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Multiple and distinct strategies of yeast SNAREs to confer the specificity of membrane fusion

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Trans-QabcR-SNARE pairing on opposing membranes is crucial for eukaryotic membrane fusion, but how selective pairs of Qabc- and R-SNARE proteins regulate membrane fusion specificity remains elusive. Here, we studied 14 purified full-length SNAREs that function in yeast endoplasmic reticulum (ER)-Golgi, intra-Golgi, endosomal, and vacuolar transport by comprehensively testing *cis*-QabcR-SNARE assembly and fusogenicity of reconstituted SNARE proteoliposomes. Strikingly, the cognate ER-Golgi and intra-Golgi SNARE-complex assemblies were highly stringent, whereas endosomal and vacuolar SNAREs assembled rather promiscuously into the non-cognate mixed complexes. However, these patterns of *cis*-SNARE assemblies cannot solely explain their potency to be fusogenic via *trans*-SNARE pairing: Only the vacuolar 3Q-SNARE combination is fusogenic in the absence of additional components; endosomal SNARE-dependent fusogenicity requires membrane-tethering factors; and ER-Golgi SNAREs can be fusogenic by synergistic actions of tethering factors and the cognate Sec1/Munc18-family protein Sly1p. Thus, our findings uncover multiple and distinct strategies of SNAREs to directly mediate fusion specificity.

Membrane fusion is a fundamental process for protein/lipid trafficking, hormone secretion, and organelle morphogenesis in eukaryotic endomembrane systems. Fusion is catalyzed and tightly regulated by numerous conserved protein families^{1–6}; SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors)⁷, SNARE chaperones such as Sec1/Munc18-family (SM) proteins^{5,8,9}, Rab GTPases, Rab effectors, and tethering complexes^{4,10,11}. Specific lipids, including sterols, diacylglycerol, phosphatidic acid (PA), phosphoinositides, and phosphatidylethanolamine (PE), are also required for efficient fusion of physiological membranes and cooperate with those essential protein components to form a fusion-competent membrane microdomain^{4,12–17}.

Of the numerous key protein components, SNARE proteins have been recognized as core machinery to catalyze membrane fusion⁷. SNAREs are integral or peripheral membrane proteins distinguished by their conserved heptad-repeat SNARE motifs that assemble into four-helical complexes in *cis* (anchored to one membrane) or in *trans* (anchored to two apposed membranes)^{7,18,19}. The central layer of the four-helical SNARE complex has three glutamine (Q) residues and one arginine (R) residue, thereby classifying SNAREs into Qa-, Qb-, Qc-, and R-SNARE subfamilies²⁰. In general, 3Q-SNAREs (Qabc-SNAREs) on one membrane and an R-SNARE on the other assemble into a *trans*-QabcR-SNARE complex, and specific sets of QabcR-SNAREs function in individual intracellular fusion steps⁷. However, despite the fact that numerous earlier *in vivo* and *in vitro* studies have characterized specific functions of SNAREs in membrane fusion, whether SNAREs mediate the fusion specificity by themselves remains elusive⁷. Indeed, two quite divergent conclusions on this issue have been established by *in vitro* experiments. Many cognate pairs of yeast t- and v-SNAREs (mostly 3Q- and R-SNAREs, respectively) can be fusogenic when reconstituted into two separate liposome populations, but the non-cognate t- and v-SNARE sets mostly have little potency to initiate lipid mixing^{21,22}. This strongly supports the concept that the intracellular compartmental specificity of membrane fusion is encoded in v- and t-SNARE pairing, as originally predicted by the SNARE hypothesis²³. However, mammalian endosomal and exocytic SNAREs have been reported to readily assemble into mixed and non-cognate *cis*-SNARE complexes in solution^{24,25} and cause promiscuous lipid mixing of the reconstituted SNARE proteoliposomes, even with the non-cognate 3Q- and R-SNARE combinations²⁶. These studies have led to the conflicting conclusion that SNAREs do not determine fusion specificity by themselves. In the present study, we now address this ambiguous question in more depth by comprehensively testing the endoplasmic reticulum (ER)-Golgi, intra-Golgi, endosomal, and



vacuolar SNAREs in the yeast *Saccharomyces cerevisiae* for their capacity to assemble into a *cis*-SNARE complex and to catalyze lipid mixing of the reconstituted SNARE proteoliposomes.

Results

Among the 24 SNARE-family members of the yeast *S. cerevisiae*²⁷, we purified 14 SNARE proteins, including three Qa-SNAREs (Sed5p, Pep12p, Vam3p), three Qb-SNAREs (Bos1p, Gos1p, Vti1p), four Qc-SNAREs (Bet1p, Sft1p, Tlg1p, Vam7p), and four R-SNAREs (Sec22p, Ykt6p, Snc2p, Nyv1p) to prepare physiological QabcR-SNARE sets for ER-Golgi, intra-Golgi, endosomal, and vacuolar transport (Figure 1a). These recombinant SNAREs were isolated as a full-length protein with an N-terminal His6-3C- or GST-His6-3C-tag (Figure 1b–e) and tested for the *cis*-SNARE complex assembly in solution (Figures 2–4). These His6-3C- and GST-His6-3C-tags were cleaved off by a human rhinovirus 3C-protease to prepare reconstituted proteoliposomes bearing untagged full-length SNARE proteins (Figures 5–8).

***Cis*-SNARE complex assemblies are not promiscuous but rather selective.** First, we thoroughly analyzed the specificity of physical SNARE-SNARE interactions by *in vitro* glutathione S-transferase (GST) pull-down assays with the yeast 3Q- and R-SNAREs (Figures 2–4). Four GST-tagged R-SNAREs (Sec22p, Ykt6p, Snc2p, and Nyv1p) were assayed with four physiological sets of 3Q-SNAREs including Sed5p/Bos1p/Bet1p, Sed5p/Gos1p/Sft1p, Pep12p/Vti1p/Tlg1p, and Vam3p/Vti1p/Vam7p, functioning in the ER-Golgi, intra-Golgi, endosomal, and vacuolar transport, respectively (Figure 2). Strikingly, we found that *cis*-assemblies of the ER-Golgi and intra-Golgi QabcR-SNARE complexes were highly specific, as these early secretory 3Q-SNARE combinations assembled into a stable *cis*-SNARE complex exclusively with Sec22p, their physiological partner R-SNARE in the ER and Golgi compartments (Figure 2a, c, lanes 1).

Moreover, these specific *cis*-assemblies of the ER-Golgi and intra-Golgi SNARE complexes require their complete 3Q-SNARE sets (Figure 2b, d, lanes 2–4), except for the Sed5p/Gos1p/Sec22p sub-complex (Figure 2d, lane 4). Unexpectedly, Golgi-resident R-SNARE Ykt6p did not bind to either ER-Golgi or intra-Golgi 3Q-SNAREs (Figure 2a, c, lanes 2), even though prior genetic and biochemical studies have reported that Ykt6p can functionally substitute for Sec22p in the ER/Golgi trafficking *in vivo*²⁸ and can stably form *cis*-SNARE complexes with the ER-Golgi or intra-Golgi 3Q-SNAREs *in vitro*²⁹. In contrast to these specific ER-Golgi/intra-Golgi SNARE assemblies, we observed that endosomal and vacuolar 3Q-SNAREs promiscuously form *cis*-QabcR-SNARE complexes either with their cognate or non-cognate R-SNAREs (Figure 2e, g, lanes 1–4), consistent with earlier *in vitro* binding studies on mammalian exocytic SNAREs^{24,25}. However, even in these promiscuous *cis*-QabcR-SNARE assemblies, the complete sets of endosomal and vacuolar 3Q-SNAREs are crucial for their association with Snc2p (Figure 2f, lanes 1–4) or Nyv1p (Figure 2h, lanes 1–4) and also more strictly required for that of Ykt6p (Figure 3a, b, lanes 1–4). Nevertheless, our current SNARE-binding data strongly suggest that *cis*-SNARE assembly of the early secretory ER-Golgi or intra-Golgi SNAREs is far more stringent compared with endosomal and vacuolar SNAREs for late secretory transport.

To further resolve the specificity of *cis*-SNARE assemblies in solution, we next studied the requirement of physiological 3Q-SNARE compositions for the formation of *cis*-QabcR-SNARE complexes (Figure 4). We employed a GST pull-down assay to test whether each single Q-SNARE in the cognate QabcR-SNARE complexes can be substituted by its non-cognate counterparts (Figure 4). Strikingly, the ER-Golgi and intra-Golgi *cis*-SNARE assemblies require a highly stringent 3Q-SNARE composition (Figure 4a–c, d–f), as we found only two non-cognate mixed 3Q-SNARE combinations that can assemble with the R-SNARE Sec22p, Sed5p/Gos1p/

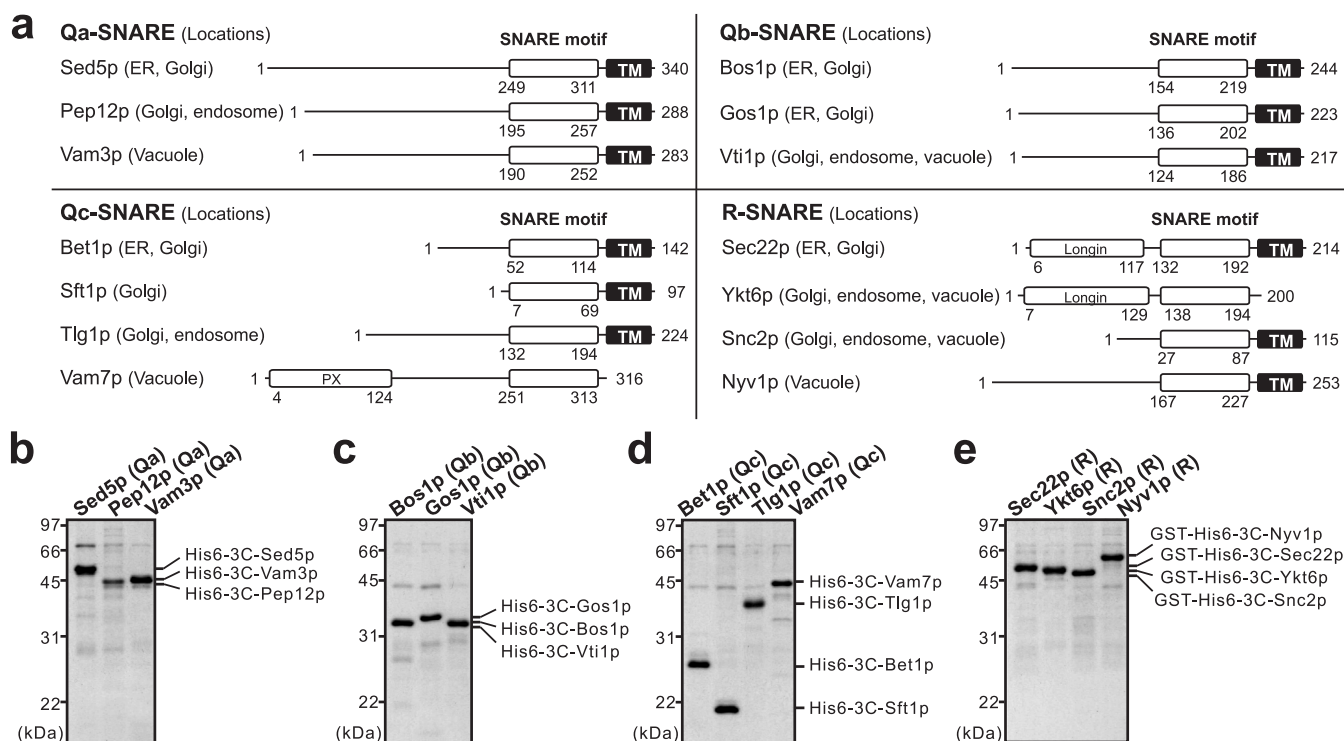


Figure 1 | Yeast ER-Golgi, intra-Golgi, endosomal, and vacuolar QabcR-SNARE proteins used in the current *in vitro* studies of *cis*-SNARE complex assembly and reconstituted SNARE-dependent proteoliposomal lipid mixing. (a) Schematic representation of the SNARE proteins in the yeast *Saccharomyces cerevisiae*, showing their subfamilies (Qa-, Qb-, Qc-, and R-SNAREs), domains including SNARE motifs and transmembrane domains (TM), and intracellular locations (endoplasmic reticulum (ER), Golgi, endosome, and vacuole). (b–e) Coomassie Blue-stained gels of purified full-length His6-3C-tagged Qa-, Qb-, and Qc-SNAREs (b–d) and GST-His6-3C-tagged R-SNAREs (e) used in Figures 2–8.

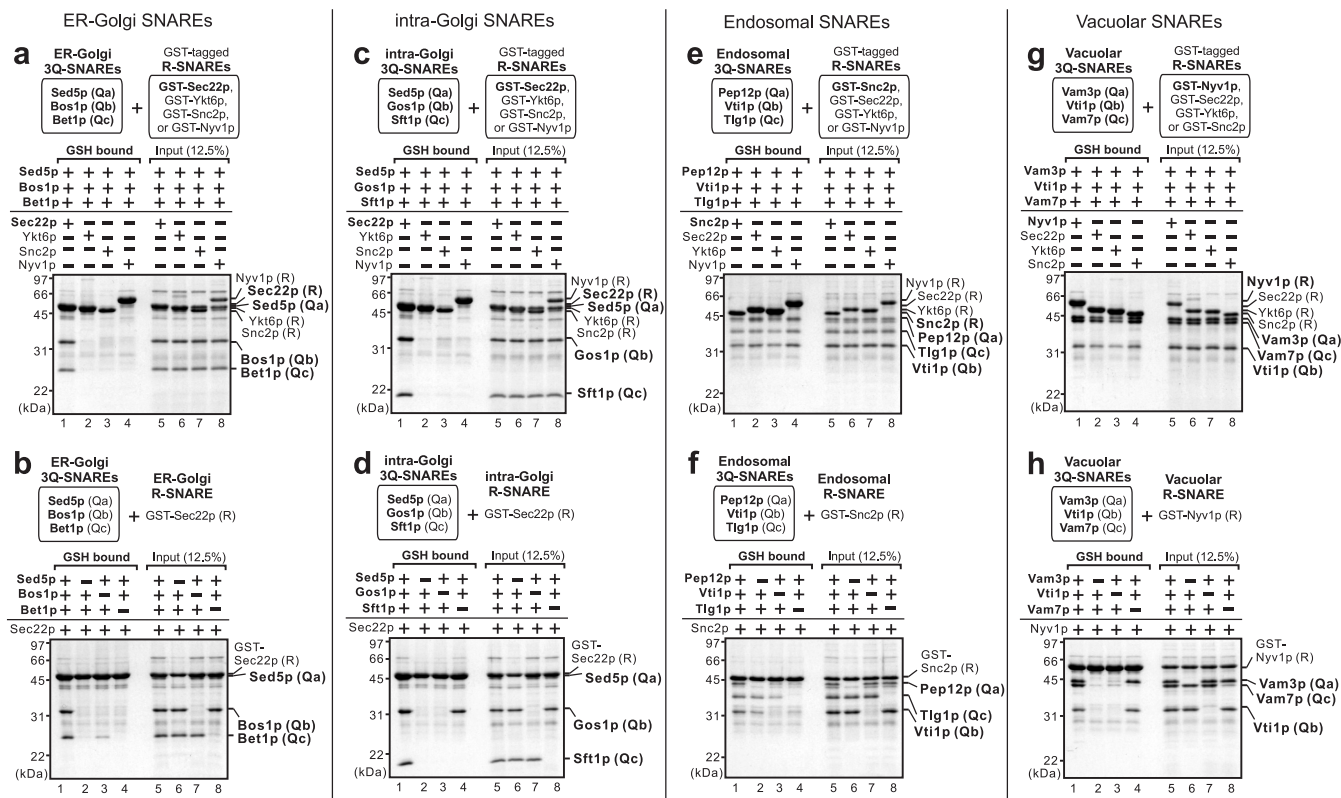


Figure 2 | ER-Golgi and intra-Golgi 3Q-SNAREs assemble into *cis*-QabcR-SNARE complexes exclusively with their cognate R-SNARE Sec22p, whereas endosomal and vacuolar 3Q-SNAREs associate promiscuously with non-cognate R-SNAREs. (a–h) Purified physiological sets of 3Q-SNAREs, ER-Golgi Sed5p/Bos1p/Bet1p (a, b), intra-Golgi Sed5p/Gos1p/Sft1p (c, d), endosomal Pep12p/Vti1p/Tlg1p (e, f), and vacuolar Vam3p/Vti1p/Vam7p (g, h), were mixed with GST-tagged R-SNAREs including GST-Sec22p, GST-Ykt6p, GST-Snc2p, and GST-Nyv1p where indicated, in RB500 (20 mM HEPES-NaOH, pH 7.4, 10% glycerol, 500 mM NaCl) containing 100 mM β -OG. These GST-tagged R-SNAREs were isolated by glutathione-Sepharose beads, followed by SDS-PAGE and Coomassie Blue staining, to analyze 3Q-SNAREs bound to the GST-tagged R-SNAREs. Initial SNARE concentrations of all the reactions in (a–h) were 4 μ M for each SNARE protein added.

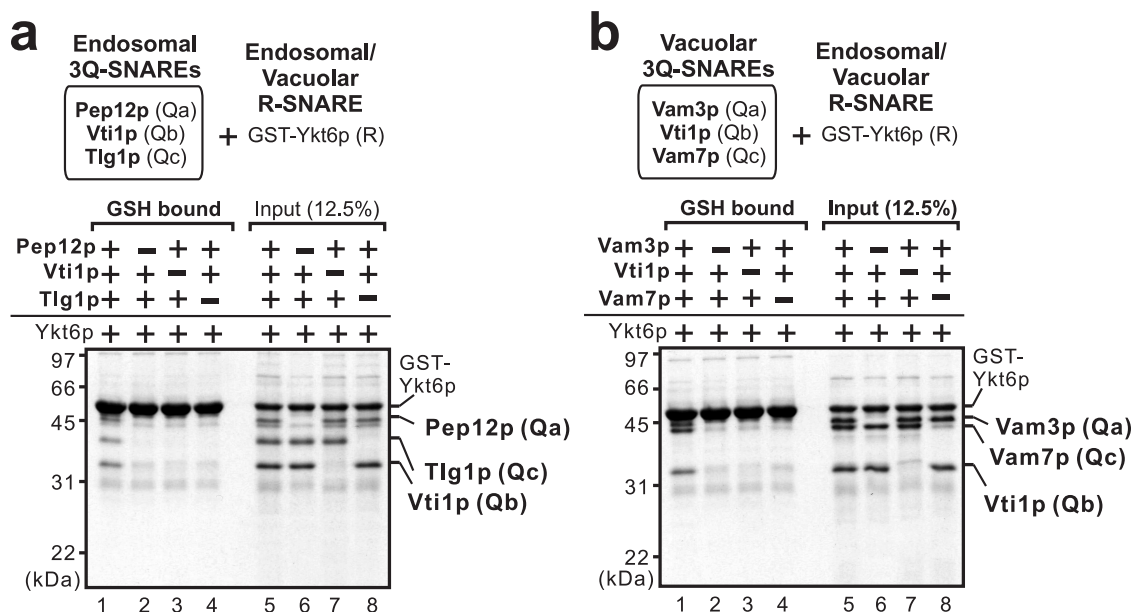


Figure 3 | The complete 3Q-SNARE sets of endosomal and vacuolar Q-SNAREs are required for the association with the R-SNARE Ykt6p. (a, b) *Cis*-SNARE-complex assemblies in solution were tested by an *in vitro* GST pull-down assay as in Figure 2. GST-tagged R-SNARE Ykt6p was incubated with endosomal (Pep12p, Vti1p, and Tlg1p) and vacuolar (Vam3p, Vti1p, and Vam7p) 3Q-SNAREs where indicated and isolated by glutathione-Sepharose beads. Q-SNAREs bound to GST-Ykt6p were analyzed by SDS-PAGE and Coomassie Blue staining. Initial SNARE concentrations in (a, b) were 4 μ M for each SNARE protein added.

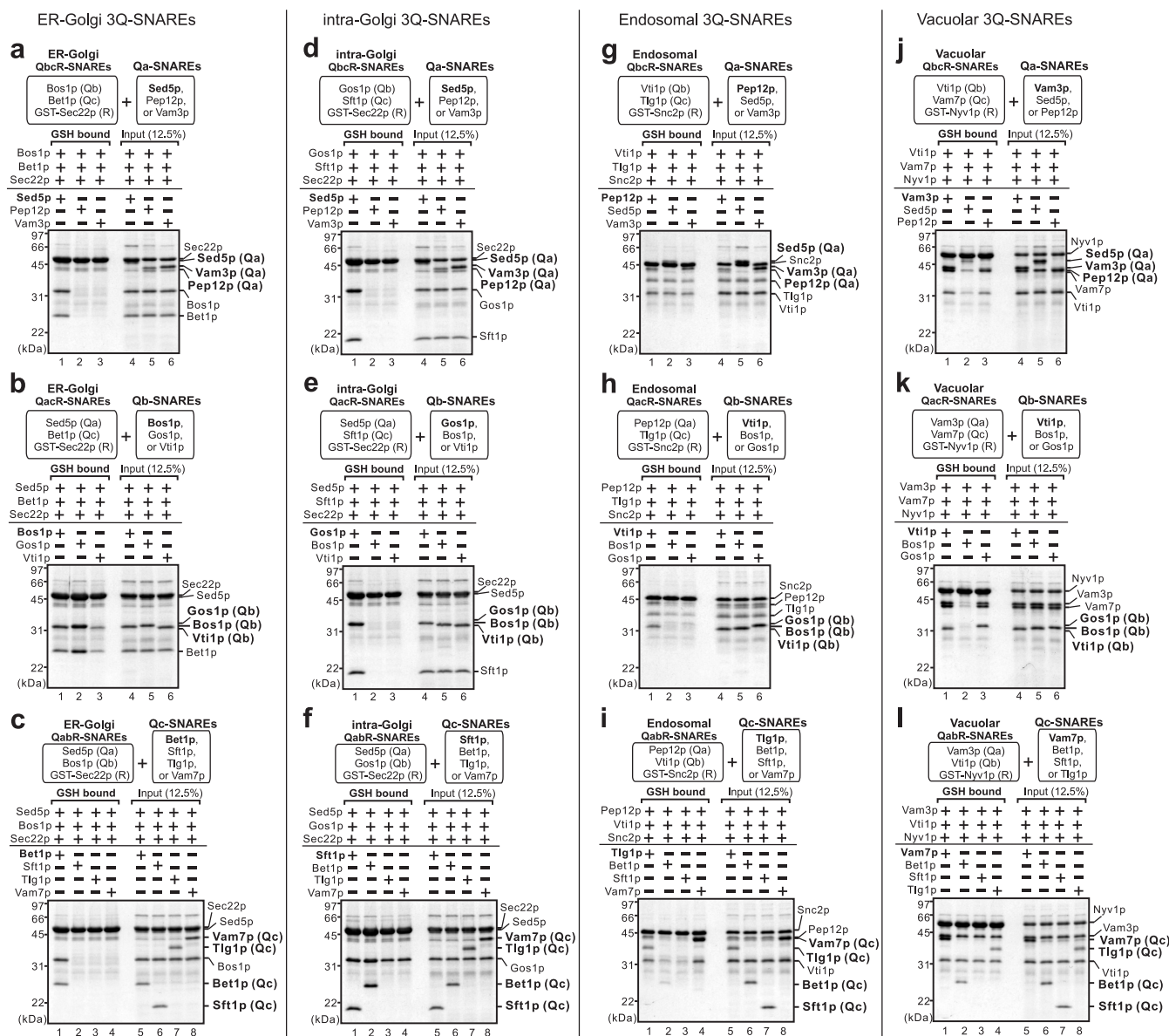


Figure 4 | Stringent 3Q-SNARE composition is required for physiological *cis*-QabcR-SNARE complex assemblies. *Cis*-SNARE-complex assemblies in solution were tested by an *in vitro* GST pull-down assay as in Figure 2. (a–c) GST-Sec22p was incubated with the cognate ER-Golgi 3Q-SNAREs (Sed5p, Bos1p, and Bet1p) and the non-cognate Qa- (a), Qb- (b), or Qc-SNAREs (c) where indicated and isolated by glutathione-Sepharose beads. Qa-, Qb-, and Qc-SNAREs bound to GST-Sec22p were assayed by SDS-PAGE and Coomassie Blue staining. (d–f) GST-Sec22p was incubated with the cognate intra-Golgi 3Q-SNAREs (Sed5p, Gos1p, and Sft1p) and the non-cognate Qa- (d), Qb- (e), or Qc-SNAREs (f) where indicated, then isolated and analyzed as in (a–c). (g–i) GST-Snc2p was incubated with the cognate endosomal 3Q-SNAREs (Pep12p, Vti1p, and Tlg1p) and the non-cognate Qa- (g), Qb- (h), or Qc-SNAREs (i) where indicated, and then isolated and analyzed as in (a–c). (j–l) GST-Nyv1p was incubated with the cognate vacuolar 3Q-SNAREs (Vam3p, Vti1p, and Vam7p) and the non-cognate Qa- (j), Qb- (k), or Qc-SNAREs (l) where indicated, then isolated and analyzed as in (a–c). Initial SNARE concentrations of all the reactions in (a–l) were 4 μ M for each SNARE protein added.

Bet1p (Figure 4b, f, lanes 2) and Sed5p/Vti1p/Bet1p (Figure 4b, lane 3). Notably, Gos1p and Vti1p have been reported to functionally and physically interact with Sed5p^{30,31}. Thus, those mixed Qabc-SNARE compositions may be also physiological 3Q-SNARE sets in the ER and Golgi compartments. The cognate 3Q-SNARE compositions of endosomal and vacuolar SNAREs appear to be less stringent than those of ER-Golgi and intra-Golgi SNAREs but yet moderately selective (Figure 4g–i, j–l). For example, Qb-SNARE Bos1p and Qc-SNARE Sft1p in the early secretory compartments cannot form *cis*-SNARE complexes either with endosomal or vacuolar SNAREs (Figure 4h, k, lanes 2, for Bos1p; Figure 4i, l, lanes 3, for Sft1p). Taken together, our results of these comprehensive pull-down assays demonstrate that appropriate 3Q-SNARE compositions are critical for

the assembly of physiological QabcR-SNARE complexes, particularly for ER-Golgi and intra-Golgi SNAREs, establishing that SNARE complex can contribute to fusion specificity by themselves, at least, through their physical SNARE-SNARE binding interactions.

Only the vacuolar 3Q-SNARE set is fusogenic in the absence of any additional factors. We next asked whether the selective *cis*-QabcR-SNARE assemblies observed in our pull-down assays reflect the capacity to initiate lipid mixing of lipid bilayers (Figure 5). We separately reconstituted four physiological cognate 3Q-SNARE sets (Figure 5a) and their partner R-SNAREs (Figure 5b) into distinct liposome populations with the specific lipid composition roughly mimicking sub-cellular membranes in yeast³², which contains

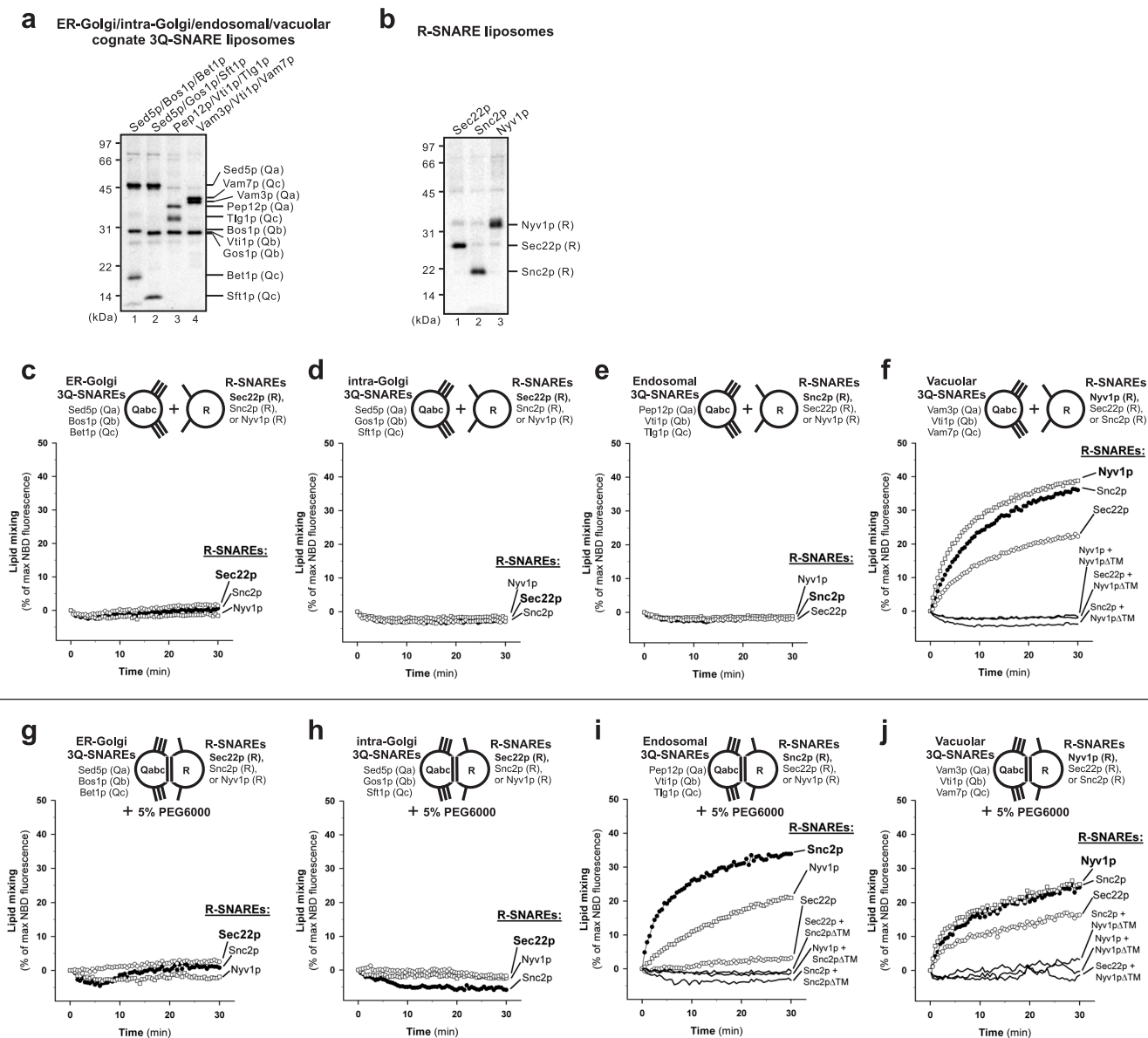


Figure 5 | Fusogenicity of reconstituted SNARE proteoliposomes bearing the physiological sets of ER-Golgi, intra-Golgi, endosomal, and vacuolar 3Q-SNAREs. (a, b) Coomassie Blue-stained gels of reconstituted proteoliposomes (20 nmol total lipids in each lane) bearing the physiological 3Q-SNARE sets (a) or R-SNAREs (b) used in (c–j). (c–f) Only vacuolar 3Q-SNAREs can catalyze lipid mixing with R-SNAREs in the absence of any additional factors. To test fusogenicity of the SNARE proteoliposomes, lipid-mixing assays were performed at 30 °C in RB150 (20 mM HEPES-NaOH, pH 7.4, 10% glycerol, 150 mM NaCl), with the 3Q-SNARE liposomes (non-labeled acceptor liposomes, 500 μ M total lipids in final) bearing the cognate ER-Golgi Sed5p/Bos1p/Bet1p (c), intra-Golgi Sed5p/Gos1p/Sft1p (d), endosomal Pep12p/Vti1p/Tlg1p (e), or vacuolar Vam3p/Vti1p/Vam7p (f) and R-SNARE liposomes (NBD-labeled donor liposomes, 200 μ M total lipids in final) bearing ER-Golgi/intra-Golgi Sec22p, endosomal Snc2p, or vacuolar Nyv1p. GST-Nyv1p lacking a transmembrane domain (Nyv1p Δ TM, 6.9 μ M in final) was added to the lipid-mixing reactions in (f) where indicated, as a competitive inhibitor. (g–j) PEG-mediated membrane tethering supports lipid mixing by endosomal 3Q-SNAREs but not ER-Golgi or intra-Golgi 3Q-SNAREs. Lipid-mixing was assayed as in (c–f), with 3Q-SNARE liposomes bearing the cognate Sed5p/Bos1p/Bet1p (g), Sed5p/Gos1p/Sft1p (h), Pep12p/Vti1p/Tlg1p (i), or Vam3p/Vti1p/Vam7p (j) and R-SNARE liposomes bearing Sec22p, Snc2p, or Nyv1p, but in the presence of 5% PEG6000. GST-tagged R-SNAREs lacking a transmembrane domain, Snc2p Δ TM (22 μ M final) and Nyv1p Δ TM (6.9 μ M final), were added as a competitive inhibitor in (i) and (j), respectively.

phosphatidylcholine (PC), PE, phosphatidylinositol (PI), phosphatidylserine (PS), PA, ergosterol, cardiolipin, and diacylglycerol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed successful and also comparable reconstitution in each case for the isolated 3Q- and R-SNARE proteoliposomes (Figure 5a, b). Fusogenicity of these reconstituted SNARE proteoliposomes was tested by a well-established fluorescence assay of liposomal lipid mixing^{33,34}. It should be noted that, in this current study, we analyzed the fusogenic capacity of

SNARE proteins only by the lipid-mixing assay (Figures 5–8), not by a content-mixing assay to monitor complete fusion of liposomal membranes. Obviously, the cognate ER-Golgi, intra-Golgi, and endosomal 3Q-SNARE sets were fully fusion-incompetent, yielding little or no lipid mixing either with their cognate or non-cognate R-SNAREs (Figure 5c–e). As all of these physiological 3Q-SNARE combinations can form a stable *cis*-QabcR-SNARE complex at least with their cognate partner R-SNAREs (Figure 2), these lipid-mixing data indicate that the ability of *cis*-SNARE assemblies does

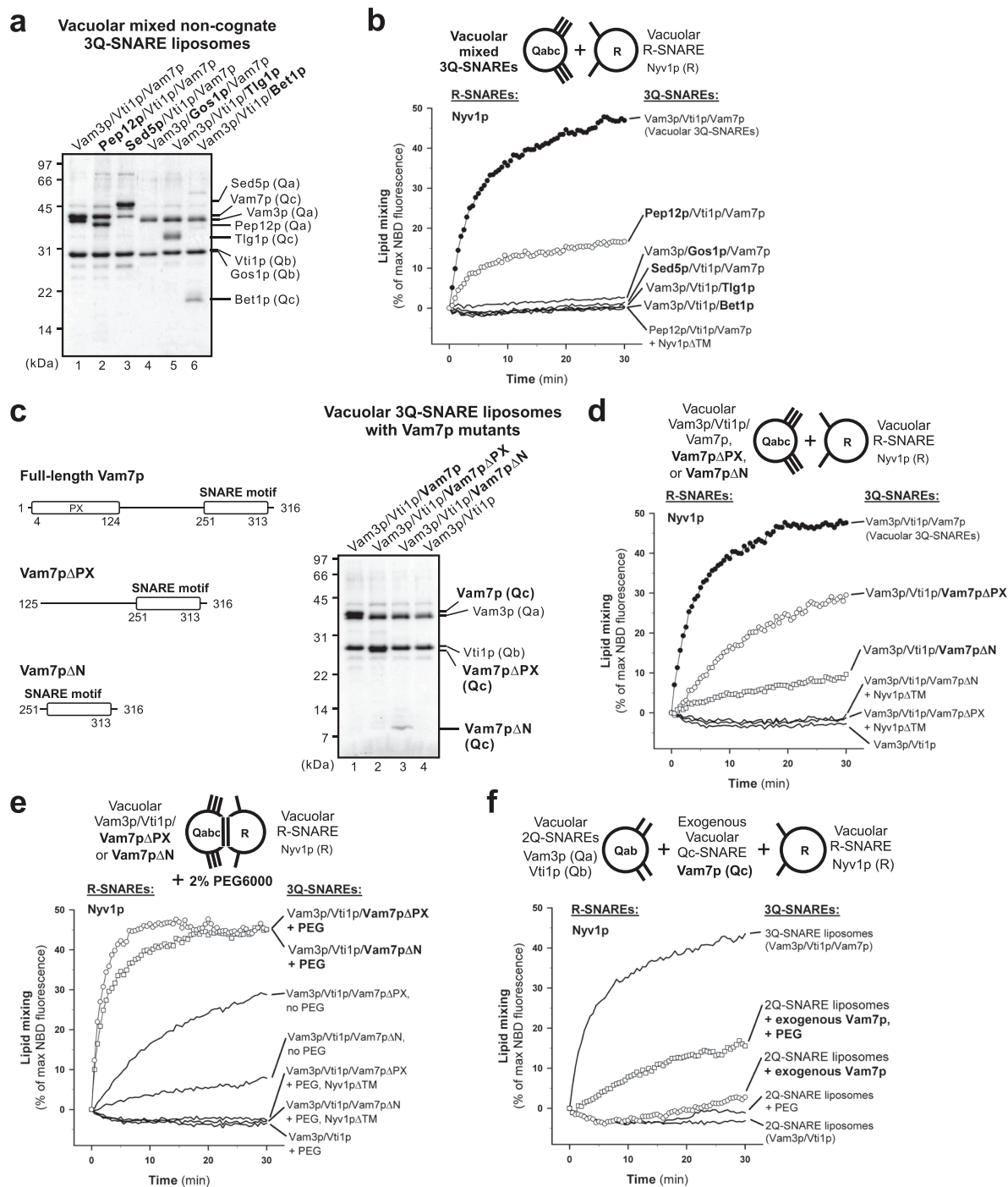


Figure 6 | Requirements for fusogenicity of vacuolar 3Q-SNAREs. (a) Coomassie Blue-stained gel of SNARE liposomes (20 nmol total lipids in each lane) bearing the vacuolar cognate 3Q-SNARE set (Vam3p/Vti1p/Vam7p) or the mixed non-cognate 3Q-SNARE sets containing vacuolar Qbc-, Qac-, or Qab-SNAREs (Pep12p/Vti1p/Vam7p, Sed5p/Vti1p/Vam7p, Vam3p/Gos1p/Vam7p, Vam3p/Vti1p/Tlg1p, and Vam3p/Vti1p/Bet1p) used in (b). (b) The vacuolar mixed non-cognate 3Q-SNARE sets except Pep12p/Vti1p/Vam7p are not fusogenic. Lipid mixing was assayed as in Figure 5c–f, with 3Q-SNARE liposomes bearing the vacuolar cognate or mixed non-cognate sets in (a) and R-SNARE liposomes bearing Nyv1p. GST-Nyv1p lacking a transmembrane domain (Nyv1pΔTM, 6.9 μM) was added where indicated, as a competitive inhibitor. (c) Schematic representation of wild-type full-length Vam7p and its mutants lacking the N-terminal residues 1–124 (Vam7pΔPX) and residues 1–250 (Vam7pΔN) (left). Coomassie Blue-stained gel of SNARE liposomes (20 nmol total lipids in each lane) bearing vacuolar Qab-SNAREs (Vam3p/Vti1p) or Qabc-SNAREs with the full-length Vam7p, Vam7pΔPX, or Vam7pΔN, used in (d–f) (right). (d) The N-terminal domain of Vam7p is critical for efficient lipid mixing by vacuolar 3Q-SNAREs. Lipid mixing was assayed as in Figure 5c–f, with 3Q-SNARE liposomes bearing vacuolar Qab-SNAREs and the full-length Vam7p, Vam7pΔPX, or Vam7pΔN in (c) and R-SNARE liposomes bearing Nyv1p. Nyv1pΔTM (6.9 μM) was added where indicated. (e) PEG-mediated tethering strongly enhances lipid mixing by vacuolar 3Q-SNARE liposomes bearing Vam7pΔPX and Vam7pΔN. Lipid mixing was assayed as in (d), but in the presence of 2% PEG6000. Nyv1pΔTM (6.9 μM) was added where indicated. (f) Exogenous vacuolar Qc-SNARE Vam7p does not support lipid mixing by vacuolar 2Q-SNARE liposomes. Lipid mixing was assayed as in (d), with R-SNARE liposomes bearing Nyv1p and 2Q-SNARE liposomes bearing Vam3p/Vti1p or 3Q-SNARE liposomes bearing Vam3p/Vti1p/Vam7p, but in the presence or absence of 5% PEG6000 where indicated. The vacuolar Qc-SNARE Vam7p (8.8 μM final), which intrinsically lacks a transmembrane domain, was added to the reactions with vacuolar 2Q-SNARE liposomes where indicated.

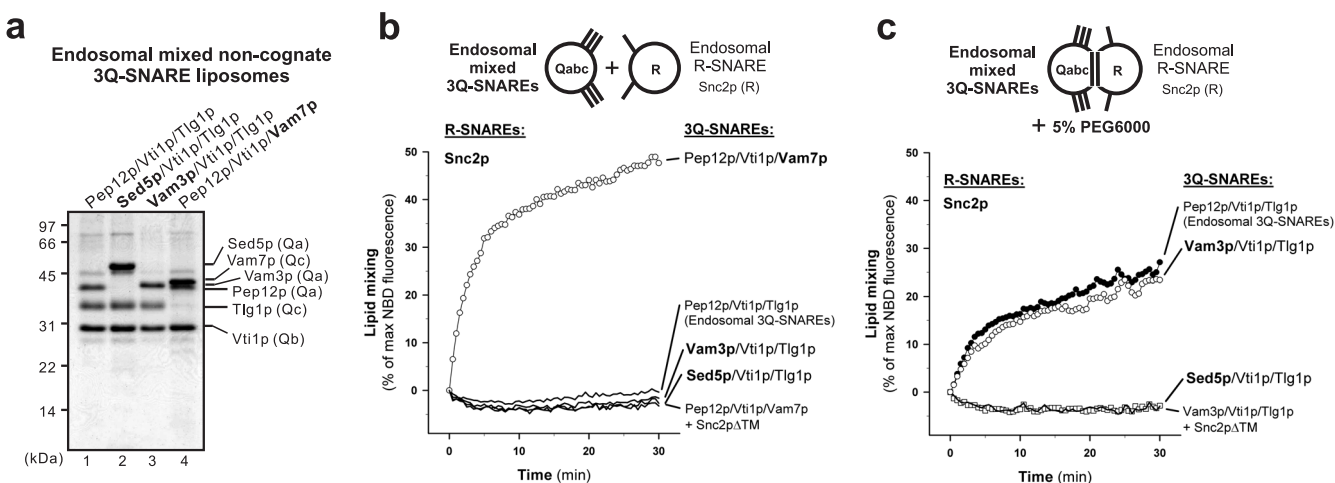


Figure 7 | Specificity of endosomal 3Q-SNARE-dependent proteoliposomal lipid mixing. (a) Coomassie Blue-stained gel of SNARE liposomes (20 nmol total lipids in each lane) bearing the endosomal cognate 3Q-SNARE set (Pep12p/Vti1p/Tlg1p) or the mixed non-cognate 3Q-SNARE sets containing endosomal Qbc- or Qab-SNAREs (Sed5p/Vti1p/Tlg1p, Vam3p/Vti1p/Tlg1p, and Pep12p/Vti1p/Vam7p) used in (b, c). (b) Only the mixed 3Q-SNARE set Pep12p/Vti1p/Vam7p is fusogenic in the absence of PEG. Lipid mixing was assayed as in Figure 5C–F, with 3Q-SNARE liposomes bearing the endosomal cognate or mixed non-cognate sets in (a) and R-SNARE liposomes bearing Snc2p. GST-Snc2p lacking a transmembrane domain (Snc2p Δ TM, 22 μ M final) was added where indicated, as a competitive inhibitor. (c) Vacuolar Vam3p, but not ER-Golgi/intra-Golgi Sed5p, can be substituted for the cognate Qa-SNARE Pep12p in endosomal SNARE-dependent lipid mixing in the presence of PEG. Lipid mixing was assayed as in (b), but in the presence of 5% PEG6000. Snc2p Δ TM (22 μ M) was added where indicated.

not directly account for the intrinsic capacity of SNAREs to initiate lipid mixing. In contrast, the vacuolar 3Q-SNARE set strikingly caused efficient lipid mixing with not only the cognate vacuolar R-SNARE Nyv1p but also with the non-cognate endosomal Snc2p and ER-Golgi Sec22p (Figure 5f), in accordance with our recent study of reconstituted SNARE proteoliposomes with vacuole-mimic lipids³⁵.

Polyethylene glycol (PEG)-mediated membrane tethering strongly activates endosomal SNARE-dependent lipid mixing. Proteoliposomes bearing the ER-Golgi, intra-Golgi, and endosomal 3Q-SNAREs had no potency to catalyze lipid mixing by themselves (Figure 5c–e), suggesting that those SNAREs strictly require additional components for *trans*-SNARE assembly and lipid mixing. To address this, we next performed a lipid mixing assay in the presence of polyethylene glycol (PEG) (Figure 5g–j). PEG is a synthetic reagent to induce nonspecific membrane tethering³⁶ and has been also applied in previous studies of reconstituted SNARE proteoliposomes^{35,37–39}. Strikingly, PEG-mediated membrane tethering selectively activated endosomal 3Q-SNAREs, driving efficient lipid mixing with the cognate R-SNARE Snc2p and non-cognate vacuolar Nyv1p (Figure 5i). Nevertheless, even when PEG promoted liposome tethering, either the ER-Golgi or intra-Golgi 3Q-SNARE set still remained fully inactive (Figure 5g, h), and vacuolar 3Q-SNARE-dependent lipid mixing was not further stimulated (Figure 5j). The PEG-supported lipid mixing by endosomal 3Q-SNAREs was fully blocked by adding the soluble cytoplasmic domain of the cognate R-SNARE Snc2p (Snc2p Δ TM) (Figure 5i), establishing that PEG does not independently trigger nonspecific lipid mixing but promotes SNARE-dependent reactions. Intriguingly, PEG-mediated tethering did not permit endosomal 3Q-SNAREs to cause significant lipid mixing with the non-cognate ER/Golgi R-SNARE Sec22p (Figure 5i), even though endosomal 3Q-SNAREs retained the ability to form a *cis*-SNARE complex with Sec22p (Figure 2e, lane 2). These findings establish that the endosomal 3Q-SNARE set critically requires tethering events for specifically forming fusogenic *trans*-SNARE complexes on opposing membranes.

Fusogenicity of vacuolar 3Q-SNAREs largely depends on the unique N-terminal domain of the Qc-SNARE Vam7p. In the

present reconstituted reactions with four physiological 3Q-SNARE combinations (Figure 5), we found that only the vacuolar 3Q-SNARE set (Vam3p, Vti1p, and Vam7p) was fusogenic in the absence of any additional factors, catalyzing efficient proteoliposomal lipid mixing with all the R-SNARE liposomes tested (Figure 5f). To resolve the molecular basis of the inherent fusogenicity of vacuolar 3Q-SNAREs, we further tested lipid mixing for various types of the 3Q-SNARE liposomes bearing the vacuolar cognate set and the mixed non-cognate sets which had retained the ability to form a *cis*-QabcR-SNARE complex with the vacuolar R-SNARE Nyv1p in solution (Figure 4j–l), including Pep12p/Vti1p/Vam7p, Sed5p/Vti1p/Vam7p, Vam3p/Gos1p/Vam7p, Vam3p/Vti1p/Tlg1p, and Vam3p/Vti1p/Bet1p (Figure 6a, b). Strikingly, most of these mixed non-cognate 3Q-liposomes, except Pep12p/Vti1p/Vam7p, were fully non-fusogenic and had no potency to initiate lipid mixing (Figure 6b). For example, either the non-cognate Qc-SNARE endosomal Tlg1p or ER-Golgi Bet1p did not substitute for the cognate Vam7p in vacuolar SNARE-dependent lipid mixing (Figure 6b). It should be noted that little Vam7p protein had been reconstituted into proteoliposomes when the Vam3p/Gos1p/Vam7p combination was applied (Figure 6a, lane 4). This indicates that the non-vacuolar, intra-Golgi Qb-SNARE Gos1p has no ability to form a stable 3Q-subcomplex with vacuolar Qac-SNAREs, even though Gos1p can form a fully-assembled *cis*-QabcR-SNARE complex with vacuolar SNAREs (Figure 4k, lane 3). Together, these results establish that an appropriate 3Q-SNARE composition is very critical for conferring fusogenicity of vacuolar 3Q-SNAREs, consistent with our prior study³⁵.

The vacuolar Qc-SNARE Vam7p is indispensable for SNARE-only lipid mixing reactions (Figure 6b). The N-terminal domain of Vam7p contains a unique Phox-homology (PX) domain for its membrane association⁴⁰ and shows no sequence similarity to any other N-terminal domains in yeast and mammal SNAREs⁷. Thus, we next ask whether this unique Vam7p N-terminal domain contributes to the fusogenicity of vacuolar 3Q-SNAREs (Figure 6c–e). Two types of the Vam7p mutants lacking either its PX domain alone (Vam7p Δ PX) or the entire N-terminal domain (Vam7p Δ N) were purified and reconstituted into 3Q-SNARE liposomes with vacuolar Qab-SNAREs (Figure 6c). Rapid lipid mixing by vacuolar 3Q-SNAREs was signifi-

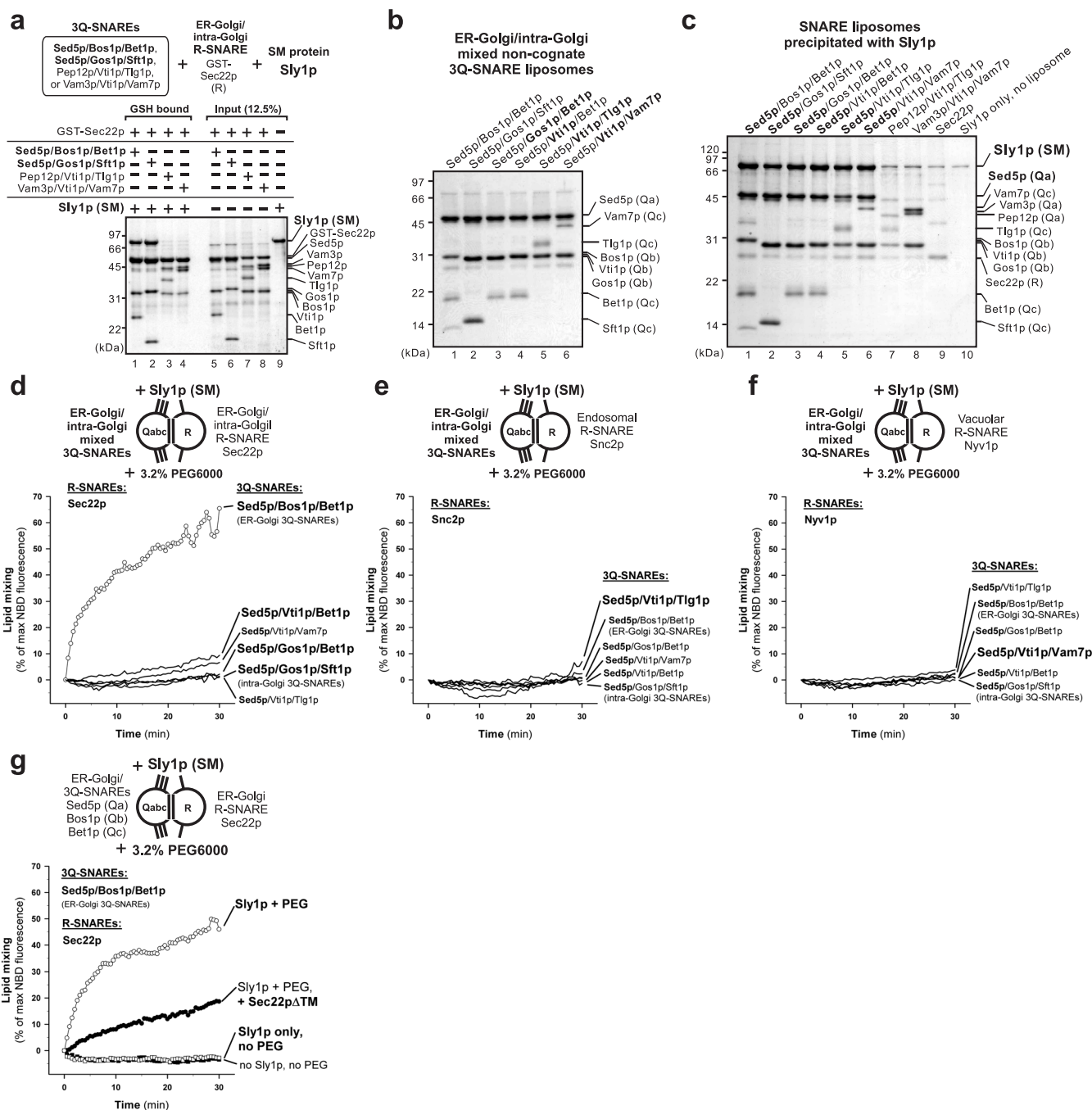


Figure 8 | The SM protein Sly1p is essential for fusogenicity of its cognate ER-Golgi SNARE complex. (a) The SM protein Sly1p specifically associates with its cognate ER-Golgi and intra-Golgi QabcR-SNARE complexes. GST-Sec22p was incubated with physiological sets of 3Q-SNAREs, which include ER-Golgi Sed5p/Bos1p/Bet1p, intra-Golgi Sed5p/Gos1p/Sft1p, endosomal Pep12p/Vti1p/Tlg1p, and vacuolar Vam3p/Vti1p/Vam7p, and Sly1p (4.6 μ M final) in RB500 containing 1% Triton X-100. After GST-Sec22p was isolated by glutathione-Sepharose beads, Q-SNAREs and Sly1p bound to the beads were analyzed by SDS-PAGE and Coomassie Blue staining. Initial SNARE concentrations of all the reactions were 4 μ M for each SNARE. (b) Coomassie Blue-stained gel of SNARE proteoliposomes (20 nmol total lipids in each lane) bearing the cognate ER-Golgi/intra-Golgi 3Q-SNARE sets or the Sed5p-containing mixed non-cognate 3Q-SNARE sets (Sed5p/Gos1p/Bet1p, Sed5p/Vti1p/Bet1p, Sed5p/Vti1p/Tlg1p, and Sed5p/Vti1p/Vam7p) used in (c–g). (c) Sly1p selectively binds to the Sed5p-containing 3Q-SNARE liposomes. SNARE liposomes (1 mM total lipids in final), which bore the Sed5p-containing 3Q-SNARE sets in (b), the endosomal/vacuolar 3Q-SNARE sets, or ER-Golgi/intra-Golgi R-SNARE Sec22p, were mixed with Sly1p (11 μ M) in RB150, incubated (4°C, 30 min), and centrifuged (20,000 g, 4°C, 30 min). Sly1p protein co-precipitated with those SNARE liposomes was analyzed by SDS-PAGE and Coomassie Blue staining. (d–f) Sly1p strongly and exclusively activate the fusogenicity of its cognate ER-Golgi SNARE complex. Lipid mixing was assayed in RB150 containing 3.2% PEG6000, with the Sed5p-containing 3Q-SNARE liposomes (400 μ M lipids final) in (b), R-SNARE liposomes (160 μ M lipids final) bearing Sec22p (d), Snc2p (e), or Nyv1p (f), and Sly1p (4.5 μ M final). (g) PEG-mediated tethering is required for Sly1p-dependent lipid mixing by ER-Golgi SNAREs. Lipid mixing between the cognate ER-Golgi 3Q- and R-SNARE liposomes (400 and 160 μ M lipids, respectively) was assayed as in (d), in the presence of Sly1p (4.5 μ M), PEG6000 (3.2%), and GST-Sec22p lacking a transmembrane domain (Sec22p^{ATM}, 10 μ M), where indicated.



cantly reduced by truncation of the Vam7p PX domain (Figure 6d, white circles for Vam3p/Vti1p/Vam7p Δ PX) and, moreover, almost fully blocked by deletion of the entire N-terminal domain (Figure 6d, white squares for Vam3p/Vti1p/Vam7p Δ N). This faithfully reflects that fusogenicity of the vacuolar 3Q-SNARE set relies upon the specific function of the Vam7p N-terminal domain. Unexpectedly, PEG-driven liposome tethering strongly accelerated lipid mixing by the 3Q-SNARE liposomes with these Vam7p mutants, Vam7p Δ PX and Vam7p Δ N (Figure 6e, white circles and squares respectively), thereby bypassing the requirement of the Vam7p N-terminal domain for driving efficient lipid mixing. Their PEG-dependent rapid lipid mixing was completely inhibited by addition of the soluble cytoplasmic domain of Nyv1p (Nyv1p Δ TM) or omission of Vam7p Δ PX or Vam7p Δ N (Figure 6e), indicating that PEG did not cause non-specific lipid mixing but definitely supported SNARE-dependent lipid mixing. Thus, these results establish that the unique N-terminal domain of Vam7p indeed permits the vacuolar 3Q-SNARE set to be fusogenic by promoting membrane tethering. This specific tethering function of the Vam7p N-terminal domain may depend in part on the affinity of the PX domain for acidic lipids^{40,41}, since phosphatidylinositol 3-phosphate (PI(3)P) was not included in the current reconstitution system.

Nevertheless, the Vam7p N-terminal domain is not the only determinant for fusogenicity of vacuolar 3Q-SNAREs. When excess Vam7p (8.8 μ M), which is a naturally soluble SNARE protein lacking a transmembrane domain (Figure 1a, Figure 6c), was exogenously added to the lipid-mixing reactions with vacuolar 2Q-SNARE liposomes, the exogenous full-length Vam7p had little potency to restore fusogenicity (Figure 6f, white circles). This very slow lipid mixing by the vacuolar 2Q-liposomes and exogenous excess Vam7p was moderately stimulated by addition of PEG but yet substantially inefficient compared to that of the vacuolar 3Q-SNARE liposomes (Figure 6f, white squares). These lipid-mixing data indicate that fusogenicity of the vacuolar 3Q-SNARE set thoroughly relies on co-reconstitution of all three vacuolar Q-SNAREs into proteoliposomes and that soluble Vam7p itself has little ability to assemble into fusogenic 3Q-SNARE complexes with Vam3p/Vti1p on membranes.

Specificity of endosomal SNARE- and PEG-dependent proteoliposomal lipid mixing. Although endosomal 3Q-SNAREs as well as ER-Golgi and intra-Golgi 3Q-SNAREs were fully inactive in the current SNARE-only lipid-mixing reactions (Figure 5c–e), PEG-mediated non-specific membrane tethering permits endosomal 3Q-SNAREs, but not ER-Golgi and intra-Golgi 3Q-SNAREs, to be fusogenic (Figure 5g–i). To further understand specificity of this endosomal 3Q-SNARE- and PEG-dependent lipid mixing, we employed lipid-mixing assays for various 3Q-SNARE liposomes bearing the cognate endosomal 3Q-SNARE set and its mixed non-cognate sets which had assembled into a stable *cis*-QabcR-SNARE complex with the endosomal R-SNARE Snc2p in GST pull-down assays (Figure 4g–i), including Sed5p/Vti1p/Tlg1p, Vam3p/Vti1p/Tlg1p, and Pep12p/Vti1p/Vam7p (Figure 7a). When PEG was not present, both of the two mixed 3Q-SNARE sets in which the endosomal Qa-SNARE Pep12p was substituted by the non-cognate Sed5p or Vam3p were yet fully non-fusogenic, as well as the endosomal cognate 3Q-SNARE set (Figure 7b). By contrast, the other mixed 3Q-SNARE set which contains vacuolar Vam7p as a Qc-SNARE, Pep12p/Vti1p/Vam7p, was fully fusogenic and catalyzed rapid lipid mixing with the cognate R-SNARE Snc2p (Figure 7b, white circles), consistent with our recent study³⁵ and the current lipid-mixing data with vacuolar Nyv1p in Figure 6b. By testing lipid mixing of the two non-fusogenic mixed 3Q-SNARE sets (Vam3p/Vti1p/Tlg1p and Sed5p/Vti1p/Tlg1p) in the presence of PEG, we next found that PEG supported lipid mixing by Vam3p/Vti1p/Tlg1p (Figure 7c, white circles), as well as the cognate 3Q set Pep12p/Vti1p/Tlg1p, but had no effect on the lipid-mixing reaction

with Sed5p/Vti1p/Tlg1p (Figure 7c, white squares). Considering that the Sed5p-containing ER-Golgi/intra-Golgi 3Q-SNARE sets also remained fully inactive in the presence of PEG (Figure 5g, h), these results indicate that PEG-mediated membrane tethering does not promiscuously promote SNARE-dependent lipid mixing and that the ER/Golgi-resident Qa-SNARE Sed5p may have the specific function to block fusogenic *trans*-assemblies between 3Q- and R-SNAREs on tethered opposing membranes.

The SM protein Sly1p is an essential component to confer fusogenicity of its cognate ER-Golgi SNARE complex. Our current reconstitution revealed that physiological sets of the ER-Golgi and intra-Golgi 3Q-SNAREs inherently had no capacity to catalyze lipid mixing by themselves (Figure 5c, d), even in the presence of PEG that promotes non-specific membrane tethering (Figure 5g, h). Therefore, we next asked whether the ER/Golgi-resident SM protein Sly1p is required for conferring fusogenicity of its cognate ER-Golgi or intra-Golgi 3Q-SNARE set (Figure 8). The SM protein Sly1p has been reported to directly bind to the ER/Golgi Qa-SNARE Sed5p and its fully assembled QabcR-SNARE complexes^{42–45}. First, to confirm the specific SNARE binding of Sly1p, we performed a GST pull-down assay in solution, with purified His6-tagged Sly1p (Figure 8a, lane 9) and four *cis*-QabcR-SNARE complexes which had been pre-assembled with the cognate ER/Golgi R-SNARE GST-Sec22p and the ER-Golgi, intra-Golgi, endosomal, or vacuolar 3Q-SNAREs (Figure 8a, lanes 5–8). Sly1p specifically associated only with its cognate ER-Golgi and intra-Golgi SNARE complexes (Figure 8a, lanes 1–2), but not with the non-cognate mixed complexes containing endosomal or vacuolar 3Q-SNAREs (Figure 8a, lanes 3–4), in accordance with the prior study⁴⁴. Moreover, to further analyze specificity of Sly1p-SNARE interactions, we next employed a liposome co-sedimentation assay by incubating Sly1p with various reconstituted SNARE proteoliposomes bearing the cognate ER-Golgi and intra-Golgi 3Q-SNARE sets (Figure 8b, lanes 1–2; Figure 8c, lanes 1–2), the Sed5p-containing mixed non-cognate 3Q-SNARE sets that had retained the capacity to form a *cis*-QabcR-SNARE complex in pull-down assays (Figure 4b, f, g, j), including Sed5p/Gos1p/Bet1p, Sed5p/Vti1p/Bet1p, Sed5p/Vti1p/Tlg1p, and Sed5p/Vti1p/Vam7p (Figure 8b, lanes 3–6; Figure 8c, lanes 3–6), endosomal and vacuolar 3Q-SNARE sets (Figure 8c, lanes 7–8), and ER/Golgi R-SNARE Sec22p (Figure 8c, lane 9). After centrifugation of these reaction mixtures with Sly1p and various SNARE liposomes, we observed that Sly1p was efficiently co-precipitated with all the six Sed5p-containing 3Q-liposomes tested (Figure 8c, lanes 1–6) but not with endosomal/vacuolar 3Q-liposomes and the cognate R-SNARE liposomes bearing Sec22p (Figure 8c, lanes 7–10). These data of liposome co-sedimentation assays establish that Sly1p can selectively recognize and bind to the Sed5p-containing 3Q-SNARE complexes on membranes. The specific interaction between Sly1p and a 3Q-SNARE sub-complex certainly depends on the presence of Sed5p. This is particularly obvious when comparing the Sly1p binding of Sed5p/Vti1p/Tlg1p and Sed5p/Vti1p/Vam7p (Figure 8c, lanes 5–6) to that of Pep12p/Vti1p/Tlg1p and Vam3p/Vti1p/Vam7p, respectively (Figure 8c, lanes 7–8).

Since we found that Sly1p stably and specifically associated with various types of the Sed5p-containing 3Q-SNARE proteoliposomes (Figure 8c, lanes 1–6), we next tested whether the SM protein Sly1p indeed selectively activates those Sed5p-containing 3Q-SNARE complexes through its SNARE binding and thereby directly catalyzes lipid mixing (Figure 8d–g). Lipid mixing was assayed by adding Sly1p to the reaction mixtures with both the R-SNARE liposomes bearing Sec22p, Snc2p, or Nyv1p and the 3Q-SNARE liposomes bearing Sed5p/Bos1p/Bet1p, Sed5p/Gos1p/Sft1p, Sed5p/Gos1p/Bet1p, Sed5p/Vti1p/Bet1p, Sed5p/Vti1p/Tlg1p, or Sed5p/Vti1p/Vam7p, which had been pre-incubated in the presence of PEG at 30°C for 10 min (Figure 8d–f). Strikingly, among the total 18 possible combinations of these three R-SNARE liposomes and six



Sed5p-containing 3Q-SNARE liposomes, Sly1p allows exclusively the cognate ER-Golgi 3Q- and R-SNARE combination (Sed5p/Bos1p/Bet1p and Sec22p) to be fusogenic, triggering rapid lipid mixing (Figure 8d, white circles). However, the other five Sed5p-containing 3Q-SNARE sets were fully inactive under the same conditions, causing little lipid mixing with all three R-SNAREs tested (Figure 8d–f), even though these 3Q-SNARE sets retained the abilities to efficiently bind Sly1p (Figure 8c) and also stably form a *cis*-QabcR-SNARE complex (Figure 4). The robust Sly1p-dependent lipid mixing by ER-Golgi SNAREs was completely abolished when omitted PEG (Figure 8g, white squares) and thoroughly inhibited by adding the soluble cytoplasmic domain of Sec22p (Sec22p Δ TM) (Figure 8g, black circles). Thus, these current lipid-mixing data with the SM protein Sly1p establish that Sly1p is certainly an essential and specific catalyst to directly confer fusogenicity of the cognate ER-Golgi SNAREs and that this Sly1p function requires not only the association with Sed5p and/or its assembled SNARE complexes but also the stringent cognate 3Q-SNARE composition (Sed5p, Bos1p, and Bet1p) and the presence of membrane-tethering agents, such as PEG that we used in the current reconstitution.

Discussion

Although SNARE proteins are undoubtedly a key component for catalyzing membrane fusion in eukaryotic endomembrane systems, whether 3Q- and R-SNAREs (or t- and v-SNAREs) have the intrinsic capacity to directly determine the specificity of membrane fusion remains a matter of debate⁷. To thoroughly resolve this fundamental but enigmatic question, we systematically tested 14 purified full-length SNAREs that function in the yeast ER-Golgi, intra-Golgi, endosomal, and vacuolar transport (Figure 1), for their *cis*-QabcR-SNARE assemblies in detergent solution (Figures 2–4, Table 1) and for their fusogenicity in a reconstituted proteoliposomal system under the physiological conditions of lipid composition and addition of other conserved fusion catalysts, a membrane tethering factor and a Sec1/Munc18-family (SM) protein (Figures 5–8, Table 1).

In contrast to prior *in vitro* SNARE-binding assays using the soluble cytoplasmic fragments of SNAREs^{24,25,29}, we studied *cis*-SNARE assemblies in detergent micellar solutions with “full-length” SNAREs that contain their C-terminal linkers and transmembrane domains, more closely resembling physiological SNARE-complex assemblies that form a continuous helical bundle into membranes throughout the SNARE motifs, linkers, and transmembrane regions⁴⁶. Our current data faithfully reflect the fact that the *cis*-assemblies of full-length QabcR-SNAREs are indeed not promiscuous but rather substantially selective (Figures 2–4), thereby establishing that SNARE proteins themselves have the inherent capacity to ensure fusion specificity, at least in part, simply through their physical SNARE-SNARE interactions. We also found significant diversity in the specificity of *cis*-SNARE assemblies: the early secretory ER-Golgi and intra-Golgi SNAREs assembled quite stringently into their cognate QabcR-SNARE complexes, allowing only very few non-cognate SNAREs to substitute for the cognate counterparts (Figure 2a, c, Figure 4a–f). By contrast, the late secretory endosomal and vacuolar SNAREs exhibited relatively moderate binding specificity (Figure 2e, g, Figure 4g–l). Those late secretory 3Q-SNARE sets non-selectively associated with both cognate and non-cognate R-SNAREs but yet lost their potency to form a stable QabcR-complex when singly replaced by particular early secretory Qb- or Qc-SNARE. These divergent characteristics of *cis*-SNARE assemblies would help us to better understand how eukaryotic cells tightly control the fusogenic activity of numerous SNAREs in their complex endomembrane trafficking systems. For example, cells select only one proper pair of 3Q- and R-SNAREs for fusion in the ER-Golgi transport while strictly silencing the other miscellaneous SNARE sets that are destined to be active in the later secretory pathways at Golgi, trans-Golgi network, endosome, vacuole, and plasma membrane.

However, the patterns of *cis*-QabcR-SNARE assemblies that we observed here do not simply reflect their intrinsic capacity to assemble into fusogenic *trans*-SNARE complexes. By thoroughly exploring SNARE-dependent lipid mixing of reconstituted proteoliposomes with the yeast 3Q- and R-SNARE sets and physiological lipid composition that resembles sub-cellular membranes in yeast cells, our current studies yielded three striking observations: (1) in the absence of any additional fusion factors, only the vacuolar 3Q-SNARE set was fusogenic, causing robust lipid mixing with all three R-SNAREs tested (the cognate Nyv1p and non-cognate Snc2p and Sec22p), whereas the other three physiological 3Q-SNARE combinations in ER-Golgi, intra-Golgi, and endosomal transport were all completely non-fusogenic (Figure 5c–f). (2) PEG-mediated membrane tethering strongly activated endosomal 3Q-SNAREs for robustly catalyzing lipid mixing, but yet PEG could not support lipid mixing by either early secretory ER-Golgi or intra-Golgi 3Q-SNAREs at all (Figure 5g–j). (3) Finally, the SM protein Sly1p and the tethering agent PEG acted synergistically to catalyze lipid mixing by the cognate ER-Golgi SNARE complex, but yet the intra-Golgi SNARE complex remained fully inactive (Figure 8). Taken together, at least for the yeast SNARE-family proteins, our findings postulate a new concept that SNARE proteins employ multiple and distinct strategies to control their inherent fusogenic capacities and thereby directly mediate the specificity of membrane fusion: highly stringent assemblies of fusogenic ER-Golgi *trans*-SNARE complexes rely on not only their very strict specificity of physical SNARE-SNARE interactions but also on the synergistic actions of their cognate SM protein Sly1p and membrane tethering factors. For endosomal SNARE complexes, their fusogenicity critically depends on membrane tethering events that facilitate fusogenic *trans*-assemblies of endosomal 3Q- and R-SNAREs on opposing membranes. This idea is consistent with the recent study by *in vitro* docking and fusion assays of isolated early endosomes in mammals, demonstrating that SNARE function is not involved in tethering and docking of early endosomes⁴⁷. For vacuolar SNARE complexes, the “pre-assembled” 3Q-SNARE complex is

Table 1 | *Cis*-QabcR-SNARE complexes identified in Figures 2–4

Qa-SNAREs	QabcR-SNARE complexes
Sed5p	Sed5p/Bos1p/Bet1p/Sec22p (ER-Golgi)^a Sed5p/Gos1p/Sft1p/Sec22p (intra-Golgi) Sed5p/Gos1p/Bet1p/Sec22p Sed5p/Vti1p/Bet1p/Sec22p Sed5p/Vti1p/Tlg1p/Snc2p Sed5p/Vti1p/Vam7p/Nyv1p
Pep12p	Pep12p/Vti1p/Tlg1p/Snc2p (endosome)^b Pep12p/Vti1p/Tlg1p/Sec22p Pep12p/Vti1p/Tlg1p/Ykt6p Pep12p/Vti1p/Tlg1p/Nyv1p ^b Pep12p/Vti1p/Vam7p/Snc2p ^a Pep12p/Vti1p/Vam7p/Nyv1p ^a
Vam3p	Vam3p/Vti1p/Vam7p/Nyv1p (vacuole)^{a,b} Vam3p/Vti1p/Vam7p/Sec22p ^{a,b} Vam3p/Vti1p/Vam7p/Ykt6p Vam3p/Vti1p/Vam7p/Snc2p ^{a,b} Vam3p/Gos1p/Vam7p/Nyv1p Vam3p/Vti1p/Bet1p/Nyv1p Vam3p/Vti1p/Tlg1p/Nyv1p Vam3p/Vti1p/Tlg1p/Snc2p ^b

The physiological cognate QabcR-SNARE complexes for ER-Golgi, intra-Golgi, endosomal, or vacuolar transport are represented in bold.

^aQabcR-SNARE combinations that catalyzed robust lipid mixing in the absence of any additional factors, in Figures 5–7.

^bQabcR-SNARE combinations that catalyzed robust lipid mixing when PEG was present, in Figures 5–7.

^cQabcR-SNARE combinations that catalyzed robust lipid mixing when both PEG and the SM protein Sly1p were present, in Figure 8.



constitutively fusogenic. However, the exogenous Qc-SNARE Vam7p in solution cannot be assembled into fusogenic 3Q-complexes on membranes by itself (Figure 6f). Thus, to ensure the specificity of vacuolar SNARE-dependent fusion, the 3Q-assembly of membrane-anchored vacuolar Qab-SNAREs with the naturally soluble Qc-SNARE Vam7p has to be tightly regulated by the other fusion components that promote assembly and disassembly of vacuolar SNAREs, including the phosphoinositide PI(3)P, Sec17p/Sec18p/ATP, and the HOPS (homotypic fusion and vacuole protein sorting) tethering complex^{15,16,39,48–50}.

Indeed, only the vacuolar cognate 3Q-SNARE set (Vam3p/Vti1p/Vam7p) has the intrinsic capacity to be fusogenic by itself, whereas all the other various cognate and non-cognate 3Q-SNARE sets we tested here are fully non-fusogenic, except for the one mixed combination of endosomal Pep12p and vacuolar Vti1p/Vam7p (Figure 5c–f, Figure 6b, Figure 7b). What specifically allows the vacuolar 3Q-SNARE complex to be fusogenic? Unexpectedly, our current reconstitution reveals that the N-terminal domain of the Qc-SNARE Vam7p is an essential component to confer the fusogenicity of vacuolar 3Q-SNAREs through its specific function to support membrane tethering (Figure 6d, e). Since the Vam7p N-terminal domain is very unique and has no sequence similarity to any other SNARE N-terminal domains, this finding leads us to postulate that all SNAREs basically have no potency to catalyze membrane tethering/docking and fusogenic *trans*-SNARE pairing by themselves and that yeast vacuolar 3Q-SNARE combination with the specialized Vam7p N-terminal domain is an exception. Nevertheless, many earlier reconstitution studies of yeast SNAREs have shown that the cognate sets of ER-Golgi SNAREs (Sed5p, Bos1p, Bet1p, and Sec22p), intra-Golgi SNAREs (Sed5p, Gos1p, Sft1p, and Ykt6p), and endosomal SNAREs (Pep12p, Vti1p, Tlg1p, and Snc2p) also led to substantial lipid mixing in the absence of any additional factors, in addition to the vacuolar SNARE set^{21,22,51–53}. In those studies, there were several marked variations in the experimental conditions: (1) a simple non-physiological PC/PS lipid composition was used for preparing SNARE proteoliposomes, unlike a complex but more physiological lipid mix used in our current reconstitution. We prefer to employ the complex lipid composition that mimics sub-cellular membranes, since prior studies on vacuolar SNAREs have established that a physiological lipid composition is a critical factor even in SNARE-only reactions of reconstituted proteoliposomes^{15,17,52}. (2) The alternative QabR:Qc SNARE topology, not the canonical Qabc:R topology, was required for initiating lipid mixing by the ER-Golgi or intra-Golgi SNARE combination^{22,51}, while we have tested only the canonical topology throughout this study (Figure 5a, b). (3) Ykt6p, but not Sec22p, was identified as an active R-SNARE protein for intra-Golgi SNARE-dependent lipid mixing²². By contrast, our data clearly indicate that Ykt6p cannot assemble into a stable *cis*-SNARE complex with intra-Golgi 3Q-SNAREs (Figure 2c), suggesting that Ykt6p will not be functional in an intra-Golgi SNARE-dependent fusion process. (4) Addition of “snc2-C-peptide”, a specific short peptide that corresponds to the C-terminal half of the cognate Snc2p R-SNARE motif, was required for endosomal SNARE-dependent lipid mixing^{53,54}. We find that this requirement of the snc2-C-peptide can be bypassed by PEG-driven membrane tethering (Figure 5e, i). Thus, in this context, there still remain several issues of the SNARE-only reactions to be further resolved.

Among numerous conserved proteins that contribute to intracellular membrane fusion, SM-family proteins are thought to be a key component to catalyze fusion *per se* together with SNAREs^{3,55}. The direct and specific functions of SM proteins in SNARE-dependent membrane fusion have been established by reconstitution studies with neuronal SM protein Munc18-1, demonstrating that Munc18-1 strongly activates lipid mixing by its cognate synaptic 3Q- and R-SNAREs, syntaxin1a, SNAP-25, and synaptobrevin^{28,56}. It should be noted that the stimulation of lipid mixing thoroughly required

lengthy pre-incubation of Munc18-1 with the cognate 3Q- and R-SNARE liposomes at 4 °C before initiating lipid mixing at the physiological temperature^{8,56}. In addition to these prior studies, we now reveal that the ER/Golgi-resident SM protein Sly1p is also an essential fusion catalyst for its cognate ER-Golgi SNARE complex (Figure 8). Strikingly, Sly1p triggered rapid lipid mixing by directly adding to the ER-Golgi 3Q- and R-SNARE liposomes without pre-incubation (Figure 8d). This robust function of Sly1p as a fusion catalyst requires highly stringent 3Q-SNARE composition and thereby further enhances fusion specificity (Figure 8d–f), as Sly1p did not activate at all the intra-Golgi 3Q-SNARE and other Sed5p-containing mixed 3Q-SNARE sets even though these 3Q-SNARE sets fully retained the ability to bind Sly1p (Figure 8b–f). Intriguingly, under the current conditions, this Sly1p-dependent fusogenicity requires PEG-mediated tethering events (Figure 8g). This supports the notion that the SM protein Sly1p by itself cannot catalyze membrane docking and *trans*-SNARE assembly, thus it needs to cooperate with tethering factors to form fusion-competent *trans*-SNARE complexes. This is consistent with the previous observations for the conserved oligomeric Golgi (COG) tethering complex, indicating that the direct interaction between the COG tethering complex and Sly1p is indispensable for Golgi SNARE assemblies⁵⁷. Since the non-specific synthetic tethering agent, PEG, was used throughout our present reconstitution studies, further studies are required to understand how the specific and physiological tethering factors guide SM proteins such as Sly1p towards catalyzing membrane docking and *trans*-SNARE assembly.

Methods

Protein expression and purification. The coding sequences of full-length yeast SNARE proteins (Sed5p, Pep12p, Vam3p, Bos1p, Gos1p, Vti1p, Bet1p, Sft1p, Tlg1p, Vam7p, Sec22p, Ykt6p, Snc2p, and Nvy1p), two mutant forms of the yeast vacuolar Qc-SNARE Vam7p (Vam7pΔPX (residues 125–316) and Vam7pΔN (residues 251–316)), and the ER/Golgi SM protein Sly1p were amplified by polymerase chain reaction (PCR) from the yeast *S. cerevisiae* genomic DNA and cloned into a pET-30 Ek/LIC or pET-41 Ek/LIC vector (Novagen, Gibbstown, NJ, USA) expressing a His6- or GST-His6-tagged protein, respectively. These PCR fragments contained the sequence encoding the human rhinovirus 3C protease site (Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro) upstream of the initial ATG codons to obtain full-length, untagged proteins with only three extra N-terminal residues (Gly-Pro-Gly) after 3C protease cleavage. SNAREs and Sly1p were produced in the *Escherichia coli* Rosetta 2(DE3) or Rosetta 2(DE3)pLysS strain (Novagen) in Terrific Broth medium (1 liter each) with kanamycin and chloramphenicol by induction with 1 mM iso-propyl 1-thio-β-D-galactopyranoside at 37 °C for 3 h for SNAREs and at 16 °C for 20 h for Sly1p. *E. coli* cells were harvested and resuspended in 30 ml each of buffer A (20 mM sodium phosphate, pH 7.0, 500 mM NaCl, 10% glycerol) containing 100 mM β-OG (β-octylglucoside, Nacalai Tesque, Kyoto, Japan), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1.0 μg/ml pepstatin A. For Sly1p, harvested cells were suspended in the same buffer but without β-OG. Cell suspensions were incubated at 4 °C for 30 min with gentle shaking, lysed by sonication (UD-201 ultrasonic disrupter; Tomy Seiko, Tokyo, Japan), and centrifuged at 50,000 rpm for 1 h at 4 °C using a 70 Ti rotor (Beckman Coulter, Fullerton, CA, USA). His6- or GST-His6-tagged SNAREs and Sly1p in the supernatants were affinity-purified by Ni-NTA agarose beads (Qiagen, Valencia, CA, USA) or COSMOGEL His-Accept beads (Nacalai Tesque) as described previously³⁵.

GST pull-down assay. GST-His6-tagged R-SNAREs and His6-tagged Q-SNAREs were mixed at 4 μM each in 400 μl RB500 (20 mM HEPES-NaOH, pH 7.4, 500 mM NaCl, 10% glycerol) containing 100 mM β-OG, incubated at 4 °C for 1 h with gentle agitation, mixed with glutathione-Sepharose 4 Fast Flow beads (200 μl, 50% slurry; GE Healthcare, Parsippany, NJ, USA) equilibrated in the same buffer, and further incubated at 4 °C for 1 h. The glutathione-Sepharose beads were isolated by centrifugation (2 min, 15,300 g, 4 °C) and washed four times in 400 μl RB500 containing 100 mM β-OG. Bound GST-tagged R-SNAREs and His6-tagged Q-SNAREs were eluted at 100 °C for 5 min with 2% SDS, followed by SDS-PAGE and Coomassie Blue staining. For the GST pull-down assays with the SM protein Sly1p, the glutathione-Sepharose beads were preincubated with Qabc-R-SNARE sets (GST-His6-tagged R-SNAREs and His-tagged Qabc-SNAREs, 4 μM for each SNARE) at 4 °C for 2 h with gentle agitation and then washed twice in 400 μl RB500 containing 1% Triton X-100 instead of 100 mM β-OG. His6-tagged Sly1p (4.6 μM final) was added to the washed beads in 500 μl RB500 with 1% Triton X-100. After a 4 °C incubation for 1 h with gentle shaking, the beads were further washed three times in 400 μl RB500 with 1% Triton X-100. Bound Qabc-R-SNAREs and Sly1p were eluted at 100 °C for 5 min with 2% SDS and analyzed by SDS-PAGE and Coomassie Blue staining.



Preparation of reconstituted SNARE proteoliposomes. Reconstitution of proteoliposomes bearing purified recombinant SNARE proteins was performed as described previously with some modifications^{15–17,35}. Non-fluorescent lipids except ergosterol (Sigma, St Louis, MO, USA) were from Avanti Polar Lipids (Alabaster, AL, USA). Fluorescent lipids, N-(7-nitro-2,1,3-benzoxadiazole-4-yl)-PE (NBD-PE), N-(lissamine rhodamine B sulfonyl)-PE (Rh-PE), and dansyl-PE, were obtained from Molecular Probes (Sunnyvale, CA, USA). Lipid mixes for SNARE liposomes contained 1-palmitoyl-2-oleoyl-PC (POPC) (40% or 42% (mol/mol) for donor or acceptor SNARE proteoliposomes, respectively), POPE (20%), soy PI (15%), POPS (6.0%), POPA (2.0%), ergosterol (12%), bovine cardiolipin (1.0%), diacylglycerol (1.0%), and fluorescent lipids (1.5% each of NBD-PE/Rh-PE or 1.0% of dansyl-PE for donor or acceptor liposomes, respectively). Dried lipid films with these lipid compositions were dissolved in RB500 containing 100 mM β -OG and mixed at 3 mM total lipids with purified R-SNAREs (final 10 μ M each, for donor liposomes) or 3Q-SNAREs (final 5 μ M each, for acceptor liposomes) that had been digested by human rhinovirus 3C protease (Novagen) at 4°C for 16 h in the same buffer to cleave off their His6- or GST-His6-tags. After incubation of these detergent-lipid-SNARE mixed micellar solutions (4°C, 1 h, gentle agitation), they were dialyzed against RB500 to remove β -OG using a Slide-A-Lyzer 20 K cut-off dialysis cassette (Thermo Scientific, Waltham, MA, USA). The SNARE proteoliposomes formed were purified by Histodenz-gradient flotation using a TLS-55 rotor (Beckman Coulter; 50,000 rpm, 2 h, 4°C), harvested from the 0%/30% Histodenz interface in RB150 (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 10% glycerol), diluted with RB150 to 2 mM total lipids in final, and stored at -80°C. Lipid concentrations of purified SNARE proteoliposomes were determined from the fluorescence of NBD-PE and dansyl-PE for the donor and acceptor liposomes respectively, as described previously¹⁵.

Lipid-mixing assay. Lipid-mixing assays were performed with NBD-labeled donor liposomes bearing R-SNAREs and non-labeled acceptor liposomes bearing 3Q-SNAREs or 2Q-SNAREs (Qab-SNAREs), as described previously^{15,33–35}, with modifications. Donor R-SNARE liposomes (final 200 μ M lipids) were mixed in RB150 with exogenous full-length Vam7p (8.8 μ M), Nyy1p Δ TM (6.9 μ M), Snc2p Δ TM (22 μ M), and PEG6000 (5.0 or 2.0% (w/v); Nacalai Tesque) where indicated, in a black 384-well plate (no. 3676; Corning, Corning, NY, USA), and pre-incubated at 30°C for 10 min in a SpectraMAX Gemini XPS plate reader (Molecular Devices). After pre-incubation, acceptor 3Q- or 2Q-SNARE liposomes (final 500 μ M lipids) were added to the reactions, followed by further incubation at 30°C for 30 min. In Figure 6E, we used the lower PEG concentrations (2.0%), in order to accurately monitor the very rapid changes of NBD fluorescence signals. For the lipid-mixing reactions with the ER-Golgi SM protein Sly1p, donor R-SNARE liposomes (final 160 μ M lipids) and acceptor 3Q-SNARE liposomes (final 400 μ M lipids) were mixed in RB150 with Sec22p Δ TM (10 μ M) and PEG6000 (3.2% final) where indicated and pre-incubated at 30°C for 10 min, followed by the addition of His6-tagged Sly1p (4.5 μ M final) and then further incubation at 30°C for 30 min. To add adequate Sly1p and Sec22p Δ TM but keeping the reaction volume constant, we assayed with the lower PEG concentrations (3.2%) in Figure 8d–g. NBD fluorescence (λ excitation = 460 nm, λ emission = 538 nm, emission cutoff = 515 nm) of donor liposomes was measured at 30-s intervals, 30 reads per well on the ‘middle’ PMT setting (arbitrary units) to monitor lipid mixing with acceptor liposomes. β -OG (100 mM final) was added after a 30 min incubation to obtain fully dequenched maximal NBD fluorescence for each reaction. The ratios of NBD fluorescence (%) were calculated as previously described¹⁵. All lipid-mixing data were from one experiment and were typical of those from more than three independent experiments.

Liposome co-sedimentation assay. SNARE proteoliposomes (1 mM total lipids in final) were mixed with His6-tagged Sly1p (11 μ M final) in RB150 (100 μ l for each) in a 1.5 ml protein low binding tube (Sarstedt, Nümbrecht, Germany), incubated at 4°C for 30 min, and centrifuged at 20,000 g at 4°C for 30 min. The pellets obtained from these proteoliposome/Sly1p solutions were washed once in RB150 (200 μ l for each), re-suspended in 2% SDS, and analyzed by SDS-PAGE and Coomassie Blue staining.

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Author contributions

J.M. designed research; J.M. and N.F. performed research; J.M. and N.F. analyzed data; J.M. wrote the paper.

Additional information

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