Functions of SNAREs in intracellular membrane fusion and lipid bilayer mixing

Christian Ungermann[1] and Dieter Langosch[2]

[1]Biochemie Zentrum der Universität Heidelberg, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany
[2]Lehrstuhl der Chemie der Biopolymere, Technische Universität München, Weihenstephaner Berg 3, 85354 Freising, Germany
Authors for correspondence (e-mail: cu2@ix.urz.uni-heidelberg.de; biopolymere@lrz.tum.de)

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Summary

Intracellular membrane fusion occurs with exquisite coordination and specificity. Each fusion event requires three basic components: Rab-GTPases organize the fusion site; SNARE proteins act during fusion; and N-ethylmaleimide-sensitive factor (NSF) plus its cofactor α-SNAP are required for recycling or activation of the fusion machinery. Whereas Rab-GTPases seem to mediate the initial membrane contact, SNAREs appear to lie at the center of the fusion process. It is known that formation of complexes between SNAREs from apposed membranes is a prerequisite for lipid bilayer mixing; however, the biophysics and many details of SNARE function are still vague. Nevertheless, recent observations are shedding light on the role of SNAREs in membrane fusion. Structural studies are revealing the mechanisms by which SNARES form complexes and interact with other proteins. Furthermore, it is now apparent that the SNARE transmembrane segment not only anchors the protein but engages in SNARE-SNARE interactions and plays an active role in fusion. Recent work indicates that the fusion process itself may comprise two stages and proceed via a hemifusion intermediate.

Key words: SNARE, Lipid bilayer, Membrane fusion, Transmembrane segment, Hemifusion

Introduction

Eukaryotic cells contain numerous compartments that are separated from the cytosol by a lipid bilayer. Exchange of lipids, metabolites or proteins across the lipid bilayer can occur through integral membrane proteins – as demonstrated for peroxisomes, mitochondria or chloroplasts. Within the endomembrane system, which includes the endoplasmic reticulum (ER), the Golgi, endosomes and lysosomes, vesicles transport cargo between organelles and thus mediate exchange (Bonifacino and Glick, 2004; Mellman and Warren, 2000). Generally, vesicle formation requires a conserved set of coat proteins that bind to cargo and induce membrane curvature. At the same time, a vesicle must incorporate proteins that target it to the right compartment and enable it to fuse with the target compartment (Fig. 1A). Thus, vesicle formation requires the uptake of targeting and fusion factors, including Rab-GTPases, tethering factors and SNAREs [soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors]. Fusion seems to involve a cascade in which a Rab-GTPase, together with tethering factors, mediates membrane contact, which is followed by SNARE pairing and lipid bilayer mixing. SNAREs seem to operate at all stages. They need to be incorporated into the right vesicle, interact with tethers, and are essential for tight membrane docking and bilayer mixing. Here, we focus primarily on their role in exocytosis and organelle fusion.

Structure of SNAREs

SNAREs are central to the fusion of endomembranes (Burri and Lithgow, 2004; Chen and Scheller, 2001; Jahn and Grubmüller, 2002; Söllner, 2004). Upon membrane contact, they form defined trans-SNARE complexes, also known as 'SNAREpins' (Weber et al., 1998). In general, SNAREs consist of a central 'SNARE domain' that is flanked by a variable N-terminal domain and a C-terminal single α-helical transmembrane anchor (Fig. 1B). SNAREs were initially classified according to their preferential localization as vesicle-localized (v)- or target-membrane-bound (t)-SNAREs (Rothman, 1994). This nomenclature turned out to be somewhat ambiguous, since t-SNAREs are also found on vesicles and v-SNAREs can be found on target membranes. A systematic sequence analysis revealed that most v-SNAREs have an arginine residue in the centre of the SNARE domain (R-SNAREs), whereas a glutamine (or aspartate) residue is found in syntaxins and SNAP-25-like proteins (Q-SNAREs) (Weimbs et al., 1997, Fasshauer et al., 1998). Further refinement of this analysis lead to the classification of three Q-SNARE groups: Q_a, Q_b and Q_c (Bock et al., 2001). The crystal structures of complexes formed by fragments of synaptic and endosomal SNAREs have been solved (Antonin et al., 2002b; Sutton et al., 1998). In each case, the complex consists of four SNARE domains forming a coiled-coil: one contributed by the R-SNARE and one each by Q_a-, Q_b- and Q_c-SNAREs. This composition of SNARE complexes is conserved: synaptic and yeast vacuole SNARE complexes each contain four helices (Sutton et al., 1998; Fukuda et al., 2000; Dietrich et al., 2005a). A closer analysis shows that the coiled-coil structure is composed of 15 hydrophobic layers, and a central hydrophilic zero-layer containing the R- and Q-

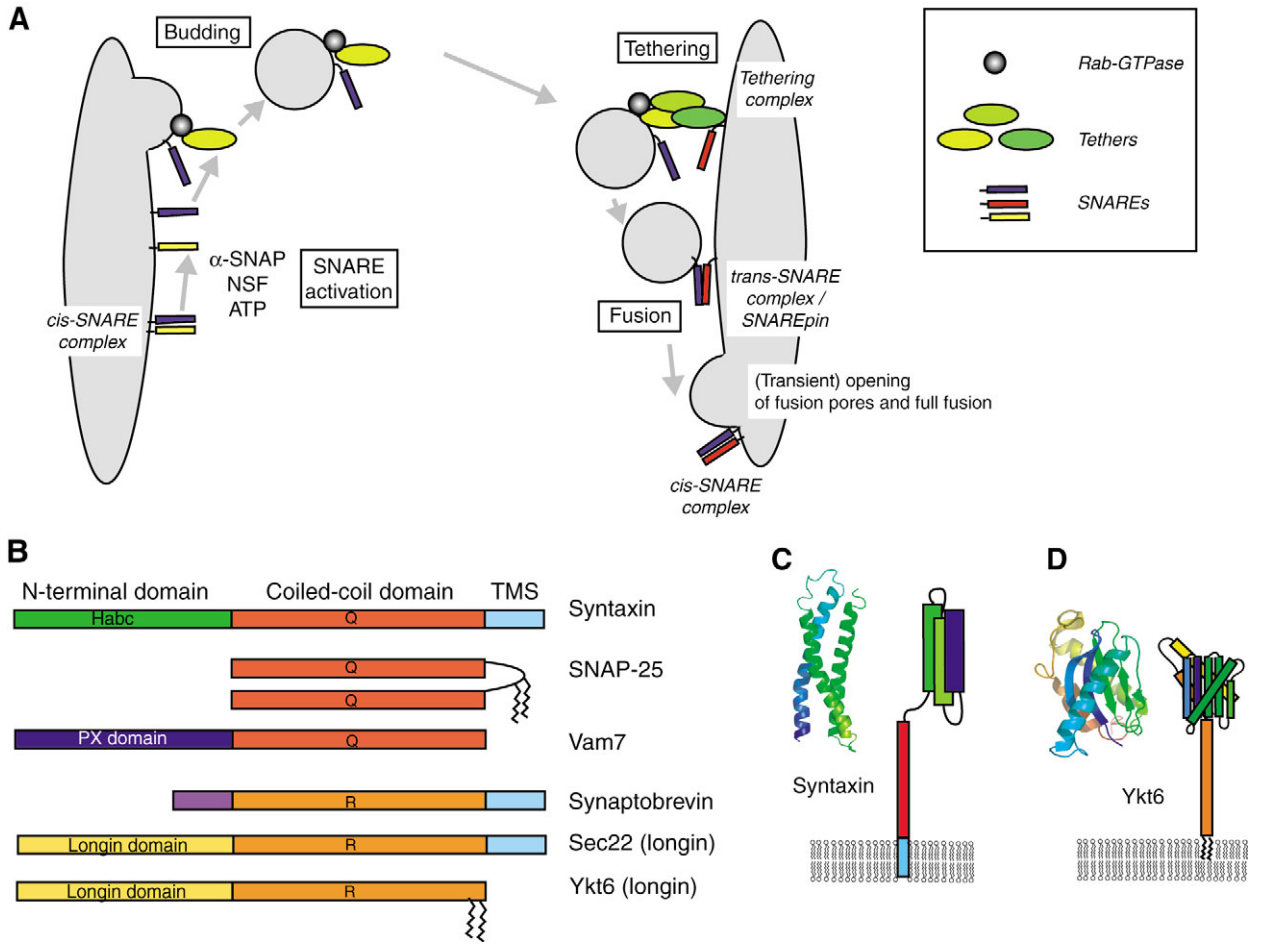

Fig. 1. SNARE function in trafficking and SNARE domain structure. (A) Vesicle trafficking. The stages of vesicle budding and fusion are shown. During SNARE activation, SNAREs become disassembled with the help of NSF, α-SNAP and ATP. Budding includes uptake of the v-SNARE, Rab proteins and tethers, which assemble before or during tethering into a complete complex. Fusion requires SNARE complex assembly and lipid mixing. (B) Domain structure of selected SNAREs. Lipid anchors on Ykt6 and SNAP-25 are indicated. (C) The H_{ABC} N-terminal domain of syntaxin (PDB accession number 1BR0) (Fernandez et al., 1998). (D) The Ykt6 N-terminal longin domain (1H8M) (Tochio et al., 2001).

residues (Sutton et al., 1998). The positions of the R and Q residues can be swapped among the different helices as long as the 3Q:1R ratio is maintained (Katz and Brennwald, 2000; Graf et al., 2005), which indicates that it is a hallmark of specificity in SNARE function.

The N-terminal domains of SNAREs show some variability (Duetruch et al., 2003). Two predominant folds are found. The N-terminus of Q-SNAREs, as far as it has been clarified structurally, consists of a three-helix bundle (Fig. 1C) (Fernandez et al., 1998; Nicholson et al., 1998; Dulubova et al., 2001; Antonin et al., 2002a). This can interact with the coiled-coil domain to control its interaction with other SNAREs and Sec1/Munc18 proteins (Fernandez et al., 1998; Nicholson et al., 1998; Rizo and Südhof, 2002). A subgroup of the R-SNAREs has a more complex longin fold, as discussed below (Fig. 1D) (Fernandez et al., 1998; Nicholson et al., 1998; Rizo and Südhof, 2002).

Among R-SNAREs, the absence of the longin-fold distinguishes brevins (synaptobrevin-like R-SNAREs) from longins (Filippini et al., 2001). Brevins have short, presumably unstructured, N-terminal extensions. The longin fold consists of five central β-sheets sandwiched by two α-helices on one face and one α-helix on the opposite face (Gonzalez et al., 2001; Tochio et al., 2001). Three longin proteins have been characterized in detail: Sec22 is required for trafficking between the ER and the Golgi, and for transport within the Golgi; Ykt6 is required along the Golgi, in endosomes and in the vacuole/lysosome system; and Ti-VAMP is required in neuronal cells (Rossi et al., 2004). The distribution of Sec22 and Ykt6 across the endomembrane system in yeast suggests that longins have a general role along the secretory pathway and in fusion reactions. The longin-fold is not unique to the three SNAREs, and has also been identified in the SEDL (Trs31) subunit of the TRAPP tethering complex (Jang et al., 2002), the σ2 and μ2 subunits of the adaptor protein (AP)-2 coat (Collins et al., 2002), the SRX domain of the α-subunit of the signal recognition particle (Schwartz and Blobel, 2003), and two endosome-associated mitogen-activated protein (MAP) kinase scaffold proteins, p15 and MP-1 (Kurzbauer et al., 2004; Lunin et al., 2004). This suggests that it is a general signature of proteins operating in trafficking reactions (for a more detailed discussion, see Rossi et al., 2004).

SNAREs are anchored to membranes by transmembrane segments (TMSs) that not only anchor them, but also contribute to SNARE-SNARE interactions and appear to play an active role in the fusion process. The synaptic R-SNARE synaptobrevin II and the Q-SNARE syntaxin 1A homo- and heterodimerize in vitro through a conserved motif within their respective α-helical TMSs (Laage and Langosch, 1997; Margittai et al., 1999; Laage et al., 2000; Roy et al., 2004) that forms a tightly packed interface (Fleming and Engelman, 2001). The long axes of these transmembrane helices are proposed to cross each other at a negative angle; by contrast, the soluble helices of the cytoplasmic SNARE domains assume a positive packing angle upon formation of the coiled-coil structure of the assembled SNARE complex. The sequence linking both domains is unstructured in syntaxin (Kim et al., 2002; Knecht and Grubmüller, 2003; Margittai et al., 2003). Homodimerization through TMS-TMS interactions, although not involving the same motif, is conserved in a yeast vacuolar Q-SNARE (R. Roy, J. Rohde, K. Peplowska, C.U. and D.L., unpublished).

A few SNAREs lack a TMS. The Q-SNAREs SNAP-25 and SNAP-23 bind to membranes through palmitate anchors (Hess et al., 1992; Vogel and Roche, 1999). Ykt6 has a palmitate and farnesyl anchor for membrane binding (Fukasawa et al., 2004; Hasegawa et al., 2004; Dietrich et al., 2005b), and the yeast Q-SNARE Vam7p has an N-terminal PX domain that binds to phosphoinositides on vacuoles (Cheever et al., 2001; Boeddinghaus et al., 2002). These alternative anchors might facilitate regulation of membrane association and the fusion reaction. Indeed, cytosolic intermediates of these SNAREs have been described. However, SNARE-mediated fusion can occur only if at least one SNARE on each bilayer is anchored by a TMS (McNew et al., 1999; Grote et al., 2000; Rohde et al., 2003).

Mechanism of membrane fusion by SNAREs

SNARE pairing

The SNARE hypothesis proposed that complex formation by SNARE proteins can explain the specificity of vesicle fusion (Rothman, 1994). SNAREs residing on the target organelle and vesicle would bind to each other, and fusion would be triggered by NSF-driven disassembly of the SNARE complex. Despite being a major breakthrough, many aspects of this hypothesis have been challenged and modified. For example, the specificity of SNARE complex formation is lower than originally thought: the cytoplasmic domains of isoforms that correspond to organelles that do not fuse to each other in vivo efficiently co-assemble in vitro (Fasshauer et al., 1999; Yang et al., 1999; Tsui and Banfield, 2000). However, the specificity of SNARE pairing appears to be higher in the cell (Scales et al., 2000) and upon reconstitution of full-length SNAREs into liposomes (McNew et al., 2000a). Nevertheless, SNAREs can functionally replace each other in vivo (Götte and Gallwitz, 1997; Liu and Barlowe, 2002; Borisovska et al., 2005) and participate in multiple reactions (Fasshauer, 2003). The specificity of organelle-organelle recognition in the cell is at least in part due to Rab proteins and tethering factors that are likely to act upstream of SNARE complex formation to establish contact between cognate membranes (Fig. 1A). Proteins discussed as tethers include Uso1p, the VFT complex, TRAPP, the exocyst, the HOPS complex and EEA1 (Gillingham and Munro, 2003; Whyte and Munro, 2002; Zerial and McBride, 2001). Tethers can bind to SNARE proteins (Shorter et al., 2002; Collins et al., 2005), and complexes including SNAREs and proposed tethers have been identified (Whyte and Munro, 2002). Since such proteins contain large coiled-coil domains, they may direct assembly of SNARE complexes at fusion sites.

Some of the reported discrepancies might be due to inherent differences in the fusion reactions of evolutionarily distant organisms such as yeast, invertebrates and mammals. Moreover, different types of fusion – such as organelle-organelle fusion or neurotransmitter release – might have different requirements. It is quite likely that evolution has fine-tuned individual SNAREs for specific functions. For example, fusion of fragmented organelles upon cell division is symmetrical since identical membranes fuse (Wickner and Haas, 2000; Shorter and Warren, 2002). Symmetrical fusion has a slow time course, involves (at least for some organelles) membranes of low intrinsic curvature and may not need a sophisticated level of regulation. By contrast, fusion of secretory vesicles to the plasma membrane is asymmetric and might occur constitutively. An extreme case is the regulated release of neurotransmitter from presynaptic nerve terminals: this is extraordinarily fast, involves small, highly curved synaptic vesicles and is precisely regulated by Ca^{2+} (Südhof, 2004).

Nature has provided a number of solutions to these different requirements. One obvious difference between fusion at or between organelles and fast secretion is that the former exclusively employs SNAREs with single SNARE motifs, whereas the latter requires SNAP-25 orthologs, which combine two SNARE domains in one protein (Fig. 1B). In organelle-organelle fusion, cis complexes of R- and Q-SNAREs exist prior to fusion (Wickner and Haas, 2000). Their dissociation by the AAA-ATPase NSF and its cofactor α-SNAP is thus required prior to assembly of trans complexes that link the membranes (Mayer et al., 1996). This is exemplified by fusion between endosomes (McBride et al., 1999), ER-derived vesicles (Barlowe, 1997) and yeast vacuoles (Nichols et al., 1997; Ungermann et al., 1998; Dietrich et al., 2005a). In neurotransmitter release, only part of synaptobrevin complexes with Q-SNAREs on the vesicle (Kretzschmar et al., 1996). The rest appears either to associate with the synaptic vesicle protein synaptophysin or to homodimerize (Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995; Pennuto et al., 2002; Roy et al., 2004). At the plasma membrane, syntaxin and SNAP-25 seem to assemble in cis prior to trans complex formation with the R-SNARE (Pabst et al., 2002; An and Almers, 2004; Fasshauer and Margittai, 2004).

The precise mechanism of trans complex formation might also depend on the type of fusion and on its level of regulation. The SNARE domains of exocytotic SNAREs from yeast appear to associate in a one-step reaction over their entire length (Zhang et al., 2004). By contrast, trans assembly of neuronal SNARE motifs probably starts at their N-termini and proceeds towards the TMSs in a ‘zippering-up’ process (Chen et al., 1999; Melia et al., 2002). Since the assembly occurs in a sequential pathway, populations of vesicles that exocytose with different kinetics might reflect different assembly stages (Xu et al., 1999). Surprisingly, trans assembly of synaptic

SNAREs reconstituted in liposomal membranes at low density is prohibited by close association of the R-SNARE domain with the bilayer through membrane-proximal tryptophan residues (Hu et al., 2002; Kweon et al., 2003). Both, zippering-up and the availability of the R-SNARE domain might thus be subject to regulation by as-yet-unknown mechanisms.

SNARE regulation

Several proteins that control SNARE complex assembly have been identified (Fig. 2). Complexins, for example, can bind to assembled SNARE complexes and modulate exocytosis (Fig. 2E) (Chen et al., 2002; McMahon et al., 1995; Pabst et al., 2002). Tomosyn and amisyn interact with syntaxin, SNAP-25 and synaptotagmin and might facilitate assembly of SNARE complexes (Fujita et al., 1998; Pobbati et al., 2004; Scales et al., 2002). Furthermore, Sec1/Munc18 (SM) proteins regulate the availability of syntaxins for SNARE complex formation at the fusion site (Rizo and Südhof, 2002). At the synapse, Munc18 binds to the closed conformation of syntaxin (Fig. 2A) (Yang et al., 2000). Displacement of Munc18 is then required prior to SNARE complex assembly (Fig. 2D) (Sutton et al., 1998; Rizo and Südhof, 2002). How this transition is mediated is still unknown. Removing the N-terminal domain of syntaxin or mutating the linker between the N-terminus and the coiled-coil domain accelerates SNARE complex assembly dramatically (Nicholson et al., 1998; Parlati et al., 1999). In yeast, the SM protein Sec1 can bind to the assembled exocytic SNARE complex and stimulate liposome fusion (Carr et al., 1999; Scott et al., 2004), providing a variation of SM protein function. Several studies have identified a small peptide at the N-terminus of syntaxins as the binding site for SM proteins (Fig. 2B) (Bracher and Weissenhorn, 2002; Dulubova et al., 2002; Dulubova et al., 2003). Surprisingly, mutations that abolish the interaction of the SM protein Sly1 and the Golgi SNARE Sed5 in vitro do not seem to result in an obvious in vivo defect (Peng and Gallwitz, 2004). Thus, establishing the precise function of SM proteins during fusion will require additional studies.

Regulation by Ca^{2+} is crucial for several fusion reactions. Calmodulin and synaptotagmin can act as Ca^{2+} sensors in several systems, although their precise roles are still debated (Südhof, 2002; Burgoyne and Clague, 2003). The role of Ca^{2+} in regulated exocytosis at synapses has been analyzed in detail.

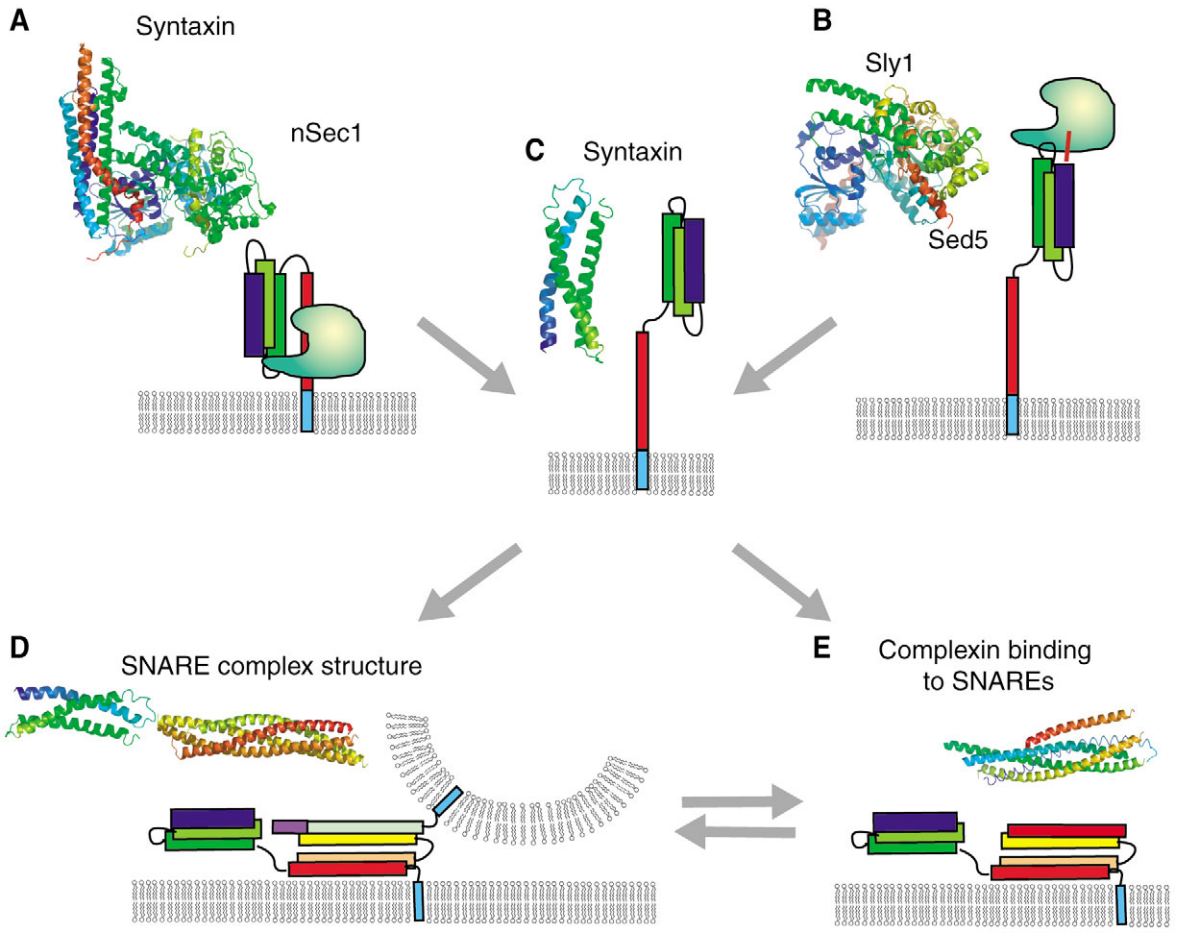

Fig. 2. SNARE complex assembly and its control. (A) Interaction of syntaxin with nSec1, also known as Munc18 (1DN1) (Yang et al., 2000). nSec1 binds to the closed conformation of syntaxin, regulating its availability. (B) Binding of Sly1 to the N-terminal peptide of the SNARE Sed5 may function similarly (1MQS) (Bracher and Weissenhorn, 2002). (C) The open conformation of the syntaxin molecule (as shown in Fig. 1C). (D) The assembled SNARE complex modeled between two membranes (1SFC) (Sutton et al., 1998). (E) Interaction of complexin with the SNARE complex might regulate exocytosis (1KIL) (Chen et al., 2002).

Regulation of neurotransmitter release by presynaptic Ca^{2+} influx is likely to be mediated by synaptotagmin in the vesicular membrane (Fernandez-Chacon et al., 2001; Tucker et al., 2004; Südhof, 2004), which is physically coupled to the SNAREs (Bai et al., 2004). Synaptotagmin is anchored to membranes by a transmembrane domain and has two Ca^{2+}-binding domains (Südhof, 2004). Synaptotagmin binds to SNAREs and lipids in a Ca^{2+}-dependent manner, which might trigger fusion (Tucker et al., 2003; Bai et al., 2004). Interestingly, in vitro reconstitution of synaptic SNAREs without synaptotagmin leads to rapid docking and slow transition to full fusion (Weber et al., 1998), whereas addition of synaptotagmin accelerates fusion, adds Ca^{2+} sensitivity and allows reduction of SNARE density on liposomes (Tucker et al., 2004).

Release of neurotransmitter might not require complete fusion under all circumstances. Rather, the 'kiss-and-run' mode could be favored (Aravanis et al., 2003), depending on the intracellular Ca^{2+} concentration (Ales et al., 1999), the synaptotagmin subtype (Wang et al., 2003) or the release probability of the respective type of nerve terminal (Gandhi and Stevens, 2003). During kiss-and-run events, neurotransmitter molecules are thought to be channeled through fusion pores (Fig. 3) (Bruns and Jahn, 1995; Borisovska et al., 2005) that extend through the vesicular and plasma membranes. These fusion pores might contain the syntaxin TMS since mutations in it alter neurotransmitter flux and pore conductance (Han et al., 2004). These mutations lie on the same face of a helix, outside the homodimerization motif (Laage et al., 2000). Such mutational analysis provides the first indication that the syntaxin TMS functions in fusion pore formation, but additional experimentation is clearly needed to clarify its precise contribution (Szule and Coorssen, 2004). In the kiss-and-run mode, the vesicles might remain tightly docked or even hemifused (see below) to the plasma membrane (Fig. 3). It is not known whether this stage is an intermediate before full fusion or whether it represents a separate pathway (Söllner, 2004). It will be interesting to see whether the synaptobrevin TMS also contributes to the fusion pore and whether TMS-TMS interactions are involved. Elucidating how the transition from hemifusion to full fusion is controlled and how this relates to kiss-and run will certainly be a major focus of future investigation.

Mechanism of lipid mixing

SNAREs probably constitute the minimal machinery necessary for membrane fusion (Fig. 3D and Fig. 4). This is underscored by their ability to mediate liposome-liposome fusion in vitro (Schütte et al., 2004; Tucker et al., 2004; Weber et al., 1998; Zhang et al., 2004). According to the original SNARE hypothesis (Rothman, 1994), trans SNARE pairing would pull the membranes together as a prerequisite of complete bilayer fusion. This simple scenario was based upon the following concept: high-curvature bilayers, such as those of secretory vesicles, are more strained than those of low-curvature bilayers that form upon fusion, and thus fusion is expected to be a downhill process. However, a shell of water molecules bound to the lipid head groups repels membranes at short distances and thus prohibits spontaneous fusion (Leikin et

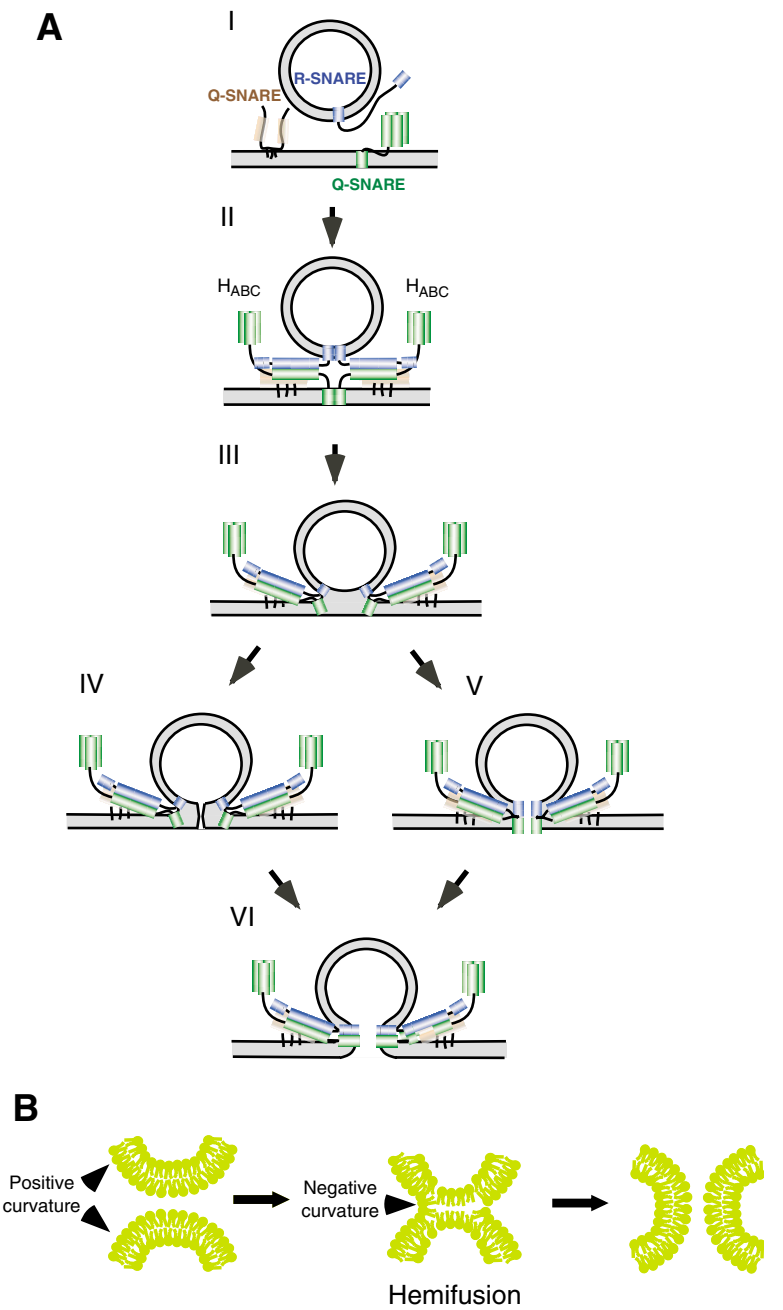

Fig. 3. Membrane fusion. (A) Putative model of SNARE-mediated membrane fusion. At stage I, Q- and R-SNAREs are separate from each other and the Q-SNARE syntaxin exists in its closed conformation. SNARE complex formation releases the Q-SNARE H_{ABC} N-terminal domain and is associated with vesicle docking (stage II). Several SNARE complexes may associate at the fusion site via their TMSs. Docking may result in hemifusion (III), which is followed by formation of lipidic (IV) or proteinaceous (V) fusion pores prior to full fusion (V). Note that proteins and membranes are not drawn to scale. (B) Lipid topology in hemifusion.

al., 1987). Therefore, close membrane apposition mediated by SNARE complex formation was thought to trigger fusion by local dehydration. However, it became evident that complete bilayer merging requires at least two major steps and that mere membrane apposition is unlikely to suffice.

Sequential steps of bilayer mixing

The first step of fusion corresponds to the mixing of the contacting monolayers while the distal monolayers stay intact. This generates the 'hemifusion' intermediate (Chernomordik et al., 1995; Chernomordik and Kozlov, 2003). The energy barrier separating non-fused membranes and the hemifusion intermediate is probably relatively small (Cohen and Melikyan, 2004) and may be overcome by the binding enthalpy released upon SNARE complex formation. Viral fusion proteins such as influenza hemagglutinin (HA) may play a similar role. Viral fusogens share basic architectural features with SNAREs, such as their single-span transmembrane topology and oligomerization through formation of membrane-extrinsic coiled-coil domains (Skehel and Wiley, 1998). A global pH-driven conformational change in the ectodomain pushes an amphipathic fusion peptide towards the target bilayer to establish initial contact between both membranes. This is followed by further conformational changes, resulting in bilayer merging. If one considers the viral fusion peptide to be the functional correlate of one SNARE TMS, the fusion mechanism elicited by viral fusogens and SNAREs is likely to share crucial features (Jahn and Südhof, 1999; Söllner, 2004). Whereas SNAREs might initiate fusion by assembly, viral fusogens achieve the same goal by conformational rearrangement (Jahn et al., 2003; Tamm et al., 2003; Söllner, 2004).

The second main step of fusion is the transition of the hemifusion intermediate to complete bilayer merging, and the corresponding energy barrier might significantly exceed that of the first step (Cohen and Melikyan, 2004). For several viral fusion proteins, it has been shown that fusion is arrested at the hemifusion stage if the membrane anchor is altered. For example, influenza HA loses its ability to mediate complete bilayer fusion when a glycosylphosphatidylinositol (GPI) membrane anchor replaces its TMS; by contrast, hemifusion (Kemble et al., 1994; Nüssler et al., 1997) and small, non-enlarging fusion pores (Markosyan et al., 2000) can still be detected. A hemifusion phenotype is also seen upon shortening of the HA TMS by more than 12 residues (Armstrong et al., 2000). Likewise, substituting the TMS of the vesicular stomatitis virus G-protein for a GPI anchor (Odell et al., 1997) or mutating it (Cleverley and Lenard, 1998) attenuates full fusion but still allows hemifusion. Several studies indicate that a hemifusion intermediate is also an authentic stage of SNARE-driven membrane fusion.

First, replacing the TMSs of synaptic SNAREs by lipid anchors (di-oleoyl phosphatidylethanolamine or C15 and C20 isoprenoids) that cannot span the lipid bilayer blocks lipid mixing of liposomes but not SNARE complex formation. Long isoprenoid chains that can span both bilayers (C45 and C55 isoprenoids) restore lipid mixing (McNew et al., 2000b). McNew et al. have therefore proposed that the SNARE complex exerts force on the anchors but these have to be of sufficient length to initiate fusion (McNew et al., 2000b). Because these long lipid anchors render the bilayers too leaky to ascertain complete fusion (McNew et al., 2000b), these results are also compatible with a hemifused state. Second, replacement of the TMSs of exocytotic and vacuolar SNAREs by C16 geranyl-geranyl moieties abolishes content mixing of yeast vacuolar membranes (Grote et al., 2000; Rohde et al., 2003) without affecting trans SNARE complex formation. The isoprenylated SNAREs might elicit hemifusion, since addition of lysolecithin (an agent that induces positive curvature in membranes) to the cells rescues the functional defect (Grote et al., 2000). Third, shortening the TMS of a yeast SNARE protein to approximately half of its original length or using low protein-to-lipid ratios in the fusion assay arrests liposomes at the hemifusion stage (Xu et al., 2005). Fourth, examining vacuole fusion, Reese et al. showed that lipid mixing can occur in the absence of content mixing in the presence of GTP-γS (Reese et al., 2005). Similarly, it has recently been demonstrated that some SNARE-mediated cell-cell fusion events, as well as in vitro liposome fusion induced by neuronal SNAREs, proceed via a hemifusion intermediate (Giraudo et al., 2005; Lu et al., 2005).

Reconstitution of fusion by TMS mimics

As outlined above, several studies indicate that altering the TMS can block fusion by showing loss of content mixing (Grote et al., 2000; Rohde et al., 2003) paralleled by arrest at the hemifusion stage (Reese et al., 2005; Xu et al., 2005; Giraudo et al., 2005; Lu et al., 2005). It is thus conceivable that one role of the TMS is to promote the hemifusion-to-fusion transition. This is supported by in vitro studies in which synthetic peptides representing synaptic SNARE TMSs can drive complete liposome fusion (Langosch et al., 2001b). Moreover, the fusogenic activity of TMS peptides that have mutated sequences decreases with increasing stability of their α-helical conformations. Thus, structural flexibility of the TMSs might support their fusogenicity, and this is consistent with their unusual amino acid compositions [i.e. over-representation of β-sheet-promoting β-branched residues (valine and isoleucine) (Langosch et al., 2001b)]. Peptides representing the TMS of the vesicular stomatitis virus G-protein yield similar results. There, mutations that diminish fusogenicity of the full-length protein in vivo (Cleverley and Lenard, 1998) also diminish fusogenicity of the TMS peptide in vitro and stabilize its helical structure (Dennison et al., 2002; Langosch et al., 2001a).

In the absence of the ectodomains, fusion by TMS peptides is probably initiated by random collisions of the liposomes and is strongly enhanced by Ca^{2+}-mediated aggregation (Langosch et al., 2001a, Langosch et al., 2001b). The suggestion that structurally flexible TMSs might contribute to lipid mixing is supported by the results of recent studies with de novo designed fusogenic TMS peptides. Depending on the ratio of helix-promoting leucine, sheet-promoting valine and helix-destabilizing proline and glycine residues, these peptides also display fusogenicities that are paralleled by various degrees of conformational flexibility (Hofmann et al., 2004). How flexibility of the TMS could translate into lipid mixing is presently unclear.

Lipid-protein interactions

Although little is known about the role of lipids in natural

fusion reactions, the interdependence of lipids and fusion proteins has been addressed in some model systems. For example, liposome fusion in vitro is promoted by cone-shaped lipids, which are thought to stabilize the hemifusion intermediate, since they favor negative membrane curvature (Chernomordik et al., 1995; Chernomordik and Kozlov, 2003). Furthermore, lipids have recently been shown to have a role in yeast vacuole fusion. Fusion of yeast vacuoles starts at the vertices of apposed bilayers, which are enriched in the Q-SNARE Vam3p and regulatory proteins including Vac8p, the GTPase Ypt7p, the HOPS/Vps-C effector complex and protein phosphatase 1 (Wang et al., 2002). In addition, several regulatory lipids, including ergosterol, diacylglycerol and 3- and 4-phosphoinositides, accumulate at the vertices with SNAREs and other fusion factors in a mutually interdependent manner (Fratti et al., 2004). The phosphoinositides appear to form acceptor sites for the soluble SNARE Vam7p and actin. Ergosterol regulates Sec17p release and diacylglycerol might accelerate fusion owing to its cone shape (Jun et al., 2004). Palmitoylated proteins such as Vac8p might facilitate fusion by intercalating into the lipids at the fusion site (Veit et al., 2001; Wang et al., 2001; Wang et al., 2002). Likewise, transmembrane proteins such as the V0 subunit of the ATPase (Peters et al., 2001) and the associated Vtc proteins (Müller et al., 2002) might support the organization of lipids and SNAREs at the fusion site. Indeed, lipid and content mixing do not occur in vacuoles lacking the V0 subunit Vph1p or Vac8p (Reese et al., 2005).

Clustering of SNAREs has also been observed in other model systems (Lang et al., 2001; Salaun et al., 2004), which suggests that lipid-SNARE communication is a ubiquitous phenomenon in vivo. Interestingly, SNAREs prefer the liquid-disordered phase and not the 'raft' phase when reconstituted into liposomes (Bacia et al., 2004). This indicates that the interaction of lipids and SNARE TMSs can support lateral sorting in membranes. It will be important to analyze the functional consequences of this observation for membrane fusion.

SNARE recycling

In contrast to viral fusion proteins, SNAREs can be reused after fusion. This requires the action of NSF and α/γ-SNAPs (Söllner et al., 1993), which dissociate SNARE complexes either prior to fusion (as in the case of in vitro organelle fusion and ER-Golgi transport reactions) (Barlowe, 1997; Mayer et al., 1996) or after fusion (as in regulated secretion) (Jahn et al., 2003). α-SNAP might serve as a folding sensor that binds to assembled SNARE complexes, mediates recruitment of NSF and supports NSF-directed disassembly. Recent studies have highlighted potential mechanisms for regulation of NSF, including its nitrosylation and phosphorylation (Huynh et al., 2004; Matsushita et al., 2003). How α-SNAP is able to bind to different SNARE subtypes is still mysterious. Interestingly, blocking the disassembly of SNAREs also interferes with vesicle budding, which suggests that the fusion and fission reactions are coupled (Littleton et al., 2001; Deak et al., 2004). This concept of SNARE function and SNARE sorting has recently been addressed in a mathematical model of the organization of the endomembrane system (Heinrich and Rapoport, 2005). The authors show that organelle identity can be explained by the specificity of the interaction of SNAREs on vesicle and target membranes and the affinity of a SNARE for a specific vesicle coat. Thus, only certain SNAREs should be packaged into vesicles and therefore be available for fusion. In turn, the nonhomogeneous distribution of SNAREs results in coat-specific vesicle flux and thus in nonidentical compartments.

Outlook

Research on SNAREs will need to address several crucial questions that are only touched on here. Dissociation of SNARE complexes by NSF and α-SNAP is well established, but has not been resolved on a mechanistic level. The interplay of SNAREs and tethers is likely to be regulated by a variety of factors, including kinases, phosphatases and other modifying enzymes, but the details are unknown. We also know little about what determines the specificity of SNARE-mediated fusion, although it is clear that distinct combinations of SNAREs are required in vitro to fuse liposomes. It is also unclear whether SNARE assembly can be stalled at intermediate stages and, if so, how stalling is mediated or reversed. The interplay of synaptotagmin and SM proteins with SNAREs is established, but the temporal sequence of interactions at the fusion site is still mysterious. Similarly, the roles of SNARE TMSs in assembly and lipid mixing are apparent but their place within the fusion mechanism has yet to be defined. Proteins and lipids might form fusion-competent zones on membranes and support recruitment of SNAREs. Only a few studies have addressed the nature of such zones, and this area also requires further research.

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