Topological arrangement of the intracellular membrane fusion machinery

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ABSTRACT **Soluble *N*-ethylmaleimide–sensitive factor attachment protein receptors (SNAREs) form a four-helix coiled-coil bundle that juxtaposes two bilayers and drives a basal level of membrane fusion. The Sec1/Munc18 (SM) protein binds to its cognate SNARE bundle and accelerates the basal fusion reaction. The question of how the topological arrangement of the SNARE helices affects the reactivity of the fusion proteins remains unanswered. Here we address the problem for the first time in a reconstituted system containing both SNAREs and SM proteins. We find that to be fusogenic a SNARE topology must support both basal fusion and SM stimulation. Certain topological combinations of exocytic SNAREs result in basal fusion but cannot support SM stimulation, whereas other topologies support SM stimulation without inducing basal fusion. It is striking that of all the possible topological combinations of exocytic SNARE helices, only one induces efficient fusion. Our results suggest that the intracellular membrane fusion complex is designed to fuse bilayers according to one genetically programmed topology.**

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INTRODUCTION

Intracellular membrane fusion is the basis of a broad range of fundamental biological processes, including neurotransmitter release, nutrient homeostasis, and receptor mobilization and internalization. The merging of intracellular bilayers is mediated by a fusion complex comprised of soluble *N*-ethylmaleimide–sensitive factor attachment protein receptors (SNAREs) and Sec1/Munc18 (SM) proteins (Figure 1A) (Sudhof and Rothman, 2009). SNAREs are small, membrane-associated proteins that contain characteristic stretches of 60–70 amino acids known as SNARE motifs. Fusion is initiated when the SNARE motifs of the vesicle-rooted SNARE (v-SNARE) and the target membrane-associated SNAREs (t-SNAREs) zipper into a four-helix coiled-coil bundle between two apposed bilayers (Sollner *et al.*, 1993; Weber *et al.*, 1998; Jahn and Scheller, 2006; Wickner and Schekman, 2008). The SNARE core bundle is arranged in a parallel manner and contains 16 hydrophobic layers of interacting side chains, except for the middle layer (zero layer), which is formed by four polar side chains, including three glutamines (Q) and one arginine (R) (Sutton *et al.*, 1998; Katz and Brennwald, 2000; Antonin *et al.*, 2002; Stein *et al.*, 2009). The SNARE core bundle is structurally conserved across fusion pathways and consists of three Q-SNARE helices (usually found in the t-SNAREs) and one R-SNARE helix (usually corresponding to the v-SNARE) (Figure 1B). Based on its position in the coiled-coil bundle, each SNARE helix is classified as Qa-, Qb-, Qc-, or R-SNARE (Jahn and Scheller, 2006; Martens and McMahon, 2008; Sorensen, 2009). N- to C-terminal zippering of the SNARE core bundle brings two membranes into close apposition and drives a basal level of fusion (Melia *et al.*, 2002; Reese *et al.*, 2005; Pobbati *et al.*, 2006; Vicogne *et al.*, 2006; Kesavan *et al.*, 2007; Li *et al.*, 2007; Domanska *et al.*, 2009; Schwartz and Merz, 2009; Walter *et al.*, 2010).

SM proteins are soluble factors of 60–70 kDa that directly interact with their cognate SNAREs to accelerate the basal fusion reaction (Novick and Schekman, 1979; Hata *et al.*, 1993; Dulubova *et al.*, 2007; Shen *et al.*, 2007). SM proteins exhibit a similar loss-of-function phenotype (abrogation of fusion) as SNAREs and are required for every pathway of intracellular vesicle transport (Latham *et al.*, 2007; Toonen and Verhage, 2007; Burgoyne *et al.*, 2009; Carr and Rizo, 2010). The conserved binding target of SM proteins is the four-helix SNARE core bundle, the principal driving force for membrane fusion. The SNARE bundle is believed to be accommodated within

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Abbreviations used: PDGFR, platelet-derived growth factor receptor; SM, Sec1/Munc18; SUMO, small ubiquitin-like receptor.

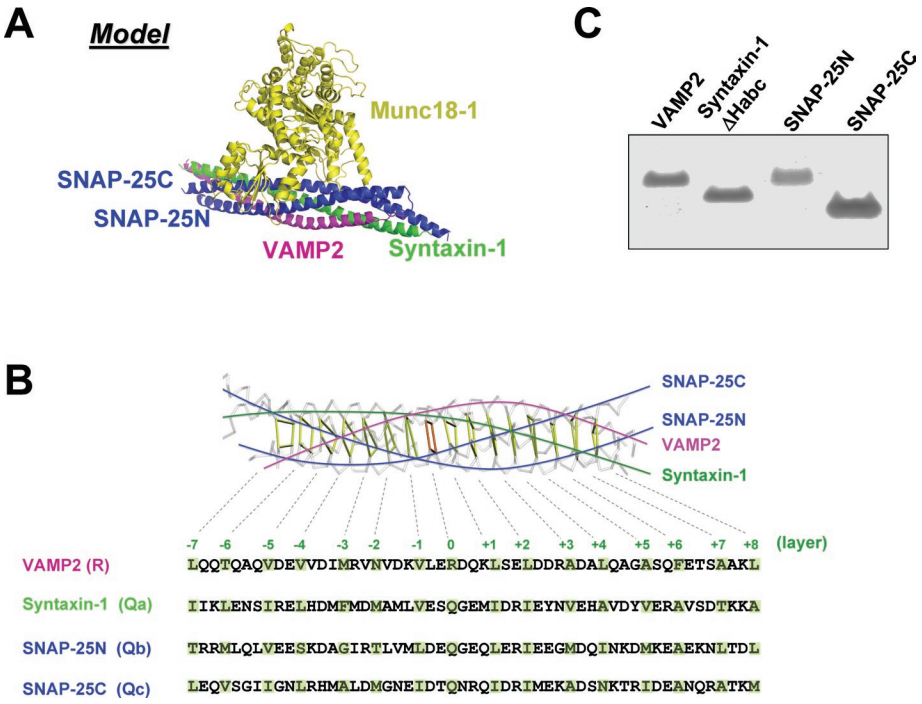

FIGURE 1: Expression of the individual helices of exocytic SNARE complex. (A) Model showing the SNARE–Munc18-1 fusion complex. Munc18-1 (the SM protein) binds to a SNARE core bundle composed of syntaxin-1 (the Qa chain), the N-terminal SNARE motif of SNAP-25 (SNAP-25N, the Qb chain), the C-terminal SNARE motif of SNAP-25 (SNAP-25C, the Qc chain), and VAMP2 (the R chain). Modeled from the crystal structures of Munc18-1 (Misura *et al.*, 2000; Bracher and Weissenhorn, 2001) and SNARE complex (Sutton *et al.*, 1998; Stein *et al.*, 2009). Structures were edited in PyMol. This model is intended to depict the hypothetical interaction between Munc18-1 and the SNARE complex. The actual binding details await high-resolution structure of the complex. (B) Top, backbone view of the SNARE core bundle with individual layers indicated. Bottom, alignment of the SNARE motifs of exocytic SNAREs with the core residues numbered, highlighted, and connected to the corresponding layers in the structure. (C). Recombinant SNARE helices were resolved on SDS–PAGE and stained with Coomassie blue.

the central cavity regions of SM proteins, which are fundamentally designed to bind four-helix bundles (Bacaj *et al.*, 2010; Hu *et al.*, 2011).

A cognate SNARE complex in the cell generally involves a defined distribution of the four SNARE helices between two bilayers, leading to the suggestion that the SNARE chains are topologically restricted by design in mediating membrane fusion (Parlati *et al.*, 2000). This concept is supported by the study of the yeast endoplasmic reticulum (ER)–Golgi transport, where only a single of all tested topological combinations is fusogenic (Parlati *et al.*, 2000). Analysis of mammalian endosomal SNAREs, however, revealed that fusion could be induced with multiple SNARE topologies (Zwilling *et al.*, 2007), although it is unclear whether this topological flexibility represents a specialized feature of homotypic membrane fusion. Moreover, in these studies it was not determined whether the SNARE topologies are compatible with the regulation by SM proteins, the other component of the vesicle fusion machinery. Thus the question of how the topological arrangement of the SNARE helices affects their fusion capacity remains unanswered.

In this study we sought to resolve this issue by examining the topological restriction of the exocytic fusion machinery, taking advantage of the large amount of functional and physiological information already available for exocytic fusion proteins. Vesicle exocytosis requires the v-SNARE VAMP2 (also known as synaptobrevin-2), the t-SNAREs syntaxin-1 and SNAP-25, and the SM protein Munc18–1/nSec1 (Figure 1, A and B) (Wu *et al.*, 1998; Verhage *et al.*, 2000; Voets *et al.*, 2001; Hu *et al.*, 2002; Weimer *et al.*, 2003; An and Almers, 2004; Jackson and Chapman, 2006). Although the Qb and Qc chains of exocytic SNAREs are normally conjoined in SNAP-25, they can be separated and independently anchored in the bilayer (Figure 1C) (Shen *et al.*, 2010). The resulting SNARE complex, which represents the typical configuration of SNAREs in the cell, is fully fusogenic (Shen *et al.*, 2010). This development presents a unique opportunity for us to use exocytic fusion as a model system to investigate the inherent topological properties of SNARE helices in the contexts of both the SNARE bundle and the SNARE–SM complex. The four helices of exocytic SNAREs—syntaxin-1 (Qa), SNAP-25N (Qb), SNAP-25C (Qc), and VAMP2 (R)—were reconstituted into two populations of proteoliposomes in all possible topological combinations. We found that certain SNARE topologies resulted in basal fusion but could not support Munc18-1 stimulation, whereas other topologies supported Munc18-1 activation without inducing appreciable basal fusion. When the overall SNARE-Munc18-1–mediated fusion was examined, however, efficient fusion was induced only when the SNARE helices were arranged in the native topology.

RESULTS

Fusion capability of exocytic SNARE complexes arranged in a 3:1 manner

Here we explored the inherent reactivity of all of the nonredundant distributions of the four exocytic SNARE helices between two proteoliposome populations. Equimolar amounts of SNAREs were reconstituted into either the acceptor or the donor liposomes, and the fusion of the liposomes was monitored by lipid mixing using fluorescence resonance energy transfer (Shen *et al.*, 2007). Three fusion parameters were measured for each topological combination of the SNARE complex: 1) the rate of the SNARE-driven basal fusion, 2) Munc18-1 activation, and 3) the overall rate of the SNARE-Munc18-1–mediated fusion reaction (the overall fusion).

In the cell, syntaxin-1 (Qa), SNAP-25N (Qb), and SNAP-25C (Qc) are located in one membrane, whereas the R-SNARE VAMP2 is in the other (Jahn and Scheller, 2006). The SNARE complex reconstituted according to this native topology (designated topology 1) induced a basal fusion reaction that was strongly activated by Munc18-1 (Figure 2, B and C). The overall SNARE-Munc18-1–mediated fusion was ~13-fold faster than the basal SNARE-driven fusion (Figure 2, B and D). When VAMP2 was replaced with VAMP8, a noncognate v-SNARE involved in lysosomal/late endosomal fusion (Antonin *et al.*, 2000), the basal fusion remained intact, but Munc18-1 activation was abolished (Figure 2, A–C), leading to the reduction of the overall fusion to ~8% of the native level (Figure 2D). When the liposomes were preincubated with the cytoplasmic fragment of VAMP2, a competitive inhibitor of SNARE complex formation, both the basal fusion and Munc18-1 activation were abrogated (Figure 2, A–D).

We next arranged the SNARE helices into other 3:1 topological combinations, in which three distinct SNARE chains were in one

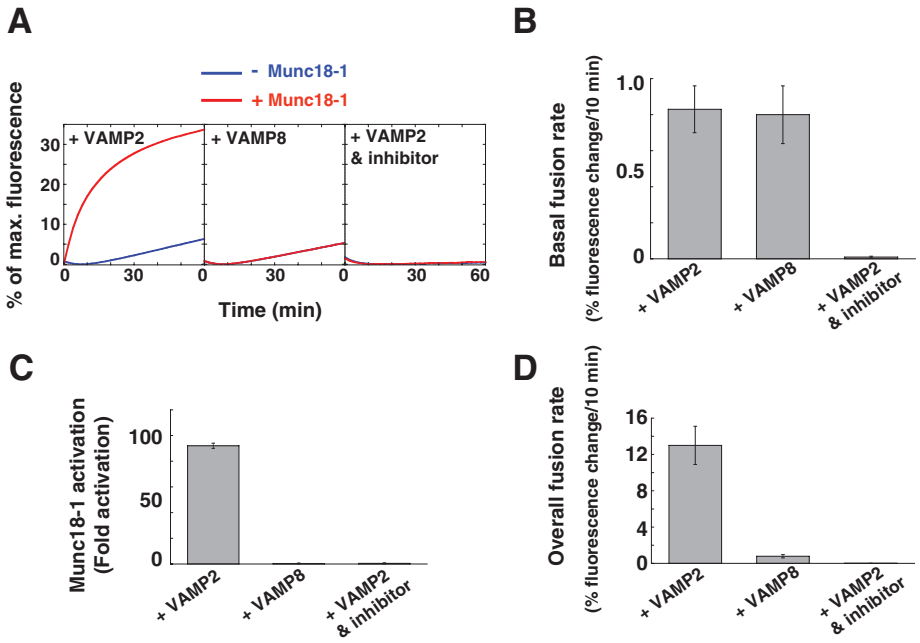

FIGURE 2: Reconstitution and characterization of exocytic SNAREs arranged in the native topology. (A) Fusion of acceptor (containing syntaxin-1, SNAP-25N, and SNAP-25C) and donor (containing VAMP2 or VAMP8) liposomes in the absence or presence of 5 μM Munc18-1. In the negative control, the cytoplasmic domain of VAMP2 (a competitive inhibitor of SNARE assembly) was added at a fivefold excess. (B) Initial rates of the basal SNARE-driven fusion reactions shown in A. Data are presented as percentage of fluorescence change per 10 min. (C) Activation of the basal fusion reactions by Munc18-1. Because some SNARE complexes support Munc18-1 activation without inducing detectable basal fusion, in this study the activation of a reaction by Munc18-1 was represented as follows: $(V_{overall} - V_{basal})/V_{overall}$, where $V_{overall}$ is the initial rate of the SNARE-Munc18-1–mediated fusion and V_{basal} is the initial rate of the basal SNARE-driven fusion. (D) Initial rates of the SNARE-Munc18-1–mediated fusion reactions shown in A. Data are presented as percentage of fluorescence change per 10 min. Error bars indicate SD.

liposome population and the fourth, distinct SNARE chain was in the other (Figure 3A). Each SNARE chain was incorporated at comparable densities across the liposome populations (Supplemental Figure S1A). We found that topology 2 (syntaxin-1 + SNAP-25N + VAMP2/SNAP-25C) and topology 3 (syntaxin-1 + SNAP-25C + VAMP2/SNAP-25N) resulted in significant levels of basal fusion (~30% of the native level; Figure 3B). However, neither of the topologies supported Munc18-1 activation, and thus the overall fusion was close to background levels (Figure 3, C and D). The SNARE complex arranged in the fourth topology (SNAP-25N + SNAP-25C + VAMP2/syntaxin-1), on the other hand, supported neither basal fusion nor Munc18-1 stimulation (Figure 3, B–D). These data suggest that multiple 3:1 SNARE topologies result in basal fusion, but only the native configuration is compatible with Munc18-1 activation. The inefficient basal fusion mediated by topologies 2–4 might be simply attributed to a slower assembly rate of the SNARE bundle. However, we found that the basal fusion kinetics of these topologies was not accelerated when the liposomes were incubated for 16 h at 4 °C (Supplemental Figure S2), suggesting that the basal fusion rate is dictated by the intrinsic property of the topological arrangement.

Fusion capability of exocytic SNARE complexes arranged in a 2:2 manner

We next examined the SNARE combinations in which two distinct SNARE helices are in one liposome population and the remaining two distinct SNARE helices are in the other (Figure 4A). Each SNARE chain was incorporated at comparable densities across the liposome populations (Supplemental Figure S1B). We found that none of these 2:2 topologies permitted appreciable basal fusion (Figure 4B). However, one of the SNARE combinations (topology 6: syntaxin-1 + SNAP-25C/SNAP-25N + VAMP2) supported Munc18-1 activation in spite of the undetectable basal fusion, although the overall fusion was an order of magnitude slower than the native level (Figure 4C-D). SNAREs arranged in other 2:2 combinations (topologies 5 and 7) were not activated by Munc18-1, and thus their overall fusion was at background levels (Figure 4D). Therefore, none of the 2:2 SNARE topologies is fusogenic, even though one of them is compatible with Munc18-1 stimulation. Again, the inefficient basal fusion of topologies 5–7 was not increased when the liposomes were incubated for 16 h at 4°C (Supplemental Figure S2). Given the overall similarity of topologies 5 and 6, it is surprising that topology 6 supports robust Munc18-1 activation but Topology 5 does not. We found that the reactivity of topologies 5 and 6 was not influenced by the Vc peptide or NSF/α-SNAP (Supplemental Figure S3), which are known to prime SNARE assembly (Weber *et al.*, 2000; Melia *et al.*, 2002; Pobbati *et al.*, 2006) or by the variations in the lipid compositions of the liposomes (Supplemental Figure S4). Thus the fusogenicity of the topological combinations is likely dictated by the intrinsic physicochemical properties of SNAREs and SM proteins.

Together these results demonstrate that, strikingly, efficient fusion is induced only when all three Q-SNARE helices are anchored in one lipid bilayer and the R-SNARE helix in the other, which precisely matches the native configuration of exocytic SNAREs in the cell.

A complete set of QabcR helices is required for the reactivity of the exocytic SNARE complex

Next we tested whether the QabcR composition is necessary for the fusion capability of exocytic SNAREs in the context of the fusogenic native topology. Each of the SNARE chains—syntaxin-1 (Qa), SNAP-25N (Qb), SNAP-25C (Qc), and VAMP2 (R)—was individually removed from the liposomes (Figure 5A). When any of the three Q-SNARE helices was absent from the acceptor bilayer, we found that the basal fusion was abolished (Figure 5B). Of interest, the SNARE complex lacking the SNAP-25N chain (No SNAP-25N) was activated by Munc18-1 despite the undetectable basal fusion, although the overall fusion was <10% of the native level (Figure 5, C and D). SNARE complexes lacking either the syntaxin-1 or the SNAP-25C helix failed to be activated by Munc18-1, and thus their overall fusion was close to background levels (Figure 5, C and D). As expected, neither basal fusion nor Munc18-1 activation was observed when the R-SNARE VAMP2 was absent from the donor liposomes (Figure 5, B–D). Hence, a complete set of QabcR helices is critical to the reactivity of the exocytic SNARE complex.

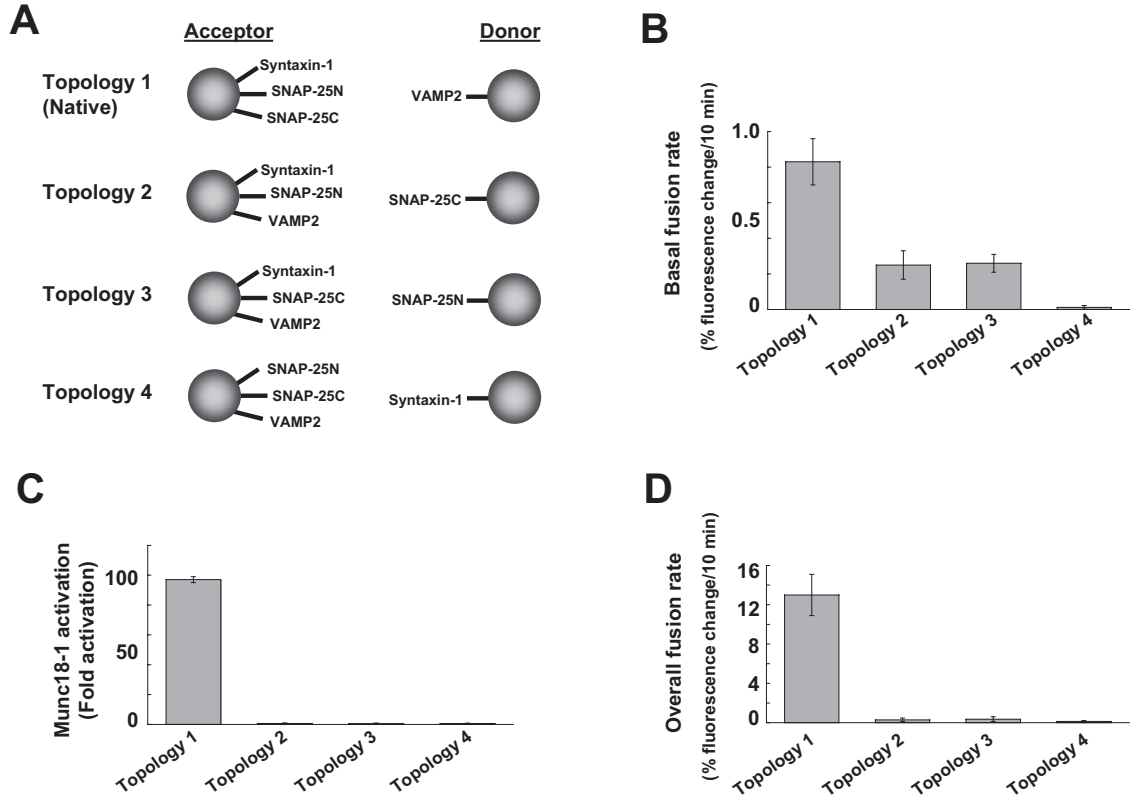

FIGURE 3: Fusion capability of exocytic SNARE complexes arranged in a 3:1 manner. (A) Diagrams showing the topological arrangements of the SNARE helices in acceptor and donor liposomes. (B) Initial rates of basal SNARE-driven fusion reactions mediated by SNARE pairs shown in A. Data are presented as percentage of fluorescence change per 10 min. (C) Activation of the basal fusion reactions by Munc18-1. (D) Initial rates of the SNARE-Munc18-1–mediated fusion reactions. Data are presented as percentage of fluorescence change per 10 min. Error bars indicate SD.

Munc18-1 activates nonnative SNARE complexes with the same compartmental specificity

It is unexpected that certain nonnative SNARE topologies support Munc18-1 activation while inducing no basal fusion. One key feature of Munc18-1 stimulation of fusion is the strict compartmental specificity (only exocytic SNAREs are activated) (Shen *et al.*, 2007). Next we tested whether this also holds true for the nonnative SNARE topologies. VAMP2 was replaced with the noncognate v-SNARE VAMP8 in the SNARE complex arranged in topology 6 or No SNAP-25N, both of which support Munc18-1 stimulation (Figure 6, A and B). We found that the substitution abrogated Munc18-1 stimulation for both topological combinations (Figure 6B). Thus SNARE complexes arranged in these nonnative topological combinations are regulated by Munc18-1 with the same compartmental specificity.

DISCUSSION

In this work we have examined how the topological arrangement of the exocytic SNARE helices affects the SNARE-SM–mediated membrane fusion. We found that there is a strict topological requirement for the Qa-, Qb-, and Qc-SNARE helices in one membrane bilayer and the R-SNARE in another. SNARE complexes arranged in other topologies result in either no appreciable fusion or a fusion reaction that is at least one order of magnitude slower than the native level. Of interest, the single fusogenic topology revealed here precisely matches the native arrangement of the exocytic SNARE helices in the cell (Jahn and Scheller, 2006), thus establishing a direct connection of this study to physiology.

Our results show that to be fusogenic a SNARE topology must first be capable of inducing an efficient level of basal membrane fusion (fusion driven by SNAREs alone). SNARE topological restriction in the basal fusion reaction has been debated due to the discrepancies in the previous studies of the ER–Golgi transport and the endosomal fusion (Parlati *et al.*, 2000; Zwilling *et al.*, 2007). With the present study on exocytic fusion, the topological restriction of the basal fusion has now been examined in three different pathways spanning nearly all major branches of the intracellular trafficking network. What can we conclude from these studies? First, SNARE complexes do exhibit certain degrees of topological restriction in the basal membrane fusion because many SNARE topologies fail to induce any fusion. One possible explanation for this restriction is that altering the topology of bilayer insertion of one SNARE helix relative to the others may slow down the rate of SNARE assembly or reduce the stability of SNARE bundles. Second, our results clearly show that the asymmetric exocytic SNAREs can induce basal fusion with multiple topologies, suggesting that the topological flexibility in the basal fusion reaction is not limited to homotypic membrane fusion. Taken together, these findings suggest that, despite the structural conservation of the SNARE bundles, at the basal fusion level there appears to be no universal rule of topological restriction.

The basal SNARE-driven membrane fusion, although serving as the basis of the overall fusion reaction, needs to be activated by its cognate SM protein to achieve physiological levels of vesicle fusion (Peng and Gallwitz, 2002; Scott *et al.*, 2004; Carpp *et al.*, 2006; Latham *et al.*, 2007; Shen *et al.*, 2007; Rodkey *et al.*, 2008; Tareste

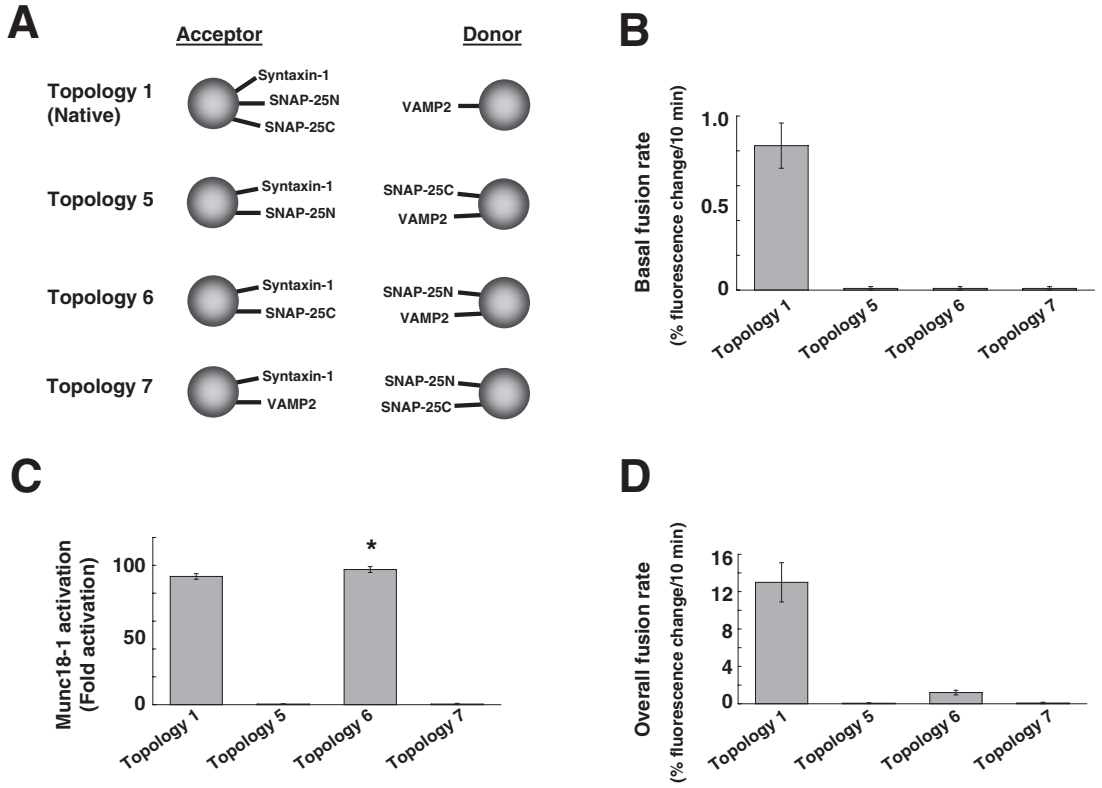

FIGURE 4: Fusion capability of exocytic SNARE complexes arranged in a 2:2 manner. (A) Diagrams showing the topological arrangements of the SNARE helices in acceptor and donor liposomes. (B) Initial rates of basal SNARE-driven fusion reactions mediated by SNARE pairs shown in A. Data are presented as percentage of fluorescence change per 10 min. (C) Activation of the basal fusion reactions by Munc18-1. Note that the Munc18-1 activation in topology 6 was close to 100% because of the low basal fusion. (D) Initial rates of the SNARE-Munc18-1–mediated fusion reactions. Data are presented as percentage of fluorescence change per 10 min. Error bars indicate SD. Note that the overall fusion rate of the indicated topological combination was still low (asterisk) despite the robust activation by Munc18-1.

et al., 2008; Furgason *et al.*, 2009; Ohya *et al.*, 2009; Diao *et al.*, 2010; Rathore *et al.*, 2010). The importance of SNARE topologies to the SM protein function was previously speculated by Reinhard Jahn and colleagues (Zwilling *et al.*, 2007) and was proved in the present study. Of all the nonredundant topological combinations of exocytic SNAREs, only two (topologies 1 and 6) support Munc18-1 activation. Notably, SNARE complexes arranged in topologies 2 and 3 induce basal fusion but are not stimulated by Munc18-1, thus reducing the overall fusion to background levels. How does the SM protein "read" the topological information of a SNARE complex? We suggest that each of the four distinct SNARE helices occupies a specific location at the asymmetric central cavity region of the SM protein, which directly grabs the SNARE core bundle (Sudhof and Rothman, 2009). As such, a SNARE complex with the wrong topology may have a reduced affinity for the SM protein even though the SNARE bundle may still form and induce basal fusion. This model is supported by the observations that Munc18-1 interacts with all exocytic SNARE subunits during fusion (Shen *et al.*, 2007; Rodkey *et al.*, 2008).

Of interest, certain SNARE topologies can support robust Munc18-1 activation without inducing detectable basal fusion. It is possible that, although the SNARE bundle forms inefficiently in these topologies, it nevertheless binds to Munc18-1 with sufficiently high affinity to allow for stimulation of fusion. These topologies, however, do not result in efficient overall fusion due to the low basal level, again highlighting the importance of the basal fusion to the overall SNARE-SM–mediated fusion reaction. It is interesting that the SNARE complex lacking SNAP-25N can be robustly activated by Munc18-1 but without eliciting appreciable basal fusion. This finding implies that the binding of Munc18-1 to the Qb chain of the SNARE bundle is dispensable for fusion activation. It is possible that a second copy of another SNARE chain such as syntaxin-1 or SNAP-25C can substitute for SNAP-25N in forming the four-helix bundle when bound to Munc18-1. Alternatively, in the absence of SNAP-25N, Munc18-1 may promote fusion by recognizing a three-helix SNARE complex containing syntaxin-1, VAMP2, and SNAP-25C. Ultimately, a SNARE topology must support both basal fusion and SM activation to be fusogenic. In this context, although endosomal SNAREs can drive basal fusion with multiple topologies, the majority of the combinations are expected to be incompatible with the asymmetric SM protein stimulation, thus likely resulting in a stringent topological restriction similar to exocytic SNAREs. The ER–Golgi fusion, on the other hand, already displays a high degree of topological restriction at the basal fusion level (Parlati *et al.*, 2000). As such, only one configuration of the ER–Golgi SNAREs is expected to induce efficient overall fusion. The sole fusogenic SNARE topology revealed in this study corresponds to the known arrangement of the exocytic fusion complex in the cell, suggesting that in vitro findings can be used to predict the native topologies of fusion complexes involved in less-well-understood transport pathways.

Taken together, our findings point to a general principle for the topological arrangement of the intracellular fusion machinery: a fusion complex is designed to fuse bilayers according to one genetically programmed topology, which is likely encoded by the inherent

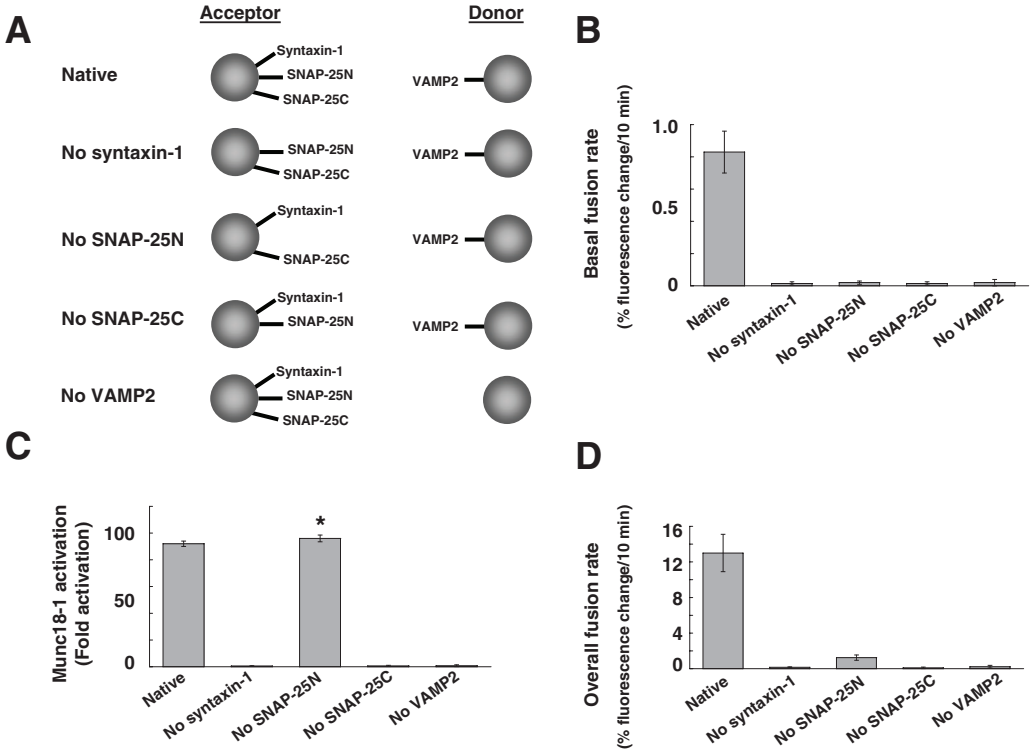

FIGURE 5: A complete set of QabcR helices is required for the reactivity of the exocytic SNARE complex. (A) Diagrams showing the topological arrangements of the SNARE helices in acceptor and donor liposomes. (B) Initial rates of basal SNARE-driven fusion reactions mediated by SNARE pairs shown in A. Data are presented as percentage of fluorescence change per 10 min. (C) Activation of the basal fusion reactions by Munc18-1. Note that the Munc18-1 activation in topology 1-SNAP-25N was close to 100% because of the low basal fusion. (D) Initial rates of the SNARE-Munc18-1–mediated fusion reactions. Data are presented as percentage of fluorescence change per 10 min. Error bars indicate SD. Note that the overall fusion rate of the indicated topological combination (asterisk) was still low despite the robust activation by Munc18-1.

physicochemical properties of SNAREs and SM proteins. This stringent topological restriction, together with the compartmental specificity of the SNARE–SM interactions, likely contributes to the overall accuracy of intracellular vesicle fusion.

MATERIALS AND METHODS

Protein expression and purification

Recombinant SNARE proteins were expressed and purified as previously described (Shen *et al.*, 2010). Syntaxin-1 ΔHabc, SNAP-25N-PDGFR, SNAP-25C-PDGFR, and v-SNAREs were expressed as SUMO-tagged proteins and had no tags left when the SUMO moieties were removed. Recombinant untagged Munc18-1 protein was produced in *Escherichia coli* as previously described (Shen *et al.*, 2007). Membrane proteins were stored in a buffer containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), 400 mM KCl, 1% *n*-octyl-β-D-glucoside, 10% glycerol, and 0.5 mM Tris(2-carboxyethyl)phosphine (TCEP) (Ji *et al.*, 2010). Soluble factors were stored in a buffer containing 25 mM HEPES (pH 7.4), 150 mM KCl, 10% glycerol, and 0.5 mM TCEP.

Proteoliposome reconstitution

All lipids were obtained from Avanti Polar Lipids (Alabaster, AL). To prepare acceptor liposomes, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), and cholesterol were mixed in a molar ratio of 60:20:10:10. To prepare donor liposomes, POPC, POPE, POPS, cholesterol, *N*-(7-nitro-2,1,3-benzoxadiazole-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine, and *N*-(Lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl phosphatidylethanolamine were mixed at a molar ratio of 60:17:10:10:1.5:1.5. SNARE proteoliposomes were prepared by detergent dilution and isolated on Nycodenz density gradient flotation (Axis-Shield, Oslo, Norway) (Shen *et al.*, 2010). Complete detergent removal was achieved by overnight dialysis of the samples in Novagen dialysis tubes (EMD, San Diego, CA) against the reconstitution buffer (25 mM HEPES, pH 7.4, 100 mM KCl, 10% glycerol, and 1 mM dithiothreitol). SNARE proteins were kept at physiologically relevant surface densities, with protein:lipid ratio at 1:200 for donor liposomes, similar to VAMP2 densities reported for native vesicles (Takamori *et al.*, 2006), and at 1:500 for acceptor liposomes. This reconstitution procedure is known to yield homogeneous populations of proteoliposomes that exhibit similar fusion properties as native membranes (Takamori *et al.*, 2006; Holt *et al.*, 2008). Reconstituted liposomes were routinely monitored by electron microscopy with negative staining.

Liposome fusion assay

Fusion reactions and data analysis were performed as previously described (Shen *et al.*, 2010). A standard fusion reaction contained 45 μl of unlabeled acceptor liposomes and 5 μl of labeled donor liposomes and was conducted in a 96-well Nunc plate (Nalge Nunc International, Rochester, NY) at 37°C. Fusion was followed by measuring the increase in NBD fluorescence at 538 nm (excitation 460 nm) every 2 min in a Synergy HT microplate reader (BioTek,

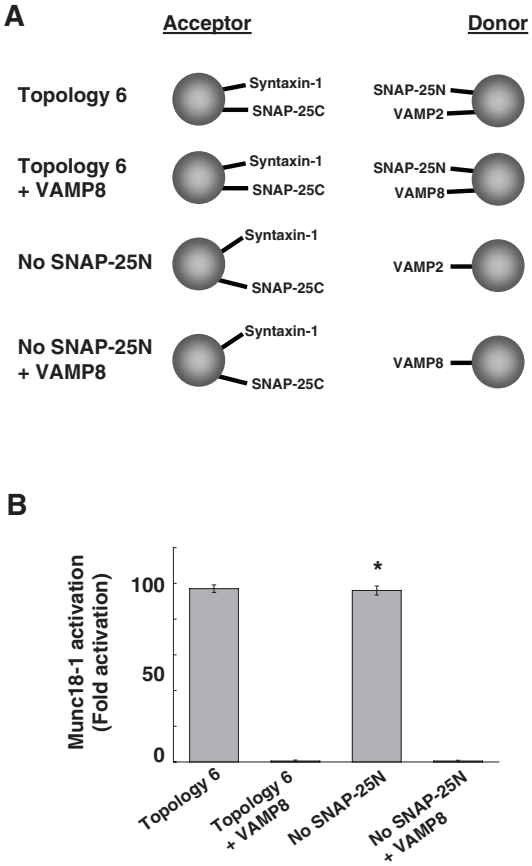

FIGURE 6: Munc18-1 activates nonnative SNARE complexes with the same compartmental specificity. (A) Diagrams showing the topological arrangements of the SNARE helices in acceptor and donor liposomes. (B) The activation of the SNARE pairs in A by Munc18-1. Data were acquired and processed in a similar way as in Figure 2C. Error bars indicate SD. Note that the overall fusion rate of the indicated topological combination (asterisk) was still low despite the robust activation by Munc18-1.

Winooski, VT). At the end of the reaction, 10 µl of 2.5% dodecyl maltoside was added to the liposomes. Fusion data were presented as the percentage of maximum fluorescence change. To assess the regulatory activity of Munc18-1, acceptor and donor liposomes were incubated with or without 5 µM Munc18-1 on ice for 1 h before the temperature was elevated to 37°C to initiate fusion. The maximum fusion rate within the first 20 min of liposome fusion was used to represent the initial rate of a fusion reaction. The fusion rate was presented as percentage of fluorescence change per 10 min. Because some SNARE complexes support Munc18-1 activation without inducing detectable basal fusion, in this study the activation of a reaction by Munc18-1 was presented as $(V_{overall} - V_{basal})/V_{overall}$, where $V_{overall}$ is the initial rate of the SNARE-Munc18-1–mediated fusion and V_{basal} is the initial rate of the basal SNARE-driven fusion. Full accounting of statistical significance was included for each figure based on at least three independent experiments.

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REFERENCES

An SJ, Almers W (2004). Tracking SNARE complex formation in live endocrine cells. Science 306, 1042–1046.

Antonin W, Fasshauer D, Becker S, Jahn R, Schneider TR (2002). Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. Nat Struct Biol 9, 107–111.

Antonin W, Holroyd C, Fasshauer D, Pabst S, Von Mollard GF, Jahn R (2000). A SNARE complex mediating fusion of late endosomes defines conserved properties of SNARE structure and function. EMBO J 19, 6453–6464.

Bacaj T, Pang ZP, Sudhof TC (2010). Testing the SNARE/SM protein model of membrane fusion. Proc Natl Acad Sci USA 107, 22365–22366.

Bracher A, Weissenhorn W (2001). Crystal structures of neuronal squid Sec1 implicate inter-domain hinge movement in the release of t-SNAREs. J Mol Biol 306, 7–13.

Burgoyne RD, Barclay JW, Ciufo LF, Graham ME, Handley MT, Morgan A (2009). The functions of Munc18-1 in regulated exocytosis. Ann NY Acad Sci 1152, 76–86.

Carpp LN, Ciufo LF, Shanks SG, Boyd A, Bryant NJ (2006). The Sec1p/Munc18 protein Vps45p binds its cognate SNARE proteins via two distinct modes. J Cell Biol 173, 927–936.

Carr CM, Rizo J (2010). At the junction of SNARE and SM protein function. Curr Opin Cell Biol 22, 488–495.

Diao J, Su Z, Lu X, Yoon TY, Shin YK, Ha T (2010). Single-vesicle fusion assay reveals Munc18-1 binding to the SNARE core is sufficient for stimulating membrane fusion. ACS Chem Neurosci 1, 168–174.

Domanska MK, Kiessling V, Stein A, Fasshauer D, Tamm LK (2009). Single vesicle millisecond fusion kinetics reveals number of SNARE complexes optimal for fast SNARE-mediated membrane fusion. J Biol Chem 284, 32158–32166.

Dulubova I, Khvotchev M, Liu S, Huryeva I, Sudhof TC, Rizo J (2007). Munc18-1 binds directly to the neuronal SNARE complex. Proc Natl Acad Sci USA 104, 2697–2702.

Furgason ML, MacDonald C, Shanks SG, Ryder SP, Bryant NJ, Munson M (2009). The N-terminal peptide of the syntaxin Tlg2p modulates binding of its closed conformation to Vps45p. Proc Natl Acad Sci USA 106, 14303–14308.

Hata Y, Slaughter CA, Sudhof TC (1993). Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin. Nature 366, 347–351.

Holt M, Riedel D, Stein A, Schuette C, Jahn R (2008). Synaptic vesicles are constitutively active fusion machines that function independently of Ca2+. Curr Biol 18, 715–722.

Hu K, Carroll J, Fedorovich S, Rickman C, Sukhodub A, Davletov B (2002). Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion. Nature 415, 646–650.

Hu SH, Christie MP, Saez NJ, Latham CF, Jarrott R, Lua LH, Collins BM, Martin JL (2011). Possible roles for Munc18-1 domain 3a and syntaxin1 N-peptide and C-terminal anchor in SNARE complex formation. Proc Natl Acad Sci USA 108, 1049–1045.

Jackson MB, Chapman ER (2006). Fusion pores and fusion machines in Ca(2+)-triggered exocytosis. Annu Rev Biophys Biomol Struct 35, 135–160.

Jahn R, Scheller RH (2006). SNAREs—engines for membrane fusion. Nat Rev Mol Cell Biol 7, 631–643.

Ji H, Coleman J, Yang R, Melia TJ, Rothman JE, Tareste D (2010). Protein determinants of SNARE-mediated lipid mixing. Biophys J 99, 553–560.

Katz L, Brennwald P (2000). Testing the 3Q:1R "rule": mutational analysis of the ionic "zero" layer in the yeast exocytic SNARE complex reveals no requirement for arginine. Mol Biol Cell 11, 3849–3858.

Kesavan J, Borisovska M, Bruns D (2007). v-SNARE actions during Ca(2+)-triggered exocytosis. Cell 131, 351–363.

Latham CF, Osborne SL, Cryle MJ, Meunier FA (2007). Arachidonic acid potentiates exocytosis and allows neuronal SNARE complex to interact with Munc18a. J Neurochem 100, 1543–1554.

Li F, Pincet F, Perez E, Eng WS, Melia TJ, Rothman JE, Tareste D (2007). Energetics and dynamics of SNAREpin folding across lipid bilayers. Nat Struct Mol Biol 14, 890–896.

Martens S, McMahon HT (2008). Mechanisms of membrane fusion: disparate players and common principles. Nat Rev Mol Cell Biol 9, 543–556.

Melia TJ, Weber T, McNew JA, Fisher LE, Johnston RJ, Parlati F, Mahal LK, Sollner TH, Rothman JE (2002). Regulation of membrane fusion by the membrane-proximal coil of the t-SNARE during zippering of SNAREpins. J Cell Biol 158, 929–940.

Misura KM, Scheller RH, Weis WI (2000). Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature 404, 355–362.
Novick P, Schekman R (1979). Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 76, 1858–1862.
Ohya T, Miaczynska M, Coskun U, Lommer B, Runge A, Drechsel D, Kalaidzidis Y, Zerial M (2009). Reconstitution of Rab- and SNARE-dependent membrane fusion by synthetic endosomes. Nature 459, 1091–1097.
Parlati F, McNew JA, Fukuda R, Miller R, Sollner TH, Rothman JE (2000). Topological restriction of SNARE-dependent membrane fusion. Nature 407, 194–198.
Peng R, Gallwitz D (2002). Sly1 protein bound to Golgi syntaxin Sed5p allows assembly and contributes to specificity of SNARE fusion complexes. J Cell Biol 157, 645–655.
Pobbati AV, Stein A, Fasshauer D (2006). N- to C-terminal SNARE complex assembly promotes rapid membrane fusion. Science 313, 673–676.
Rathore SS, Bend EG, Yu H, Hammarlund M, Jorgensen EM, Shen J (2010). Syntaxin N-terminal peptide motif is an initiation factor for the assembly of the SNARE-Sec1/Munc18 membrane fusion complex. Proc Natl Acad Sci USA 107, 22399–22406.
Reese C, Heise F, Mayer A (2005). Trans-SNARE pairing can precede a hemifusion intermediate in intracellular membrane fusion. Nature 436, 410–414.
Rodkey TL, Liu S, Barry M, McNew JA (2008). Munc18a scaffolds SNARE assembly to promote membrane fusion. Mol Biol Cell 19, 5422–5434.
Schwartz ML, Merz AJ (2009). Capture and release of partially zipped trans-SNARE complexes on intact organelles. J Cell Biol 185, 535–549.
Scott BL, Van Komen JS, Irshad H, Liu S, Wilson KA, McNew JA (2004). Sec1p directly stimulates SNARE-mediated membrane fusion in vitro. J Cell Biol 167, 75–85.
Shen J, Rathore S, Khandan L, Rothman JE (2010). SNARE bundle and syntaxin N-peptide constitute a minimal complement for Munc18-1 activation of membrane fusion. J Cell Biol 190, 55–63.
Shen J, Tareste DC, Paumet F, Rothman JE, Melia TJ (2007). Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. Cell 128, 183–195.
Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993). SNAP receptors implicated in vesicle targeting and fusion. Nature 362, 318–324.
Sorensen JB (2009). Conflicting views on the membrane fusion machinery and the fusion pore. Annu Rev Cell Dev Biol 25, 513–537.
Stein A, Weber G, Wahl MC, Jahn R (2009). Helical extension of the neuronal SNARE complex into the membrane. Nature 460, 525–528.
Sudhof TC, Rothman JE (2009). Membrane fusion: grappling with SNARE and SM proteins. Science 323, 474–477.
Sutton RB, Fasshauer D, Jahn R, Brunger AT (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature 395, 347–353.
Takamori S *et al*. (2006). Molecular anatomy of a trafficking organelle. Cell 127, 831–846.
Tareste D, Shen J, Melia TJ, Rothman JE (2008). SNAREpin/Munc18 promotes adhesion and fusion of large vesicles to giant membranes. Proc Natl Acad Sci USA 105, 2380–2385.
Toonen RF, Verhage M (2007). Munc18-1 in secretion: lonely Munc joins SNARE team and takes control. Trends Neurosci 30, 564–572.
Verhage M *et al*. (2000). Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science 287, 864–869.
Vicogne J, Vollenweider D, Smith JR, Huang P, Frohman MA, Pessin JE (2006). Asymmetric phospholipid distribution drives in vitro reconstituted SNARE-dependent membrane fusion. Proc Natl Acad Sci USA 103, 14761–14766.
Voets T, Toonen RF, Brian EC, de Wit H, Moser T, Rettig J, Sudhof TC, Neher E, Verhage M (2001). Munc18-1 promotes large dense-core vesicle docking. Neuron 31, 581–591.
Walter AM, Wiederhold K, Bruns D, Fasshauer D, Sorensen JB (2010). Synaptobrevin N-terminally bound to syntaxin-SNAP-25 defines the primed vesicle state in regulated exocytosis. J Cell Biol 188, 401–413.
Weber T, Parlati F, McNew JA, Johnston RJ, Westermann B, Sollner TH, Rothman JE (2000). SNAREpins are functionally resistant to disruption by NSF and alphaSNAP. J Cell Biol 149, 1063–1072.
Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE (1998). SNAREpins: minimal machinery for membrane fusion. Cell 92, 759–772.
Weimer RM, Richmond JE, Davis WS, Hadwiger G, Nonet ML, Jorgensen EM (2003). Defects in synaptic vesicle docking in unc-18 mutants. Nat Neurosci 6, 1023–1030.
Wickner W, Schekman R (2008). Membrane fusion. Nat Struct Mol Biol 15, 658–664.
Wu MN, Littleton JT, Bhat MA, Prokop A, Bellen HJ (1998). ROP, the *Drosophila* Sec1 homolog, interacts with syntaxin and regulates neurotransmitter release in a dosage-dependent manner. EMBO J 17, 127–139.
Zwilling D, Cypionka A, Pohl WH, Fasshauer D, Walla PJ, Wahl MC, Jahn R (2007). Early endosomal SNAREs form a structurally conserved SNARE complex and fuse liposomes with multiple topologies. EMBO J 26, 9–18.