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At the Junction of SNARE and SM Protein Function

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Summary

Sec1/Munc18 (SM) proteins bind to and function with soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) at each vesicle fusion site in the cell. The purpose for these interactions is becoming clearer, as what had been interpreted as functional divergence between SM proteins acting at different vesicle trafficking steps, or in specialized cells, is giving way to more recent evidence for common functions among all SM proteins. What is emerging is a picture of SM proteins acting not merely as SNARE regulators, but also as central components of the membrane fusion apparatus. The available data suggest sequential models that describe how the soluble SM protein might first regulate SNARE complex assembly and then cooperate with SNAREs to stimulate membrane fusion.

Introduction

Membrane-enclosed vesicles traffic protein and lipid cargo for a wide variety of functions fundamental to eukaryotic cells, including organelle formation and maintenance, neurotransmitter secretion, protein targeting and cell growth. For accurate trafficking, these vesicles rely on conserved families of both soluble and membrane-bound intracellular proteins, which assemble together to form vesicle attachment and membrane fusion complexes in order to merge vesicles with specific target membrane compartments. The conserved nature of intracellular vesicle trafficking proteins suggests that common principles underlie the mechanism of vesicle attachment and membrane fusion [1].

Vesicle trafficking proteins fall into four major categories: i) vesicle-anchored (v) and target-membrane-anchored (t) soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), which bring the two membranes together and catalyze fusion by assembling into tight SNARE complexes through alpha-helical sequences called SNARE motifs [2]; ii) N-ethylmaleimide-sensitive factor (NSF) and NSF attachment proteins (SNAPs), which disassemble SNARE complexes to recycle the SNAREs for another round of fusion [reviewed in [3]; [1]; [4]; [5]]; iii) Rab GTPases and multicomponent vesicle tethering complexes, which orchestrate vesicle attachment and the subsequent assembly of cognate v-t SNARE complexes [reviewed in [6]]; and iv) Sec1/Munc18 (SM) proteins,

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which are soluble factors that may act with the SNARE proteins before and after vesicle attachment.

The exact function(s) of SM proteins has been enigmatic, in part because multiple roles in the steps that lead to membrane fusion have been attributed to SM proteins [reviewed in [7]; [8]], and in part because there are likely some differences in their mechanisms of action. Initial studies supported functional differentiation among SM proteins, as diverse modes of interactions were observed between SM proteins and SNAREs from the syntaxin family, as well as with assembled SNARE complexes [reviewed in [7]]. Yet none of the interactions appeared to be universal: an unsatisfying result, considering the conservation of four SM protein subfamilies throughout the evolution of all eukaryotes [9] [10] [11] and their essential functions for exocytosis (Sec1/Munc18), endocytosis (Vps45), protein biosynthesis (Sly1) and degradation (Vps33).

A string of exciting new results is leading to a more cogent picture of how the functions of SM proteins and SNAREs are coupled. While SM proteins have been viewed in the past as regulators of SNARE function, the emerging picture suggests that SM proteins also cooperate with SNARE complexes to induce vesicle membrane fusion. In this review, we describe some of the recent studies, placing them in the overall context set by the earlier studies, and we discuss potential models for the mechanism of action of SM proteins.

Architecture of SM proteins

The primary amino-acid sequence of SM proteins predicts hydrophilic proteins of approximately 600 amino acids with no recognizable domains or motifs to suggest how SM proteins could promote vesicle attachment or fusion. Structural studies have revealed that the overall fold of SM proteins is highly conserved between different organisms and at different vesicle trafficking steps, supporting a commonality of function(s) [12]; [13,14]. The SM protein structure includes three domains (called domains 1–3) (Figure 1A) that together form an arch shape with a large cavity on one side, and a deep groove on the opposite side, both of which have been implicated in interactions with the SNAREs (Figure 1). Comparison of diverse SM proteins structures reveals a conformational flexibility in the orientations of domain 1 and part of domain 3 around the central cavity [13], which is likely to impact the interactions with SNAREs.

An SM protein bound to the closed conformation of syntaxin

The first connection between SM proteins and SNAREs came from the identification of a tight interaction between the synaptic protein Munc18-1 and its cognate t-SNARE, syntaxin-1, which are both required for neurotransmitter release [15]. This interaction requires a closed conformation of syntaxin-1, whereby an N-terminal three-helix bundle called the H_{abc} domain [16] binds to the SNARE motif, preventing SNARE complex assembly [17]; [18]. A co-crystal structure revealed how closed syntaxin-1 is held by the flexible cavity of Munc18-1 [12] (Figure 1). In this configuration, syntaxin-1 is prevented from premature reassembly with the other SNAREs after disassembly, and the binding interaction stabilizes both Munc18-1 and syntaxin-1 *in vivo* [19]; [20]. At the same time, these findings were puzzling, because an inhibitory role for Munc18-1 is at odds with its essential role in neurotransmitter release [19].

To address this apparent paradox, it was proposed that the Munc18-1/syntaxin-1 structure represented an intermediate in the opening reaction that stimulates SNARE assembly [17]; [12]; [21]. However, recent NMR data indicates that isolated syntaxin-1 forms a weak closed conformation that is only stabilized when Munc18-1 is bound [22], supporting an inhibitory role. Moreover, a mutation designed to open syntaxin-1 increases the probability

of release of synaptic vesicles [20], and another synaptic protein with a key role in release, Unc13/Munc13, has been implicated in opening syntaxin-1 [23]. The MUN domain [24] of Munc13 binds to the SNARE complex and has been proposed to promote assembly [25]; [26]. Other domains of Unc13/Munc13 and diverse factors that bind to them [27]; [28] control distinct forms of presynaptic plasticity, likely by regulating the activity of the MUN domain in opening syntaxin-1 [24]. Hence, the transition from closed to open syntaxin-1 may be a key point of regulation for brain function.

A function for SM proteins in gating SNARE assembly does not, however, appear to be universal. By contrast to syntaxin-1, the closed conformation of the yeast homolog, Sso1p, is highly stable [29], and Sso1p does not bind to its cognate SM protein, Sec1p [30]; [31]. Furthermore, at the yeast vacuole, the syntaxin Vam3p does not adopt a closed conformation [32]. Moreover, several studies showed that most syntaxins bind to their cognate SM proteins using a different mode, involving the syntaxin amino terminus [14]; [33]; [34] (see below).

Taken together, these findings suggested that the Munc18-1/syntaxin-1 interaction arose from specific requirements of regulated exocytosis in higher eukaryotes. However, enticing new data indicate that the regulatory function is not limited to the synapses of higher eukaryotes: the yeast endosomal syntaxin Tlg2p also adopts a closed conformation that binds to its cognate SM protein Vps45p [35], and the same conclusion may apply to their mammalian homologues [36]. Moreover, the Unc13/Munc13 MUN domain was recently found to have homology with components of tethering complexes involved in diverse types of membrane traffic [37], suggesting that the mechanism of syntaxin-1 opening may be shared in other systems. Hence, even if some mechanisms of regulation of the conformational transition of neuronal syntaxin-1 are specific to the synapse, these recent data suggest the Munc18-1/closed syntaxin-1 structure may represent a more general complex than previously suggested.

An interaction with the syntaxin N-terminus

Although it seems clear that binding of SM proteins to closed syntaxin conformations plays an important regulatory role, a more general function for SM proteins may involve an interaction at the syntaxin amino terminus, with a conserved N-peptide motif [reviewed in [38]]. Multiple findings have supported the functional importance of syntaxin N-peptide binding to SM proteins *in vivo*, including the disruption of the Golgi structure and of neurotransmitter release caused by interference with this interaction [34]; [39]; [40]; [41]. However, other studies have revealed milder or no functional effects upon disruption of syntaxin N-peptide binding to SM proteins [42]; [43]; [44]. Although widespread, binding of SM proteins to the syntaxin N-peptide motif is not universal. For example, it is clear that syntaxin N-peptide motifs are not involved in the function of Sec1p [31] and Vps33p [45], two SM proteins that also do not interact with closed syntaxin.

In a recent study, the N-peptide interaction between syntaxin 1 and Munc18-1 was shown to inhibit assembly of syntaxin with other SNAREs, preventing formation of the SNARE complex [36]. Re-examination of the original Munc18-1/syntaxin 1 X-ray diffraction data [12] has revealed how this SM protein simultaneously binds to the syntaxin N-peptide and the helical bundle of the closed conformation [36] (Figure 1A). A view from the membrane end suggests how this configuration might influence SNARE assembly (Figure 1B). While the connection between the N-peptide and the first helix of the H_{abc} domain is not resolved, the SM protein-bound syntaxin may overlap a binding site for an assembled SNARE complex, which has recently been mapped for yeast Sec1p to a nearby “groove” region [11]. Note that, although the interaction of the syntaxin-1 N-peptide with Munc18-1 hinders the

transition from the Munc18-1/closed syntaxin-1 complex to the SNARE complex [36], other factors in the cell, such as Munc13, likely facilitate this transition [25]; [26], and interactions with the N-peptide and H_{abc} domain could keep Munc18-1 bound to syntaxin-1 after opening the closed conformation [39]; [46].

How might N-peptide binding be required for synaptic vesicle fusion? One possibility places the assembly regulation function of syntaxin-binding SM proteins as an obligatory intermediate on the pathway to SM protein stimulated vesicle fusion (Figure 2A, B). Capture of the SM protein by either a tight N-peptide or closed-conformation interaction would not only ensure efficient and highly regulated SNARE complex assembly (and prevention of rapid, inappropriate re-assembly after disassembly), but would also concentrate the SM protein at vesicle docking sites, where it could act on SNARE complexes to stimulate membrane fusion.

SM proteins and membrane fusion

In addition to a regulatory role with syntaxin at the SNARE complex assembly step, there is functional evidence that SM proteins are required at a later step, for fusion of the vesicle and target membranes. In *S. cerevisiae*, *sec1* mutants have been isolated with a temperature sensitive block after SNARE complex assembly, but before secretory vesicle fusion [11]; [47]. These mutations cluster in the groove region of Sec1p (Figure 1B), a region distinct from the sites of *sec1* mutations that block secretion at a step before SNARE complex assembly [11]. Evidence from studies in chromaffin cells also indicates that Munc18-1 acts at multiple steps in exocytosis [48]. Overexpression of the t-SNARE, SNAP-25, rescues the vesicle attachment defect, but not the vesicle fusion defect observed in chromaffin cells from Munc18-1 KO mice [49]. These and other observations [reviewed in [7]] underscore a fundamental requirement of SM proteins for membrane fusion.

Biochemical studies have uncovered a direct interaction with assembled SNARE complexes, placing SM proteins at the site of the membrane fusion reaction. Binding to SNARE complexes was initially observed for two yeast SM proteins, Sly1p [50]; [43] and Sec1p [30]. More recently, a direct interaction with SNARE complexes has been observed for Munc18-1 [51]; [52], Munc18c [53] and Vps45 [42]; [36]; [35]. For many SM proteins, the discovery of an interaction with the N-peptide motif offered a trivial explanation: SM proteins could remain bound to the syntaxin amino terminus, while the carboxy-terminal SNARE motif helix participated in the four-helix bundle that forms the SNARE complex. In this way, the function of the conserved N-peptide interaction would be to concentrate SM protein at membrane fusion sites (Figure 2A). However, as mentioned above, N-peptide binding is not universally required and, therefore, may not represent the configuration required for the membrane fusion function of SM proteins. Moreover, mutations that disrupt binding of Munc18-1 to the syntaxin-1 N-terminal region disrupt vesicle priming at a release-ready state (thought to be the SNARE complex assembly step), but do not affect fusion of primed vesicles, suggesting that the N-peptide interaction represents a function required upstream of membrane fusion [46]. A strong clue for the functional significance of an SM protein-SNARE complex interaction was provided by the finding that yeast Sec1p does not bind the N-peptide or closed conformation of its cognate syntaxin, Sso1p, but instead binds the helical bundle of the assembled SNARE complex [31]. Although Munc18-1 binding to the SNARE four-helix bundle has also been reported [51]; [54]; [55], more studies are required to determine whether the Sec1p result represents a general interaction required for SM protein function in membrane fusion.

Recently, several groups have reported SM protein-stimulation of SNARE-mediated lipid mixing, a limited assay for membrane fusion [56]; [51]; [57]. At high concentrations,

SNAREs promote lipid mixing in the absence of other factors [58], although lipid mixing depends on the physical state of the vesicles and may be accompanied by disruption of membrane integrity [59]; [60]. Uncovering the stimulatory effect of Munc18-1 on SNARE-dependent lipid mixing required lowering SNARE concentrations [51]; [61]. In similar experiments, but with giant unilamellar vesicles, Munc18-1 was found to be required for lipid mixing [62]. In these experiments, the effect of Munc18-1 depended on its interaction with the syntaxin-1 N-peptide [51]; [62]. Subsequent studies of single vesicle fusion events revealed a stimulatory role for Munc18-1 that does not require the syntaxin N-peptide or H_{abc} domain [63], suggesting strongly that Munc18-1 binds and acts with the SNARE complex helical bundle to stimulate fusion. Recent data suggest that Munc18-1 can bind to the membrane-proximal region of the v-SNARE, synaptobrevin [55], which would place Munc18-1 at the end of the SNARE complex adjacent to the membrane, where it could perform its required function in membrane fusion.

In another system, the yeast vacuolar membrane fusion machinery was reconstituted in liposomes to recapitulate the protein and lipid requirements for homotypic vacuole fusion in the cell [57]; [64]. Experiments using this comprehensive reconstitution assay revealed a strict dependence of lipid mixing on the Vps33p-HOPS tethering complex, which is required to promote formation of *trans* SNARE complexes between fusing membranes. In a similar analysis, the reconstitution of the endosomal fusion machinery in a contents mixing assay revealed a strong dependence on a protein complex including the SM protein, Vps45 [54]. However, the specific roles of the SM protein within the vacuolar and endosomal protein complexes remain to be discovered.

Models for SM protein and SNARE induced membrane fusion

Based in part on the results presented above, a consensus is building that SM proteins function both as regulators of SNARE complex assembly at the vesicle attachment step, and as a part of the core machinery at the membrane fusion step. Syntaxin interactions and the involvement of other factors, such as Rab-GTPases and tethering complexes, are likely to underlie the regulatory function, but how might a soluble SM protein cooperate with SNARE complexes to induce membrane fusion?

We envision two hypothetical models. In both models, we assume that the SM protein is attracted to the site of fusion by virtue of binding to the cognate syntaxin or through some other nearby interaction, and that subsequent translocation to a binding site on the SNARE complex helical bundle is key for membrane fusion (Figure 2A,B).

One model (Figure 2A) proposes that the bulk of the SM protein bound to the assembling SNARE complex prevents diffusion of the SNAREs to the center of the intermembrane space, where they could hinder fusion. In this configuration, the SM protein provides a key asymmetry that helps to apply torque to the two membranes [65]. Note that, if Munc18-1 were bound to the membrane adjacent region of syntaxin, it would be difficult to complete C-terminal assembly of the SNARE complex without strongly bending the membranes; in addition, two basic regions of Munc18-1 could participate in membrane bending to induce fusion [55].

A second model (Figure 2B,C) proposes that SM proteins stimulate vesicle membrane fusion by destabilizing a hemi-fusion intermediate state. The conserved helical-bundle shape of the SNARE complex could be responsible for inducing the negative curvature required for the stalk and hemi-fusion membrane intermediates, and there is evidence to support stabilization of hemifusion by *trans* SNARE complex assembly [66–68].

Resolving the hemi-fusion intermediate to complete membrane fusion, however, has been difficult to envision. At low concentrations of fusion proteins, the hemi-fusion intermediate has been shown to become quite stable, if not resolved in a timely fashion [69]; [68]. By binding and changing the shape of the imbedded SNAREs (see legend, Figure 2C), SM proteins could transiently disfavor negative curvature for more positive curvature, much like the effect induced on local positive membrane curvature when attachment proteins bind to receptors [for a review, see [70]]. If the shape change occurs simultaneously for SNARE complexes that rim the intersection of two fusing membranes, a reversal in curvature preference could generate enough outward force to destabilize the hemi-fusion diaphragm and complete the membrane-fusion reaction. Protein-induced curvature at the membrane fusion site has been proposed previously as a mechanism for stimulation of membrane fusion [71]; [72].

Conclusions

Recent work has simplified the picture of SM proteins acting with SNAREs to control vesicle attachment and membrane fusion, yet several questions remain to be answered. Is binding to the syntaxin N-peptide inhibitory or activating? A sequential model for regulation of SNARE assembly, followed by cooperation with SNARE complexes in vesicle fusion, might explain how SM proteins could be both inhibitory and stimulatory, especially in tightly regulated, rapid-response systems, such as synaptic transmission. Is a steric hindrance model or one that involves changes in membrane curvature informative for SM protein activation of membrane fusion, or is the correct mechanism altogether something else? Here, there may be lessons to be learned from membrane fission, where analogous membrane intermediates may be involved. Before vesicle docking, are SM proteins required at an early step, acting with vesicle tethering complexes and Rab-GTPases for selective vesicle attachment to target membranes? The isolation and structural mapping of additional SM protein mutants in model systems will help to separate early and late functions for SM proteins and provide a closer look at the surfaces used for selective vesicle trafficking to and fusion with target membranes. In the near future, we can expect high-resolution structures of SM protein-SNARE complex assemblies to help inform the mechanisms of regulation and fusion activation. The ultimate challenge will be to determine the sequence of steps taken as dynamic interactions between soluble and membrane-anchored proteins force two membranes through the dramatic changes that result in the irreversible and vital consequences of membrane fusion.

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shows that Munc18-1 stimulates adhesion and lipid mixing of t-SNARE containing vesicles with giant vesicles containing v-SNAREs. Under the conditions of these experiments, Removal of the syntaxin H_{abc} domain prevented vesicle adhesion (and lipid mixing), and a mutation in the syntaxin-1 N-peptide disrupted lipid mixing but not adhesion. [PubMed: 18268324]

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Figure 1. Structure of Munc18-1 bound to syntaxin-1

(A) A ribbon diagram illustrates the architecture of the conserved three-domain SM protein, Munc18-1, bound to the helical bundle of syntaxin-1 in the closed conformation. Munc18-1 domain 1 (blue) binds the syntaxin N-peptide (red) and forms an arch with domains 2 (light blue) and 3 (teal). The cavity of the arch binds the syntaxin-1 helical bundle formed by the SNARE motif helix (light pink) bound to the H_{abc} domain (hot pink) in the closed conformation. The location of the groove between domains 1 and 2 and the syntaxin-binding cavity are indicated with arrows.

(B) Rotated view reveals two syntaxin-binding sites and a groove implicated in SNARE complex binding. SM protein Munc18-1 [domains colored as in (A)] is viewed from the membrane end of the closed, 4-helix bundle of syntaxin-1 [colored as in (A)]. The syntaxin N-peptide is bound to domain 1 of Munc18-1, and is connected to the syntaxin-1 H_{abc} helical bundle by an unresolved linker (aa 10–26; hatched line). Mutations in the groove region of a yeast SM protein, Sec1p, abolish SNARE complex binding. The syntaxin-binding cavity and domain 3a are tilted into the page and are, therefore, not visible in this view. Images were created using Pymol™ (DeLano Scientific, Palo Alto, CA) and Protein Data Bank accession number 3C98.

Figure 2. Simplified models for SM protein function in vesicle fusion

Before fusion of the secretory vesicle (1), the t-SNARE syntaxin (magenta) adopts a closed conformation and is sequestered from the other SNAREs by an SM protein (blue ellipse). When the secretory vesicle comes into close apposition with the plasma membrane, the SM protein-bound t-SNARE assembles with the v-SNARE (green), pinning together the two membranes. The SM protein then binds to the SNARE complex and activates membrane fusion using an unknown mechanism.

(A) In one model, the SM protein translocates from the syntaxin N-terminal domain (2) to bind the assembled SNARE complexes at the membrane-proximal end before membrane

mixing (3). Here, the SM protein is proposed to provide leverage for the SNAREs in order to achieve membrane fusion (4) [see ref. 65].

(B) In another model, SNARE complex assembly induces hemifusion (2), before the SM protein translocates from the syntaxin N-terminal domain to the membrane-proximal end of the assembled SNARE complexes, where it stimulates full fusion (3) by altering the curvature preference of the membrane fusion intermediate (C).

(C) How protein shape can change membrane curvature at key steps in fusion. A planar segment of a vesicle membrane (vm) containing unpaired v-SNAREs is shown below the unpaired t-SNAREs in a segment of the plasma membrane [pm; “apposition,” in (B)]. The luminal leaflet of the vesicle and the extracellular leaflet of the plasma membrane are distal (blue), whereas the leaflets facing the cytoplasm are proximal (red) to each other. Each SNARE is anchored across both leaflets of a membrane bilayer by a helical transmembrane domain (black rectangle). In step (1), SNAREs assemble between the two membranes to form *trans* SNARE complexes, and the proximal leaflets fuse to form a transient stalk intermediate. In step (2), the distal leaflets meet in the center of the stalk to form a new bilayer, called the hemifusion diaphragm (hd). The cone shape (triangle with cytoplasmic apex) of the *trans* SNARE complexes stabilizes the hemifusion intermediate by promoting negative curvature in the proximal leaflets [“hemifusion,” in (B)]. In step (3), hemifusion is resolved to full fusion, and the vesicle lumen becomes continuous with the extracellular space [“full fusion,” in (B)]. The SM protein changes the geometry of the SNARE complex from a cone to an inverted cone shape, simultaneously inducing positive membrane curvature (exaggerated to emphasize an outward pulling force) and formation of the *cis* SNARE complex, in order to destabilize the hemifusion diaphragm and complete membrane fusion.