suffice to induce vigorous fusion. This is a specific recruitment of Vam7p rather than an indirect stimulation of SNARE complex formation through tethering, as HOPS does not facilitate fusion with a low amount of a soluble form of another vacuolar SNARE, Vti1p. Our findings establish yet another function among the multiple tasks that HOPS performs to catalyze the fusion of yeast vacuoles.**

Monitoring Editor
Thomas F. J. Martin
University of Wisconsin

Received: Jul 26, 2013
Revised: Sep 16, 2013
Accepted: Sep 26, 2013

INTRODUCTION

The compartmentation of eukaryotic cells through their endomembrane system ensures a spatial separation of cellular functions that prevents undesired interference between different metabolic pathways. Dynamic remodeling of those membranes through fusion and fission events allows a regulated transport of proteins, lipids, and solutes between organelles. Transport vesicles bearing selected cargo tether to their target membrane and then dock stably and fuse.

To understand the mechanisms that govern such membrane fusion events, we study the homotypic fusion of yeast vacuoles (reviewed in Wickner, 2010). A quantitative assay of the fusion of isolated vacuoles (Haas *et al.*, 1994) has led to a basic understanding of the processes that precede the fusion of vacuolar membranes. Multiple proteins participate in these steps. Four vacuolar soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins, Vam3p, Vti1p, Vam7p, and Nyv1p, play a central role. They form a membrane-anchored four-helical bundle, initially in *cis* (bound to one membrane). This complex becomes disassembled by Sec17p/Sec18p, an ATP-dependent chaperone system, in the early step of priming. On disassembly, Vam7p—a peripheral membrane protein—is released into the cytosol (Boeddinghaus *et al.*, 2002). Pairs of vacuoles are tethered by the homotypic fusion and vacuole protein sorting (HOPS) complex. The four SNAREs need to form a new complex in *trans* (i.e., anchored to two apposed membranes) in order to dock stably and proceed to fusion. A central question for our mechanistic understanding of membrane fusion is whether the assembly of *trans* 4-SNARE complexes occurs spontaneously at a meaningful rate or needs to be catalyzed by other proteins.

The heterohexameric tethering complex HOPS is a candidate for performing this catalysis. HOPS is an effector of the vacuolar Rab Ypt7p (Seals *et al.*, 2000; Stroupe *et al.*, 2006; Wickner, 2010),

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E13-07-0419) on October 2, 2013.

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Abbreviations used: FRET, fluorescence resonance energy transfer; HOPS, homotypic fusion and vacuole protein sorting complex; PEG, polyethylene glycol; PhycoE, R-phycoerythrin; RPL, reconstituted proteoliposome; SNARE, soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor.

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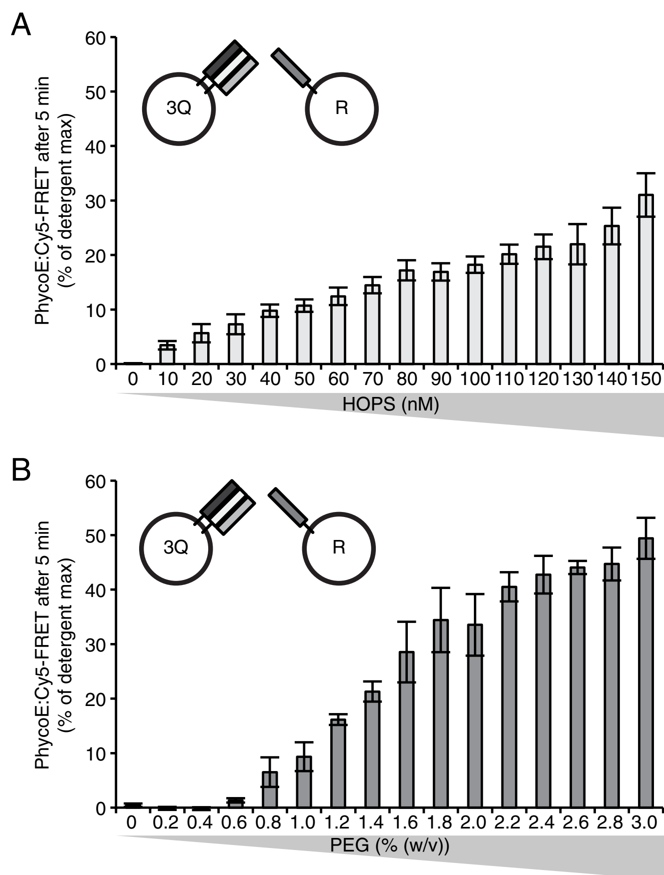

FIGURE 1: Tethering by either HOPS or PEG facilitates fusion in 3Q-1R reactions. 3Q-RPLs (Vam3, Vti1, and Vam7, which assembled into 3Q-SNARE complexes during the dialysis step of proteoliposome formation; Fukuda *et al.*, 2000) and 1R-RPLs (Nvy1) were assayed for content mixing with increasing concentrations of either HOPS (A) or polyethylene glycol (B). Percentages of content mixing after 5 min. Data are the means of three independent experiments. In this and subsequent figures, error bars represent SDs.

associates with SNARE complexes on vacuoles (Collins *et al.*, 2005), and has direct affinity for the PX domain of the SNARE Vam7p (Haas *et al.*, 1994; Stroupe *et al.*, 2006). Two of the HOPS subunits, Vps39p and Vps41p, have affinity for Ypt7p (Ostrowicz *et al.*, 2010; Bröcker *et al.*, 2012), providing a mechanistic rationale for the capacity of HOPS to dramatically cluster reconstituted proteoliposomes (RPLs) bearing only the purified Ypt7p protein (Hickey *et al.*, 2009). HOPS may fulfill additional functions, such as proofreading the composition of *trans*-SNARE complexes (Starai *et al.*, 2008) and protecting them from disassembly by Sec17p and Sec18p (Hickey and Wickner, 2010; Xu *et al.*, 2010). Nevertheless, it has been unclear whether HOPS participates in the formation of *trans*-SNARE complexes by means other than tethering membranes. We now report an additional function of the HOPS complex, the catalysis of Vam7p participation in fusion.

RESULTS

Proteoliposomes were prepared bearing the three vacuolar Q-SNAREs (Vam3p, Vti1p, and Vam7p), which had preassembled into a 3Q-SNARE complex (Fukuda *et al.*, 2000) during the lengthy dialysis used in proteoliposome preparation. The proteoliposomes can fuse with those bearing the R-SNARE Nyv1p when tethering is supported by either HOPS (Figure 1A) or polyethylene glycol (PEG; Figure 1B). All proteoliposomes used in this study bore Ypt7p to enable high-affinity HOPS binding. Fusion is rigorously defined as the mixing of lumenal contents that are continuously shielded by intact membranes from the surrounding aqueous environment (Zucchi and Zick, 2011). Proteoliposomes are prepared by dialysis of detergent mixed micellar solutions of vacuolar-composition lipids, the vacuolar Rab Ypt7p, and the vacuolar SNAREs. To allow content mixing to be assayed, either Cy5-labeled streptavidin or biotinylated R-phycoerythrin (PhycoE) is added to the detergent micellar solutions, causing a small portion of either to become entrapped in the proteoliposomal lumen. After flotation of proteoliposomes, they are mixed with an excess of nonfluorescent streptavidin before coincubation. Fusion is measured as the mixing of their lumenally entrapped proteins, which bind to each other with high affinity and then exhibit fluorescence resonance energy transfer (FRET). Each population of proteoliposomes also bears a fluorescent lipid, which also exhibit FRET upon lipid mixing. The availability of alternative tethering agents, such as PEG, allowed us to ask whether HOPS is required beyond tethering when SNAREs have not been fully preassembled during the lengthy dialysis that is required for proteoliposome formation. When 2Q-SNARE (Vam3p and Vti1p) RPLs are incubated with 1R-SNARE (Nyv1p) RPLs, Vam7p addition is strictly required for either fusion (Figure 2A) or for the formation of SNARE complexes, which include Nyv1p and Vam3p, as assayed by coimmunoprecipitation of Vam3p and the associated Nyv1p (Figure 2B). Some fusion can be seen without added tethering agent, but only at very high levels (>2 μM) of added Vam7p (Figure 2A, black bars). At such high concentrations, added Vam7 or its PX domain alone can suffice to promote the fusion of 3Q- and 1R-SNARE proteoliposomes (Supplemental Figure S1), in accord with earlier studies (Xu and Wickner, 2012). Although reactions with 2Q- and 1R-RPLs need less Vam7p for fusion in the presence of 2% PEG than reactions without tethering agent (compare black and gray bars in Figure 2A, e.g., 0.25 vs. 4 μM), HOPS dramatically lowers the concentration of Vam7p needed for fusion to the low nanomolar range (Figure 2A, white bars). Given that 2% PEG promotes the fusion of 3Q- to 1R-RPLs better than 100 nM HOPS (Figure 1) and thus can be presumed to have greater tethering activity at these concentrations, the greater potency of 100 nM HOPS in promoting the fusion of 2Q- and 1R-RPLs at low Vam7p levels (Figure 2A) suggests that HOPS may act beyond tethering to catalyze the association of Vam7p with the other SNAREs. This is supported by a direct assay of the Vam7p dependence of the association of Vam3p and Nyv1p in *trans* in these reactions (Figure 2B). At low levels of Vam7p (<0.25 μM), such *trans* association is only seen in the presence of HOPS, whereas at higher levels of Vam7p it can also be seen for reactions containing PEG, paralleling the observations made in fusion assays (Figure 2, compare A and B). The well-characterized affinity of Vam7p PX domain for phosphatidylinositol 3-phosphate (PI(3)P; Cheever *et al.*, 2001) still makes an important contribution under these fusion conditions, as Vam7p bearing the Y42A mutation, which specifically ablates its affinity for PI(3)P (Cheever *et al.*, 2001), is needed at substantially higher levels for fusion (Figure 3).

Although these data show that HOPS can function beyond tethering to catalyze SNARE complex assembly, it was unclear whether this was specific for Vam7p as the unpaired SNARE. To seek fusion conditions in which SNAREs would initially be entirely unpaired, we prepared proteoliposomes that each bore only one of the membrane-bound SNAREs. These were assayed in pairs with the two complementary SNAREs added as soluble forms without their membrane anchors (Figure 4). Only one such combination gave fusion. In

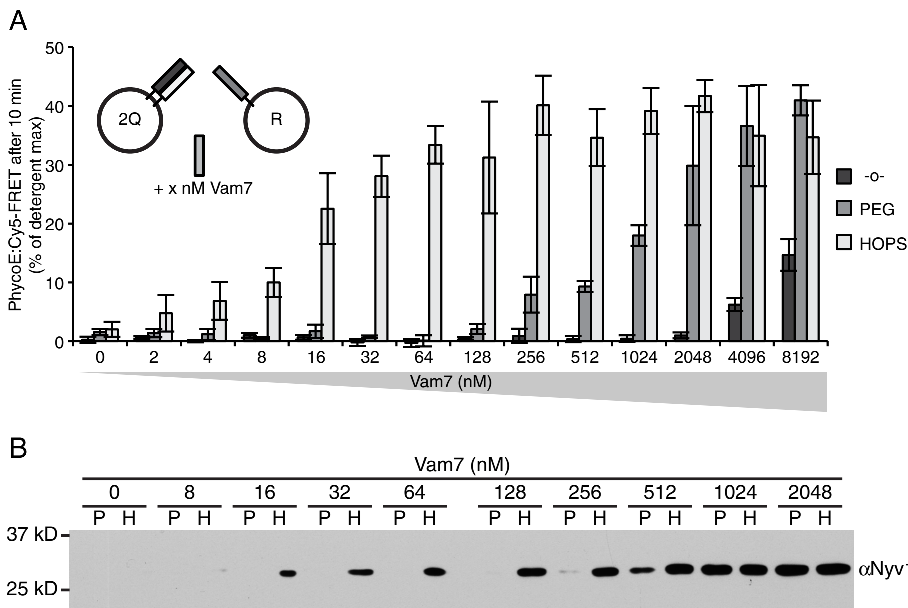

FIGURE 2: Only HOPS facilitates fusion in 2Q-1R reactions at low levels of Vam7. (A) Content-mixing reactions of 2Q-RPLs (Vam3, Vti1) and 1R-RPLs (Nvy1) with increasing concentrations of Vam7 in the presence of either 2% (wt/vol) PEG or 100 nM HOPS. Percentages of content mixing after 10 min. (B) Western blot showing the amount of Nyv1 that coimmunoprecipitates with Vam3 (see *Materials and Methods* for details) after 10 min in reaction conditions as in A. P, H, incubations containing PEG [2% (wt/vol)] or HOPS (100 nM), respectively. Data are the means of three independent experiments.

this combination, proteoliposomes bore Ypt7p and either Vam3p or Nyv1p, with HOPS, Vam7p, and soluble-Vti1p (sVti1p) lacking the normal apolar membrane anchor added to initiate fusion. Fusion depended on the concentration of the two soluble SNAREs, with HOPS supporting more fusion at intermediate soluble SNARE levels than PEG (Figure 5A). To determine whether HOPS simply catalyzes the assembly of any combination of separate SNAREs or exhibits a specificity for the Qc SNARE Vam7p, we performed fusion assays with 100 nM HOPS or 2% PEG and with varied Vam7p concentrations in the presence of a constant, high level of sVti1p (Figure 5B) or with varied sVti1p in the presence of a constant, high level of Vam7p (Figure 5C). Whereas both PEG- and HOPS-supported fusion diminished in parallel at lower concentrations of sVti1p (Figure 5C), only HOPS supported fusion at low levels of Vam7p (Figure 5B).

When the 3 Q-SNAREs were fully preassembled into a 3Q-SNARE complex (Fukuda *et al.*, 2000) during the lengthy dialysis of proteoliposome preparation, 2% PEG was as or more capable than 100 nM HOPS of supporting fusion (Figure 1). Thus HOPS catalysis (Figure 5) enables low levels of Vam7p (but not sVti1p) to function in fusion.

Whereas PEG promotes liposome tethering, it also can precipitate proteins (Ingham, 1990) and is capable of triggering fusion even in the absence of proteins (Lentz, 2007). We therefore sought to confirm our findings with an independent tethering system. Proteoliposomes were prepared with Ypt7p and a biotinylated lipid, as well as with Nyv1p, the three vacuolar Q-SNAREs, two Q-SNAREs (Vti1p and Vam3p), or one Q-SNARE (Vam3p). Because the content mixing assay used throughout this study is also based on the tight interaction of biotin and streptavidin, it is incompatible with tethering via streptavidin and biotinylated lipid. Therefore lipid mixing was used as readout of fusion for the experiments with this alternative tethering system. Comparable rates of fusion of the 3Q- and 1R-RPLs were supported by either HOPS or added streptavidin but not by streptavidin that had been presaturated with free biotin (Figure 6A). When fusion incubations did not have fully preassembled 3Q-SNARE subcomplexes (Figures 6, B–D), fusion in the absence of HOPS was seen only at the very high Vam7p concentrations (e.g., 4 µM) characteristic of tethering-agent-independent fusion (compare Figure 2A, black bars), as confirmed by the equivalence of the fusion signals seen with streptavidin or biotin-saturated streptavidin (Figure 6, B–D, gray and black bars, respectively). HOPS-mediated fusion of either 2Q-RPLs (with added Vam7p) or 1Q (Vam3p)-RPLs (with added Vam7p and sVti1p) with 1R-RPLs was supported at equivalent levels over a broad range of added Vam7p (Figure 6, B and C), whereas the rate of fusion of 1Q- and 1R-RPLs declined steadily as the sVti1p level declined below 1 µM (Figure 6D). Thus the inability of tethering systems other than HOPS to support fusion at low concentrations of free Vam7p cannot be ascribed to a peculiar property of one or another tethering system; instead, HOPS is specifically able to catalyze the PI(3)P-facilitated entry of low concentrations of Vam7p into fusion-competent SNARE complexes.

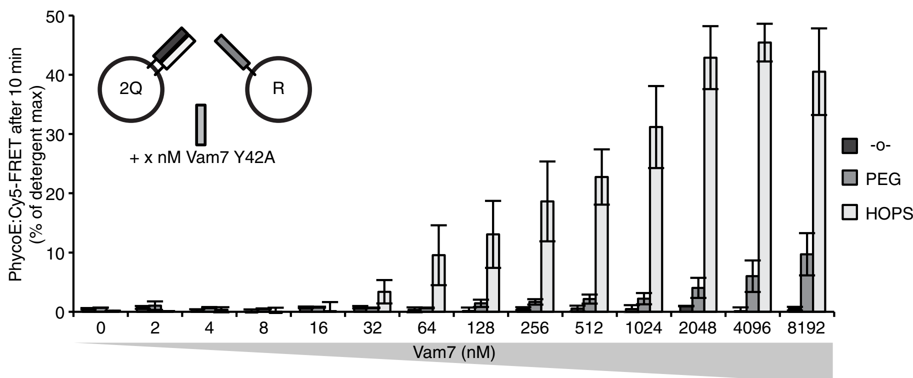

FIGURE 3: Vam7p recognition of PI(3)P promotes tethering-dependent 2Q-1R fusion reactions. Content-mixing reactions of 2Q-RPLs (Vam3, Vti1) and 1R-RPLs (Nvy1) with increasing concentration of mutant Vam7 (Y42A) in the presence of either control buffer (-o-), 2% (wt/vol) PEG, or 100 nM HOPS. Percentages of content mixing after 10 min. Data are the means of three independent experiments.

DISCUSSION

Although *trans*-SNARE complexes can form spontaneously, there are several indicators that catalysis of SNARE assembly may provide physiological speed and specificity. First, in model reconstitutions in which proteoliposomes are formed during prolonged dialysis, SNARE proteins can assemble into complementary *cis*-SNARE subcomplexes, although the uncatalyzed assembly of such SNARE complexes proceeds rather slowly (Hickey and Wickner,

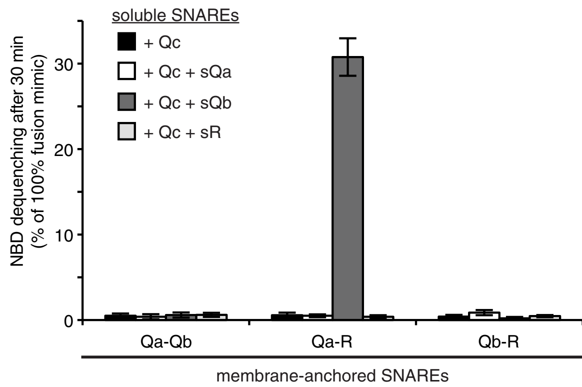

FIGURE 4: The Qb SNARE Vti1 can support fusion without its transmembrane domain. Lipid-mixing reactions of pairs of donor and acceptor proteoliposomes, each bearing one membrane-anchored SNARE (Qa, Vam3; Qb, Vti1; R, Nyv1), in the presence of Vam7 (Qc) and soluble versions of SNAREs without their transmembrane domains as indicated (4 µM each). All reactions contained 100 nM HOPS. Percentages of lipid mixing after 30 min as mean of three independent experiments.

2010). Once preassembled, vacuolar 3Q-SNARE RPLs can rapidly fuse with 1R-RPLs as long as some means of tethering is provided (Figures 1 and 6A). Previous results (Fukuda *et al.*, 2000; Mima *et al.*, 2008; Izawa *et al.*, 2012) suggested that proteoliposomes with vacuolar SNAREs alone could readily fuse (as measured by fluorescent lipid dequenching) in such a 3Q-1R setup without tethering agents. Our recent data (unpublished), however, reveal that the observed lipid dequenching does not necessarily reflect fusion activity. As measured by protected lumenal compartment mixing, tethering is an essential prerequisite for fusion and can be achieved by the physiological combination of Ypt7p and HOPS (Figure 1A), the addition of PEG (Figure 1B), biotinylated lipid and streptavidin (Figure 6A), or even by Vam7p or its PX domain alone at unphysiologically high concentrations (Supplemental Figure S1) as previously indicated (Xu and Wickner, 2012). Yet when vacuolar 2Q-SNARE RPLs are mixed with R-SNARE RPLs and physiological concentrations of Vam7p are added along with a synthetic tethering agent, fusion is extremely slow (Figures 3 and 6B). Second, Sec18p (NSF) and Sec17p (α-SNAP) are constantly present and can disassemble functional SNARE complexes, blocking fusion (Mima *et al.*, 2008). HOPS, but not synthetic tethering agents, can provide relief from inhibition by these SNARE-disassembly chaperones (Hickey and Wickner, 2010; Xu *et al.*, 2010). A third reason for SNARE assembly catalysis is that spontaneous SNARE complex assembly can be promiscuous, creating nonfusogenic complexes of the wrong SNAREs (Fasshauer *et al.*, 1999; Yang *et al.*, 1999). Because rapid and efficient fusion can be reconstituted with only the four vacuolar SNAREs, the Rab GTPase Ypt7p, HOPS, Sec17p, and Sec18p (Stroupe *et al.*, 2009), the search for SNARE complex assembly catalysts is narrowed to a few candidate proteins. Our present studies show that HOPS catalyzes the entry of Vam7p into fusogenic SNARE complexes.

HOPS acts at each stage of docking and fusion, fulfilling several distinct functions. HOPS and the Rab Ypt7p are necessary for tethering (Mayer and Wickner, 1997; Stroupe *et al.*, 2006; Hickey and Wickner, 2010) and can fulfill this function independent of any SNARE (Hickey and Wickner, 2010). HOPS also proofreads the SNAREs for vacuole fusion, with respect to both their 0-layer composition and position and their N-domains (Starai *et al.*, 2008). We

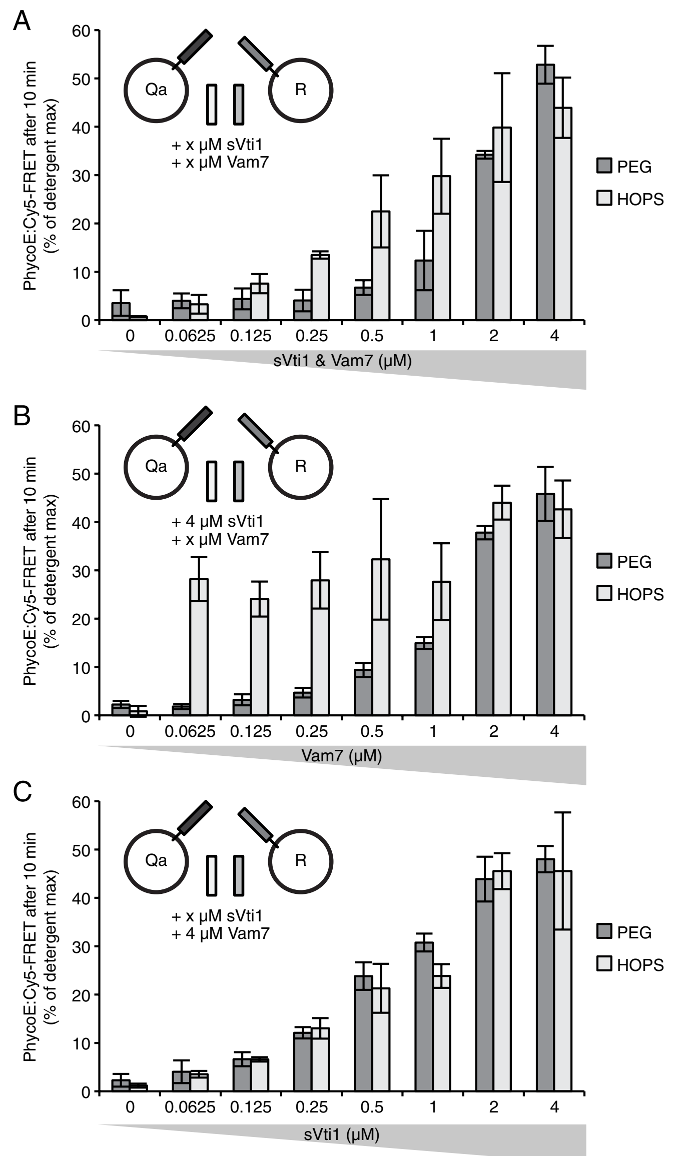

FIGURE 5: HOPS specifically recruits Vam7. Content-mixing reactions of Qa-RPLs (Vam3) and 1R-RPLs (Nvy1) in the presence of either 2% (wt/vol) PEG or 100 nM HOPS, with increasing levels of Vam7 and a soluble version of Vti1 (sVti1; A), a constant high level of sVti1 and varied levels of Vam7 (B), or a constant high level of Vam7 and varied levels of sVti1 (C). Percentages of content mixing after 10 min. Data are the means of three independent experiments.

reported that HOPS protects *trans*-SNARE (but not *cis*-SNARE) complexes from disassembly by Sec17p and Sec18p (Xu *et al.*, 2010); this may also underlie its proofreading function. In RPL fusion studies, HOPS radically alters the effect of addition of Sec17p/Sec18p, changing them from agents that block fusion to agents that strongly promote fusion (Mima *et al.*, 2008). Other tethering agents, such as PEG, do not prevent Sec17p/Sec18p inhibition of fusion (Hickey and Wickner, 2010). This fusion synergy of HOPS and Sec17p/Sec18p is even seen when fusion is measured with RPLs bearing complementary SNAREs (mixtures of R-SNARE RPLs and 3Q-SNARE RPLs or of R-SNARE RPLs and 2Q-SNARE RPLs plus Vam7p), in which case there is no obvious reason for *cis*-SNARE complex disassembly (Mima *et al.*, 2008). We have now reported a

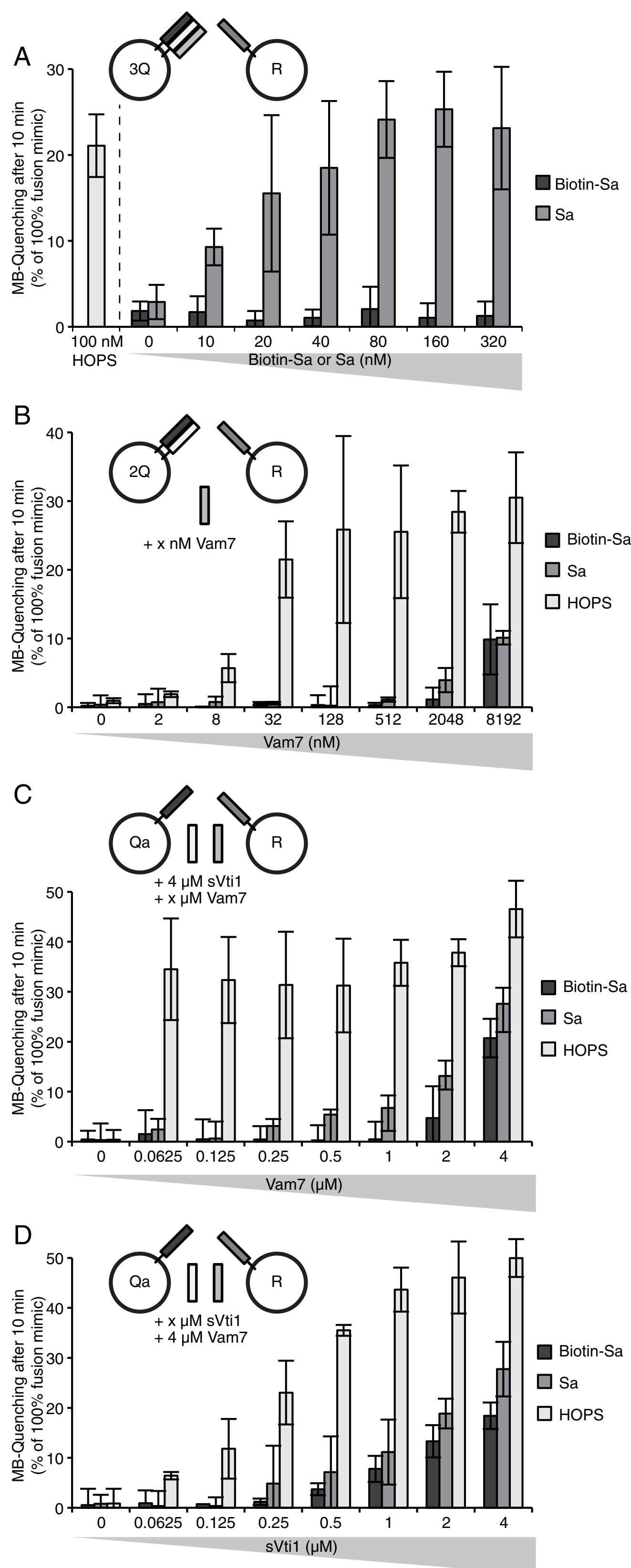

FIGURE 6: Alternative tethering, via streptavidin and biotinylated lipid, cannot replace HOPS for Vam7p functional recruitment. (A) Lipid-mixing reactions of 3Q-RPLs (Vam3, Vti1, Vam7) and 1R-RPLs (Nvy1) in the presence of increasing concentrations of streptavidin (Sa) or biotin-saturated streptavidin (Biotin-Sa); a reaction with 100 nM HOPS is shown for comparison. (B) Fusion reactions of 2Q-RPLs (Vam3, Vti1) and 1R-RPLs (Nvy1) with increasing concentration of Vam7 in the presence of 100 nM biotin-saturated streptavidin (Biotin-Sa), 100 nM streptavidin (Sa), or 100 nM HOPS. (C) Fusion reactions of Qa-RPLs (Vam3) and 1R-RPLs (Nvy1) with a constant high level of sVti1 and increasing levels of Vam7 in the presence of 100 nM biotin-saturated streptavidin (Biotin-Sa), 100 nM streptavidin (Sa), or 100 nM HOPS. (D) Fusion reactions of Qa-RPLs (Vam3) and 1R-RPLs (Nvy1) with a constant high level of Vam7 and increasing levels of sVti1 in the presence of 100 nM biotin-saturated streptavidin (Biotin-Sa), 100 nM streptavidin (Sa), or 100 nM HOPS. Percentages of lipid mixing after 10 min as the mean of two independent experiments.

fifth function of HOPS, catalyzing the entry of limiting concentrations of Vam7p into fusogenic SNARE complexes. At a minimum, the catalysis includes that the direct affinity of HOPS for Vam7p allows HOPS to provide a direct and essential contribution to high-affinity binding of Vam7p to the membrane. This is in full accord with prior studies, which showed selective (Stroupe *et al.*, 2006), but by no means exclusive (Lobingier and Merz, 2012), affinity of HOPS for Vam7p. HOPS, along with PI(3)P (Cheever *et al.*, 2001) and acidic lipids (Karunakaran and Wickner, 2013), is required for Vam7p membrane association. This may be the totality of HOPS catalysis of Vam7p assembly into SNARE complexes, or HOPS may bind the other SNAREs (Krämer and Ungermann, 2011; Lobingier and Merz, 2012) simultaneously and catalyze their association with membrane-bound Vam7p by inducing conformational changes. Further studies will be essential to understand the nature of this catalysis. A previous study from our lab (Hickey and Wickner, 2010) suggested that HOPS facilitates *trans*-SNARE complex formation only indirectly by tethering membranes. This interpretation, however, needs to be revised in light of the present findings, which in fact fully agree with earlier data that already indicated that HOPS is better than PEG at supporting the fusion of 1R- and 2Q-SNARE RPLs with added Vam7p (Hickey and Wickner, 2010, Figure 6F).

It is unclear which combinations of the six HOPS subunits (Vps 11, 16, 18, 33, 39, and 41p) fulfill each of the multiple HOPS functions. Although individual subunits and a number of subcomplexes have been isolated and shown to have relevant binding affinities, full HOPS function for fusion has yet to be reconstituted with a mixture of subcomplexes and/or individual subunits. Two HOPS subunits, Vps39p and Vps41p, have direct affinity for Ypt7p (Ostrowicz *et al.*, 2010), creating the potential for one HOPS complex to bind to two membrane-bound Ypt7p and effect tethering. Vps16p and Vps18p bind to the PX domain of Vam7p, whereas Vps16, 18, and 33 bind to Q-SNARE N-domains (Krämer and Ungermann, 2011; Lobingier and Merz, 2012). Vps33p alone, a Sec1-Munc18 family member, can bind to the 4SNARE complex or to the SNARE domain of several individual vacuolar SNAREs (Lobingier and Merz, 2012). These initial mappings will require extensive genetic and structural analysis for a more sophisticated understanding of HOPS to emerge. Additional questions include whether HOPS has direct interactions with other SNAREs or SNARE chaperones, whether its associations with the Rab and with SNAREs are exclusive of each other, whether it has a direct role in the lipid rearrangements whereby two bilayers fuse, and when and how it is released from its Rab and SNARE associations.

There are other large protein complexes with parallel functions to HOPS in other organelle fusion systems. Although HOPS is the only reported large tethering complex with a bound Sec1-Munc18 subunit, the Sec6 subunit of the exocyst will directly bind Sec1 (Morgera *et al.*, 2012), and the COG tethering complex will bind its

Sec1-Munc18 family protein, Vps45p (Laufman *et al.*, 2013). In both cases, this interaction was proposed to have functional significance for the formation of competent SNARE complexes. Other tethering complexes may associate with their respective Sec1-Munc18 proteins as well, but apparently this association is often transient and only persists during the current isolation protocols for HOPS. Two other fusion reactions have been reconstituted with all-purified components—the neuronal synaptic fusion (Ma *et al.*, 2013) and homotypic endosome fusion (Ohya *et al.*, 2009). Strikingly, neither of these reconstitutions included a large tethering complex, although the Munc13-1 MUN domain is related to subunits of tethering complexes from diverse membrane compartments such as exocyst, Dsl1, COG, and GARP (Li *et al.*, 2011). Hence the MUN domain may be performing a related function, although it is unclear whether this function requires formation of oligomers that may include the SM protein Munc18-1 (a homologue of the HOPS Vps33p subunit). Many fusion reactions require large tethering complexes, such as Dsl1 at the endoplasmic reticulum, TRAPP, COG, and GARP complexes at the Golgi, CORVET at the endosome, and the exocyst at the plasma membrane (reviewed in Yu and Hughson, 2010). Functional in vitro assays will be needed to allow direct comparisons of their respective functions to those of HOPS.

We propose that SNARE complex assembly for most or all organelles will require catalysis to achieve adequate rates, ensure nonpromiscuous pairings, and resist futile cycles of premature Sec17p/Sec18p (NSF/αSNAP) disassembly. This postulate is supported by our present findings and those of Ma *et al.* (2013), who showed that syntaxin associated with Munc18-1 (instead of with SNAP-25) could assemble into fusion-competent SNARE complexes in an NSF/SNAP-resistant manner. Further studies will be needed to test the generality of this idea.

MATERIALS AND METHODS

Proteins and reagents

The SNARE proteins GST-TEV-Vam3p, Vti1p, and Nvy1p were isolated as previously described (Mima *et al.*, 2008; Zucchi and Zick, 2011). Vam7 (wild type or Y42A mutant) was purified as described (Schwartz and Merz, 2009), except that the secondary purification via nickel nitrilotriacetic acid affinity chromatography was omitted.

A soluble variant of Vti1 without its transmembrane domain was purified as a MBP-fusion protein, MBP-TEV-Vti1(1-194). pMBP-Parallel1_sVti1 (a kind gift of Youngsoo Jun [Gwangju Institute of Science and Technology, Gwangju, South Korea]) was transformed into Rosetta (DE3) cells (EMD Millipore, Billerica, MA). Luria–Bertani medium, 200 ml, containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol was inoculated with a single colony. After overnight growth at 37°C, the culture was added to 2× 3l of Terrific Broth medium containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol and shaken at 37°C to an OD_{600} of 1.5. Isopropyl β-D-1-thiogalactopyranoside was added to 0.5 mM. After 3 h of growth at 37°C, bacteria were collected by centrifugation (4000 × *g*, 5 min, 4°C). The pellet was resuspended in 50 ml of 20 mM TrisCl, pH 8.0, 200 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride and snap-frozen in liquid nitrogen. Thawed cells were subjected to three passes through a French press at 5 kpsi. The lysate was centrifuged (2°C, 1 h, 50,000 rpm, SW 60Ti rotor [Beckman Coulter, Brea, CA]). The supernatant was added to 30 ml of amylose resin (New England BioLabs, Ipswich, MA) preequilibrated with wash buffer (20 mM TrisCl, pH 8.0, 200 mM NaCl) and nutated at 4°C for 2 h. The resin was then poured into a 2.5-cm-diameter column and washed with five volumes of wash buffer containing 5 mM 2-mercaptoethanol. MBP-sVti1 was eluted with wash buffer containing 10 mM maltose. Protein fractions were pooled, dialyzed against RB150 (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]–NaOH, pH 7.5, 150 mM NaCl, 10% [vol/vol] glycerol), aliquoted, and snap-frozen in liquid nitrogen.

GST-Ypt7p was isolated as described (Hickey *et al.*, 2009) with modifications. After 36 l of culture was harvested, the CSY29 cells were resuspended in DTT wash buffer (100 mM TrisCl, pH 9.4, 10 mM dithiothreitol; 2.5 ml per 3×10^{10} cells) and incubated at room temperature for 10 min. Cells were sedimented (5000 rpm; JLA 10.500 [Beckman Coulter], 5 min, 23°C), and the pellet was resuspended in spheroplasting buffer (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 0.2% [wt/vol] dextrose, 600 mM sorbitol, 50 mM potassium phosphate [KPi], pH 7.5) to an OD_{600} of ~150. Lyticase was added to 75 µg/l, and the suspension was incubated at 30°C for 60–90 min with agitation. Spheroplasts were collected at 10,000 rpm in a JA-14 rotor (Beckman Coulter) for 5 min, then resuspended in 325 ml of extraction buffer (20 mM HEPES-NaOH, pH 7.4, 500 mM NaCl, 10% [vol/vol] glycerol, 1 mM $MgCl_2$, 0.5% [wt/vol] Thesit). After incubation on ice for 1 h with agitation, lysates were cleared via two consecutive centrifugations (4°C, 45 Ti rotor [Beckman Coulter]; 15,000 rpm for 10 min, then 40,000 rpm for 35 min). The supernatant was added to 60 ml of glutathione–Sepharose 4B (GE Healthcare, Pittsburgh, PA) preequilibrated with extraction buffer and nutated for 2–3 h at 4°C. After the resin was washed (in 200 ml, 4°C, 3 min at 2000 × *g*) three times with extraction buffer and twice more with wash buffer (20 mM TrisCl, pH 8.0, 50 mM NaCl, 10% [vol/vol] glycerol, 1 mM $MgCl_2$, 0.5% [wt/vol] β-octylglucoside), it was poured into a 2.5-cm-diameter column. Elution was with wash buffer containing 20 mM glutathione. The peak fractions were pooled, aliquoted, and snap-frozen in liquid nitrogen. The degree of prenylation was determined after SDS–PAGE and colloidal Coomassie staining with UN-SCAN-IT gel 5.3 software (Silk Scientific, Orem, UT).

The purification of HOPS (Hickey and Wickner, 2010) was modified as follows. Strain CHY61 was inoculated into 50 ml of CSM-his-leu-trp-ura plus 200 µg/ml G418 plus 100 µg/ml clonNAT. After growth overnight at 30°C, the culture was diluted into 600 ml of fresh growth medium and grown for another 8 h at 30°C. Four 6-l flasks with 2.3 l of yeast extract/peptone plus 2% (wt/vol) galactose were inoculated from this culture to an OD_{600} of 0.06, and growth was continued overnight at 30°C. After no more than 16 h, the cells were harvested by centrifugation at 5200 rpm in a JLA-10.500 rotor for 5 min at 23°C, resuspended in 2 l of DTT wash buffer, and incubated for 10 min at 30°C. Cells were again collected by centrifugation at 5200 rpm in a JLA-10.500 rotor for 5 min at room temperature and resuspended in 600 ml of spheroplasting buffer (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 0.2% [wt/vol] dextrose, 600 mM sorbitol, 50 mM KPi, pH 7.5). Lyticase was added to 110 µg/l, and the suspension was incubated at 30°C for 60 min with agitation. Additional components were added to bring the suspension to 1 l in extraction buffer (20 mM HEPES-NaOH, pH 7.8, 400 mM NaCl, 10% [vol/vol] glycerol, 1% [wt/vol] Triton X-100, 5 mM 2-mercaptoethanol). The suspension was then incubated at 4°C with agitation for 1 h. After centrifugation (45,000 rpm, 45 Ti rotor, for 25 min, 4°C), the supernatants were passed through a 0.22-µm filter and frozen dropwise in liquid nitrogen. The supernatants from two growths were thawed, pooled, added to 60 ml of glutathione–Sepharose 4B, preequilibrated with extraction buffer, and nutated for 60–90 min at 4°C. The resin was then poured into a 5-cm-diameter column and washed with three column volumes of extraction buffer. After washing with four column volumes of wash buffer (20 mM HEPES-NaOH, pH 7.8, 400 mM NaCl, 200 mM

sorbitol, 10% [vol/vol] glycerol, 0.004% [wt/vol] Triton X-100, 1 mM 2-mercaptoethanol), 120 ml of elution buffer (wash buffer + 10 mM glutathione) was applied, and fractions (4 ml each) were collected. Peak fractions were pooled and concentrated with a 100K MWCO Amicon Ultra-15 (EMD Millipore) centrifugal filter device. After overnight incubation with TEV protease (equimolar concentration) at 4°C, samples were aliquoted and snap-frozen in liquid nitrogen.

Reconstituted Rab/SNARE proteoliposomes

Proteoliposomes for content-mixing assays were prepared by detergent dialysis in RB150/Mg^{2+} (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 10% glycerol [vol/vol]) as described (Zucchi and Zick, 2011) from lipid mixes mimicking the vacuolar composition (43.6 mol% 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine, 18 mol% 1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine, 18 mol% soy L-α-phosphatidylinositol, 4.4 mol% 1,2-dilinoleoyl-*sn*-glycero-3-phospho-L-serine, 2 mol% 1,2-dilinoleoyl-*sn*-glycero-3-phosphate, 1 mol% 16:0 1,2-dipalmitoyl-*sn*-glycerol [all from Avanti Polar Lipids, Alabaster, AL]; 8 mol% ergosterol [Sigma-Aldrich, St. Louis, MO], 1 mol% each of di-C16 phosphatidylinositol 3-phosphate and phosphatidylinositol 4,5-bisphosphate [Echelon Biosciences] and 3 mol% 7-nitrobenz-2-oxa-1,3-diazole [NBD]–1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine [DPPE; Life Technologies, Carlsbad, CA]) for donor liposomes or 3 mol% Marina-Blue-DPPE for acceptor, subsets of the four vacuolar SNAREs, and Ypt7p, entrapping Cy5-labeled streptavidin or biotinylated R-phycoerythrin. Molar protein:lipid ratio was 1:2500 for SNAREs and 1:2000 for Ypt7p. Isolation after reconstitution was achieved by floatation on a three-step Histodenz gradient (35, 25% Histodenz [wt/vol] and RB150/Mg^{2+}). Histodenz (Sigma-Aldrich) solutions were prepared as 70% stock solution in modified RB150/Mg^{2+} with a reduced concentration (2% [vol/vol]) of glycerol to compensate for the osmotic activity of the density medium; lower-concentration solutions were obtained by dilution with RB150/Mg^{2+}. RPLs for experiments based on lipid dequenching were prepared with 1.5 mol% of NBD-DPPE and rhodamine-DPPE for donor RPLs and without fluorescent lipids for acceptor RPLs. RPLs for experiments based on tethering via streptavidin were prepared with 0.1 mol% 18:1 Biotinyl Cap PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(cap biotinyl) [Avanti Polar Lipids]) and without entrapped content markers.

RPL fusion assays

Proteoliposome fusion was primarily measured by the resulting lumenal content mixing. Fusion reactions of 20 µl were assembled from four premixes of 5 µl each: two mixes of donor or acceptor RPLs (250 µM lipid each) in RB150/Mg^{2+} with 5 µM streptavidin, a mix of soluble SNAREs (or respective buffers), and tethering agents (HOPS, PEG 8000). All components were incubated individually at 27°C for 10 min and then combined in wells of 384-well plates to initiate the reaction. The plates were incubated at 27°C in a fluorescence plate reader for 30–60 min, and FRET between PhycoE and Cy5 (excitation, 565 nm; emission, 670 nm; cutoff, 630 nm) was measured at intervals of 5–15 s in a SpectraMax Gemini XPS (Molecular Devices, Sunnyvale, CA) fluorescent plate reader. Maximal values were determined after addition of 0.1% (wt/vol) Thesit to samples that had not received streptavidin. NBD/rhodamine lipid-dequenching reactions were performed in the same way, but the NBD fluorescence (excitation, 460 nm; emission, 538 nm; cutoff, 515 nm) was measured and its intensity increase expressed as percentage of fluorescence intensity recorded for 100% fusion mimic RPLs (i.e., containing 0.75 mol% of NBD-DPPE and rhodamine-DPPE).

For reactions based on tethering via streptavidin, fusion was assessed as lipid mixing through the quenching of Marina-Blue fluorescence by NBD. In this case, RPL premixes did not contain any streptavidin, but streptavidin (or streptavidin that had been saturated with a 10-fold molar excess of free biotin as control) was added as tethering agent. Reactions were also incubated in 384-well plates at 27°C for 30 min, and Marina-Blue fluorescence (excitation, 370 nm; emission, 465 nm; cutoff, 420 nm) was continuously measured at intervals of 5–15 s. To estimate the fraction of liposomes that underwent fusion, RPLs that mimic full fusion (i.e., with 1.5 mol% Marina-Blue-PE and NBD-PE) were prepared and used as a 100% fusion mimic.

trans-SNARE complex assay

To estimate the amount of *trans*-SNARE complex that formed during a reaction, the amount of Nyv1 that coimmunoprecipitated with Vam3 was determined. A 40-µl fusion reaction was incubated for 10 min at 27°C, placed on ice, and diluted 10-fold in IP buffer (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 1% [wt/vol] bovine serum albumin, 1% [wt/vol] β-octylglucoside) containing 50 µg/ml of affinity-purified anti-Vam3 antibody. The mix was incubated nutating at room temperature for 2 h before 20 µl of protein A magnetic beads (Thermo Scientific, Portsmouth, NH) were added, and nutation was continued for 2 h. After the beads were washed three times with 500 µl of IP buffer, samples were eluted in 100 µl of reducing SDS sample buffer at 95°C for 10 min. Aliquots (20 µl) of each sample were subjected to SDS–PAGE and immunoblotting with anti-Nyv1 antibody.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Heath Grant R01 GM23377-37. We thank Amy Orr, Holly Jakubowski, and Deborah Douville (Geisel School of Medicine at Dartmouth) for expert assistance and Youngsoo Jun for providing expression plasmids. M.Z. was supported by a research fellowship (ZI 1339/1-1) from the German Research Foundation.

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