



Tethering guides fusion-competent *trans*-SNARE assembly

Hongki Song^a and William Wickner^{a,1}

^aDepartment of Biochemistry and Cell Biology, Geisel School of Medicine at Dartmouth, Hanover, NH 03755-3844

Contributed by William Wickner, May 23, 2019 (sent for review May 3, 2019; reviewed by Josep Rizo and Philip D. Stahl)

R-SNAREs (soluble *N*-ethylmaleimide-sensitive factor receptor), Q-SNAREs, and Sec1/Munc18 (SM)-family proteins are essential for membrane fusion in exocytic and endocytic trafficking. The yeast vacuolar tethering/SM complex HOPS (homotypic fusion and vacuole protein sorting) increases the fusion of membranes bearing R-SNARE to those with 3Q-SNAREs far more than it enhances their *trans*-SNARE pairings. We now report that the fusion of these proteoliposomes is also supported by GST-PX or GST-FYVE, recombinant dimeric proteins which tether by binding the phosphoinositides in both membranes. GST-PX is purely a tether, as it supports fusion without SNARE recognition. GST-PX tethering supports the assembly of new, active SNARE complexes rather than enhancing the function of the fusion-inactive SNARE complexes which had spontaneously formed in the absence of a tether. When SNAREs are more disassembled, as by Sec17, Sec18, and ATP (adenosine triphosphate), HOPS is required, and GST-PX does not suffice. We propose a working model where tethering orients SNARE domains for parallel, active assembly.

membrane fusion | yeast vacuoles | SNARE | HOPS

Intracellular membrane fusion requires conserved proteins and lipids (1). Rab-family GTPases bind effector complexes which tether membranes to initiate docking (2). SNARE (soluble *N*-ethylmaleimide-sensitive factor receptor) proteins often consist of an N domain, a characteristic heptad-repeat SNARE domain, and a C-terminal membrane anchor (3). SNARE domains assemble into a 4-subunit complex of coiled-coils with each α -helical SNARE domain burying a largely apolar face into the interior of the complex. Arginyl and glutaminyl residues, termed the 0-layer, form a polar center of this otherwise apolar interior. The SNARE domains associate in parallel, with their N termini at one end of the coiled-coil and their C termini at the other (4–6), although an antiparallel association has been demonstrated as well (7, 8). SNAREs are in four conserved families, termed R, Qa, Qb, and Qc, and SNARE complexes have an RQaQbQc composition (9). SNARE complexes are either in *trans*, when anchored to two apposed membranes before fusion, or in *cis* on one membrane after fusion. SNAREs can assemble spontaneously, but their assembly can also be catalyzed by Sec1/Munc18 (SM) proteins, alone or as part of a multisubunit protein (10–12). Synaptic membrane fusion uses SNAREs and SM proteins, but also has additional proteins (e.g., synaptotagmin, complexin, and Munc13) which confer parallel assembly of the four SNARE domains (8), Ca²⁺ regulation, and speed. The interplay between fusion proteins has received extensive study, yet central aspects of how they cooperate for fusion are unclear.

We study membrane fusion with vacuoles (lysosomes) from *Saccharomyces cerevisiae*. Vacuoles undergo fission and homotypic fusion. At steady state, many yeast strains have one to three large vacuoles which are readily visualized in the light microscope. Mutants which block fusion but allow unimpeded fission allow cell growth on rich media with strikingly small vacuole morphology and were termed *vam* mutants (13). The *VAM* genes encode all six subunits of the HOPS (homotypic fusion and vacuole protein sorting) complex, which is required for homotypic fusion and vacuole protein sorting, as well as the vacuolar Rab-family GTPase Ypt7 and two of the SNAREs. Fusion also

requires several proteins that are shared with trafficking in the exocytic pathway; being essential for growth, they were not identified by the *VAM* screen. Vacuole fusion has been studied *in vivo*, with the isolated organelle *in vitro*, and with purified proteins reconstituted into proteoliposomes of physiological lipid composition and fluidity. Fusion requires Ypt7 (14) and its large hetero-hexameric effector complex HOPS, comprised of Vps11, 16, 18, 33, 39, and 41p subunits (15–17). The Vps33 subunit is the SM family member of the vacuole and has been shown to have conserved grooves which bind the R- and Qa-SNARE domains, in parallel and in register (10). Vps39 and Vps41 subunits of HOPS have direct affinity for Ypt7 (18), explaining how HOPS and Ypt7 on each membrane can suffice for membrane tethering (19). Vacuoles have an R-SNARE (Nyv1), Qa-SNARE (Vam3), Qb-SNARE (Vti1), and Qc-SNARE (Vam7), referred to hereafter as simply R, Qa, Qb, and Qc. Each has a C-terminal apolar transmembrane anchor except for Qc, which has an N-terminal PX domain which contributes to membrane binding by its affinities for PI3P (20) and for HOPS (21). As purified, vacuoles have SNAREs as 4-SNARE complexes, subcomplexes, and as membrane-anchored individual SNAREs, although Qc is only present as part of SNARE complexes (22). Fusion begins with ATP (adenosine triphosphate)-dependent disassembly of the *cis*-SNARE complexes on each vacuole by Sec18/NSF and Sec17/ α SNAP (23), freeing the SNAREs (including Qc; ref. 24) for later *trans*-SNARE complex assembly. The initial association of primed vacuoles, termed tethering, is mediated by HOPS and Ypt7 on each membrane (19, 21). SNAREs then assemble into *trans*-complexes linking R-SNAREs on one membrane to QaQbQc (3Q) complexes on its fusion partner (25). Fusion is enhanced by Sec17 and Sec18 binding to *trans*-SNARE complexes (26–28), which may provide a bulky wedge (29) and facilitate complete SNARE zippering (28).

Significance

R-SNARE (soluble *N*-ethylmaleimide-sensitive factor receptor) and the set of 3Q-SNAREs, anchored in separate membranes, can associate in *trans* in a coiled-coil tetramer. Proteoliposomes bearing only these SNAREs form abundant *trans*-complexes but fuse very poorly, while membrane association through even a synthetic tether allows rapid and functional SNARE complex assembly without catalysis by Munc18 or Munc13 family proteins. Tethering guides *trans*-SNARE associations to active conformations instead of the inactive *trans*-SNARE complexes which form in the absence of tethers.

Author contributions: H.S. and W.W. designed research, performed research, and wrote the paper.

Reviewers: J.R., UT Southwestern Medical Center; and P.D.S., Washington University School of Medicine.

Conflict of interest statement: W.W. and J.R. are coauthors on a 2017 Perspective article.

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¹To whom correspondence may be addressed. Email: William.Wickner@Dartmouth.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1907640116/-DCSupplemental.

Published online June 24, 2019.

Despite extensive study, it remains unclear when *trans*-SNARE complex assembly is spontaneous and when it is catalyzed by SNARE-binding sites on HOPS, including its SM subunit Vps33. It has been shown that coincubation of proteoliposomes bearing Ypt7 and the R-SNARE with other proteoliposomes bearing Ypt7 and the 3Q-SNARE supports substantial *trans*-SNARE pairing but only very slow fusion (30). When HOPS was added after 30 min, there was a sudden burst of fusion at ~100 times the prior rate, although HOPS only increased the level of *trans*-SNARE complex two- to fourfold (30). Proteoliposomes bearing concentrated SNAREs (1:2,000 molar ratio to lipid) spontaneously assembled more *trans*-SNARE complex than was seen after HOPS addition to proteoliposomes with dilute SNAREs (1:16,000 molar ratio to lipid), yet the latter had a far greater rate of fusion. It was not clear in this study whether HOPS was using the accumulated *trans*-SNARE pairs for fusion or whether the new SNARE complexes which HOPS elicited were more active. If the latter, was the capacity of HOPS for SNARE recognition and binding essential for it to catalyze the assembly of new, active *trans*-SNARE complexes?

We now report that rapid fusion of membranes bearing R-SNARE to those with 3Q-SNAREs requires neither catalysis of SNARE complex assembly nor activation of the accumulated spontaneous *trans*-SNARE complexes by a membrane tether. R- and 3Q-SNARE proteoliposomes fuse almost as well with optimal levels of the synthetic tether GST-PX as with HOPS, even though GST-PX lacks binding sites for SNAREs. GST-PX is a dimeric protein (31) which relies on each PX domain in the dimer binding to PI3P in each proteoliposome to support fusion. Without GST-PX, there is only very slow fusion of coincubated R- and 3Q-SNARE proteoliposomes, but addition of GST-PX triggers rapid fusion, as had been seen for HOPS. Fusion remains fully sensitive to competition by added sR, a soluble form of the R-SNARE without its membrane anchor, and thus this fusion uses SNARE complex assembled after the addition of the GST-PX tether rather than stable preassembled *trans*-SNARE complex. GST-PX does not replace HOPS in the presence of Sec17 and Sec18, SNARE chaperones which may inhibit (32) or promote (27, 28) fusion. Thus, tethering is required for efficient assembly of R with 3Q-SNAREs into a fusogenic structure, and HOPS or GST-PX provides such tethering. The SNARE-recognition functions of HOPS are not required for productively assembling R- with the preassembled complex of 3Q-SNAREs if the membranes are first tethered. Based on pioneering studies with neuronal SNAREs (7), we propose a working model of antiparallel SNARE assembly without prior membrane tethering and functional, parallel assembly when membranes are first tethered.

Results

Separate proteoliposome stocks were prepared with either of two luminal fluorescent protein markers, Cy5-streptavidin or biotinylated-phycoerythrin. Fusion mixes the luminal compartments (33), and the ensuing binding of biotin to streptavidin brings the Cy5 and phycoerythrin fluorophores into intimate contact, assayed by FRET (fluorescence resonance energy transfer). No fusion is seen without a tethering agent when the proteoliposomes bear R- or 3Q-SNAREs at a molar ratio to lipids of 1:16,000 (Fig. 1A). To test whether tethering per se would suffice for fusion, we employed a recombinant fusion between GST, the inherently dimeric (31) glutathione transferase, and a PX domain which binds phosphatidylinositol 3-phosphate. The GST-PX dimer could bind PI3P on the R- and 3Q-SNARE proteoliposomes to tether them together. Fusion is supported by the addition of GST-PX (Fig. 1A). Fusion only relies on the capacity of PX to recognize PI3P and not some inherent fusogenic capacity of the PX domain, as fusion is also seen with GST-FYVE (Fig. 1B). GST-PX fusion is ablated by the Y42A mutation in the PX domain (Fig. 2A), removing its

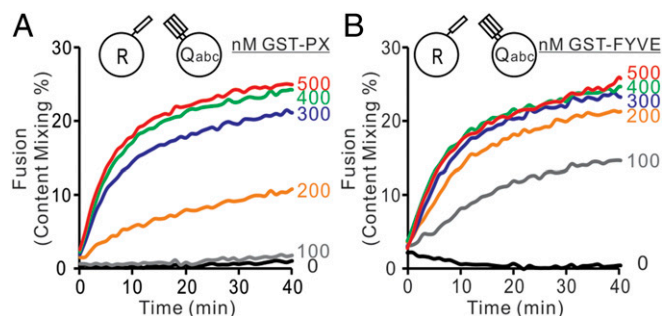


Fig. 1. GST-PX supports fusion through tethering. (A and B) Fusion reactions contained proteoliposomes bearing R or QaQbQc (3Q) at 1:16,000 SNARE:lipid molar ratios. Mixed proteoliposomes were incubated as described (34), but without albumin, glutathione, or DTT with 0–500 nM GST-PX (A) or GST-FYVE (B) for 40 min at 27 °C, and content mixing was assayed by FRET between the luminal markers (33). Kinetic curves in this figure are representative of $n \geq 3$ experiments; means and SDs for each experiment are presented in *SI Appendix, Fig. S1*.

PI3P affinity (20). Fusion relies on the presence of PI3P on both the R- and 3Q- bearing proteoliposomes (Fig. 2B). Liberation of the PX domain from GST dimer by thrombin cleavage (*SI Appendix, Fig. S2D*) removes its capacity to support fusion (Fig. 2C).

While GST-PX can tether membranes, it lacks the R- and Qa-SNARE domain recognition sites found in the Vps33 SM-family subunit of HOPS (10) as well as the HOPS affinity for Ypt7 (15); thus dimeric GST-PX has a purely tethering function. To compare the efficiency of fusion between R- and 3Q-proteoliposomes with optimal levels of HOPS or GST-PX, we prepared R- and 3Q-proteoliposomes with physiologically low 1:16,000, 1:32,000, and 1:64,000 molar ratio of SNAREs to lipid phosphorus and the HOPS receptor protein Ypt7 at 1:8,000 molar ratio to lipid (34). Optimal levels of HOPS and of GST-PX (*SI Appendix, Fig. S4*) gave comparable rates and extents of fusion (Fig. 3 and *SI Appendix, Fig. S3*), suggesting that HOPS largely functions here as a tether and that its SNARE recognition functions are not required in this intentionally simplified context, with the three preassembled Q-SNAREs and without Sec17 or Sec18.

trans-SNARE complex assembly can be competitively blocked by the addition of a soluble form of the R-SNARE, termed sR, which lacks its transmembrane anchor [Fig. 4, no sR (a, black) vs. with sR (b, gray); also ref. 35]. Ample *trans*-SNARE complexes have been shown to assemble during coincubation of R- and 3Q-SNARE proteoliposomes without tether (35); are these *trans*-SNARE complexes being used by the added GST-PX tether for fusion, or is fusion supported by the new *trans*-SNARE complexes assembled after GST-PX addition? R- and 3Q-SNARE proteoliposomes were preincubated together (Fig. 4, c, d; also see *SI Appendix, Fig. S5*) or separately (e, f) in the same buffer and at the same temperature for 30 min, then mixed with each other where separate and supplemented with buffer (c, e) or sR (d, f) immediately before GST-PX addition. Coincubation neither enhanced the kinetics of subsequent fusion after GST-PX addition (a vs. c) nor altered the sensitivity of the ensuing fusion to sR (b, d, and f), and thus fusion relied on the new *trans*-SNARE complexes rather than those which had been assembled before the addition of the tether.

Tethers Promote the Assembly of Functional *trans*-SNARE Pairs. HOPS addition to Ypt7/R- and Ypt7/3Q proteoliposomes only increased *trans*-SNARE levels two- to fourfold (35), and the synthetic tether of dimeric GST-PX had a similar effect. With R- and 3Q-SNARE proteoliposomes at molar ratios of SNARE:lipid of 1:4,000, 1:8,000, and 1:16,000, we directly assayed the rates

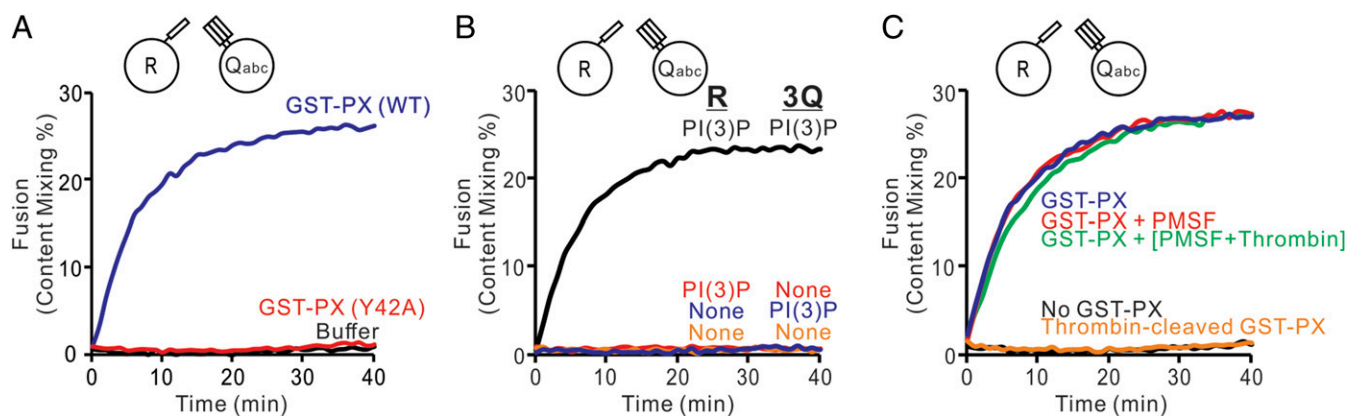


Fig. 2. Membrane tethering is essential for fusion. Fusion of proteoliposomes bearing R or 3Q at 1:16,000 molar ratio to lipid. (A) Incubations were performed with 500 nM GST-PX (WT), GST-PX(Y42A), or their buffer for 40 min at 27 °C. (B) Reactions had 500 nM GST-PX as in A, but with R- and 3Q-proteoliposomes with or without PI(3)P as indicated. (C) GST-PX has a thrombin cleavage sequence between its GST and PX domains. Cleavage by thrombin was terminated by adding PMSF (phenylmethyl sulfonyl fluoride) as indicated. Fusion incubations were supplemented with: buffer alone (black), GST-PX which had been incubated for 2 h at 4 °C with 1 unit/mL thrombin followed by addition of 200 μ M PMSF to block further thrombin activity (orange), GST-PX incubated for 2 h at 4 °C with thrombin that had been premixed with PMSF (green), GST-PX (blue), or GST-PX incubated with PMSF without thrombin (red). Kinetic curves of content-mixing assays in this figure are representative of $n \geq 3$ experiments; means and SDs for each experiment are presented in *SI Appendix, Fig. S2*.

of fusion and the extent of association of R-SNARE with the Qa member of the 3Q complex in *trans* after incubation without any tether or with GST-PX (Fig. 5). There was a very slow linear rate of fusion in reactions with high SNARE concentrations (1:4,000 molar ratio to lipid) and without tether, but the fusion rate was higher with GST-PX (Fig. 5A). At each SNARE:lipid ratio, fusion was more rapid and more SNARE complex had assembled in the presence of GST-PX (Fig. 5B and C, lanes 2, 4, and 6 vs. 1, 3, and 5). However, fusion was 23-fold more rapid for proteoliposomes with dilute SNAREs incubated with GST-PX than for proteoliposomes with concentrated SNAREs in the absence of GST-PX (Fig. 5B, lanes 6 vs. 1), while the latter actually had more *trans*-SNARE association than the former (Fig. 5C, lanes 1 vs. 6). The total SNARE complex measured here is the sum of the *trans*-SNARE complex, SNARE complex that had been *trans* but became *cis* as a result of fusion, and *cis*-

SNARE complex which had never been *trans* but assembled after fusion from R- and Q-SNAREs which were brought into the same membrane by fusion. Thus, a greater portion of the total SNARE complex will be “*trans*” when there has been very little fusion (lane 1, no GST-PX) than when there has been fusion (lane 6, plus GST-PX), and the relative amount of truly *trans*-SNARE complex will be even less where more fusion had occurred (Fig. 5C, lane 6 relative to lane 1). The presence of even a synthetic tether therefore allows the assembly of *trans*-SNARE complexes which are far more functional.

Is SNARE recognition by HOPS required in other contexts? GST-PX does not support the fusion of R- and 3Q-proteoliposomes in the presence of the physiological SNARE chaperones Sec17, Sec18, and ATP (Fig. 6 and *SI Appendix, Fig. S6*), which can disassemble *trans*-SNARE complexes (36) and block fusion without HOPS (37). Sec17, Sec18, and ATP are physiological

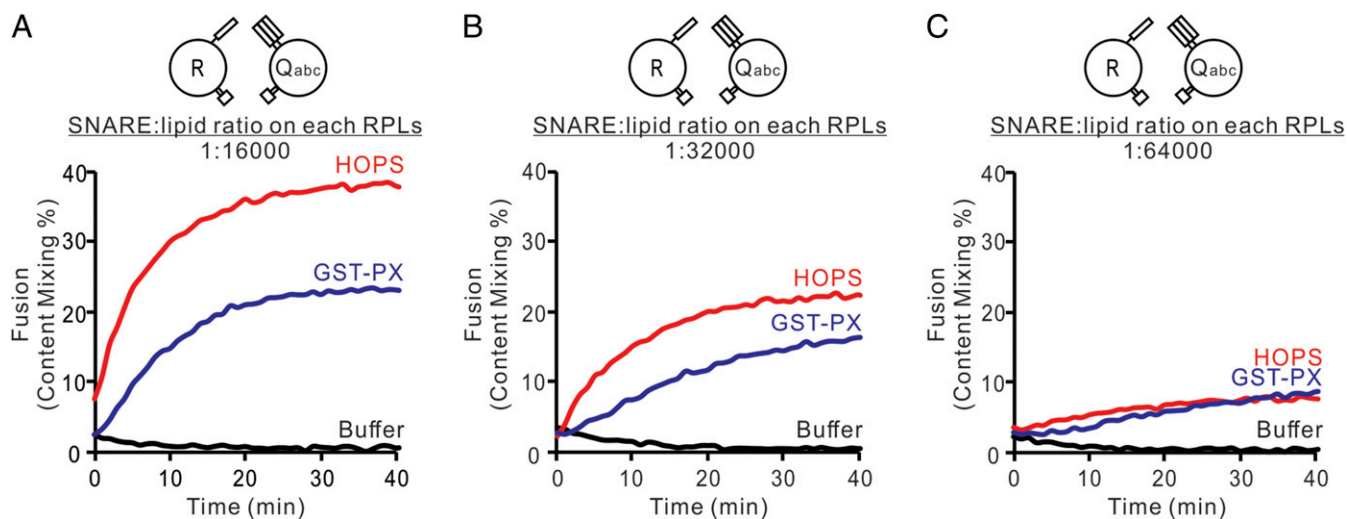


Fig. 3. GST-PX and HOPS are comparable tethers. (A–C) Fusion incubations had proteoliposomes bearing R or QaQbQc (3Q) at 1:16,000 (A), 1:32,000 (B), or 1:64,000 (C) SNARE:lipid molar ratios. Incubations were with 50 nM HOPS or 500 nM GST-PX for 40 min at 27 °C. All proteoliposomes had Ypt7-TM at a 1:8,000 protein:lipid molar ratio. Kinetic curves of content-mixing assays in this figure are representative of $n \geq 3$ experiments; means and SDs for each experiment are presented in *SI Appendix, Fig. S3*.

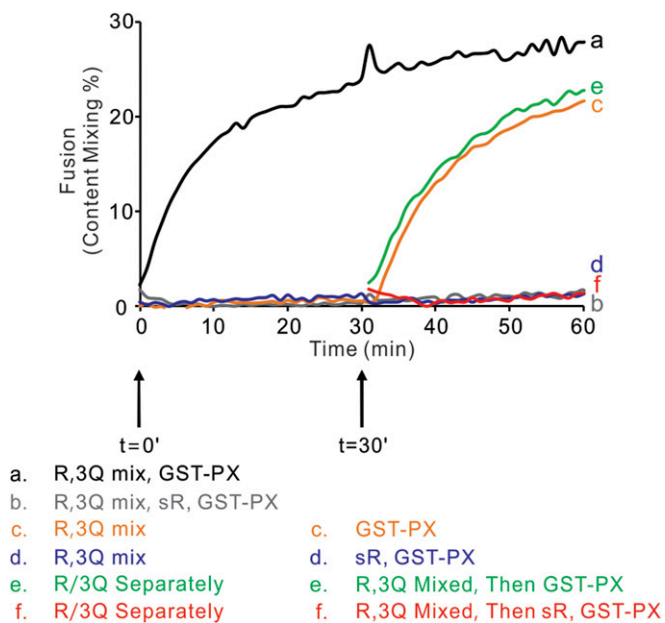


Fig. 4. Fusion induced by the GST-PX tether is from new *trans*-SNARE pairing. Fusion reactions had proteoliposomes bearing either the R or QaQbQc (3Q) at 1:16,000 SNARE:lipid molar ratio. Fusion incubations received 500 nM GST-PX at $t = 0$ min (a, b) or $t = 30$ min (c–f) with 1 μ M soluble R-SNARE (sR) where indicated (b, d, and f). Content-mixing assays in this figure are representative of $n \geq 3$ experiments; means and SDs for each experiment are presented in *SI Appendix, Fig. S5*.

factors which may largely keep SNAREs disassembled between cycles of docking and fusion, although the model reaction of fusion between liposomes bearing R-SNARE and 3Q-SNAREs has received extensive study. This model reaction, with pre-assembled 3Q-proteoliposomes in the absence of Sec17 and Sec18, reveals that SNAREs which assemble spontaneously between untethered membranes are largely inactive for fusion, while it is tethering per se which permits the spontaneous assembly of fusion-competent *trans*-SNARE pairs.

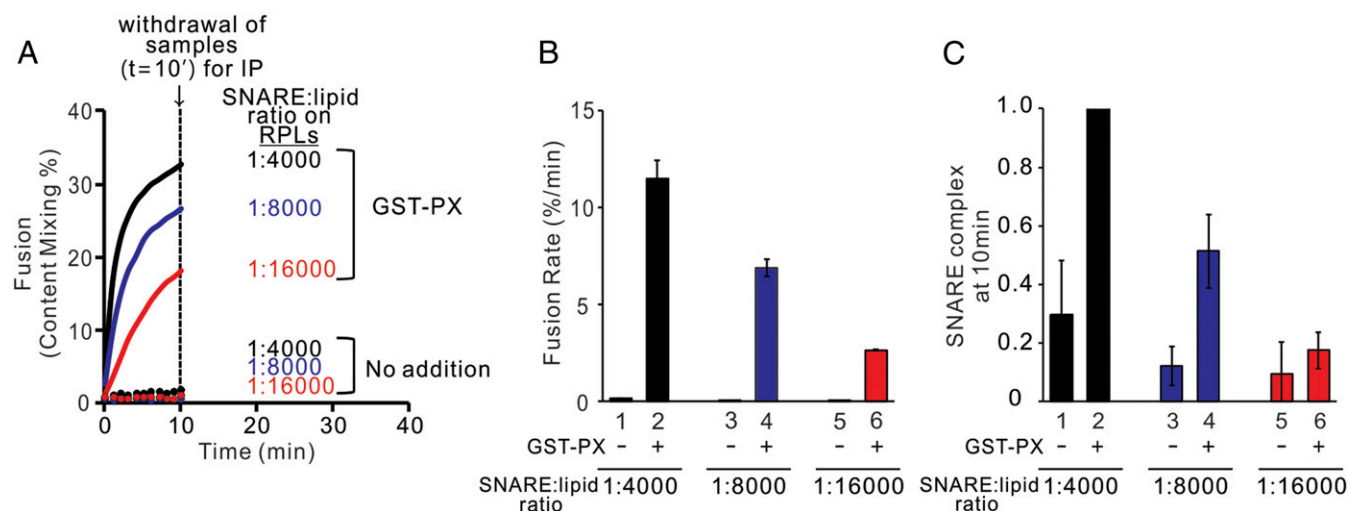


Fig. 5. *Trans*-SNARE complexes formed without a tether give far less fusion. (A) R- and 3Q-SNARE reconstituted proteoliposomes (RPLs) at 1:4,000, 1:8,000, or 1:16,000 SNARE:lipid molar ratio were incubated together without a tethering agent or with 500 nM GST-PX and assayed for fusion. (B) R- and 3Q-SNARE RPLs at SNARE:lipid molar ratios of 1:4,000, 1:8,000, or 1:16,000 were incubated together without tether (odd lanes) or with 500 nM GST-PX (even lanes) and assayed for the rate of fusion. (C) After a 10-min incubation, samples were solubilized in detergent and assayed for R bound to Qa as described (27). The signal intensity of condition 2 (1:4,000 proteoliposomes with 500 nM GST-PX) was normalized to 100%.

Discussion

SNAREs are essential for fusion. The fundamental discovery that R, Qa, Qb, and Qc SNAREs are complementary and can self-assemble in equimolar stoichiometry is a foundation for unraveling how Rabs, tethers, SM proteins, and SNARE chaperones cooperate with SNAREs and regulatory lipids for fusion. Do tethering factors merely spatially concentrate SNAREs by bringing membranes into close apposition to facilitate SNARE assembly in *trans*, and are SM proteins always required to catalyze rapid R and Qa SNARE assembly in *trans*? Our current findings show that tethering per se strikingly enhances the fusion potency of *trans*-SNARE complexes which form between R- and 3Q-SNARE complexes without further catalysis by SM protein functions. A working model of this is shown in Fig. 7. We suggest that the collision of membranes bearing R- and 3Q-SNAREs without prior tethering may commonly lead to antiparallel SNARE complexes (7) which do not support fusion, plus fewer parallel SNARE complexes which support the fusion seen at unphysiologically high SNARE levels (Fig. 7A). In contrast, the R- and 3Q-SNAREs on membranes with tethers (T in Fig. 7B) are oriented with all of their SNARE domain N termini distal to the bilayer and C termini proximal. Lateral movement of SNAREs along the surfaces of the bilayers of the two tethered membranes (Fig. 7B) will lead to SNARE collisions in which the SNARE domains are all parallel, giving parallel coiled-coil assemblies. It is possible that the initial, fusion-incompetent *trans*-SNARE complexes which form without the aid of a tether might then link the two membranes and thus fulfill the tethering function, allowing later *trans*-SNARE complexes to assemble in a fusion-competent conformation and thereby supporting the low rate of fusion seen with SNAREs alone. Direct assay of SNARE complex structure as formed in the presence or absence of tethers will be essential to test this model. This is technically challenging, as physiological levels of SNAREs are low, and *trans*-SNARE complexes are only a minor fraction of SNAREs (38) and are formed in the context of other proteins such as HOPS at the vertex ring domain where bilayers come into apposition (39).

HOPS has direct affinity for vacuolar SNAREs (10, 21), but HOPS may bind SNAREs for purposes other than catalysis of R:Qa association. These might include 3Q assembly, SNARE complex protection from Sec17 inhibition (32), protection from

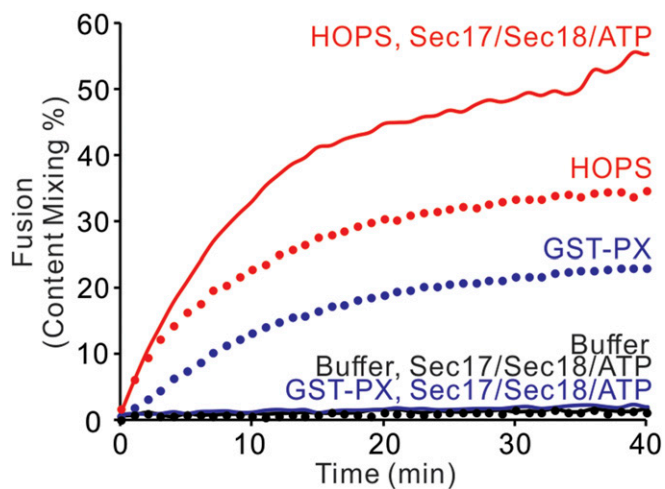


Fig. 6. HOPS is far better than GST-PX in fusion incubations with Sec17, Sec18, and ATP. Fusion reactions had proteoliposomes bearing Ypt7-TM at a molar ratio to lipid of 1:8,000 and either the R- or 3Q-SNAREs at 1:16,000 SNARE:lipid molar ratio. Fusion incubations received either buffer, 500 nM GST-PX, or 50 nM HOPS for 40 min at 27 °C (dotted lines) and Sec17 (300 nM), Sec18 (100 nM), and ATP (1 mM) where indicated (solid lines). Kinetic curves of content-mixing assays in this figure are representative of $n \geq 3$ experiments; see *SI Appendix, Fig. S6*.

disassembly by Sec17, Sec18, and ATP (36), or to provide protein bulk immediately adjacent to the *trans*-SNARE complexes at the fusion microdomain (29). Even at physiological 1:32,000 or 1:64,000 molar ratios of SNAREs to lipids (30), synthetic tethering by GST-PX gives comparable fusion to HOPS (Fig. 3), suggesting that there is little need for direct catalysis of functional R:3Q assembly once the 3Q-SNARE complex is assembled and tethering occurs.

Other pathways of SNARE assembly in which the Q-SNAREs are not preassembled may exploit the SM capacity to template R:Qa association. Munc18-1 has been shown by force microscopy

(40) to catalyze the assembly of neuronal syntaxin (Qa) and synaptobrevin (R), forming an intermediate which can go on to full 4-SNARE domain complex upon association with SNAP-25 (Qb, Qc). In this same study, the recombinant Vps33 subunit of HOPS was shown to catalyze the assembly of vacuolar R- and Qa-SNAREs into a complex which can further assemble with Qb and Qc. In the context of membranes, SNARE complex formation does not always equate to fusion (Fig. 5; also ref. 30). Fusion can be reconstituted with proteoliposomes bearing R-SNARE and others bearing either one, two, or all 3Q-SNAREs plus hexameric HOPS and the remaining Q-SNAREs in soluble form without their membrane anchor (41). While HOPS supports each of these fusion reactions, fusion with Vps33 has not been reported. Further studies will be needed to establish conditions where HOPS templates the association of membrane-bound R- and Qa-SNAREs in *trans* as a favorable intermediate on the path to fusion.

Neuronal SNAREs can assemble in parallel or antiparallel, active or inactive, configurations (7, 8). Unc13 and Unc18 ensure the parallel assembly of the 3Q-SNARE domains of syntaxin and SNAP-25, and Unc13 further aligns the R-SNARE synaptobrevin (8). While there is no Unc13 for vacuoles, we now find that tethering aligns R- and 3Q-SNAREs for productive SNARE complex assembly, which we hypothesize to be a parallel conformation. Unc13 is also a tethering factor (42); perhaps this tethering activity per se favors parallel SNARE complex assembly in a similar manner to the GST-PX tether.

Experimental Procedures

Proteins and Reagents. Most lipids were from Avanti Polar Lipids. Ergosterol was from Sigma, and PtdIns(3)P was from Echelon. Biotinylated R-phycoerythrin was purchased from Life Technologies, Cy5-streptavidin was from SeraCare, and underivatized streptavidin was from Thermo Fisher. Protein was assayed by the method of Bradford (43).

Protein expression and purification. HOPS (19), vacuolar SNAREs (26, 33, 37, 44), GST-FYVE(Hrs) (45), and GST-PX(Vam7) (46) were isolated as described. Ypt7-TM, bearing the transmembrane anchor of Vam3, was derived from a construct kindly provided by Christian Ungermann, University of Osnabrück, Osnabrück, Germany.

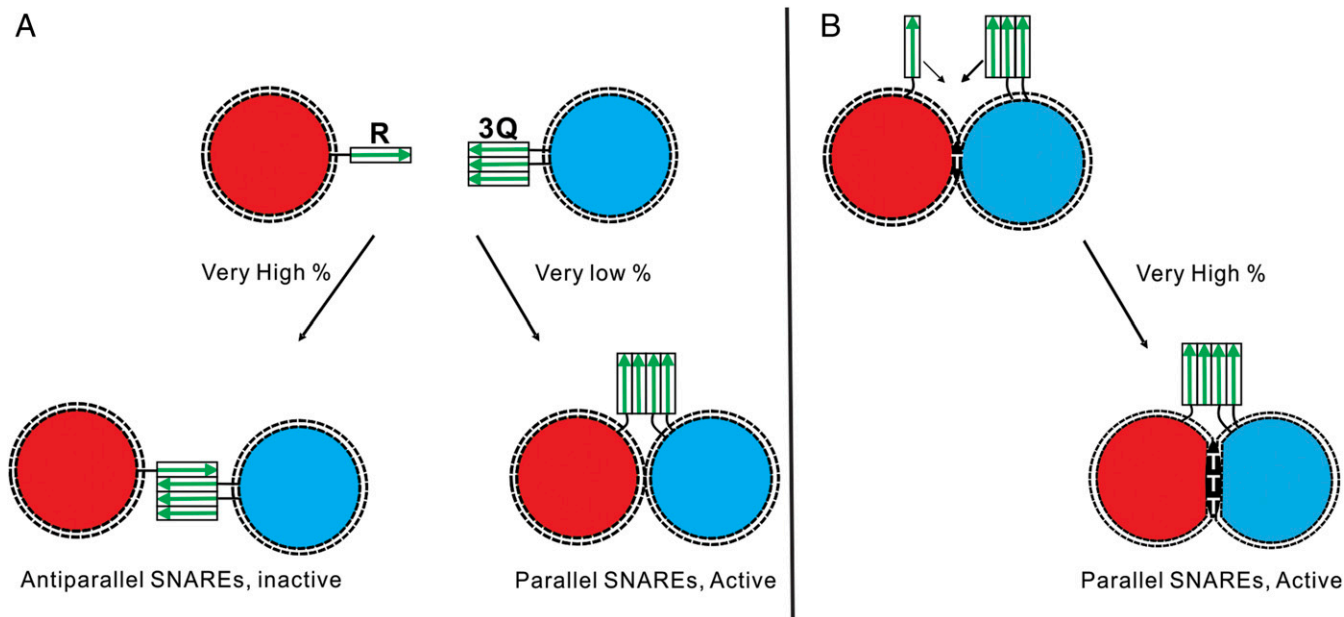


Fig. 7. Working model. (A) Membrane contact without prior tethering may give antiparallel *trans*-SNARE complexes which are inactive or even inhibitory. (B) Initial membrane contact by a tether (T) and lateral movement of oriented SNAREs in each membrane may favor parallel, active complex assembly. Some inactive antiparallel SNARE complexes, shown in A, may serve as a tether, allowing limited parallel SNARE complex assembly and slow fusion.

Proteoliposome Preparation. Proteoliposomes with mixed vacuolar lipids (VMLs) (37) were prepared as described (30) with or without Ypt7-TM at a 1:8,000 molar ratio to lipid and individual SNAREs at the indicated molar protein:lipid ratios. For VML (18:2) compositions, lipids in chloroform were mixed with β -OG in the following proportions: 47.6 mol % DLPC (1,2-Dioleoyl-sn-glycero-3-phosphocholine), 18 mol % DLPE (1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine), 18 mol % soy PI (L- α -phosphatidylinositol), 4.4 mol % DLPS (1,2-Dioleoyl-sn-glycero-3-phospho-L-serine), 2 mol % DLP A (1,2-Dioleoyl-sn-glycero-3-phosphate), 1 mol % 16:0 DAG (1,2-Dipalmitoyl-sn-glycerol), 8 mol % ergosterol, and 1 mol % PI(3)P [1,2-dipalmitoyl-sn-glycero-3-phospho-(1-myoinositol-3-phosphate)].

Membrane Fusion Assay. Fusion incubations (20 μ L) bore Ypt7/R-SNARE proteoliposomes and Ypt7/Q-SNARE proteoliposomes (each 250 μ M lipid) which had been separately preincubated for 10 min at 27 $^{\circ}$ C in RB150 [20 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 10% (vol/vol) glycerol], 5 μ M streptavidin, 1 mM EDTA, and 10 μ M GTP, then mixed with 1.25 mM MgCl₂ to complete the exchange of Ypt7 to its GTP-bound form, required on the R-SNARE proteoliposomes for fusion (34). These preincubated proteoliposomes were mixed in equal parts, and 10- μ L portions were placed in wells. A mixture of other proteins (10 μ L; e.g., HOPS, GST-PX, GST-FYVE, Sec17, Sec18, and

sSNAREs) were added at time 0. Content-mixing signals were measured in plates reader [PhycoE:Cy5 FRET: excitation (ex): 565 nm; emission (em): 670 nm; cutoff: 630 nm] every minute, using SpectraMax Gemini XPS (Molecular Devices) instrument. Maximal content mixing was measured by adding 0.2% (wt/vol) Thesit to parallel samples without streptavidin.

Coimmunoprecipitation. R-SNARE, which coimmunoprecipitated with antibody to Qa, was measured as described (30). After assaying FRET for 30 min, samples were chilled, mixed with 10 volumes of 0 $^{\circ}$ C modified RIPA buffer [20 mM Hepes-NaOH, pH 7.4, 150 mM NaCl, 0.2% (wt/vol) BSA, 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium cholate, 0.1% (wt/vol) SDS] bearing protein A magnetic beads (ThermoFisher) and 5 μ M GST-R to prevent further association of wild-type R with Qa in the extract. After nutation (2 h, 23 $^{\circ}$ C), resin was thrice washed in 1 mL modified RIPA buffer. Proteins were recovered with 100 μ L of SDS sample buffer (5 min, 95 $^{\circ}$ C). SDS/PAGE was followed by immunoblotting with antibody to R, Qa, or Qc. Bound protein was quantified by UN-SCAN-IT (Silk Scientific).

ACKNOWLEDGMENTS. We thank Axel Brunger, Gustav Lienhard, and Charles Barlowe for stimulating discussions and Amy Orr for expert technical assistance. This work was supported by NIH grant R35GM118037.

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