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Three steps forward, two steps back: Mechanistic insights into the assembly and disassembly of the SNARE complex

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Abstract

Membrane fusion is a tightly controlled process in all eukaryotic cell types. The SNARE family of proteins is required for fusion throughout the exocytic and endocytic trafficking pathways. SNAREs on a transport vesicle interact with the cognate SNAREs on the target membrane, forming an incredibly stable SNARE complex that provides energy for the membranes to fuse, although many aspects of the mechanism remain elusive. Recent advances in single-molecule and high-resolution structural methods provide exciting new insights into how SNARE complexes assemble, including measurements of assembly energetics and identification of intermediates in the assembly pathway. These techniques were also key in elucidating mechanistic details into how the SNARE complex is disassembled, including details of the energetics required for ATP-dependent α -SNAP/NSF-mediated SNARE complex disassembly, and the structural changes that accompany ATP hydrolysis by the disassembly machinery. Additionally, SNARE complex formation and disassembly are tightly regulated processes; innovative biochemical and biophysical characterization has deepened our understanding of how these regulators work to control membrane fusion and exocytosis.

Exocytosis and membrane fusion

Most eukaryotic cells carry out exocytosis for the delivery of lipids and proteins to facilitate cellular growth, as well as signaling to the cell's exterior environment. Exocytic vesicle fusion allows the plasma membrane to expand, often in a specific location in the cell (i.e. polarized growth). Additionally, proteins that were embedded in the vesicle membrane are delivered to the plasma membrane to serve as signaling receptors, transport proteins, and channels. The soluble contents of the vesicles are also delivered to the extracellular space upon fusion. Exocytic vesicles originate from the trans-Golgi network or recycling endosomes, and are transported on the cytoskeleton to the plasma membrane. Upon arrival at the plasma membrane, SNARE (Soluble *N*-ethylmaleimide sensitive factor protein receptor) complexes form between the vesicle and the plasma membrane, leading to membrane fusion and cargo delivery.

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Certain cell types have specialized forms of exocytosis that require additional regulation, such as neurotransmitter release in neurons, histamine release in mast cells, and transport of the glucose transporter GLUT4 to the plasma membrane in muscle and adipose cells. It is important to note that although regulation of these specific pathways is different from one another and from the growth pathway, they share many common core components—the different pathways use the cytoskeleton and motor proteins for transport of vesicles, and membrane fusion is carried out by the SNARE family of proteins [1–5]. This review will focus primarily on new information regarding the mechanism of SNARE-mediated membrane fusion at the plasma membrane and its regulation. More detailed reviews of the general steps and components of exocytosis and endocytosis can be found in [6–10] and references therein.

The fundamental machinery of trafficking

Exocytosis is a multi-step process that can be regulated at many points by many factors. Cargo transport begins when macromolecules are packaged into vesicles that bud from the trans-Golgi network. These vesicles are then carried by motor proteins of the myosin and kinesin families that walk towards the plasma membrane using actin filaments and microtubule tracks, and ATP as fuel [7]. The specificity of the cargo-motor protein interaction is controlled by the Rab family of small GTPases [10]. Once at the cell periphery, a multisubunit tethering complex called the exocyst facilitates SNARE-mediated membrane fusion. The exocyst has been suggested to tether the vesicle to the target membrane, bringing the SNARE proteins into close proximity. Additionally, the yeast exocyst plays a more direct role through its interaction with the SNARE subunit Sec9, the yeast homolog of SNAP-25 [11]. The exocyst does not appear to be required for synaptic vesicle fusion in neurons [12], but is essential in other cell types [13]. Other SNARE assembly and fusion regulators include the Sec1/Munc18 family [3,14], neuronal specific proteins including synaptotagmin and complexin (discussed below), and Munc13 [15]. After the membranes have been fused by the SNAREs, the SNARE complex must be either disassembled and recycled for another round of fusion, or degraded. Therefore, regulation of exocytosis occurs at the levels of cargo loading, vesicle transport, and membrane fusion. The studies highlighted below will focus specifically on the mechanisms of SNARE complex formation and disassembly, and how these processes can be regulated.

SNARE-mediated membrane fusion

Membrane fusion results from binding of the vesicle-associated (v-) SNARE VAMP (also called synaptobrevin) to the target membrane-associated (t-) SNAREs syntaxin and SNAP-25 [8]. Biochemical and biophysical characterization of these proteins indicated that their unassembled structures are comprised of random coils, which gain significant helicity when they form the assembled ternary complex (Fig. 1) [16]. The neuronal SNARE complex is very stable; it is resistant to both heat (with a melting temperature of greater than 90°C) and sodium dodecyl sulfate [16,17]. Analyses using both electron microscopy and spin labeling electron paramagnetic resonance spectroscopy showed that these helices formed a parallel coiled-coil, providing the first clue that these proteins might be not be antiparallel receptors for vesicle recognition, but rather “zipper” and pull the vesicle and target

membranes together for fusion [18,19]. Moreover, the crystal structure established that the post-fusion SNARE complex is a parallel four-helix-bundle, with two helices contributed by SNAP-25 and one each by the membrane-embedded syntaxin and synaptobrevin [20]. This structure identified an ionic layer (three glutamine residues from SNAP-25 and syntaxin that interact with an arginine from synaptobrevin) buried at the interface of the complex and surrounded by hydrophobic interactions. These combined interactions may help to stabilize the ternary complex, properly align the register of the complex, and/or be important for complex disassembly, although this has been debated [20,21]. These data, together with the finding that SNARE complexes were necessary and sufficient to catalyze liposome fusion [22], support the hypothesis that v- and t-SNARE zippering from the N-termini to the membrane-embedded C-terminal helices [17,23,24] provides the energy for membrane fusion [25].

SNARE complex formation originally appeared to be a simple two-state process: uncomplexed v- and t-SNAREs would directly form ternary complexes with no kinetic intermediates [25]. However, recent work challenged this idea, at least for neuronal SNAREs, but perhaps for others as well [26–29]; intermediates in the SNARE assembly pathway were identified that might be key to the regulation of assembly and fusion. The first line of evidence comes from experiments using optical tweezers, which allowed researchers to directly pull on the C-terminal regions (normally embedded in the membrane) of the complexed neuronal SNAREs [26]. Gao *et al.* used this optical tweezers and Hidden Markov modeling to identify four distinct states: (1) ternary complex is completely formed; (2) application of force allows the SNAREs to partially unzip, oscillating between the partially unzipped form and the folded form; (3) increasing the application of force on the C-termini causes state 2 to further unfold to a half-zipped state; and (4) the half-zipped state crosses a threshold and completely unfolds. This process is shown in Figure 1, but in reverse order. When trap distances were analyzed, the half-zipped state corresponded to formation of the complex up to, and including, the ionic layer. An additional study by Min *et al.* using magnetic tweezers independently isolated this half-zipped state, but not the partially unzipped state 2 [27**]. Interestingly, both studies observed the same hysteresis behavior; this suggested that they may have been characterizing the same intermediate state, and was reminiscent of the assembly and disassembly hysteresis observed biochemically by Fasshauer *et al.* [30]. Taken together, these data suggest that neuronal SNARE complex assembly occurs with at least one intermediate.

Do these folding intermediates play a role in the regulation of SNARE complexes in different cell types? The authors of the studies above suggest that the half-zipped state is important for the fusion priming observed in neurons (see below), but its relevance in other cell types was not clear. To answer this, Zorman *et al.* compared the energetics for SNARE complex formation from four different origins: synaptic vesicle fusion in neurons, those involved in exocytosis of GLUT4 vesicles, endosomal SNAREs and the yeast exocytic SNAREs [28]. Using artificially concatenated SNARE proteins, the authors isolated the half-zipped state in *all four* SNAREs tested, although the energetics differed. The N-terminal half of the complexes all had similar folding energies. However, differences were discovered between the C-terminal halves of each complex. Additionally, formation of the

C-terminal half of each complex was more favorable than their N-terminal halves, leading the authors to postulate that this evolved to counteract the increase in repulsive energies that arises as the membranes are moved closer together. Furthermore, the authors predict that these differences in energetics are not the key determinants in the regulatory mechanisms employed, although it was noted that the two SNAREs with the most favorable C-terminal domain folding energies (neuronal and GLUT4) are regulated by functionally similar fusion priming mechanisms.

Regulation of SNARE zippering

Neuronal fusion reactions are incredibly fast *in vivo* (on the order of ms); however, *in vitro* measurements previously reported a fusion rate of minutes [22]. A handful of more recent studies demonstrated fusion rates in the sub-second regime, but each of these studies have specific caveats, including a lack of dependence on a fully assembled t-SNARE [31,32]; the requirement for a polyethylene glycol (PEG) tether between the vesicle and target membrane [33]; or use of artificial PEGylated membranes [34]. A key difference between these *in vitro* studies and synaptic vesicle fusion *in vivo* is that the SNARE complexes likely exist in a primed pre-fusion state at presynaptic sites. Indeed, docked vesicles are directly observed in neurons prior to stimulation [35,36].

How does priming occur? Priming results from the cooperation between complexin and the Ca^{2+} -sensor synaptotagmin to regulate neuronal SNAREs [25,37]. Complexin binds trans-SNARE complexes and appears to wedge a helix into the C-terminal region of the forming complex, thus sterically inhibiting the completion of SNARE folding [38,39]. Upon arrival of an action potential and Ca^{2+} influx, Ca^{2+} binds to synaptotagmin, a conformational change occurs that leads to the release of complexin, completion of SNARE assembly and fusion of the membranes. Recently, it was shown that synaptotagmin draws the opposing membranes together via electrostatic interactions while simultaneously removing complexin from blocking the completion of SNARE zippering [40*]. The exact nature of this synaptotagmin-induced structural change remains elusive. Additionally, crystallographic studies suggested that complexin might cross-link adjacent clamped SNARE complexes. Release of a single complexin may thus destabilize the remaining complexin-SNARE interactions, allowing for cooperative membrane fusion by the adjoining SNARE complexes [39,41]; while intriguing, this idea remains controversial [42].

SNARE disassembly

The post-fusion assembled SNARE complex is very stable. Given this stability, the complex will not spontaneously disassemble, which is required for the recycling and/or turnover of v- and t-SNAREs. As a result, the post-fusion SNARE complex must be mechanically disassembled. Two studies on the two proteins responsible for this disassembly reaction, the AAA+ ATPase N-ethylmaleimide sensitive factor (NSF) and α -SNAP (soluble NSF-attachment protein), provide exciting new insights into this mechanism [43**,44].

A combination of structural and single molecule microscopic experiments elucidate how post-fusion SNAREs are disassembled. High-resolution cryogenic electron microscopy was used by Zhao *et al.* to determine structures of the SNARE- α SNAP-NSF supercomplex in

both the ADP- and ATP-bound states, resolving conformational changes between the two nucleotide states (Fig. 2) [44]. The structure reveals that NSF can adopt two states, an ATP-bound split washer conformation and an ADP-bound flat, open-washer conformation. This spring-loaded state change forces α -SNAP to alter its conformation as well, inducing a shearing motion in the SNARE complex. The authors propose that this motion induces SNARE unwinding and dissociation.

In a complementary study by Ryu *et al.*, single molecule microscopy revealed that SNARE disassembly can be induced by a single round of ATP hydrolysis; the actual number of subunits that must hydrolyze ATP remains unclear [43**]. Another recent biochemical study suggests that hydrolysis of as few as 10 molecules of ATP are needed per disassembly reaction [45]. Additionally, single molecule Förster Resonance Energy Transfer experiments on SNAREs during the α -SNAP/NSF-mediated disassembly reaction [43**] supported the shearing motion described in [44**]. A more detailed inspection of the kinetics from the single molecule experiments in [38] will be necessary to delve deeper into the mechanism, particularly with respect to conformations observed by Zhao *et al* [44**].

Disassembly regulation and moonlighting functions of the machinery

The requirement for α -SNAP/NSF in SNARE disassembly has been known for decades [46,47], but recent evidence suggests that their involvement in membrane fusion might be more complicated than originally proposed. Studies of the yeast homologs to α -SNAP and NSF (Sec17 and Sec18, respectively) demonstrated that Sec17 can associate with SNARE complexes and recruit Sec1/Munc18 regulatory proteins to inhibit disassembly by Sec18 [48]. Furthermore, bulk fusion assays demonstrated that Sec17 can enhance SNARE-mediated membrane fusion in the absence of Sec18 [49*]. Mutational analysis indicated that this stimulatory effect was dependent on a hydrophobic loop on Sec17, leading to the proposal that the effect was caused by a direct interaction of Sec17 with both target and vesicle membranes [40*]. The *in vivo* implications of this work are unknown, but it is clear that our understanding of the disassembly machinery remains incomplete.

Conclusions

Recent biophysical and structural characterizations of SNARE proteins and their regulators provided critical insights into how these molecules orchestrate the fundamental process of membrane fusion. Single molecule studies defined potentially important regulatory intermediates in the SNARE assembly process and showed that these intermediates are conserved in different cell types. Extension of these studies to include regulatory factors, such as synaptotagmin and complexin, will be important to our understanding of highly regulated membrane fusion reactions. Moreover, high-resolution structural electron cryomicroscopy and single molecule microscopy were integral in elucidating key aspects of the SNARE complex disassembly process. These studies revealed that a single round of ATP hydrolysis coupled to conformational changes in α -SNAP/NSF led to a shearing and twisting motion that could unravel and destabilize the incredibly stable SNARE complex. Surprisingly, biochemical characterization found moonlighting functions for the disassembly machinery: they appear to *promote* assembly under specific conditions. Further

improvements in single molecule microscopy and electron cryomicroscopy, combined with careful quantitative analyses, will improve our understanding of the molecular mechanisms of the membrane fusion and SNARE complex disassembly machinery.

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Highlights

- SNARE complex formation drives membrane fusion
- Complex formation occurs with intermediates, potentially important for regulation
- α -SNAP/NSF disassemble SNAREs by inducing shearing/rotation with ATP hydrolysis
- Complexin/Synaptotagmin regulate vesicle release via conformational changes

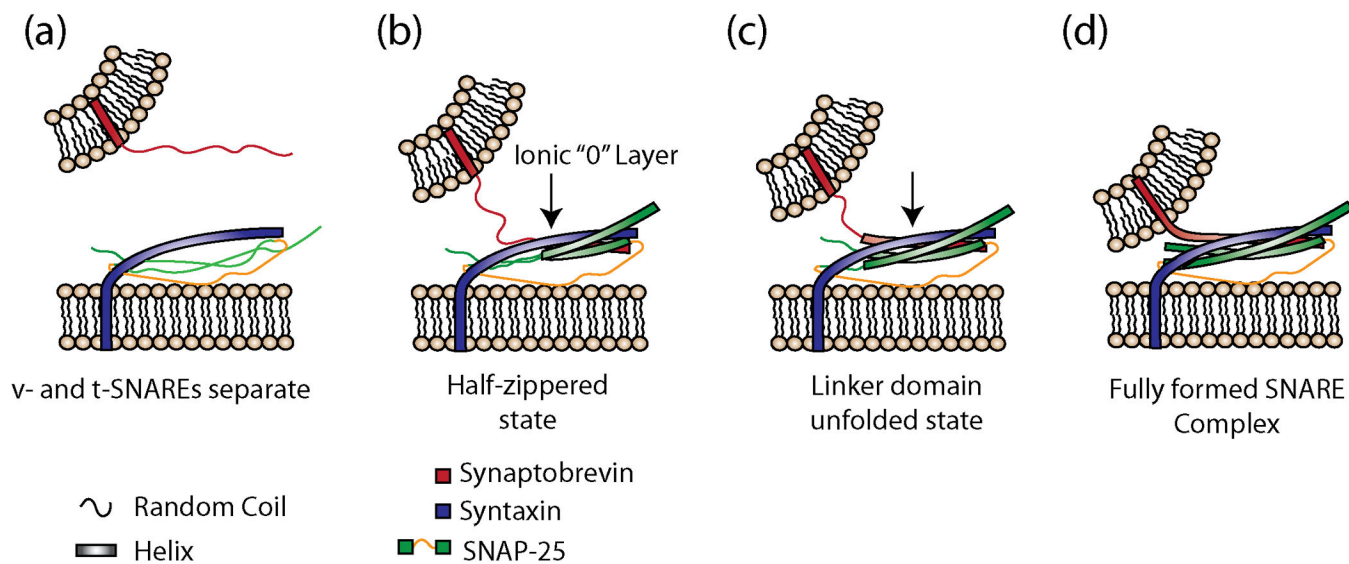


Fig 1. SNARE complex structure formation during membrane fusion. (a) v- and t-SNAREs are separate. (b) Complex formation begins via association of the N-terminal regions of the individual SNARE proteins forming the half-zipped state [26,27**]. This association includes the ionic (0) layer. (c) A second intermediate state has been postulated [26], here called the partially unzipped state. (d) SNARE complex assembly and fusion complete. Here, the SNARE complex is drawn to reflect the conformation as observed in the crystal structure of the neuronal SNAREs [20]. It is still unclear at which point in these steps that membrane hemifusion and fusion occur.

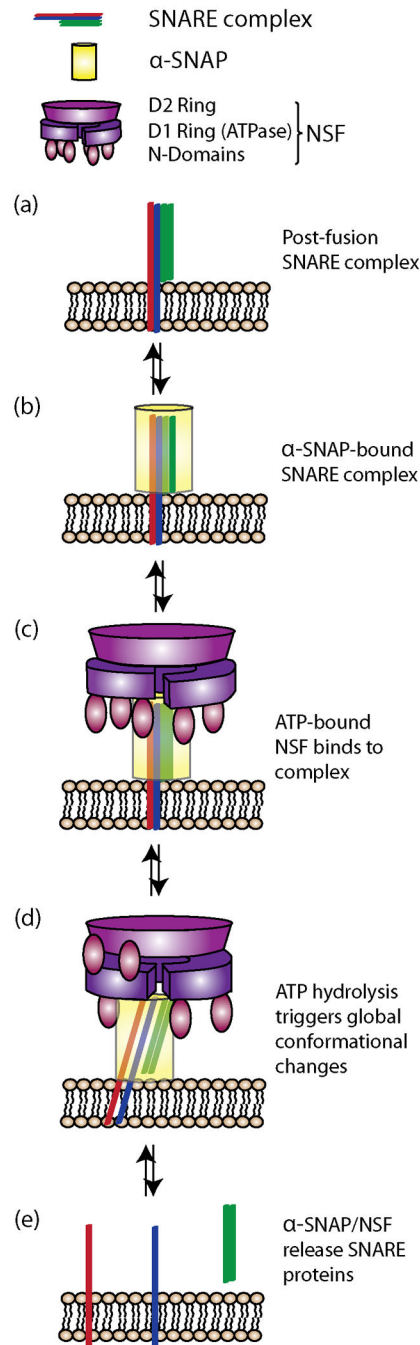


Fig 2. α -SNAP/NSF drive ATP-dependent cis-SNARE complex disassembly via shearing motions. (a) Post-fusion SNARE complex is shown as four parallel rods (omitting the SNAP-25 loop) for clarity of presentation. (b) Four α -SNAP monomers combine to form a collar around the SNARE complex [44**]. (c) ATP-bound NSF binds to the SNARE- α -SNAP complex. (d) A single round of ATP hydrolysis is sufficient to induce structural changes in NSF and α -SNAP, including two of the N-domains binding the D2 ring and the concomitant shifting of α -SNAP. These motions are sufficient to induce shearing and twisting motions in the

SNARE complex. The shearing and twisting motions disrupt the inter-helical interactions and destabilize the four-helix bundle, driving disassembly (e). The exact order of release of the SNARE proteins remains unknown.

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