

Published in final edited form as:

Science. 2009 January 23; 323(5913): 474–477. doi:10.1126/science.1161748.

Membrane Fusion: Grappling with SNARE and SM Proteins

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Abstract

The two universally required components of the intracellular membrane fusion machinery, SNARE and SM (Sec1/Munc18-like) proteins, play complementary roles in fusion. Vesicular and target membrane-localized SNARE proteins zipper up into an α -helical bundle that pulls the two membranes tightly together to exert the force required for fusion. SM proteins, shaped like clasps, bind to trans-SNARE complexes to direct their fusogenic action. Individual fusion reactions are executed by distinct combinations of SNARE and SM proteins to ensure specificity, and are controlled by regulators that embed the SM/SNARE fusion machinery into a physiological context. This regulation is spectacularly apparent in the exquisite speed and precision of synaptic exocytosis where the Ca^{2+} -sensor synaptotagmin cooperates with the clamp-activator complexin to control the precisely timed release of neurotransmitters that initiates synaptic transmission and underlies brain function.

Life in eukaryotes depends on the fusion of membranous organelles. Every vital process relies on the orderly execution of membrane fusion, from the exquisite compartmental organization of all cells to the precise timing of synaptic transmission in brain. SNARE and SM proteins have long been known to be required for fusion, but precisely how they co-operate has been unclear until very recently. Moreover, because this universal fusion machinery is constitutively “on”, the necessity for control of fusion – as needed for all of biology, from cell division and migration to hormone signaling and synaptic transmission – requires a superimposed dynamic control mechanism that grapples with the SNARE and SM proteins, clamps them down when not needed, and activates them when they are.

Here we review recent advances and suggest a simple and unified view of the mechanisms by which SNARE and SM proteins function together as the universal fusion machinery, responsible for all intracellular membrane fusion except that involving mitochondria. We present a simple and coherent picture of how membrane fusion is executed and controlled, providing a foundation for understanding physiology and its chronic imbalances which contribute to diseases as diverse as diabetes, immune deficiency, and Parkinson’s disease.

SNARE proteins – the force generators

NSF (for N-ethylmaleimide Sensitive Factor) and SNAP (for Soluble NSF Attachment Protein – note that this protein is not related to ‘SNAP’ type SNARE proteins described below) were purified based on their requirement for transport vesicle fusion in a cell-free system (1–3). SNARE proteins were identified as receptors for SNAP and NSF (hence the name SNARE, which abbreviates SNAp REceptor) as a complex of three membrane proteins proposed to bridge the exocytic vesicle to the plasma membrane (4). These proteins, syntaxin-1 and SNAP-25, emanating from the pre-synaptic plasma membrane, and vesicle-

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associated membrane protein (VAMP)/synaptobrevin, located in the synaptic vesicle, had previously been individually sequenced and localized (5–9). They were also recognized along with many synaptic vesicle proteins and yeast secretion genes to be members of conserved gene families that were directly or indirectly implicated in vesicle transport (10–15). Consistent with their proposed central importance in fusion (4), the synaptic SNARE proteins were identified as targets for botulinum and tetanus toxins, exquisitely specific proteases that block synaptic vesicle fusion (16–18). Finding the membrane-bridging SNARE complex at the synapse focused attention on these three proteins (and their homologues in other organelles and tissues) as being at the heart of membrane fusion and suggested that synaptic SNARE proteins and their ubiquitously expressed homologues are universal fusion proteins – a concept broadly referred to as the SNARE hypothesis (4). The SNARE hypothesis also postulated that SNAREs fall into two broad categories, v-SNAREs in transport vesicles and t-SNAREs in target membranes, that pair specifically to add compartmental specificity to membrane fusion. A comprehensive test of hundreds of combinations of SNAREs derived from the yeast genome indicated that the compartmental specificity of the yeast cell correlates in almost every case with the physical chemistry of isolated SNAREs. Only a dozen or so SNARE combinations are fusogenic, corresponding to the known transport processes in the cell (19–21), demonstrating that SNAREs can impart considerable specificity to membrane fusion.

The structure of SNARE proteins and the architecture of SNARE complexes illustrate their mechanism (Fig. 1). Individual SNARE proteins are unfolded, but they spontaneously assemble into a remarkably stable (22) four-helix bundle (23) which forms between membranes as a ‘trans-SNARE complex’ (also known as a ‘SNAREpin’) that catalyzes fusion by forcing membranes closely together as it zippers up, exerting force against any attempted separation of its helices from each other (Fig. 2A)(24, 25). The force required to rupture trans-SNARE complexes is estimated to be in the range of 100–300 pN, and each SNAREpin releases about 35 k_BT of energy (equivalent to about 20 kcal/mole) as it zippers up (26). The activation energy for lipid bilayer fusion is in the range of 50–100 k_BT (27), and so three or more individual SNAREpins suitably arranged will provide enough energy to drive fusion, in line with current estimates (28). In the post-fusion state (Fig. 2B), the fully-zipped SNARE complex (emanating from the fused membrane) is termed the ‘cis-SNARE complex’.

Current evidence suggests that SNARE complex formation promotes membrane fusion by simple mechanical force because their normally polypeptide membrane anchors can be replaced by passive lipid structures that span both leaflets (29). Moreover, the linker region between the SNARE motif and the trans-membrane region is critical (30, 31) as a force transducer that translates the energy released upon trans-SNARE complex zippering into a catalytic force that fuses the apposing bilayers.

Overall, fusion is driven by an ATP-dependent cycle of SNARE association and dissociation. In this cycle, the bilayer merger is thermodynamically coupled to exergonic folding of SNARE proteins, followed by their endergonic unfolding by a specialized ATPase (NSF) that returns them to their initial state for another round. This simple thermodynamic mechanism has been demonstrated in the spontaneous fusion of artificial lipid vesicles containing purified v- and t-SNARE proteins (25). Once assembled, SNARE complexes are recycled by the ATPase NSF and its adaptor protein, SNAP, the latter binding directly to the SNARE complex (32,33). NSF is a hexamer that presumably uses 3–6 ATP’s with each catalytic cycle (totaling about 20–40 kcal/mole to disrupt the SNARE complex).

SNARE proteins are diverse (typically 20–30% protein sequence identity as a super-family), but each contains a characteristic ~70 residue “SNARE motif” with heptad repeats (34). It is this motif that forms the four-helix bundle. Most but not all SNARE proteins are membrane anchored (at their carboxy terminal ends) and contain a single SNARE motif, except for SNAP-class SNAREs which contain two motifs and are specialized for exocytosis. Within the four-helix bundle, four classes of SNARE motifs are structurally distinguished (referred to as R-, Qa-, Qb, and Qc-SNARE motifs; 34). All SNARE complexes contain one member of each class, which is referred to as the R/Q-rule, with the R-SNARE usually corresponding to the v-SNARE, and the Q-SNAREs usually corresponding to the t-SNAREs. Frequently, the v-SNARE is uniquely positioned in a separate membrane from the three t-SNAREs in order for fusion to occur (19). This topological restriction reveals distinct but not well-understood roles for the v- and t-SNARE components in the force-generating mechanism.

While it is thus clear that SNAREs drive fusion thermodynamically, estimates of catalytic potency vary widely among the kinds of defined systems where isolated SNARE kinetics can be studied. Fusion kinetics range from about 10's of msec for single events (35,36) to 10's of minutes for populations in the earliest studies (25), and depend strongly on SNARE concentration and local membrane architecture, indicating that an additional protein(s) may be needed under physiological conditions. In fact, while SM proteins can be dispensed with in vitro at high SNARE concentrations, as we will now see, the system in vivo universally requires an SM protein as a subunit of the t-SNARE complex to clasp the assembling SNARE complexes.

SM proteins – Clasping SNAREpins

SM proteins have been linked to membrane fusion since the synaptic SM protein (Munc18-1) was isolated bound to the synaptic t-SNARE syntaxin-1 (37), but only recently has a clear view emerged of how SM proteins work in fusion. SM proteins associate with SNARE proteins in multiple ways, including as clasps binding both the v-SNARE and t-SNARE components of zippering SNARE complexes. It now seems likely that SM proteins organize trans-SNARE complexes (i.e., SNAREpins) spatially and temporally.

SM proteins (Fig. 1B) are composed of a conserved ~600 amino acid sequence that folds into an arch-shaped ‘clasp’ structure (38). SM proteins interact with SNAREs in different ways. First, they bind to the individual synaptic t-SNARE subunit syntaxin-1, forming a complex that includes part of the SNARE motif, thus disabling the formation of SNARE complexes (Fig. 3A). Here, the SM protein embraces a four-helix bundle formed exclusively within the syntaxin. In addition to its SNARE motif, Syntaxin-1 also contains a three helix bundle which comprises its globular, N-terminal ‘Habc’ domain that folds back and binds the helical SNARE motif to form the ‘closed’ syntaxin conformation (38,39). In this arrangement, the SM-protein clasps these four helices – the three from the Habc domain, and the fourth from the SNARE motif. Only syntaxins among the SNARE superfamily assume such a stable intramolecular closed conformation, yet this structure reveals a general feature of SM proteins: they are fundamentally designed to clasp a four helix bundle. As we will shortly see, this can also be the four-helix bundle of a zippering SNAREpin.

The early discovery of this mode of binding to the closed conformation of syntaxin-1 led to the suggestion that SM proteins act as negative regulators. However, an SM protein is positively required in all fusion reactions, and all genetic screens involving fusion reactions identified, among other genes, those encoding SM proteins (e.g., see 10, 40). As well, reverse genetic deletion of the major synaptic SM protein (Munc18-1) blocks exocytosis without altering synapse formation (41), even more completely than the strong effect of

deleting VAMP/synaptobrevin (42). Thus, SM proteins could not only be negative regulators.

Recently this mechanistic gap was resolved when a second, distinct mechanism of interaction between SM and SNARE proteins was found (Fig. 3B), explaining how SM proteins could promote fusion. Here, the SM protein is anchored by its N-terminal lobe to a specific N-terminal peptide sequence of the syntaxin (43, 44). This binding leaves the arch-shaped body of the SM protein free to fold back on the SNAREpin and clasp across the zippering four helix bundle near the membrane (Fig. 3C).

Of course, this can only happen when the v-SNARE (one helix) combines with the t-SNARE (three helices) to comprise four helices, potentially enabling SM proteins to cooperate in trans-SNARE complex assembly and organization, spatially and temporally, thereby stimulating SNARE-mediated fusion upon tethering to syntaxin's N-terminus (45,46). Targeted mutagenesis and biophysical studies indicate that the SM protein contacts residues on the surface of both the v- and the t-SNARE in the SNARE complex (45,46), as expected from clasping (Fig. 3C).

Thus, SM proteins are – together with SNARE proteins – the universal components of the fusion machinery, equally essential for membrane fusion in the cell (Fig. 4) and capable of promoting compartmental specificity (47). Yet, this clear-cut *in vivo* requirement for SM proteins was not evident in defined fusion assays, which in retrospect had utilized un-physiologically high concentrations of SNAREs. By maximizing fusion by SNAREs in the absence of SM proteins, defined systems indeed established the inherent thermodynamic sufficiency of SNARE proteins for fusion, but at the same time somehow by-passed the vital requirement for SM proteins in the complexity of a cellular environment. We ascribe this difference to the relatively low SNARE concentrations in cells, presumably kept low to allow effective regulation of their activity (47).

Exactly how SM proteins cooperate with SNARE complexes for fusion is not yet known. We suggest a kinetic role in which SM proteins co-operate with SNAREs by helping them assemble into productive topological arrangements at the interface of two membranes (such as ring-like arrangements that could facilitate the opening of fusion pores), possibly by restricting the diffusion of SNAREs into the space between fusing membranes (48). Thus, SM proteins likely act as catalysts for SNAREs which in turn are catalysts for membrane fusion. The HOPS complex containing the SM protein Vps33 appears to act in this manner (49). We also note that SM-protein binding to SNARE proteins likely performs additional functions in fusion that seamlessly merge with their universal roles in fusion, for example in vesicle tethering and in regulating the speed of fusion (50).

In sum, the universal fusion machinery (Figure 3C) consists of a v-SNARE protein and a t-SNARE complex, the latter comprised of a syntaxin 'heavy chain' with one or two associated non-syntaxin SNARE 'light chains', and a cognate SM protein bound to the N-terminus of the syntaxin. The t-SNARE complex engages the cognate v-SNARE in the opposing membrane, and as these two SNAREs zipper-up towards the membrane, the SM protein cooperates in fusion at least in part by circumferentially clasping the assembling trans-SNARE complex.

Complexins – Grappling with SNAREs for synaptic transmission

Different intracellular fusion reactions are subject to distinct regulatory processes that adapt the universal fusion machinery to organismal physiology. These regulators prevent rampant fusion events that would otherwise occur because membrane fusion is driven by a thermodynamically spontaneous process of protein folding. Equally importantly, these

regulators poise the fusion machinery in an active state to allow rapid and synchronous fusion in response to a trigger. By grappling (i.e. “seizing at close quarters”) the SNAREs, regulatory proteins can accomplish orderly clamping and activation, holding the machinery in a ‘cocked’ state that only needs a small triggering stimulus to burst forward. A grapple can be used either to prevent or induce an action; in other words, by their nature, grapples are capable of inhibiting a process, activating a process, or both under differing conditions.

Complexin and synaptotagmin are probably the best understood grappling proteins in membrane fusion (51). Together, these two proteins account for the precise timing and regulation of the secretion of hormones like insulin from the pancreas and neurotransmitter release at the synapse, the latter underlying all information processing in the brain. As we will describe now, synaptic and other exocytic SNAREs are first activated and then clamped by complexin (52–54), and finally triggered by Ca^{2+} binding to synaptotagmin, which reverses the action of complexin and allows fusion to be completed (55, 56).

At the synapse, at any one time, there are only a handful of synaptic vesicles docked at the pre-synaptic plasma membrane, and these are the most advanced in the fusion process – referred to as being “primed for fusion” or “readily releasable”. When Ca^{2+} enters the nerve terminal as the result of an arriving action potential, this ion selectively triggers fusion of these few vesicles, often in less than a millisecond, faster than any other membrane fusion event (57). The primed vesicles are distinguished from the rest – and kinetically the most advanced – because their v-SNAREs have already formed partially zippered trans-SNARE complexes with the plasma membrane t-SNAREs, as evidenced by the fact that complexin acts upstream of Ca^{2+} -triggered fusion, but nevertheless requires SNARE-complex binding for function (52,58). Complexin acts as the quintessential grappling protein that elevates zippered SNARE complexes into this activated but frozen state, and releases it when Ca^{2+} enters and binds to synaptotagmin.

Synaptotagmin (Fig. 1D) is a synaptic (or secretory) vesicle protein containing two protein kinase C-like C_2 domains, leading to the suggestion that it acts as the Ca^{2+} -sensor for exocytosis (59). The fact that synaptotagmin binds Ca^{2+} (60) and to SNARE proteins (5, 61, 62), and that its C_2 domains function as autonomous Ca^{2+} -binding domains – indeed, were the first C_2 domains for which this was revealed (63) – gave credence to this hypothesis. Synaptotagmin is required in mice for the tightly regulated, synchronous (i.e. rapid and coordinated) synaptic exocytosis characteristic of neurotransmission, but not for synaptic vesicle fusion *per se* (63). Reducing the Ca^{2+} -binding affinity of synaptotagmin in mice caused a correspondingly reduced Ca^{2+} -sensitivity of fusion which is thus determined by Ca^{2+} -binding to synaptotagmin (55,56), formally proving that synaptotagmin is the sensor. In triggering synaptic fusion, synaptotagmin binds to both phospholipids and to SNARE complexes in a Ca^{2+} -regulated manner (56).

Strikingly, deletion of complexin causes a precise phenocopy of the synaptotagmin deletion: a loss of Ca^{2+} -triggered synchronous release, but not of fusion because asynchronous release is unimpaired (52), suggesting that complexin somehow functions to activate SNARE complexes for subsequent synaptotagmin action. In addition, complexin clamps fusion, as evidenced both inhibition of SNARE-mediated fusion *in vitro* (54, 64), and by increased spontaneous synaptic fusion in complexin-deficient synapses (58, 65). Then, Ca^{2+} -binding to synaptotagmin releases the complexin clamp and triggers fusion by binding to SNARE complexes and phospholipids.

Very recent work has revealed how precisely complexin might control fusion in cooperation with synaptotagmin. Complexin contains a central α -helix that binds at the interface of the v- and t-SNARE proximal to the membrane (Fig. 1C; 66). It also contains an accessory helix

and an unstructured N-terminal sequence that are located proximal to the membrane – where the final stages of zippering take place. In this issue of *Science* it is reported that SNARE binding by the central helix of complexin and its accessory helix are required for activation and clamping of fusion, whereas the N-terminal unstructured sequence is only required for activation but not clamping (58, 67). The accessory helix may clamp fusion by forming an alternate four helix bundle with the membrane-proximal portion of the t-SNARE, thereby preventing the v-SNARE from completing its zippering and triggering fusion (67). This creates a “toggle switch” that can reversibly clamp fusion at a late stage. The N-terminal complexin sequence, in turn, may independently interact with the trans-SNARE complex where it inserts into the fusing membranes, because a point mutation in synaptobrevin at the membrane prevents activation by complexin (65).

How might complexin and synaptotagmin interface with each other during Ca^{2+} -triggered fusion to control this toggle switch? Synaptotagmin competes with complexin for binding to assembled SNARE complexes, releasing complexin in a Ca^{2+} -dependent manner (54), the simplest possible molecular mechanism for Ca^{2+} -coupling. However, the details of how complexin and synaptotagmin act on SNARE complexes in a pas-de-deux that is choreographed by Ca^{2+} and enables the supreme speed and precision of synaptic transmission remain for the future.

Perspective

Intracellular membrane fusion in eukaryotes is executed by a conserved and universal fusion machinery composed of SNARE and SM proteins. Fusion results from the thermodynamic coupling of protein folding (assembly of v-SNAREs with t-SNAREs, spatially and temporally organized by SM proteins) to bilayer perturbation. Energy made available from folding is productively channeled into the bilayer, so that on balance fusion is the favored, spontaneous reaction. Nevertheless, fusion is tightly regulated in a spatial and temporal manner, most strikingly at the synapse where the regulation of fusion enables information processing by the brain. We are just beginning to understand how this regulation works, but in the case of the synapse the molecular details have recently been de-mystified with the elucidation of the interplay between complexin, SNAREs, and synaptotagmin. There are a plethora of proteins and compounds that more fragmentary evidence suggests may regulate synaptic and other fusion processes, including the large families of Rab GTPases, tethering proteins, and phosphoinositides, but the underlying principles are likely the same, driven by the simple mechanism described in this review.

Acknowledgments

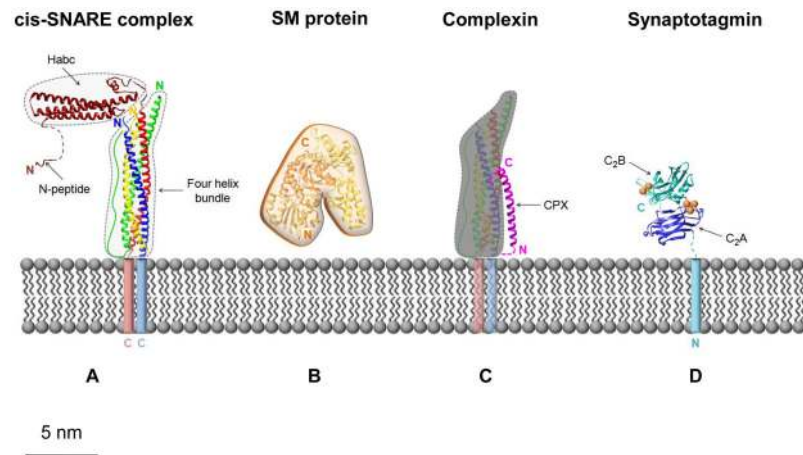
Work described in this review was supported by grants by the NIH to J.E.R. and T.C.S., and by an investigatorship of the HHMI to T.C.S.

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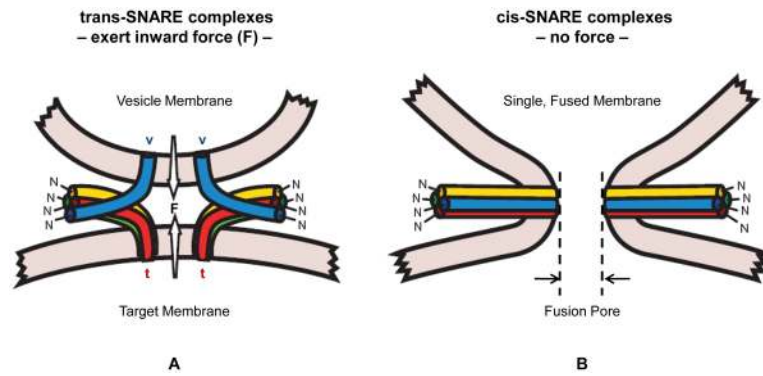
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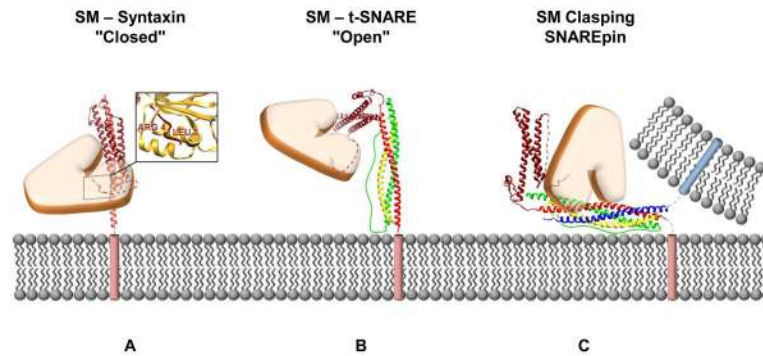
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**Figure 1.**

Structure of SNARE and SM proteins and some proteins that grapple with them. **(A)** SNARE complex (also called cis-SNARE complex) of VAMP/synaptobrevin-2 (blue helix), Syntaxin-1A (red helix), and SNAP-25 (green and yellow helices for the N- and C-terminal domains, respectively; adapted from (23)). The Habc domain of Syntaxin-1A (brown helices, adapted from (68)) is positioned arbitrarily. **(B)** An SM protein, highlighting its arch-like structure (Munc18-1, adapted from (38)). **(C)** Complexin, bound to the SNARE complex (Complexin-1, shown in magenta, adapted from (66)), has a helical region that binds at the interface of v- and t-SNARE in an anti-parallel orientation. **(D)** Synaptotagmin, the calcium sensor for synchronous synaptic transmission (Synaptotagmin-1, adapted from (69, 70)), with its membrane-proximal (C₂A) and membrane-distal (C₂B) C₂ domains labeled and the position of critical bound calcium ions (orange) shown. All proteins are at the same scale and the bilayer thickness is approximately on the same scale.

**Figure 2.**

(**A**) The zippering model for SNARE-catalyzed membrane fusion. Three helices anchored in one membrane (the t-SNARE) assemble with the fourth helix anchored in the other membrane (v-SNARE) to form trans-SNARE complexes, or SNAREpins. Assembly proceeds progressively from the membrane-distal N-termini towards the membrane-proximal C-termini of the SNAREs. This generates an inward force vector (**F**) that pulls the bilayers together, forcing them to fuse. Complete zippering is sterically prevented until fusion occurs, so that fusion and the completion of zippering are thermodynamically coupled. (**B**) Therefore, when fusion has occurred, the force vanishes and the SNAREs are in the low energy cis-SNARE complex.

**Figure 3.**

SM proteins are designed to bind four helix bundles. **(A)** The “closed” conformation of Syntaxin-1A, in which the SM protein Munc18-1 binds the four helix bundle composed of syntaxin’s own Habc domain (three helices, in brown) and its own SNARE motif helix (fourth helix, in red; adapted from (38)). This closed state has so far only been found with syntaxins involved in exocytosis. Inset: SM proteins are universally attached to Habc domains by a specialized sequence at the N-terminus of Habc (labeled as N-peptide; adapted from 38, 43, 44). **(B)** The “open” conformation of a t-SNARE complex, consisting of a t-SNARE and its cognate SM protein bound to the N-peptide of its syntaxin’s Habc domain. This is believed to be the universal state in which t-SNAREs are open (i.e. reactive) with cognate v-SNAREs to form trans-SNARE complexes **(C)** resulting in fusion. Positioning of the protein domains in B and C are arbitrary. Panel C illustrates SNAREs and SM proteins, the universal fusion machinery.