invited review

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What is the role of SNARE proteins in membrane fusion?

Joseph G. Duman and John G. Forte
Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

Duman, Joseph G., and John G. Forte. What is the role of SNARE proteins in membrane fusion? *Am J Physiol Cell Physiol* 285: C237–C249, 2003; 10.1152/ajpcell.00091.2003.—Soluble *N*-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins have been at the forefront of research on biological membrane fusion for some time. The subcellular localization of SNAREs and their ability to form the so-called SNARE complex may be integral to determining the specificity of intracellular fusion (the SNARE hypothesis) and/or serving as the minimal fusion machinery. Both the SNARE hypothesis and the idea of the minimal fusion machinery have been challenged by a number of experimental observations in various model systems, suggesting that SNAREs may have other functions. Considering recent advances in the SNARE literature, it appears that SNAREs may actually function as part of a complex fusion "machine." Their role in the machinery could be any one or a combination of roles, including establishing tight membrane contact, formation of a scaffolding on which to build the machine, binding of lipid surfaces, and many others. It is also possible that complexations other than the classic SNARE complex participate in membrane fusion.

soluble *N*-ethylmaleimide-sensitive factor activating protein receptor

AMONG THE MOST IMPORTANT and widely studied proteins in membrane trafficking, docking, and fusion are the soluble *N*-ethylmaleimide-sensitive factor activating protein receptors, or SNAREs. SNARE proteins are membrane-associated proteins that contain characteristic SNARE domains: heptad repeats ~60 amino acids in length that are predicted to form coiled-coils. Since their discovery, it has become evident that they facilitate membrane fusion in numerous eukaryotic systems, and an extensive literature concerning their role in this process has developed (28, 40, 54). Two especially salient features of SNARE proteins recommend them as fundamental fusion factors. The first is their subcellular localization: distinct SNAREs are localized to distinct membrane compartments and domains in all eukaryotic cells. This feature led to the proposal of the SNARE hypothesis, which proposed that SNAREs dictate the specificity of membrane fusion events (116). The review by Jahn and Sudhof (54) contains an extensive list of SNARE proteins and their intracellular localizations. The second feature is the ability of a given set of SNAREs in two adjacent membranes to form extremely stable, even SDS-resistant, complexes through interactions among their SNARE domains that bring the membranes into close apposition. As we see, this property has been invoked to link SNAREs to the energetics of fusion. The pressing and unanswered question is: how? How do the distinctive and compelling properties of SNAREs relate to the process of membrane fusion? This question relates directly to another pressing question, namely, what is the mechanism of intracellular membrane fusion itself? By understanding the role of SNAREs, we can take an enormous step forward in our attack of this question. In this review, we briefly examine the basis for the most prevalent modes of thought regarding SNARE function in membrane fusion. We highlight some experimental evidence that challenges these modes and move on to a discussion of some alternate roles for SNAREs and emerging ideas regarding them.

LESSONS FROM NEURONAL SNARES

The two features enumerated in the Introduction, subcellular localization and complex formation, are perhaps most clearly illustrated in the widely studied neuronal SNARE proteins. The presynaptic membrane of a neural axon is the site of rapid exocytic events upon cell stimulation by an action potential. In response to Ca^{2+} influx, synaptic vesicles containing neurotransmitter fuse with the presynaptic membrane and release their contents into the synaptic cleft, where they diffuse to the postsynaptic cell (71). Three SNARE proteins are involved in this process: vesicle-associated membrane protein (VAMP)-2, a single-pass transmembrane protein located primarily in the vesicular membrane; synaptosome-associated protein (SNAP)-25, which contains two SNARE domains flanking a region of palmitolyated cysteines by which it associates with the plasma membrane; and syntaxin 1A, a single-pass transmembrane protein that resides primarily in the plasma membrane (71). All SNARE domains have a cytoplasmic orientation. At some time

Address for reprint requests and other correspondence: J. G. Forte, Dept. of Molecular and Cell Biology, Univ. of California, Berkeley, CA 94720 (E-mail: jforte@uclink.berkeley.edu).

during the exocytic process, the SNAREs assemble into an SDS-resistant trans (i.e., membrane spanning) complex with a 1:1:1 stoichiometry. This complex is apparently required for fusion, because preventing complex formation by cleaving SNAREs with clostridial neurotoxins (47) completely inhibits neurotransmitter exocytosis (53), though it does not inhibit targeting of synaptic vesicles to the presynaptic membrane (52). After fusion, the SNARE complex recruits α-SNAP and *N*-ethylmaleimide-sensitive factor (NSF) (115, 116), which disassemble the now cis (i.e., resident in one membrane) complex and allow individual SNAREs to be recycled.

The structure of the core part of neuronal SNARE complex, consisting of the SNARE domains assembled into an SDS-resistant complex (in the absence of transmembrane or other domains), has been solved by both spin labeling electron paramagnetic resonance and X-ray crystallography. In both cases, this revealed a long (~12 nm), twisted, parallel four-helix bundle composed of SNARE domains oriented with their COOH termini toward the membrane (30, 99, 119). SNAP-25 contributes two helices to the bundle, and VAMP-2 and syntaxin 1A contribute one apiece. The surface of the bundle has four prominent grooves and is highly polar, with some very localized patches of charge (30, 119); these features provide putative binding sites for associated proteins. The coiled bundle is 16 layers deep, and a layer near the middle, called the ionic central layer, contains three glutamines and one arginine, the arginine being donated by VAMP-2 and every other SNARE motif contributing one glutamine. The ionic central layer is also commonly referred to as the "zero layer." The structure of the core complex and the geometry of the ionic central layer are shown in Fig. 1.

The neuronal SNAREs represent only one of many SNARE complexes that exist. How much do other SNARE complexes resemble this archetype? Sequence alignment of many SNAREs reveals that the ionic nature of the central layer may be a critical feature of complex development. Glutamine residues are highly conserved in ionic central layer positions in relatives of SNAP-25 and syntaxin 1A, and arginine residues are likewise conserved in these positions in VAMP relatives (33). The exocytic SNARE complex in yeast has been examined by deep-etch electron microscopy. The results of this investigation indicated that the yeast complex members Snc2p, Sec9p, and Ssop, corresponding to VAMP-2, SNAP-25, and syntaxin 1A, respectively, form a very similar parallel bundle complex at the yeast plasma membrane (59), albeit at lower resolution than X-ray crystallography. Another mammalian SNARE complex, the late endosomal complex, also provides some insight into the generality question. The SNARE complex in late endosomes has a low overall sequence homology to the neuronal SNARE complex and consists of four proteins: syntaxin 7, syntaxin 8, Vti1b, and VAMP-8. Biochemical studies of this complex, however, reveal a marked similarity to the neuronal complex: both complexes contain one copy of each participating protein, both complexes are resistant to dissolution by SDS, the constituents of both complexes

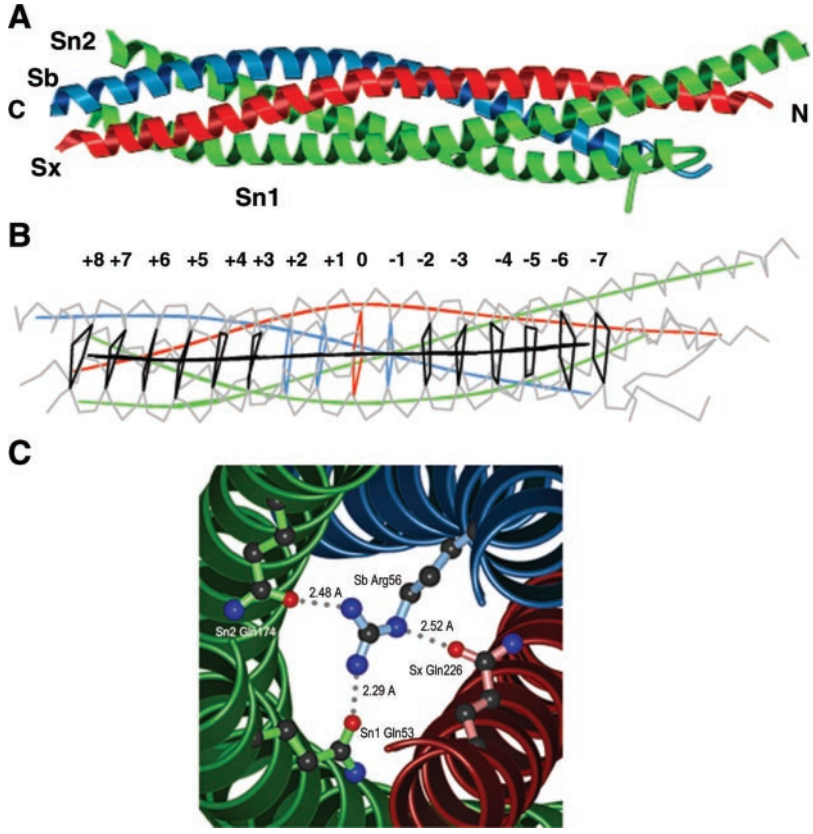

Fig. 1. Topology and organization of the SNARE fusion complex reconstructed from crystallographic data. *A*: backbone ribbon drawing of the synaptic fusion complex: VAMP-2, also known as synaptobrevin-II (Sb; blue); syntaxin-1A (Sx; red); SNAP-25B (Sn1 and Sn2; green). The synaptic fusion complex is arranged as a cylinder 120 Å in length with a circular cross section (see Fig. 2). All 4 components of the heterotrimer are arranged in parallel, with the NH_2 termini (N) at one end of the bundle and the COOH termini (C) at the membrane-anchor end. *B*: organization of the SNARE fusion complex with regions of interaction shown as layers drawn perpendicular to the superhelical bundle axis. Layers are numbered and indicated by virtual bonds between corresponding Cα positions: Cα traces (gray); local helical axes (blue, red, and green for synaptobrevin-II, syntaxin-1A, and SNAP-25B, respectively); the superhelical axis (black); and layers (0, red; −1, +1, and +2, blue; all others black). *C*: expanded view of ionic central layer (0 layer) of the SNARE fusion complex. Side chains involved in the layer are shown as balls and sticks; backbone is shown as a ribbon. The total buried surface area for the side chain atoms in this layer is 742 Å. Reprinted from Sutton et al. (119) with permission.

are largely disordered before complex formation but assume an α-helical structure upon complex formation, and both complexes can be disassembled by the ATPase NSF in the presence of α-SNAP (5). Moreover, the authors of this study found that proteins could be switched between the two complexes without significantly changing properties of the complex, as long as syntaxin 7 was substituted for syntaxin 1A, Vti1b for the NH_2-terminal SNARE domain of SNAP-25, syntaxin 8 for the COOH-terminal SNARE domain of SNAP-25, and VAMP-8 for VAMP-2 (5). Finally, when the crystal structure of the late endosomal SNARE core complex was solved, it was found to be markedly similar to that of the neuronal SNARE complex, with the ionic central layer being conserved and most variations occurring on the surface (4). These observations indicate that many of the features of the neuronal SNARE complex could well be conserved throughout the entire SNARE family.

THE SNARE HYPOTHESIS AND THE "MINIMAL FUSION MACHINERY"

What, then, do SNARE complexes accomplish? The so-called SNARE hypothesis has served as the de facto answer to this question for some time. It proposes that SNAREs comprise the specificity determinants for membrane fusion: if SNAREs were localized to distinct membrane compartments and only competent to form complexes with appropriate cognate SNAREs, this would impart a lock-and-key specificity to SNARE-mediated fusion (116). SNAREs resident on vesicles are therefore referred to as v-SNARES, and SNAREs resident on target membranes as t-SNAREs. [These designations generally correspond to another SNARE nomenclature, that of q- and r-SNAREs. The q/r designations are based on the amino acid donated by the SNARE to the ionic central layer. Generally, t-SNAREs—SNAP-25, syntaxin 1A, and their relatives—correspond to q-SNAREs, and v-SNAREs—VAMP and its relatives—correspond to r-SNAREs (33). We use the v/t-SNARE designations in this article.]

A dramatic set of experiments conducted by Rothman and colleagues has sought to confirm the SNARE hypothesis and has resulted in a bold addendum to the hypothesis. Initially, the group reconstituted the neuronal SNAREs into liposomes. When liposomes containing VAMP-2 were mixed with liposomes containing syntaxin 1A and SNAP-25, they observed membrane fusion (131). This led to the conclusion that SNAREs are sufficient for membrane fusion, the "minimal machinery" for this process. This property of the system allowed the group to test the specificity posited in the SNARE hypothesis. Using proteins from *Saccharomyces cerevisiae* because of its completely sequenced genome, they created a combinatorial panel of liposomes containing v- and t-SNAREs known to be involved in three distinct membrane trafficking steps in the cell. With one exception, only the v- and t-SNARE combinations that function in vivo mediated membrane fusion in vitro (76). Furthermore, when liposomes containing the three sets of t-SNAREs were used against an expanded panel of all 11 v-SNAREs found in the yeast genome, only in vivo-identified complexes displayed fusion, again with one exception (76). They went on to show that complexes only form and lead to fusion when the v-SNARE resides on one membrane and the t-SNAREs reside on the other; the system does not tolerate topological switching within a functional complex (91). The group has since used the system to show that the SNARE complex forms in an orderly manner, "zippering" from NH_2 terminus to COOH terminus, and that this zippering must be completed for membrane fusion to occur (78). Thus, in addition to the conclusion confirming the SNARE hypothesis, the group has asserted that SNAREs are also the minimal machinery for biological membrane fusion. These roles are illustrated in Fig. 2.

Numerous criticisms have confronted these experiments. First, many have pointed out that the speed with which the Rothman fusion reactions proceed is far too slow to be physiological. The group responded by repeating the experiments with a truncated version of syntaxin 1A that contains the SNARE domain but lacks a regulatory domain at its NH_2 terminus (92). This approach did speed the in vitro fusion reaction from one round of fusion per hour to almost three rounds of fusion per hour (92). Though this is clearly an improvement, it is still nowhere near the speed with which the neuronal fusion reaction occurs in vivo. Second, the in vitro system does not use biological membranes. It is well established that synthetic membranes can be induced to fuse with each other under a number of conditions (12), some of which require no protein (45, 101, 128). The differences between biological and synthetic membranes manifest in both components of the membrane. First, consider the lipid composition of the membrane. Does the composition of the synthetic membrane reflect a genuine membrane? Do the supermolecular structures of a natural membrane form in the synthetic membrane (130)? What effects do they have on membrane merging? Second, the contribution of protein to the biophysical properties of the membrane should not be ignored. Many biological membranes are 50% or more protein by mass, obviously exerting a large effect on the properties of the membranes. Related to this is the concern that the membranes employed in the system simply need to be held in close apposition to undergo fusion. The Rothman group addressed this criticism by replacing the transmembrane domains of SNAREs with phospholipid anchors. These modified SNAREs formed complexes but were unable to mediate membrane fusion (77). Finally, the Rothman system was attacked by many who doubted whether the lipid-mixing assay of fusion employed by the group actually reflected genuine, complete membrane fusion. The group repeated the original experiments using a content-mixing assay and showed that the events that they reported reflected complete fusion of liposomes and mixing of aqueous contents (84).

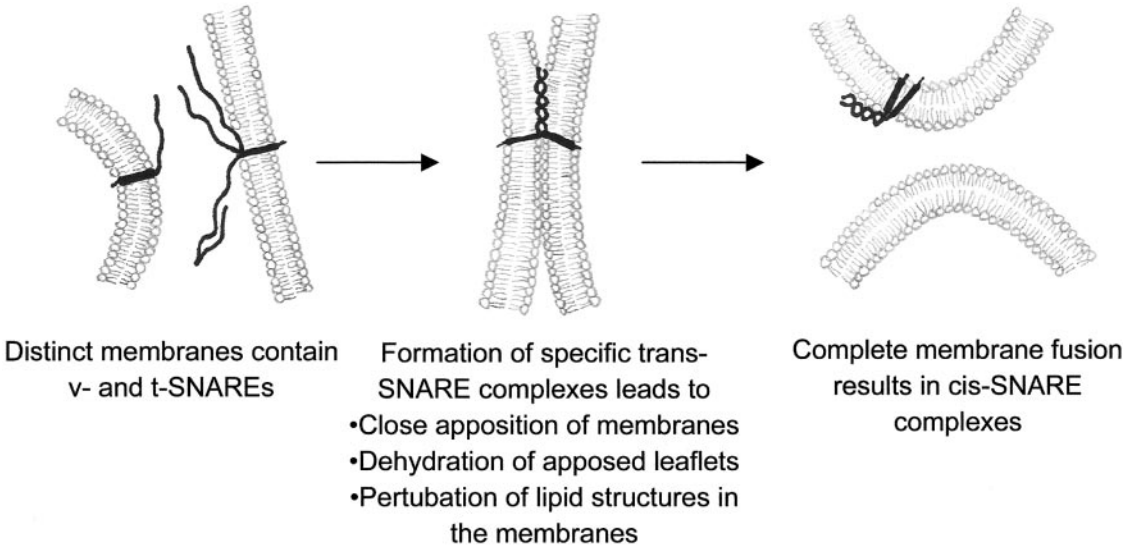

Fig. 2. SNARE hypothesis and minimal fusion machinery. Membranes that are to undergo fusion contain v-SNAREs or t-SNAREs. As a step in fusion, v-SNAREs in one membrane form a highly stable complex with t-SNAREs in the other membrane. This membrane-connecting complex is called a trans-SNARE complex and, according to the SNARE hypothesis, only forms among specific sets of SNAREs. Once the membranes are so bound, the minimal fusion machinery hypothesis states that the SNARE complex causes membrane fusion. In addition to its role in pinning membranes together, the SNARE complex may cause dehydration of the membranes, thus removing an important energetic barrier to fusion. SNARE complex formation may also exert a force on membrane lipids, causing them to transition into a fusion intermediate (for simplicity, not shown here). After complete fusion of the membranes, SNAREs reside in the same membrane as a cis-SNARE complex. This complex must be disassembled and recycled for its components to catalyze further rounds of fusion.

DO SNARES MEDIATE SPECIFICITY IN VIVO?

With these striking in vitro results, the SNARE hypothesis has acquired a conceptual partner, that of the minimal fusion machinery. Are SNAREs the determinants of specificity in vivo? Are they also the minimal machinery for membrane fusion? Are they both? First, let us further examine the issue of specificity. Some observations have suggested that SNAREs really do mediate the specificity of membrane fusion in living cells. When SNARE proteins are overexpressed in COS cells and then immunoprecipitated, VAMP-2 is found to form complexes with syntaxins 1 and 4, but not with syntaxins 2 or 3 (14). Furthermore, overexpression of wild-type syntaxin 3 in Madin-Darby canine kidney (MDCK) cells selectively inhibits trans-Golgi-to-apical membrane traffic (72). Although both observations suggest that SNAREs can provide at least some degree of specificity in living cells, an accruing body of evidence suggests that this degree is actually quite low in vivo.

SNAREs have been shown to be fairly promiscuous in their interactions. In fact, even SDS-resistant complexes readily form between noncognate SNAREs in vitro (31, 137). However, the Rothman group proposed that the apparent nonspecificity was due to the soluble SNARE fragments that were used in these studies; membrane association, they argued, would impose additional constraints on the proteins and lead to interactive specificity (76). Nevertheless, examples from cellular systems argue that SNAREs do not account for the specificity of membrane fusion. Certain SNAREs mediate more than one transport step and, in so doing, incorporate into more than one SNARE complex. The v-SNARE Vti1p forms a complex with the t-SNARE Vam3p in mediating two distinct biosynthetic pathways (36). It also pairs with two other syntaxin-like t-SNAREs: with Sed5p to mediate retrograde traffic to the cis-Golgi, and with Pep12p to mediate traffic from the Golgi to the prevacuolar compartment (73, 125). Vam3 and Pep12 deletions cause distinct trafficking defects that confirm their assignment to the specific steps described above. However, a Vam3 deletion can be rescued by Pep12 overexpression and vice versa (23), indicating that, in a pinch, either of these t-SNAREs can substitute for the other in vivo and the secretory pathway still functions normally. Moreover, besides its complex with Vti1p, Vam3p pairs with the v-SNARE Nyv1p to mediate homotypic fusion of the vacuole (83). A similar situation exists for Bet1p, a SNARE that can function in both ER-to-Golgi and intra-Golgi retrograde transport (123). In animals, the t-SNARE syntaxin 7 can interact with the v-SNAREs VAMP-7 and VAMP-8 (126). Furthermore, in *Drosophila melanogaster*, the neuronal VAMP-2 homolog can be functionally replaced in vivo by a v-SNARE that normally operates in an earlier stage of the secretory pathway and vice versa (10). Moreover, in *Drosophila*, syntaxin 1A is expressed throughout the axonal plasma membrane, yet synaptic vesicles only fuse with the plasma membrane at the synapse (110). These observations imply that SNAREs simply do not provide the level of specificity that is needed for cellular integrity. Additional mechanisms are clearly required.

Although SNARE complex formation does not critically determine the specificity of intracellular membrane fusion events, some degree of subunit composition specificity is encoded therein. The SNARE complex does not form if its components are mutated so that more than one arginine is present in the ionic central layer, though it does not matter which SNARE donates it. If the ionic central layer glutamine in the syntaxin 1A homolog or either glutamine in the SNAP-25 homolog is mutated to arginine, the SNARE complex will still form normally as long as the VAMP-2 homolog arginine is mutated to glutamine (58, 89). The ionic central layer can therefore help prevent formation of complexes without the appropriate subunit composition by preventing multiple v-SNAREs from incorporating into complex. This subunit composition specificity may not be absolutely conserved, however, because functional SNARE complexes can sometimes still form if the ionic central layer arginine is not present at all (58, 89). One must therefore conclude that the ability of the ionic central layer to mediate even subunit composition specificity is not complete. In fact, the primary purpose of the central layer may be to assist in complex disassembly by NSF and α-SNAP (108).

ARE SNARES THE MINIMAL FUSION MACHINERY?

Turning to the partner of the SNARE hypothesis, we must consider whether SNAREs comprise the minimal fusion machinery, as the Rothman group's experiments assert. Theirs is not the only argument that this is indeed the case. It was noted some time ago that SNAREs might be able to contribute the energy necessary to overcome the biophysical barriers to fusion by forming the ultra-stable SNARE complex (44, 113). Determining whether the free energy gained by complex formation is sufficient has been more challenging, but it is widely thought that several SNARE complexes working in concert would clearly be able to contribute the requisite energy through formation of complex multimers, perhaps forming a "fusion pore" (55). Indeed, SNAREs are not randomly distributed throughout membranes but are concentrated at certain sites in a cholesterol-dependent manner (64) and are known to be active only in certain restricted sites within a fusing membrane (64, 129). Such regional concentrations support the idea that SNAREs work in concert and should generally allay any concerns about the free energy available in SNARE complex formation to pay the energetic "fusion debt." Another series of observations supporting the idea that SNARE complexes directly power membrane fusion is the qualitative correlation between the stability of the SNARE complex and its ability to mediate fusion. Mutations in SNAREs that allow complexes to form, but with reduced stability, generally lead to reduced exocytic activity in live *Drosophila* (34).

The idea that SNAREs might be the minimal fusion machinery was proposed with knowledge of another well-studied fusion system, that of viral fusion proteins. A number of enveloped viruses, including influenza and human immunodeficiency virus (HIV), use these proteins to invade host cells through a membrane fusion process. The proteins responsible for this event, which include influenza hemagglutinin and HIV gp41, are single integral membrane proteins that undergo a pH-dependent conformational change, often a proteolytic processing step, and invade the host membrane by inserting into it a short sequence known as the fusion peptide (50). There are remarkable structural similarities between the fusogenic state of viral fusion proteins and the core SNARE complex, suggesting a related function (13, 50, 51). It has been proposed that one of the functions of viral fusion proteins is to bring the fusing membranes close together, a role that these proteins could very well share with SNAREs. Many other aspects of the structure and biology of viral fusion proteins, including the nature of the fusion peptide (120, 121), are active and productive areas of research and beyond the scope of this review. However, they do provide a strong rationale for the minimal fusion machinery idea, as was appreciated in the proposal of this idea (131).

Complex formation is closely followed temporally by exocytic fusion in permeabilized PC-12 cells (18), and the authors of this study assert that a tight temporal correlation makes a fusion mechanism in which SNAREs directly mediate membrane fusion the most likely. This is somewhat unconvincing because of the time resolution in the study, which is much slower than the exocytic events that were being measured. More significantly, the invocation of the temporal and/or spatial correlation of SNARE complex formation with fusion as evidence that SNAREs are the minimal fusion machinery in vivo underscores the difficulties in proving this hypothesis. Studies correlating complex formation with fusion in vivo show that the SNARE complex is important for fusion, not that it is the minimal fusion machinery because many other components are also present. This was a part of the rationale for undertaking the in vitro experiments of the Rothman group (131), and these experiments remain the most compelling evidence that SNAREs fulfill the role of minimum fusion machines. One must remain cognizant of the aforementioned difficulties in extending these results to in vivo systems, however.

Empirical difficulties with the proposal that SNAREs are minimal fusion machines arise in the permeabilized PC-12 cell study cited above, in which SNARE complexes with markedly lower stability than wild type supported exocytosis at or above wild-type levels (18). Other studies also indicate that SNAREs are not the cell's minimal fusion machinery. In both isolated sea urchin cortical granules (20) and yeast vacuoles (124), SNARE complexes form as a part of the fusion process but can then be disassembled before completion of the process without affecting the kinetics or frequency of fusion. In fact, at least two requisite fusion steps occur downstream of SNARE complex formation in the yeast vacuole: one is dependent on protein phosphatase 1 activity (94), and one is sensitive to Ca^{2+}/calmodulin

(96). The fusion events facilitated by VAMP-2 continue to occur in VAMP-2 knockout mice, though with lower frequency (109). Moreover, the thermodynamic and kinetic properties of SNAREs may not be suitable for driving fusion as it is observed in vivo. In vitro, SNARE complex formation is a two-step process with a half-time ($t_{1/2}$) of 1 min for the first step and a $t_{1/2}$ of 1 h for the second step (32). Without the help of other proteins, complex formation simply cannot support the millisecond fusion observed at the synapse or even the slower events observed in secretory cells. Furthermore, the unusual stability of the SNARE complex is difficult to reconcile with the fast reversibility of kiss-and-run-type fusion events, in which the vesicular contents are released into the outer environment and the vesicle is reformed on an extremely fast (subsecond) time scale (1, 25, 117).

ALTERNATE ROLES FOR SNARES IN FUSION

If SNAREs are not the minimal fusion machinery, bringing membranes together and directly causing their fusion, then what do they do? Alternate roles have been proposed for them outside of actually causing the terminal fusion step. First, they may, in fact, tie membranes together, but only to allow or facilitate subsequent events that actually fuse the membranes. This type of role has been suggested in the yeast vacuole system, in which SNARE complex formation allows close apposition of membranes, upstream of trans-association of v-type ATPases, which also acidify the vacuolar interior (95). The association of v-type ATPases may then drive the actual membrane fusion event by creating a proteinacious fusion pore that expands in a Ca^{2+}/calmodulin-dependent manner and may ultimately lead to membrane fusion (95). Second, they may act primarily as licensing or signaling factors, connecting the fusion machinery to other events in the cell (35). For example, SNAREs could tie fusion to upstream signaling events. In the sea urchin cortical granule system, the intact SNARE complex is not required for fusion, yet SNAREs are present and the complex has been shown to form before membrane fusion (20). In this case, it has been proposed that SNAREs modulate the sensitivity of the fusion reaction to cytosolic Ca^{2+}; this role has also been proposed in the neural synapse (118). Playing a role in the Ca^{2+} sensitivity of fusion could be an important role for SNAREs in many systems but cannot be universal, because the Ca^{2+} requirement for yeast vacuole fusion is downstream of SNARE complex formation (96). Also, some exocytic systems have no demonstrable Ca^{2+} requirement. For example, secretion by the gastric parietal cell requires SNAREs for exocytic activity (2, 56, 57, 96), yet the system functions well in the absence of any cytosolic Ca^{2+} increase (82). Finally, a more general way for the SNARE complex to direct fusion may be through providing a platform for the assembly of the remaining fusion machinery. In this way, SNAREs could connect upstream signaling events with requisite downstream steps. The SNARE complex can then be seen as a signaling molecule itself, bringing together other fusion reaction participants in a constructive manner. We refer to this idea as the "SNARE signaling hypothesis" as we discuss it further. This model is not exclusive of the close apposition model, and, indeed, SNAREs may fulfill more than one function in a given fusion event.

The idea that SNAREs may serve a signaling role is supported by the fact that the surface of the SNARE complex contains many distinctive subsurfaces (e.g., grooves, charged patches) that are putative binding sites for other proteins (4, 119). Given this hypothesis, however, it would not then be necessary for the complex to be embedded in both fusing membranes to function. As long as the complex were targeted to the correct site and formed at the appropriate time, it would be able to support membrane fusion. There are hints of this in the literature. First, though fusion steps are inhibited in yeast whose t-SNARE Pep12 is deleted, the phenotype can be rescued by expressing a truncated version of Pep12 whose transmembrane domain has been deleted (41). In permeabilized PC-12 cells, a mutation in SNAP-25 that eliminates a feature at the surface of the SNARE complex (i.e., the signaling part) is deficient in its ability to support exocytosis, despite the wild type stability of the complex (18). Certainly, the signaling hypothesis presents an exciting possibility.

If the SNARE complex is to organize the fusion machinery, then there have to be other components of the machinery. Proteins must be identified that interact with SNAREs and/or the SNARE complex. At the current time, a number of proteins have been shown to interact with these proteins and play a role in membrane fusion. One group of such proteins is the SM proteins. SM proteins are a family of soluble proteins with the ability to bind to t-SNAREs (38). They are known to regulate membrane fusion in a variety of systems, including the neural synapse and the yeast secretory pathway (7, 88). SM proteins were originally thought to bind to syntaxin and make it unavailable for complex formation (26), though it has become clear that, at least in some cases, they can bind the entire SNARE complex (15). Moreover, this binding can have effects quite distinct from negative regulation of complexation. In yeast, the SM protein Sly1p binds the t-SNARE Sed5p and readily allows SNARE complexes to form, while apparently controlling the specificity of SNARE associations (93). Sly1p can also bind preformed SNARE complexes in vitro (93). This is not always the case for SM proteins, because binding nSec1, a neuronal SM protein, precludes incorporation of syntaxin 1A into SNARE complexes (138). Furthermore, various SM proteins can bind to different domains of the SNAREs with which they associate (26, 60, 61). These data indicate that the mechanism of SM and SNAREs in vivo may not be entirely universal. Gallwitz and Jahn (38) have hypothesized that SM proteins also interact with non-SNARE proteins in the process of membrane fusion and that these interactions are just as important as SM protein-SNARE

Table 1. *SNARE-interacting proteins*

Protein or Component	Source	SNAREs Bound	Comments	Refs.
Amisyn	M	Syntaxins 1A, 4	Homology to VAMP; involved in PC-12 cell exocytosis	107
Calmodulin	M	VAMP-2	Ubiquitous Ca^{2+} sensor	100
CaM kinase II	M	Syntaxin 1A	Protein kinase involved in neural processes	86
CFTR	M	Syntaxin 1A	Cl^- channel; regulated salt and water balance in epithelia	22
Complexins	M	SNARE complexes	May regulate closure of the fusion pore	48,90
ENaC	M	Syntaxin 1A	Epithelial Na^+ channel important in salt and water balance	19
α-Fodrin	M	Syntaxins 1,3,4	Nonerythroid spectrin	81
GAP-43	M	Synaptic SNARE complexes	Localized to axonal growth cones and presynaptic membranes	46
Hrs2	M	SNAP-25	Ca^{2+}-regulated ATPase	8
Myosin V	M	VAMP	ATP driven motor protein implicated in vesicle traffic	87
N-type Ca^{2+} channels	M	Syntaxin 1A	Participate in fast neurotransmitter release	9
Ocsyn	M	Syntaxin 1A	Localized to organ of Corti	106
p115	M	Various	Membrane tethering protein localized to Golgi	39
Pallidin	M	Syntaxin 13	Highly charged novel protein; also present in higher order complex	49
Phospholipids	M, Y	Various (v and t)	SNAREs tend to bind negatively charged lipids	100,127
Q-type Ca^{2+} channels	M	Syntaxin 1A	Participate in fast neurotransmitter release	9
Rim	M	SNAP-25, syntaxin 1A	Also interacts with GTP-bound rab3	21
Sec20p	Y	Ufelp (t)	Functions in ER-Golgi transport	102
SIP30	M	SNAP-25	Mostly expressed in brain	68
Spring	M	SNAP-25	Ring-finger containing protein	69
Synaptophysin	M	VAMP	Tends to prevent complex formation	29
Synaptotagmin I	M	SNAP-25/syntaxin 1A binary complex	May couple exocytosis to Ca^{2+} signals	104
Synaptotagmin I	M	VAMP	Modulates O-glycosylation of synaptotagmin	37
Synaptotagmin IX	M	SNAP-25	May couple exocytosis to Ca^{2+} signals	27,139
Synip	M	Syntaxin 4	SNARE-binding is insulin dependent	79
Syntaphilin	M	Syntaxin I	Expressed neuronally	66
Taxilin	M	Syntaxins 1A and 4	Restricted to neutoendocrine cells	85
Tip20p	Y	Ufelp (t)	Funcions in ER-Golgi transport	102
VAP-33, A, B	A, M	VAMPs	Also binds to occludin in tight junctions in mammalian cells	114,132,133
VFT docking complex	Y	Tlglp (t)	Docking complex	112
VSM1 gene product	Y	Snclp, Snc2p (v)	May negatively regulate constitutive exocytosis	75
Vtc docking complex	Y	Nyvlp (v)	Ties fusion machinery to v-ATPase	80

The proteins listed have been shown to directly interact with individual SNAREs and SNARE complexes. New SNARE-interactive proteins are continually being discovered, and intractions are being discovered for known proteins. Several of the proteins are proposed to regulate SNARE interactions, though some may have additional functions. Many of the proteins listed here have homologues in other species. The emphasis here is on mammalian proteins (including proteins identified in mammal-derived tissue cultures), though several yeast proteins are included to illustrate the breadth of SNARE-binding proteins. Source: M, mammalian; Y, yeast; A, *Aplysia californica*. In the case of SNAREs from yeast, a (t) or (v) after the name indicates a t-SNARE or v-SNARE, respectively. Many proteins that exist in higher order complexes with SNAREs, but for which no direct interaction has been demonstrated, are not listed here. SM proteins are not shown here; a table summarizing their binding and functions can be found elsewhere (38).

interactions. In the end, it is currently difficult to say whether SM proteins are generally regulators of SNARE function, components of the fusion machinery (see below), or both.

Synaptotagmins are proteins with Ca^{2+}- and phospholipid-binding C2 domains that reside in plasma membranes and vesicles. Their requirement for fast synaptic transmission has led to the proposal that they are the Ca^{2+} sensors in this process (17). Syntaxin 1A and SNAP-25 isolated from bovine brain bind endogenous synaptotagmin I (104). Moreover, binding of synaptotagmins I and IX to SNAP-25 is required during Ca^{2+}-dependent exocytosis in PC-12 cells (27, 139). A study has suggested that synaptotagmins bind Ca^{2+} and transduce a signal to associated t-SNAREs, which then interact with v-SNAREs (67). Synaptotagmins also associate with v-SNAREs, a phenomenon that may regulate posttranslational O-glycosylation of synaptotagmin (37). The necessity of the SNARE-synaptotagmin interaction for membrane fusion, however, has not been unequivocally established. For instance, there is evidence in PC-12 cells that the process of membrane fusion critically depends on the ability of synaptotagmins to bind phospholipids and not SNARE complexes (111). This is consistent with the hypothesis that SNAREs are involved in regulating the fusion machine, rather than being its minimal component.

A variety of other fusion components also interact with SNAREs in ways indicating that the fusion machine may be built on the SNARE complex. Complexins are proteins that rapidly and tightly bind to the SNARE complex (48, 90) and are also required for fusion in the neural synapse, though their exact function is unclear (105). It has been suggested that complexins regulate closure of the SNARE-based fusion pore and could be critical for kiss-and-run exocytosis

(6, 103). Calmodulin kinase II (CaMKII) interacts with syntaxin at the neural synapse exclusive of the SM protein munc18 (86). This interaction increases the ability of syntaxin to bind both synaptotagmin and SNAP-25, suggesting that the CAMKII/syntaxin 1A complex may regulate or coordinate a step in core complex formation. In the yeast vacuole system, a t-SNARE was found to interact with proteolipid subunits (V0) of the v-ATPase (95), and a v-SNARE was found to indirectly associate with V0 through a complex of vacuolar transport chaperone (Vtc) proteins (80). Though these results do not establish that V0 binds the entire SNARE complex, they do indicate that the function of this putative component of the fusion machinery is affected by its binding (or not) to SNAREs. In PC-12 cells, binding of calmodulin and phospholipids to VAMP is required for exocytosis, though not for formation of the SNARE complex (100). [This is not the only indication that binding of the fusion machinery to phospholipids is important for fusion. In the yeast vacuole, the SNAP-25 analog Vam7p cycles off of the membrane and is re-recruited to the fusion machinery via binding to phosphatidylinositol 3-phosphate (11).] A number of other proteins are known or implicated to regulate exocytosis, interact with one or more individual SNAREs, and could be regulated by interactions with either individual SNAREs or SNARE complexes. Among these proteins are the recently described syntaphilin (66), the VSM1 gene product (75), and the Chediak-Higashi protein (122). Ion channels, a class of proteins of high interest to physiology, are also known to associate with certain SNARE proteins. N- and Q-type Ca^{2+} channels were first observed to behave this way (9), though epithelial Na^{+} channels (ENaC) (19) and cystic fibrosis transmembrane conductance regulator (CFTR) (22) also participate in interactions with SNAREs. The role that this phenomenon plays in the fusion process (if any) is unclear, though it is clear that SNARE binding to ion channels can alter the open probability (16) and/or affect the slow inactivation process of the channel (24). Table 1 lists several proteins that have been shown to interact directly with SNAREs.

In several cases, knowledge of the fusion machinery has expanded beyond elucidation of single proteins that interact with SNAREs and/or SNARE complexes. In the yeast vacuolar sorting pathway, the t-SNARE Pep12p interacts with the SM protein Vps45p, which interacts with an adaptor (Vac1p) that associates with the rab-like GTPase Vps21p in its GTP-bound form (97). Similarly, in the yeast Golgi, the t-SNARE Tlg1p and GTP-bound Ypt6p (another rab GTPase) interact indirectly through binding different subunits of a proteinaceous complex that mediates membrane association before trans-SNARE complex formation, the VFT (Vps fifty-three) docking complex (112). These results are provocative because they link two protein families required for trafficking, SNAREs and rabs, more precisely and convincingly than previous studies (70, 74). The indirect and transient nature of these connections also highlights the technical challenges involved in identifying all components of the fusion machinery. A summary of the roles that SNAREs may play in some or all complex fusion machines can be found in Table 2.

The identification of SNARE-interacting proteins does not in itself prove the SNARE signaling hypothesis. These proteins could also reflect an elaborate cellular scheme by which to regulate formation of the minimal fusion machinery. However, in addition to the data summarized in the previous section, the SNARE signaling hypothesis is recommended by its resolution of some issues in the fusion area. For example, it can reconcile the permissiveness of SNARE-SNARE interactions with the specificity of the fusion events in which they participate, implied by their distinct subcellular localizations. Rather than being a lock-and-key determinant of specificity, SNAREs may fit specific fusion reactions by the surfaces they create upon complexation, thereby directing the assembly of specific fusion machines, though the cohorts of fusion machine participants may overlap. The SNARE signaling hypothesis can thus explain why SNAREs are required for so many mechanistically and kinetically distinct fusion steps in a more satisfying way than the minimal fusion machinery idea can. Also, it is much easier to reconcile the fact that SNARE complexes with submaximal stabilities can support exocytosis very well, while some complexes with high stability cannot at all (18, 43) within the framework of the signaling idea. It is important to remember, however, that not everything known about SNAREs may be equally applicable to different systems. Two of the best-studied fusion systems, the neural synapse and the yeast vacuole, have many similarities in terms of the types of proteins involved yet have apparently very distinct mechanisms

Table 2. *Roles for SNAREs in a complex fusion machine*

Role	Ref.
Before fusion	
Determination of the fusion site	64, 129
Modulation of fusion sensitivity to cytosolic $[Ca^{2+}]$	20, 118
Maintenance of close membrane apposition, allowing for further fusion steps	95
Licensing of fusion (i.e., detection of SNARE complex by fusion machinery increases likelihood of fusion)	35
During fusion	
Recruitment of fusion machinery into fusion machine	4, 15, 48, 90, 93, 95, 97, 112, 119
Releasing proteins upon formation of the SNARE complex, which could inactivate regulatory proteins or activation proteins involved in fusion	88, 138
Binding of lipids, causing or facilitating their transition to a fusion state	11, 100
After fusion	
Imparting vectorality to fusion events that are not kiss-and-run	42

Table 3. *Comparison of well-studied fusion events*

	Synaptic Vesicle Fusion	Yeast Vacuole Fusion
Components		
Tethering proteins	Bassoon? Piccolo? Aczonin?	HOPS complex
Rab GTPase	Rab3	Ypt7p
SM protein	nSec1	None known
t-SNAREs	Syntaxin 1A	Vam3p
	SNAP-25	Vam7p
v-SNARE	VAMP-2	Nyvlp
Ca^{2+}-binding proteins	Synaptotagmin	Calmodulin
	Calmodulin? RIM? Annexin?	
Ca^{2+} source	Extracellular space, through plasma membrane Ca^{2+} channels	Vacuolar interior
Other proteins		V-type ATPase, protein phosphatase 1
Process		
Priming		Vacuoles undergo complex priming process involving disassembly of cis-SNARE complexes by Sec17/18 (NSF/α-SNAP)
Membrane association	Synaptic vesicles associate with the plasma membrane, perhaps through one of the docking complexes listed above	Vam7p·GTP mediates tethering of vacuoles after activation by HOPS
	Rab3·GTP mediates displacement of nSec1 from syntaxin	Trans-SNARE complexes form as tethered vacuoles dock
	Ca^{2+} influx, caused by plasma membrane depolarization, causes zippering of SNAREs to form trans-SNARE complexes	Trans-SNARE complexes lead to Ca^{2+} release from vacuole; Ca^{2+} binds to calmodulin leading to formation of trans-complexes between calmodulin, Vam3p, and the V_0 subunit of the v-type ATPase
Membrane fusion	Membrane fusion occurs, perhaps as a direct result of trans-SNARE complex formation	Action of protein phosphatase 1 on an unknown target triggers membrane fusion
Recycling	Cis-SNARE complexes in membrane are disassembled by NSF/α-SNAP and recycled	
Geometry	Vesicle fuses with plasma membrane; sometimes quickly reversible (i.e., kiss-and-run)	Vacuoles fuse along large sites of contact (vertices) leading to formation of small intravacuolar membranes as a consequence of membrane fusion.
Time course	Milliseconds to milliseconds	Seconds to minutes

Comparison of two of the best-known SNARE-mediated fusion processes, fusion of synaptic vesicles with the axonal membrane and homotypic fusion of vacuoles in yeast. Many of the components of the tethering, docking, and fusion machinery are homologous between these systems. The process, however, is much different. This comparison was constructed from a pair of excellent reviews on the respective fusion events. The mechanism of the vacuolar fusion event is known in great detail—many of these details were omitted to facilitate comparison between vacuolar fusion and the more speculative process described for synaptic fusion. See these reviews for full details (71, 134).

of fusion. Table 3 provides a brief comparison of these two events, highlighting some of what is known about SNARE-interacting proteins and the potential role of SNAREs in each system.

IS THE SNARE COMPLEX NECESSARY FOR FUSION?

The alternative models of SNARE complex function that have been described thus far all retain an important facet of the SNARE hypothesis. This is the assumption that the super-stable complex observed in cells has some direct role in fusion. It has also been proposed that this complex has no physiological function per se but is an end state reached after SNARE function is completed (42). In this model, the functional SNARE complex is of a much higher free energy than the traditional complex and forms quickly and reversibly. These features would reconcile, at least to some extent, the discrepancy between SNARE complex formation kinetics and the kinetics of physiological membrane fusion. Speed and reversibility in complex formation also provide an attractive mechanism by which kiss-and-run exocytosis could be mediated by SNAREs. Interestingly, there is evidence for a so-called "loose" SNARE complex that can support membrane fusion (136). This complex is made of the same SNAREs that form the traditional "tight" SDS-resistant complex, but its components are susceptible to cleavage by clostridial neurotoxins, establishing it as clearly distinct from the classic complex (47). One marked disagreement between the experiments and the model, however, is that the loose complex supported a slower rate of fusion than the tight complex (136). This may simply reflect the fact that the apparently tight complexes were only end products of previous fusion reactions. In the light of structural data and the signaling idea, it is plausible that a loose SNARE complex could help to direct fusion and that proceeding to the tight complex lends vectorality to fusion, at least in cases that are not kiss-and-run.

Another alternative SNARE complex is the so-called t-SNARE complex. The t-SNARE complex is a binary association of, for example, syntaxin 1A and SNAP-25.

The structure of the neuronal t-SNARE complex has been determined, revealing a four-helix parallel bundle consisting of two copies of syntaxin 1A and one copy of SNAP-25 (135). It is generally thought that the t-SNARE complex represents a starting point for formation of the classic ternary SNARE complex, though a recent observation questions this view. In permeabilized chromaffin cells, stimulation of exocytosis led to increased formation of a variety of t-SNARE complexes, having different sizes and, presumably, states of oligomerization and quaternary structure (67). Interestingly, inhibition of secretion by botulinum neurotoxin A, which cleaves SNAP-25, did not inhibit the formation of t-SNARE complexes in response to Ca^{2+}, though it did alter the composition of t-SNARE complexes and increase the number of ternary SNARE complexes drastically (67). These results suggest that there may be a role for the t-SNARE complex(es) in membrane fusion besides, or in addition to, a precursor to formation of the classic SNARE complex.

SNARES BEYOND THE SNARE DOMAIN

Another assumption that has been maintained throughout this discussion is that the SNARE domains comprise the most critical features of SNARE function. Though fairly small, SNARE proteins do contain other domains, especially a three-helix bundle that is present at the NH_2 terminus of many different SNAREs (3). Interactions between these domains and other proteins and/or the SNARE domains of the complex may serve a purpose that is quite as important as the function of the SNARE domains. However, this role is probably regulatory and does not directly participate in the fusion reaction (26). Alternatively, it has been proposed that the transmembrane segments of SNAREs mediate an important step in membrane fusion (65), perhaps by driving dimerization (63) or assisting in complex formation (98), though this is not true in all cases, because transmembrane segments of SNAREs can be removed without affecting function in yeast (41). The COOH-terminal calmodulin- and phospholipid-binding domain of VAMP is distinct from the SNARE domain (100) and does seem to be required for exocytosis in PC-12 cells. The cysteine-rich region that bridges the two SNARE domains of SNAP-25 has been implicated in recruiting SNAP-25 to SNARE complexes (62). Overall, the prevalent feeling in the field, whether the complete SNARE hypothesis is accepted or not, is that the assumption regarding the preeminence of the SNARE domain is supported by the weight of evidence.

Conclusion

In summary, SNAREs have captured the imagination of virtually everyone in the field of membrane fusion. They possess features that recommend them as specificity determinants as well as possibly the minimal fusion machinery. From the Rothman SNARE experiments, both of these roles are forwarded, though a growing body of evidence suggests that the actual physiological process of membrane fusion is much more complicated. Proteins that interact with SNAREs, many of which may facilitate complex formation, are still being discovered today (54). Understanding the functions of these proteins may give us the insight we need to finally appreciate the contribution of SNAREs to cell function. We may also discover that another assumption that is widely held, explicitly and implicitly, that the function of SNAREs is perfectly analogous in every organism and transport step is not actually true, despite the dramatic similarities in structure and biochemistry between divergent SNARE complexes. In any case, the groundwork for an exciting and contentious continuing debate has been laid, and SNAREs will likely continue to make cell biology headlines for the foreseeable future.

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