A short region upstream of the yeast vacuolar Qa-SNARE heptad-repeats promotes membrane fusion through enhanced SNARE complex assembly

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ABSTRACT **Whereas SNARE (soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor) heptad-repeats are well studied, SNAREs also have upstream N-domains of indeterminate function. The assembly of yeast vacuolar SNAREs into complexes for fusion can be studied in chemically defined reactions. Complementary proteoliposomes bearing a Rab:GTP and either the vacuolar R-SNARE or one of the three integrally anchored Q-SNAREs were incubated with the tethering/SM protein complex HOPS and the two other soluble SNAREs (lacking a transmembrane anchor) or their SNARE heptad-repeat domains. Fusion required a transmembrane-anchored R-SNARE on one membrane and an anchored Q-SNARE on the other. The N-domain of the Qb-SNARE was completely dispensable for fusion. Whereas fusion can be promoted by very high concentrations of the Qa-SNARE heptad-repeat domain alone, at physiological concentrations the Qa-SNARE heptad-repeat domain alone has almost no fusion activity. The 181–198 region of Qa, immediately upstream of the SNARE heptad-repeat domain, is required for normal fusion activity with HOPS. This region is needed for normal SNARE complex assembly.**

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INTRODUCTION

Membrane fusion is essential for cell compartmentation and growth, hormone secretion, and neurotransmission. The catalysts of fusion are conserved in all eukaryotic cells. Rab-family GTPases in their GTP-associated conformations are specific to each organelle (Grosshans *et al.*, 2006). They bind "effector" tethering proteins, which draw membranes into stable proximity (Baker and Hughson, 2016). Sec1/Munc18 (SM) proteins catalyze the assembly of soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins anchored to each membrane into a four-SNARE complex (Rizo and Südhof, 2012; Baker *et al.*, 2015; Orr *et al.*, 2017).

SNARE proteins consist of N-domains and SNARE heptad-repeat domains, often bound to membranes by C-terminal transmembrane anchors (Jahn and Scheller, 2006). The SNARE heptad-repeat domains assemble into a four-SNARE coiled coils structure of four α-helices twisted around each other, with largely apolar, internally oriented amino acyl residues and largely polar, externally oriented residues. The exception to this pattern is at the center of the coiled-coil rod, where there is an arginyl residue from one SNARE and three glutaminyl residues from the three other SNAREs, each internally oriented in the four-SNARE complex. The SNAREs are in four conserved families, termed R, Qa, Qb, and Qc according to their central, internally oriented polar residue (Fasshauer *et al.*, 1998). SNARE complexes have a conserved composition of RQaQbQc. These complexes are in-*cis* if each SNARE is anchored to the same membrane bilayer or in-*trans* if they are anchored to apposed membranes. Although SNARE complexes can slowly form spontaneously, physiological assembly is catalyzed by SM proteins or by tethering complexes such as homotypic fusion and vacuole protein sorting complex (HOPS; which has an SM protein subunit) at the vacuole/lysosome (Seals *et al.*, 2000; Stroupe *et al.*, 2006; Zick and Wickner, 2013; Baker *et al.*, 2015) or the COG complex at the Golgi (Miller *et al.*, 2013). Fusion can be triggered by SNARE complex–bound proteins such as synaptotagmin (Fernandez-Chacon *et al.*,

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Abbreviations used: FRET, fluorescence resonance energy transfer; HOPS, homotypic fusion and vacuole protein sorting complex; PtdIns(3)P, phosphatidylinositol 3-phosphate; RPL, reconstituted proteoliposome; SNARE, soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor; VML, vacuolar mimic lipid.

2001) or Sec17/α-SNAP (Zick *et al.*, 2015). After fusion, *cis*-SNARE complexes are disassembled by Sec18/NSF using the energy of ATP hydrolysis and stimulated by the cochaperone Sec17/ α-SNAP, freeing the SNAREs for subsequent rounds of fusion.

We study membrane fusion through the lens of the homotypic fusion of yeast vacuoles (Wickner, 2010). Although many yeast strains have one to three vacuoles at steady state, vacuoles undergo constant fission and fusion. Mutations that block fusion allow continued fission, giving rise to a vacuolar morphology (*vam*) phenotype of many tiny vacuoles. Screens of mutated cells identified *vam* mutations in each gene that encodes a protein exclusively dedicated to vacuole fusion (Wada *et al.*, 1992). Among these are genes encoding the vacuolar Rab, several SNAREs, and six large polypeptides, which include the vacuolar SM protein and together (Nakamura *et al.*, 1997; Seals *et al.*, 2000; Stroupe *et al.*, 2006) form the vacuolar tethering complex termed homotypic fusion and vacuole protein sorting (HOPS). Extensive study of the fusion of the purified organelle (Haas *et al.*, 1994; Wickner, 2010) and reconstitution of fusion with physiological levels of purified proteins and defined lipids (Mima *et al.*, 2008; Stroupe *et al*, 2009; Zick and Wickner, 2016) has led to a working model of the fusion pathway. *cis*-SNARE complexes, composed of the R-SNARE Nyv1, the Qa-SNARE Vam3, the Qb-SNARE Vti1, and the Qc-SNARE Vam7 (referred to hereafter as R, Qa, Qb, and Qc, respectively) are disassembled by Sec18, Sec17, and ATP. This priming step somehow signals to the tethering machinery (Mayer and Wickner, 1997). Tethering is largely due to the association between the Rab-family GTPase Ypt7 on each membrane with Vps39 and Vps41, two subunits of HOPS (Brett *et al.*, 2008; Bröcker *et al.*, 2012). HOPS then catalyzes the assembly of the four vacuolar SNAREs into a *trans*-SNARE complex, using the direct affinity of its subunits Vps16 and Vps18 for Qc (Krämer and Ungermann, 2011) and of its Vps33 SM subunit for the R- and Qa-SNARE domains (Baker *et al.*, 2015). Fusion is promoted by several factors: the zippering of the SNARE domains in the N- to C-direction, the Sec18-assisted association of Sec17 and insertion of the Sec17 apolar N-domain loop into the bilayers (Schwartz and Merz, 2009; Zick *et al.*, 2015), and the local enrichment (Fratti *et al.*, 2004) of key lipids, including small head-group non–bilayer-prone lipids (Zick *et al.*, 2014) and lipids of fluid fatty acyl chains (Zick and Wickner, 2016). Several partially redundant interactions also promote fusion: for example, HOPS binds to several SNAREs (Stroupe *et al.*, 2006; Baker *et al.*, 2015) and vacuolar phosphoinositides (Stroupe *et al.*, 2006) as well as to Ypt7 (Seals *et al.*, 2000), and tethering is also promoted by *trans*-interactions of phosphatidylinositol 3-phosphate (PtdIns(3)P) with the Phox domain of the Qc-SNARE (Cheever *et al.*, 2001; Zick and Wickner, 2013, 2014).

Whereas the canonical SNARE domains have received extensive physical and functional study, less is known of the roles of the N-domains, which are upstream of the SNARE heptad-repeats. The Qc N-domain has strong Phox homology and has direct affinity for PtdIns(3)P (Cheever *et al.*, 2001) and HOPS (Stroupe *et al.*, 2006). The N-domain of the vacuolar R-SNARE is not required for fusion activity (Jun *et al.*, 2007). We now report studies of the role of the Qa and Qb N-domains in fusion. Whereas the Qb N-domain has little apparent role in fusion, the Qa N-domain is required at all but the very highest SNARE concentrations. Strikingly, the most important part of the Qa N-domain is the ~20 residues immediately upstream of the Qa-SNARE heptad-repeat motifs. This short upstream region has features of amino acyl charge and size that are conserved among the Qa-SNARE family members.

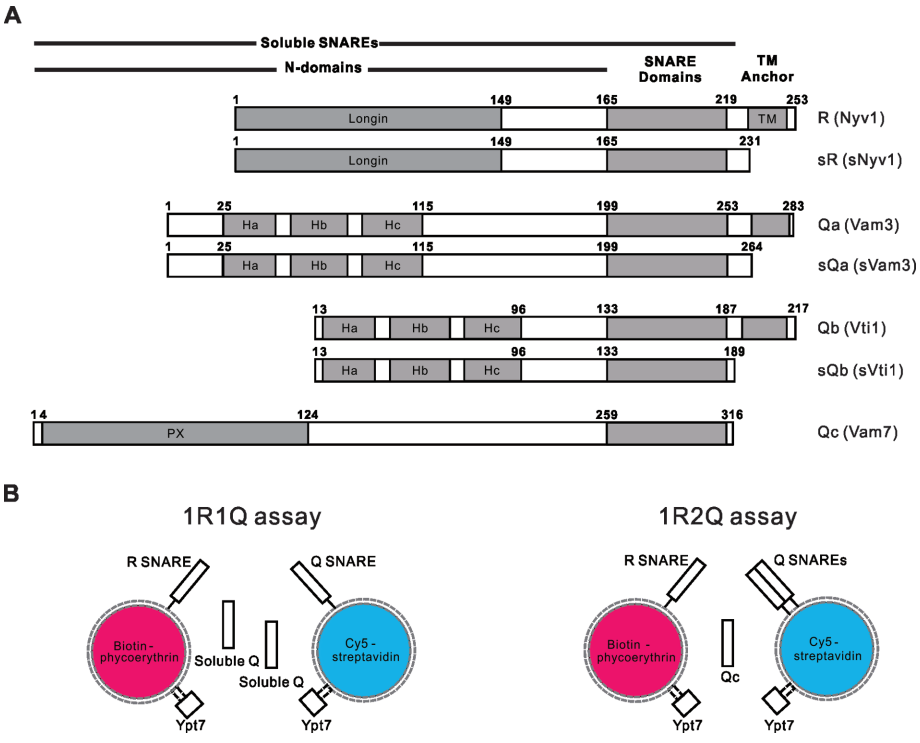

FIGURE 1: SNAREs and assays of content mixing. (A) The yeast vacuolar R-SNARE Nyv1 and the Qa- and Qb-SNAREs (Vam3 and Vti1) have transmembrane regions. The Qc-SNARE Vam7 is a soluble protein. For some experiments, the transmembrane regions of the Qa- and Qb-SNAREs were removed to make soluble Qa- and Qb-SNAREs (sQa and sQb). (B) Proteoliposomes lumenally marked with fluorescent protein Cy5-derivatized streptavidin or biotinylated phycoerythrin were reconstituted with integrally bound SNAREs and the Rab Ypt7. On membrane fusion, the Cy5-streptoavidin binds to biotinylated phycoerythrin, yielding a strong FRET signal (Zucchi and Zick, 2011).

RESULTS

Yeast vacuolar SNAREs have N-domains and canonical R-, Qa-, Qb-, and Qc-SNARE domains (Figure 1A). Three of these SNAREs have apolar transmembrane anchors, whereas the Qc-SNARE Vam7 binds membranes by the affinity of its SNARE domain for other SNAREs and of its N-domain for PtdIns(3)P (Cheever *et al.*, 2001), HOPS (Stroupe *et al.*, 2006), and acidic lipids (Lee *et al.*, 2006; Karunakaran and Wickner, 2013). Each SNARE has a polar, water-soluble domain, which we term the soluble SNARE or sSNARE (Figure 1A), consisting of its SNARE heptad-repeats as well as its N-domain, which is upstream of the SNARE heptad-repeats. To study membrane fusion, we reconstituted membrane-anchored SNAREs and the prenylated vacuolar Rab Ypt7 into proteoliposomes (Zucchi and Zick, 2011), which we lumenally marked with either of two fluorescent proteins, Cy5-derivatized streptavidin or biotinylated phycoerythrin (Figure 1B). The bilayers of each liposome keep these two fluorescent proteins too far apart for any fluorescence resonance energy transfer (FRET) signal. On incubation with fusion factors such as HOPS,

the proteoliposomes fuse, mixing their contents and allowing the biotinylated phycoerythrin to bind Cy5-streptavidin, bringing these two fluorophores into close proximity and yielding a strong FRET signal (Zucchi and Zick, 2011; Zick and Wickner, 2014). A large molar excess of nonfluorescent streptavidin is present in the assay outside the proteoliposomes to prevent any signal due to lysis (Zick and Wickner, 2014). This same assay scheme can be used with proteoliposomes bearing all combinations of the vacuolar Rab and SNAREs.

Role of membrane anchors in fusion

We prepared proteoliposomes bearing Ypt7:GTP and lumenal fluorescent marker proteins that either lacked integrally bound SNAREs entirely (Figure 2A) or had at least one fusion partner with one or more integrally bound SNAREs (Figure 2, B–K). These were mixed in combinations with HOPS and the soluble forms of each of the SNAREs, which were not integrally bound to the proteoliposomes. Protected lumenal content mixing was assayed as the development of FRET signal (Figure 2). Fusion occurred only when one set of proteoliposomes bore the anchored R-SNARE and the other bore the membrane-anchored form of at least one of the three Q-SNAREs (Qa, Qb, or Qc-t.m.—the entire Qc protein plus the transmembrane domain of Vti1 [Qb]; Figure 2, H–K). To explore the roles of SNARE N-domains, we incorporated just one integrally bound Q-SNARE into proteoliposomes and added the other two as "soluble SNAREs" lacking a transmembrane anchor.

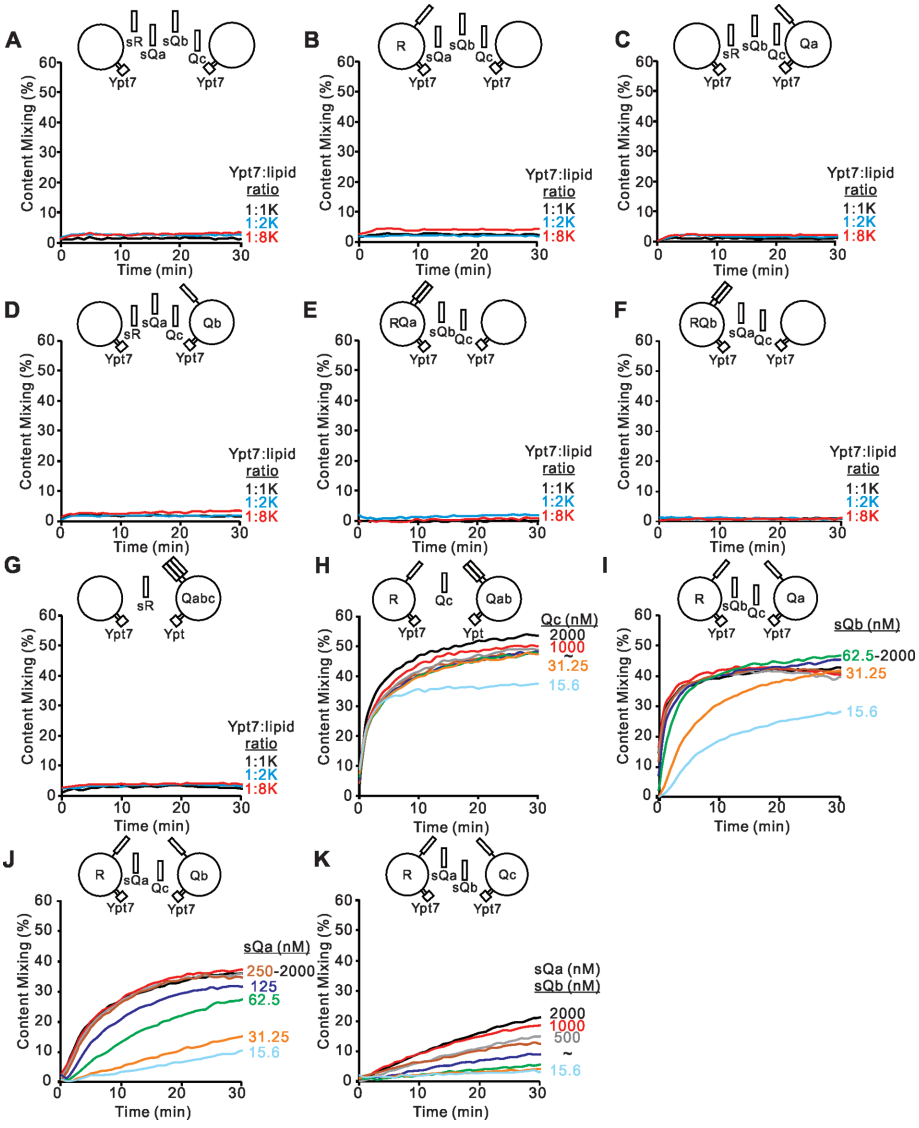

FIGURE 2: Fusion needs a membrane-anchored form of the R-SNARE on one fusion partner and at least one Q-SNARE on the other. Fusion reactions had proteoliposomes of VMLs (18:2), the Rab Ypt7, and SNAREs as indicated, each at a 1:8000 molar ratio to lipid. Fusion was initiated by adding sSNAREs, Qc, and HOPS. (A–F) Proteoliposomes had three different concentrations of Ypt7p (1:1000, 1:2000, or 1:8000 protein:lipid ratio). (A) Proteoliposomes bore no membrane-anchored SNAREs. Fusion incubations had three sSNAREs, Qc, and HOPS. (B–D) One proteoliposome fusion partner had a single membrane-anchored SNARE. (E) One proteoliposome fusion partner had both the R-SNARE Nyv1 and the Qa-SNARE Vam3. (F) One proteoliposome fusion partner had both the R-SNARE Nyv1 and the Qa-SNARE Vti1. (G) One set of proteoliposomes had the 3Q-SNAREs. (H) Membrane fusion reactions of 1R-proteoliposomes and QaQb-proteoliposomes with the indicated concentrations of Qc-SNARE. (I–K) Membrane fusion reactions of 1R-proteoliposome with Q-proteoliposomes with the indicated concentrations of soluble SNAREs. (K) The naturally soluble Qc-SNARE Vam7 was joined at its C-terminus to a transmembrane domain from the Qb-SNARE Vti1. Kinetic curves of content mixing assays in this figure are representative of at least three experiments. Soluble SNAREs were 2 μM in A–G. Qc was 50 nM in I and J.

N-domain requirements for fusion

To explore the functions of the Qb N-domains, we prepared proteoliposomes bearing Ypt7:GTP and either R or Qa and then assayed their fusion in the presence of HOPS, Vam7, and either soluble Qb (sQb 1–189—the entire protein minus its C-terminal transmembrane anchor) or the Qb SNARE heptad-repeat domain (sQb 133–189). There was comparable fusion at high or low levels of sQb (1–189) or sQb (133–189) (Figure 3, A–C); thus the Qb N-domain was not required for fusion under these reaction conditions.

To explore the importance of the Qa N-domain, we generated soluble Qa-SNARE (sQa—the entire protein minus its C-terminal transmembrane anchor) and derivatives with an eight–amino acid substitution to introduce a 3C protease cleavage site immediately upstream of the SNARE heptad-repeats. With purified proteins, we tested protein cleavage by 3C protease. Coomassie-stained SDS–PAGE showed that sQa-3CSD and Qa-3CSD (the sQa and Qa with a 3C protease site immediately upstream of the SNARE heptad-repeats) were cleaved by 3C protease treatment, but sQa and Qa were not affected (unpublished data). Incubation of proteoliposomes bearing Ypt7:GTP and either R or Qb supplemented with sQa or sQa-3CSD and buffer, Qc, and HOPS yields membrane fusion (Figure 4A, black and red lines). The fusion activity of sQa was not affected by incubation with 3C protease (Figure 4A, blue line). However, sQa-3CSD fusion activity is abolished after cleavage by 3C protease (Figure 4A, green line). To test whether the N-domain is also required for fusion with membrane-anchored Qa, we prepared

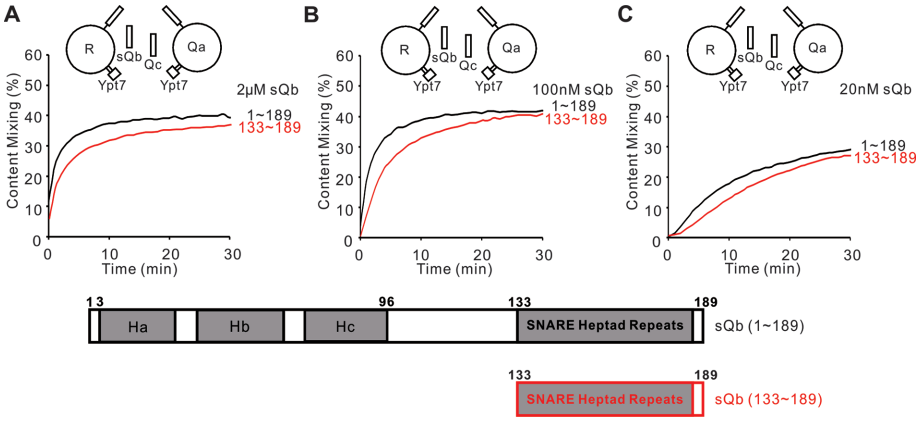

FIGURE 3: The Qb N-domain is not required for fusion. The Qb-SNARE heptad-repeat domain (sQb 133–189) has comparable fusion activity to full-length sQb (sQb 1–189). Fusion reactions had a mixture of proteoliposomes bearing Ypt7 and either the R-SNARE (1:8000 protein:lipid ratio) or the Qa-SNARE (1:8000 protein:lipid ratio). Full-length sQb or the Qb-SNARE heptad-repeat domain (sQb 133–189) and Qc (50 nM) were added, and fusion was initiated by adding 50 nM HOPS. Fusion assays were performed with 50 nM Qc and (A) 2 μM, (B) 100 nM, or (C) 20 nM sQb 1–189 (black line) or sQb 133–189 (red line). All proteoliposomes had Ypt7 at a 1:8000 protein:lipid ratio. Kinetic curves of content mixing assays are representative of at least three experiments.

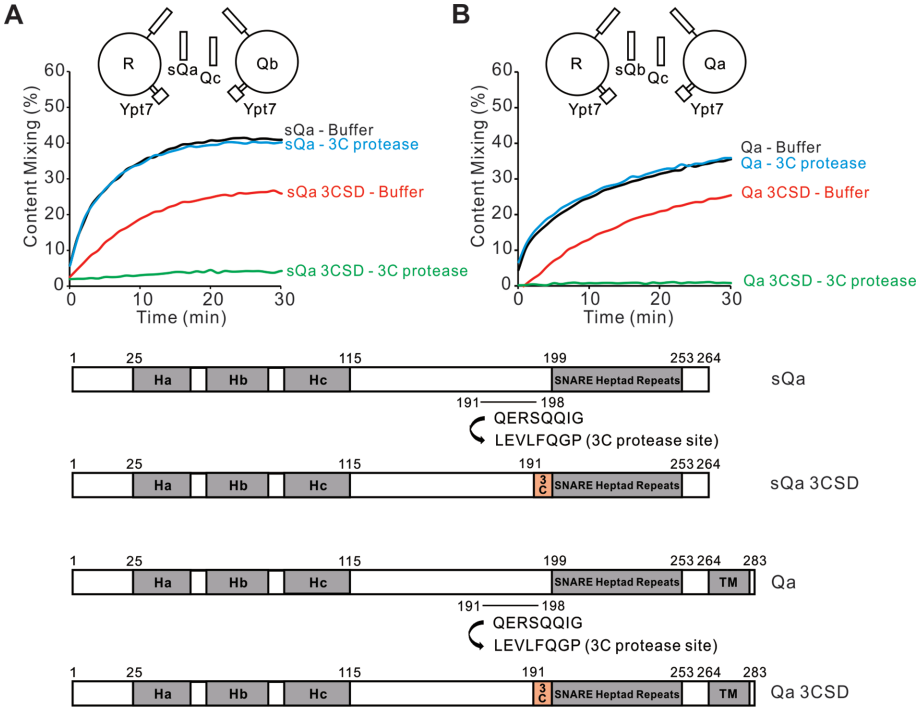

FIGURE 4: Proteolytic removal of the N-domain of Vam3 abolishes its fusion activity. An eight–amino acid 3C protease cleavage sequence was substituted upstream of the Qa-SNARE heptad-repeats (sQa-3CSD and Qa-3CSD). Cleavage was by 3C protease where indicated. (A) The fusion of R-SNARE and Qb-SNARE proteoliposomes was performed with 50 nM HOPS, 50 nM Qc, 100 nM sQa (black line), or sQa-3CSD (red line) or with sQa (blue line) or sQa-3CSD (green line) incubated with 3C protease (sQa-3CSD in 200 μl and incubated with 3 U of 3C protease or its buffer (50 mM TrisCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 50% glycerol) at 4°C for 1 h in Rb150 buffer. (B) Incubations of R-SNARE and Qa-SNARE (black line) or Qa-3CSD (red line) proteoliposomes that had been incubated with 3C buffer only or Qa (blue line) or Qa-3CSD (green line) proteoliposomes that had been incubated with 3C protease (1 U in 100 μl at 4°C overnight) were performed with 50 nM Qc, 50 nM sQb, and 50 nM HOPS. Kinetic curves of content mixing assays are representative of at least three experiments.

proteoliposomes bearing Ypt7:GTP and Qa or Qa-3CSD. These proteoliposomes were treated with 3C protease buffer as a control or with 3C protease. Membrane fusion occurred between proteoliposomes bearing the membrane-anchored R-SNARE and those bearing the membrane-anchored Qa-SNARE or Qa-3CSD SNARE that was incubated with the buffer of 3C protease rather than the protease itself (Figure 4B, black and red lines). Fusion activity was abolished for proteoliposomes bearing Qa-3CSD that had been treated with 3C protease (Figure 4B, green line), although 3C protease treatment did not inhibit the fusion of proteoliposomes bearing wild-type Qa (Figure 4B, blue line). Thus the fusion function of the N-domain of sQa and Qa requires its covalent attachment to the SNARE heptad-repeats.

We used a nested set of deletions to determine which regions of the Qa N-domain were most important for fusion. Qa (127–264) is a deletion of the Habc domain of sQa that has been reported to be important for its binding to HOPS and for fusion (Laage and Ungermann, 2001; Lürick *et al.*, 2015). More extensive deletion proteins were prepared as well; sQa (199–264) deletes all of the N-domain of sQa, leaving only the SNARE heptad-repeats. Proteoliposomes bearing Ypt7:GTP and integrally bound R- or Qb-SNAREs were incubated in the presence of HOPS, Qc, and sQa or its derivatives (Figure 5). At 2 μM, most of the shortened versions of sQa were as active as full-length sQa (1-264), although the SNARE heptad-repeats alone, sQa (199–264), had only half the fusion activity (Figure 5A). When assayed at 100 nM, however, sQa (1–264) was more active than shorter derivatives, and the SNARE heptad-repeats (sQa 199–264) showed virtually no fusion activity (Figure 5B). Only full-length sQa was active at 20 nM (Figure 5C). These data suggest that each region of the Qa N-domain, including the Habc domain, affects the concentration of Qa needed for fusion (Figure 5C) and highlight an especially important role of the 19–amino acyl region (181–198) of Qa just upstream of the SNARE heptad-repeats (Figure 5B).

Because the N-domain was not required for fusion at high sQa concentrations (Figure 5A), it was possible that its sole function was to target sQa to the membrane and that this targeting function might be dispensable for Qa, which retains its physiological transmembrane anchor. We therefore generated Qa-proteoliposomes bearing membrane-anchored Qa or its N-terminally shortened derivatives, as well as 2Q-proteoliposomes

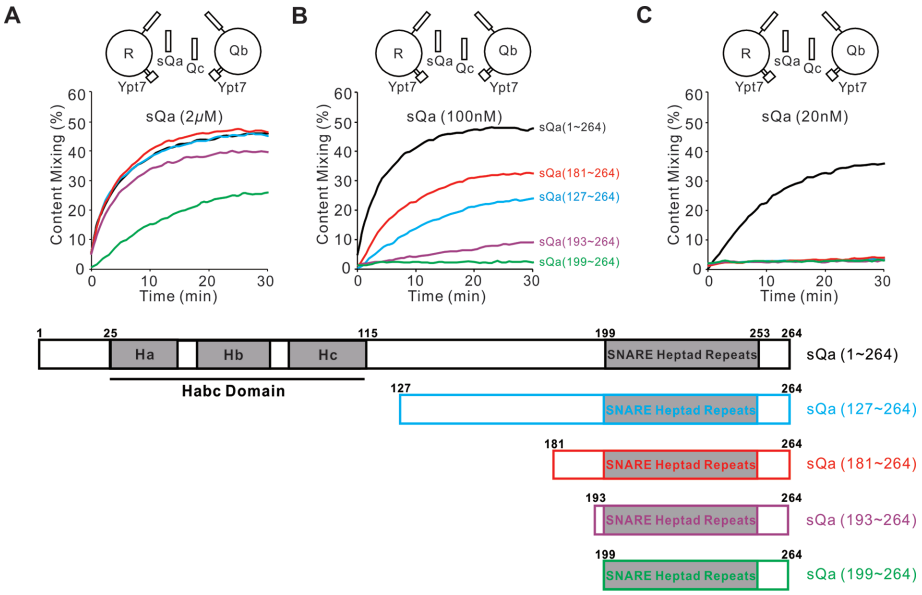

FIGURE 5: The N-domain region of sQa is important for membrane fusion. Fusion reactions had mixed proteoliposomes bearing either R- or Qb-SNAREs (1:8000 protein:lipid ratio). The indicated concentrations of full-length sQa or its N-terminally shortened derivatives and 50 nM Qc were added, and fusion was initiated by adding 50 nM HOPS. (A) Fusion reactions with 2 μM sQa or its truncated derivatives. (B) Reactions with 100 nM sQa or its derivatives. (C) Reaction with 20 nM sQa or its derivatives. All proteoliposomes had Ypt7p at a 1:8000 protein:lipid ratio. Kinetic curves of content mixing assays are representative of at least three experiments.

with Qb and these same wild-type or shortened Qa SNAREs. With 50 nM sQb, Qc, and HOPS, proteoliposome pairs with membrane-anchored R-SNARE and Qa (1–264), Qa (127–264), and Qa (181–264) had similar fusion activity (Figure 6A). However, proteoliposomes with Qa (199–264) were inert (Figure 6A and Supplemental Figure S1A). Proteoliposomes were also prepared with Ypt7:GTP and 2Q-SNAREs (Qa and Qb). 2Q-proteoliposomes bearing Qa (1–264), Qa (127–264), or Qa (181–264) and the Qb-SNARE that were incubated with R-SNARE proteoliposomes in the presence of HOPS and Qc supported fusion, but proteoliposomes with Qa (199–264) and Qb SNAREs had very low fusion activity (Figure 6B and Supplemental Figure S1B). Thus the region 181–198 of Qa is important for fusion per se and not simply for targeting the Qa SNARE to the membrane. Qa requires its N-domain, and especially the region 181–198 just upstream of the SNARE heptad-repeats, for full fusion activity. We did not observe any restoration of fusion when even 2 μM synthetic peptide corresponding to Qa (181–198) was added to a fusion incubation with Ypt7:1R and Ypt7:Qa (199–264) proteoliposomes with HOPS, sQb, and Qc (unpublished data).

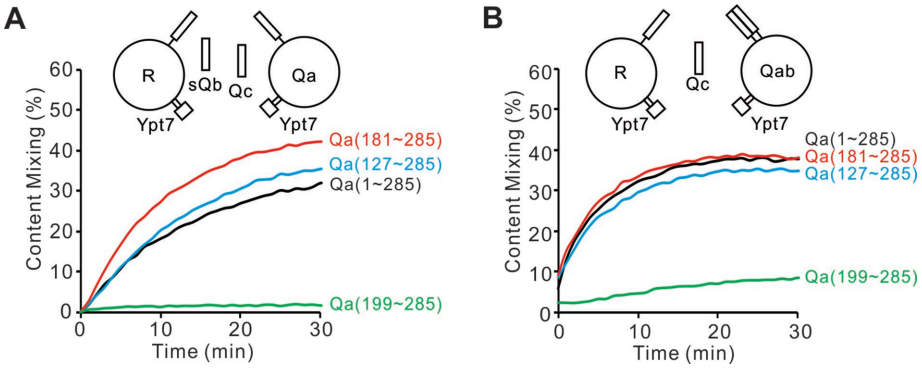

FIGURE 6: The 181–198 region of membrane-anchored Qa is critically important for membrane fusion. (A) Fusion reactions had a mixture of proteoliposomes bearing either the R-SNARE (1:16,000 protein:lipid ratio) or the Qa-SNARE or its derivatives (1:16,000 protein:lipid ratio). A mixture of the soluble Qb-SNARE (100 nM), the Qc-SNARE Vam7 (50 nM), and HOPS (50 nM) was added to initiate fusion. (B) Fusion reactions had a mixture of proteoliposomes bearing either the R-SNARE or the Qa-SNARE or its derivatives and the Qb-SNARE (1:16,000 ratio of each SNARE:lipid). Qc (50 nM) and HOPS (50 nM) were added to initiate fusion. All RPLs had Ypt7p at a 1:8000 protein:lipid ratio. Kinetic curves of content mixing assays are representative of at least three experiments.

Role of the N-domain of Qa in SNARE complex assembly

We measured both fusion and SNARE complex formation with sQa and its derivatives. To quantify SNARE complexes, we performed fusion incubations with hexahistidine (his_6)-tagged Qc, which has a comparable fusion activity to wild-type Qc (unpublished data). Fusion was initiated by the addition of soluble protein mixtures, either 100 nM sQa or its derivatives, 50 nM his_6-Qc, and 50 nM HOPS (Figure 7A). As controls, some incubations (Figure 7A, f–h) were performed without sQa, his_6-Qc, or HOPS. After 30 min, mixtures were dissolved in detergent, and the extracts were mixed with Ni–nitriloacetic acid (NTA) magnetic beads. After washing, the bead-bound proteins were released by SDS sample buffer and assayed by immunoblot for bound R and Qb SNAREs. R and Qb association with Qc was supported by full-length sQa (Figure 7, B and C, sample a). These interactions were decreased by successive deletions of the N-domain of sQa or omission of Qa, Qc, or HOPS (Figure 7, B and C). There is an especially marked reduction in SNARE pairing as the deletion of the N-domain proceeds from amino acyl residue 181 to 199, when the Qc-associated R- and Qb-SNAREs (sample e) are equivalent to the background levels seen in the absence of the Qa SNARE (sample f). Although there is substantial background association of the R-SNARE with Qc, seen in the absence of Qa or of HOPS (Figure 7B, f and h), the binding of the Qb SNARE to his_6-Qc requires the R-SNARE (Supplemental Figure S2), as well as Qa residues 181–199 (Figure 7C, b vs. e), Qc, and HOPS (Figure 7C, g and h). Thus the N-domain of Qa is important for the assembly of the SNARE complex and its attendant fusion activity.

Because the region 181–198 of the Qa-SNARE is of particular importance for membrane fusion, we compared this region of Vam3 with that of other Qa SNAREs from *Saccharomyces cerevisiae*, *Homo sapiens*, *Mus musculus*, and *Drosophila melanogaster*. Using the CLC sequence viewer program with the Clustal Omega plug-in (Sievers *et al.*, 2011), we aligned sequences of 14 Qa-SNAREs (Figure 8); the SNARE heptad-repeats are highly conserved within these

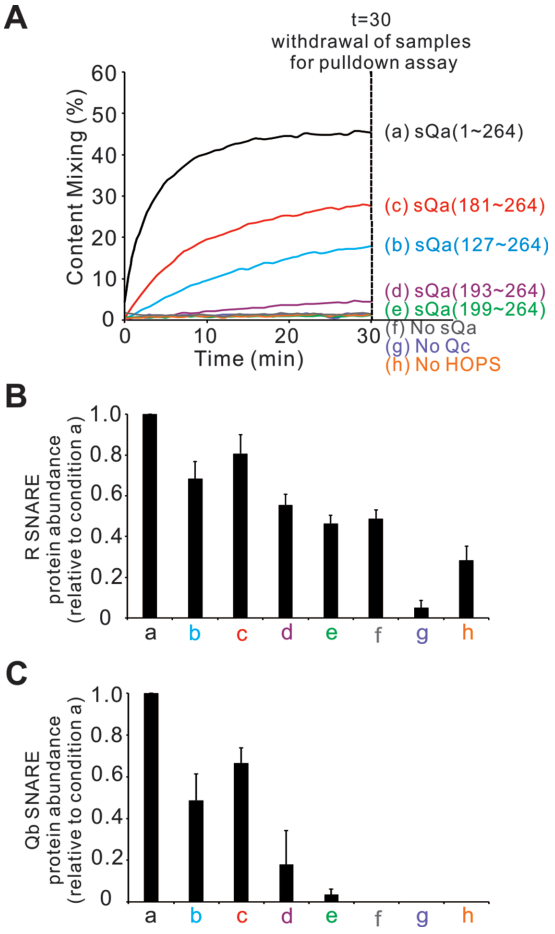

FIGURE 7: Deletions in the Qa N-domain affect SNARE complex formation. (A) Fusion reactions had proteoliposomes bearing either the R-SNARE (1:8000 protein:lipid ratio) or the Qb-SNARE (1:8000 protein:lipid ratio) and 100 nM sQa (1–264), sQa (127–264), sQa (181–264), sQa (193–264), or sQa (199–264). Fusion was initiated by adding 100 nM sQa or its derivatives, 50 nM his_6-tagged Qc, and 50 nM HOPS or their respective buffers. All RPLs had Ypt7p at a 1:8000 protein:lipid ratio. (B, C) After 30 min of fusion incubation, samples were solubilized in detergent and subjected to his_6-Qc SNARE pull down with Ni-NTA magnetic beads and then analyzed by SDS–PAGE and immunoblotting with anti-R or -Qb antibodies. Data were analyzed by immunoblot with standard curves of each SNARE and represent the average and SD of three independent experiments. The signal intensities of condition a (with sQa (1–264)) were normalized to 1.

14 Qa-SNAREs sequences (Fasshauer *et al.*, 1998). Of interest, the conservation extends at least 15 amino acyl residues upstream from the N-terminal end of the heptad-repeats of these Qa-SNAREs. This suggests that the conserved region of Qa-SNAREs upstream of the SNARE heptad-repeats may support the fusion activity of other Qa-SNAREs by enhancing SNARE complex assembly.

DISCUSSION

Our studies of vacuolar SNARE N-domains show that the Qb SNARE N-domain is dispensable for fusion under our assay conditions, whereas the Qa-SNARE N-domain has important functions. Fusion needs at least one membrane-anchored Q-SNARE and, on the fusion partner, a membrane-anchored R-SNARE. Although the Qa and Qb vacuolar SNAREs have transmembrane anchors, these are apparently redundant, as only one anchor is required. Our findings are in accord with the well-studied human neuronal SNAREs, which also have apolar membrane-spanning anchor domains for the R- and Qa-SNAREs, whereas the Qb- and Qc-SNARE domains are part of the same protein, SNAP-25, which is prenyl anchored to the lipid bilayer (Jahn and Scheller, 2006).

Although Qb is required for several membrane transport steps (Gossing *et al.*, 2013), the N-domain of Qb is dispensable for fusion under our assay conditions. It contains a helical Habc bundle (Tishgarten *et al.*, 1999; Antonin *et al.*, 2002), and a thermosensitive mutation in this domain renders the protein labile in vivo, resulting in decreased levels of complexes with other SNAREs (Gossing *et al.*, 2013). Because no direct affinity has been reported of Qb for Vps33 or HOPS (Baker *et al.*, 2015; Orr *et al.*, 2017), its assembly and function with the other SNAREs might rest strictly on the interaction of its SNARE domain with the other vacuolar SNARE domains.

The Qa-SNARE also has an N-terminal Habc domain, which has received considerable study. In addition to the Qa-SNARE domain binding to a conserved pocket in the Vps33 subunit of HOPS (Baker *et al.*, 2015), its Habc domain has been report to have direct HOPS affinity (Lürick *et al.*, 2015). Deletion of the Habc domain has little effect on steady-state vacuolar structure in vivo (Laage and Ungermann, 2001) but has variably been reported to have no effect on the fusion of purified vacuoles in vitro (Wang *et al.*, 2001) or a small stimulatory effect (Lürick *et al.*, 2015). We find no effect of the presence or absence of Habc on the fusion activity of membrane-anchored Qa, even when assayed at physiological SNARE levels of a 1:32,000 molar ratio to lipids (Figure 6 and Supplemental Figure S1). There is, however, a concentration-dependent need for Habc for sQa function; the deletion of residues 1–126 (which includes Habc) abolishes fusion with 20 nM sQa, inhibits it twofold at 100 nM sQa, and has no measurable effect at 2 μM sQa (Figure 5). When the Qa SNARE is integrally anchored, the affinity of its Habc region for HOPS may be unnecessary for functional SNARE complex assembly.

In contrast to the modest effects on fusion of most of the Qa N-domain, fusion is strongly affected by residues 181–198, just upstream of the SNARE heptad-repeats (residues 199–253). At 100 nM, sQa (181–264) has ~70% of the fusion activity of full-length sQa, whereas sQa (199–264) has no detectable activity (Figure 5). This short domain also controls fusion when Qa is membrane anchored (Figure 6). This region, just preceding the SNARE heptad-repeat domain, exhibits substantial conservation among Qa SNAREs of yeast, humans, *Drosophila*, and mice (Figure 8) and is needed for SNARE complex assembly (Figure 7). A similar domain of Qa, residues 184–198, affects the direct binding of the Vps33 HOPS SM-family subunit to the Qa-SNARE domain (Lobingier and Merz, 2012). Although further studies will be needed to evaluate the role of this region in other SNARE-dependent fusion systems, it is noteworthy that the crystal structure of the neuronal SNARE complex (Sutton *et al.*, 1998) shows that this region (residues 181–198 of Qa) is helical, the helix is continuous with the Qa heptad-repeat domain helix, and it is packed in the crystal next to the Qb helix. Our findings suggest that the zippering of the SNARE domain may begin upstream of the canonical heptad-repeats.

MATERIALS AND METHODS

Proteins and reagents

Most lipids were obtained from Avanti Polar Lipids. Ergosterol was from Sigma-Aldrich, PtdIns(3)P was from Echelon, and the fluorescent lipids Marina Blue–1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (DHPE) and nitrobenzoxadiazole (NBD)-DHPE were from Life Technologies. Biotinylated R-phycoerythrin was purchased

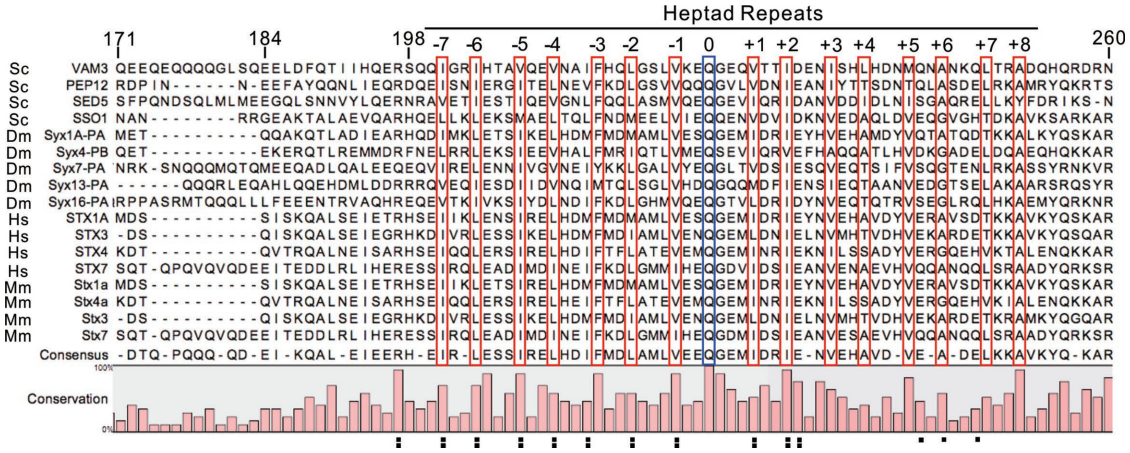

FIGURE 8: Conserved domains of Qa SNAREs. Sequences of representative Qa-SNAREs from *H. sapiens* (STX1A; NP_004594.1, STX3; NP_004168.1, STX4; NP_004595.2, and STX7; NP_001313508.1), *M. musculus* (Stx1a; NP_058081.2, Stx3; NP_689344.1, Stx4a; NP_033320.1, and Stx7; NP_058077.2), *S. cerevisiae* (Vam3; CAA99304.1, Pep12; CAA99226.1, Sed5; CAA97549.1, and Sso1; CAA97949.1), and *D. melanogaster* (Syx1A-PA; NP_524475.1, Syx4-PB; NP_001259195.1, Syx7-PA; NP_730632.1, Syx13-PA; NP_524054.1, and Syx16-PA; NP_523420.1) were aligned and compared by CLC sequence viewer with Clustal Omega plug-in. The SNARE heptad-repeats were highly conserved in various Qa-SNAREs. The 184–198 amino acid region of Qa is also highly conserved across species and protein variants of Qa. Red box: hydrophobic heptad-repeats. Blue box: conserved glutamines of Q-SNAREs. Hs, *H. sapiens*; Mm, *M. musculus*; Sc, *S. cerevisiae*; Dm, *D. melanogaster*. The "**:**" and "**.**" characters indicate strongly or weakly conserved residues by the default setting of Clustal Omega (Sievers *et al.*, 2011).

from Life Technologies, Cy5-derivatized streptavidin was from KPL, and underivatized streptavidin was from Thermo Fischer.

Ypt7p (Hickey *et al.*, 2009), HOPS (Hickey and Wickner, 2010), and vacuolar SNAREs (Mima *et al.*, 2008; Schwartz and Merz, 2009; Zucchi and Zick, 2011) were isolated as described. The N-domain deletion mutants of Qb or Qa were amplified by PCR from full-length Qb or Qa clones with the Phusion high-fidelity DNA polymerase from NEB and cloned into pMBP parallel1 or pGST parallel1 vectors. All plasmids were confirmed by sequencing. Soluble SNAREs and derivatives were purified by following the MBP-TEV-Qb (1–194) protocol (Zick and Wickner, 2013). Proteins were frozen in aliquots in liquid nitrogen and stored at –80°C.

Proteoliposome preparation

Proteoliposomes of vacuolar mixed lipid (VML) composition (Mima *et al.*, 2008) were prepared from mixed micellar solutions (containing 50 mM β-octyl-glucoside) as described (Zick *et al.*, 2014), with prenylated Ypt7 at a 1:8000 molar ratio to lipid and individual SNAREs at the indicated molar protein:lipid ratios. Lipids dissolved in chloroform were mixed with β-octyl-glucoside in the following proportions: for VML (18:2) compositions, 44.8–47.6 mol% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 18 mol% 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, 18 mol% soy L-α-phosphatidylinositol, 4.4 mol% 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine), 2 mol% 1,2-dioleoyl-*sn*-glycero-3-phosphate, 1 mol% 16:0 1,2-dipalmitoyl-*sn*-glycerol), 8 mol% ergosterol, and 1 mol% 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1-myo-inositol-3-phosphate). All proteoliposomes were prepared with 0.2 or 3% mol% NBD-PE as a lipid marker.

Membrane fusion assay

Fusion reactions were assembled in 20 µl. Fusion assay pairs of reconstituted proteoliposomes (RPLs; each 250 µM lipid) in RB150 (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]/NaOH, pH 7.4, 150 mM NaCl, 10% [vol/vol] glycerol), 5 µM streptavidin, 1 mM EDTA, and 10 µM GTP were preincubated for 10 min at 27°C before addition of 1.25 mM $MgCl_2$ to exchange Ypt7 to its GTP-bound form. A 10-µl portion was transferred to 384-well plates. Soluble components (10 µl of 100 nM HOPS, 100 nM Vam7, and soluble SNAREs as noted) or their respective buffers were added to initiate the reactions. All reactions contained 0.5% (wt/vol) defatted bovine serum albumin [BSA], 5 mM reduced glutathione, and 1 mM dithiothreitol. Plates were incubated at 27°C in a fluorescence plate reader for 40 min, and content mixing signals (PhycoE–Cy5-FRET: excitation 565 nm; emission 670 nm; cutoff 630 nm) were recorded at intervals of 1 min in a SpectraMax Gemini XPS (Molecular Devices, Sunnyvale, CA) fluorescence plate reader. Maximal content mixing values were determined after addition of 0.2% (wt/vol) Thesit to samples that had not received streptavidin.

Determination of SNARE associations by pull-down assay

To measure SNARE complex that formed during a fusion reaction, we assayed the Qb or R that was bound to his_6-Qc by magnetic nickel Dynabeads (ThermoFisher). A 40-µl reaction was incubated for 30 min at 27°C during assay of FRET signal, placed on ice, and diluted 10-fold with ice-cold 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, 20 mM imidazole) containing 10 µl of RIPA buffer–washed Dynabeads and 500 nM GST-sR to prevent nonspecific interactions. The mixture was nutated at room temperature for 1 h. The beads were then washed three times with 1 ml of modified RIPA buffer, and bound proteins were eluted with reducing SDS–PAGE sample buffer for 5 min at 95°C. All samples were analyzed by SDS–PAGE and Western blotting with anti-R or -Qb antibodies.

Amino acid sequence comparisons

Amino acid sequences of the Qa-SNAREs from *S. cerevisiae, H. sapiens, M. musculus*, and *D. melanogaster* were obtained from the

National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov/protein/). Amino acid sequences were analyzed by the CLC viewer program (Qiagen) with Clustal Omega plug-in (Sievers *et al.*, 2011).

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