Review

Regulation of SNARE-Mediated Membrane Fusion during Exocytosis

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Regulation of SNARE-Mediated Membrane Fusion during Exocytosis

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Contents

1. Introduction

The primary delivery mechanism for proteins and lipids within the cell is the secretory pathway where vesicular transport intermediates ferry cargo from one membrane-bound compartment to the next. This process requires a carefully orchestrated method to generate these transport vesicles and ensure that they are delivered to the appropriate destination by membrane fusion. Many of the molecular details regarding vesicle production and consumption are known. This Review will focus on the mechanism and regulation of exocytosis, the final step in the secretory pathway. Molecular genetics and cell biology in lower eukaryotes such as the baker's yeast *Saccharomyces cerevisiae* have been complemented with biochemical, electrophysiological, and morphological studies in vertebrate systems, primarily neurons or neuroexocrine cells. The union of these diverse methods and experimental models has identified the basic machinery of exocytosis and provided general mechanisms of action. The additional regulatory mechanisms found in more complex cells appear to be built upon a common foundation found in all eukaryotes. Every eukaryotic cell conducts the business of exocytosis. While model systems have been studied extensively, our knowledge of this process in many other systems remains rudimentary. However, given that the same general principles appear to be operational in yeast and man, one can expect that the same paradigms apply.

James A. McNew was born in Nocona, Texas. He received a B.S. in Biochemistry from Texas A&M University and Ph.D. in Pharmacology from The University of Texas, Southwestern Medical Center, in Dallas. Following postdoctoral training at the Memorial Sloan-Kettering Cancer Center in New York with Jim Rothman, he joined the Department of Biochemistry and Cell Biology at Rice University in Houston, Texas. His laboratory studies protein and lipid trafficking, as well as membrane fusion.

2. Membrane Fusion

The final step of exocytosis is the merger of the transport vesicle membrane and the plasma membrane. This event marks the release of vesicle content to the outside of the cell. In the case of neurotransmission, this is the final step in the conversion of the electrical signal of membrane

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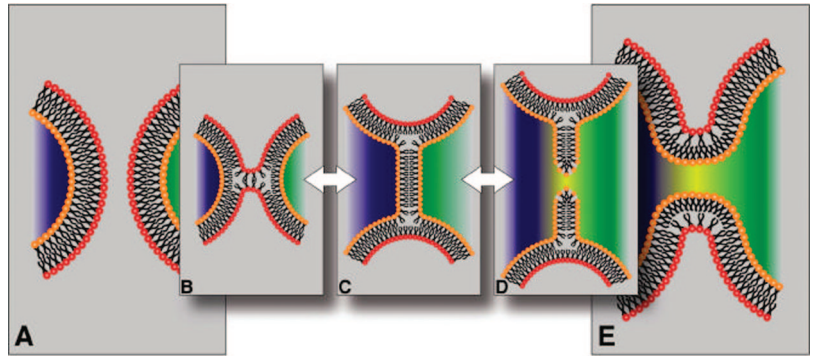

Figure 1. Schematic model of membrane fusion from the perspective of membrane lipids. (A) Close approach of two vesicles with only a portion of each shown. (B) The outer leaflets (red spheres) from both bilayers merge, generating a hemifusion intermediate. (C) The stalk expands to form the hemifusion diaphragm. (D) The hemifusion diaphragm ruptures and inner leaflets mix (orange spheres) resulting in aqueous continuity. (E) Full fusion proceeds to generate a continuous phospholipid bilayer and aqueous content mixing (mixing of green and blue content).

depolarization into a chemical signal deposited into the synaptic cleft.

2.1. Fusion with Model Membranes

The physical process of lipid bilayer fusion has been studied extensively for many years.[1–6] Model membranes containing the appropriate composition of lipids and specific environmental conditions will fuse spontaneously.[7–10] However, all biological membrane fusion is driven by protein catalysts.[11–15] The first protein fusogen to be studied in detail was the hemagglutinin (HA) protein from the enveloped influenza virus.[16,17] Influenza HA is responsible for merging the viral membrane with a cellular membrane during infection. Studies with model membranes, theoretical analysis, and experimental examination of HA-mediated fusion have outlined the model for membrane fusion shown in Figure 1.

2.2. Fusion Intermediates

The bilayer nature of membranes suggests that a multistep process is required for fusion to occur. This hypothesis is supported by a large amount of experimental evidence. First, the outer monolayers of the merging membranes must achieve intimate contact (Figure 1A). After contact, the outermost contacting monolayers (red spheres) merge and progress to a "stalk" intermediate called hemifusion (Figure 1B). The hemifusion stalk expands to form a region where the inner monolayer of lipids (orange spheres) form a pseudobilayer structure (Figure 1C). Rupture of the hemifusion diaphragm (Figure 1D) results in full fusion, enabling complete lipid and aqueous content mixing (Figure 1E). This model presupposes that the initial connection between merging bilayers is lipidic in nature. An alternative view is that a proteinaceous channel forms between membranes during fusion[18] and that the transmembrane segment of the SNARE proteins may serve this function.[19,20]

While many of the details involved in the formulation of the model shown in Figure 1 are derived from theory,[21,22] model membranes,[23] and HA fusion,[3,4,8,24,25] emerging evidence strongly suggests that SNARE protein-mediated membrane fusion transitions through the same spectrum of intermediates indicative of a lipidic connection.[12,26,27]

3. SNARE Proteins

The fusion of all intracellular transport vesicles is mediated by a protein family collectively known as SNAREs (soluble NSF attachment protein receptors).[28,29] It is now widely accepted that SNARE proteins are responsible for membrane fusion in the secretory pathway[30–32] (see section 5 for details.).

3.1. Identification and Classification

The founding members of the SNARE superfamily were originally identified from bovine brain and participate in synaptic transmission.[13,33] SNAREs are operationally divided into two groups: those that are found primarily on the transport vesicle called v-SNAREs and those found primarily on the target membrane, called t-SNAREs. The neuronal SNARE complex contains two t-SNAREs localized to the presynaptic plasma membrane called Syntaxin1A[34] and SNAP25 (synaptosome associated protein of 25 kDa),[35] as well as one v-SNARE located on the synaptic vesicle known as VAMP2 (vesicle associated membrane protein or synaptobrevin).[36,37]

3.2. Nomenclature

While the v- and t-SNARE nomenclature appropriately describes SNAREs that function in the exocytic pathway, this distinction is sometimes blurred with regard to fusion between internal membranes. The distinction of v- and t-SNARE is further confounded when all of the participating SNAREs are resident in the same membrane, as is the case in homotypic fusion, best characterized in yeast vacuolar fusion.[38,39] Other efforts have been made to describe SNAREs on the basis of structural landmarks within various SNARE family members.[40,41] This designation is largely based on the so-called "ionic layer" found in the neuronal SNARE complex.[42] This region of the canonical coiled coil sequence contains polar or charged residues in place of hydrophobic residues. In the neuronal SNARE complex, Syntaxin 1A and both SNAP25 chains have a glutamine (Q) in this location, while VAMP2 has an arginine (R) residue. The "Q-SNAREs" or t-SNAREs are further subdivided into Qa (Syntaxin1a), Qb (Helix A of SNAP25), and Qc (Helix B of SNAP25), and v-SNARE VAMP2 is considered to be an "R-SNARE"[40,43] (see Figure 2A for domain structure). The precise function of this feature is unclear, although its presence is not absolutely required *in vivo*.[44,45]

The Q-SNARE and R-SNARE designation does not hold for all functional SNARE complexes because the yeast ER-Golgi v-SNARE Bet1p has neither a Q nor an R, but an S

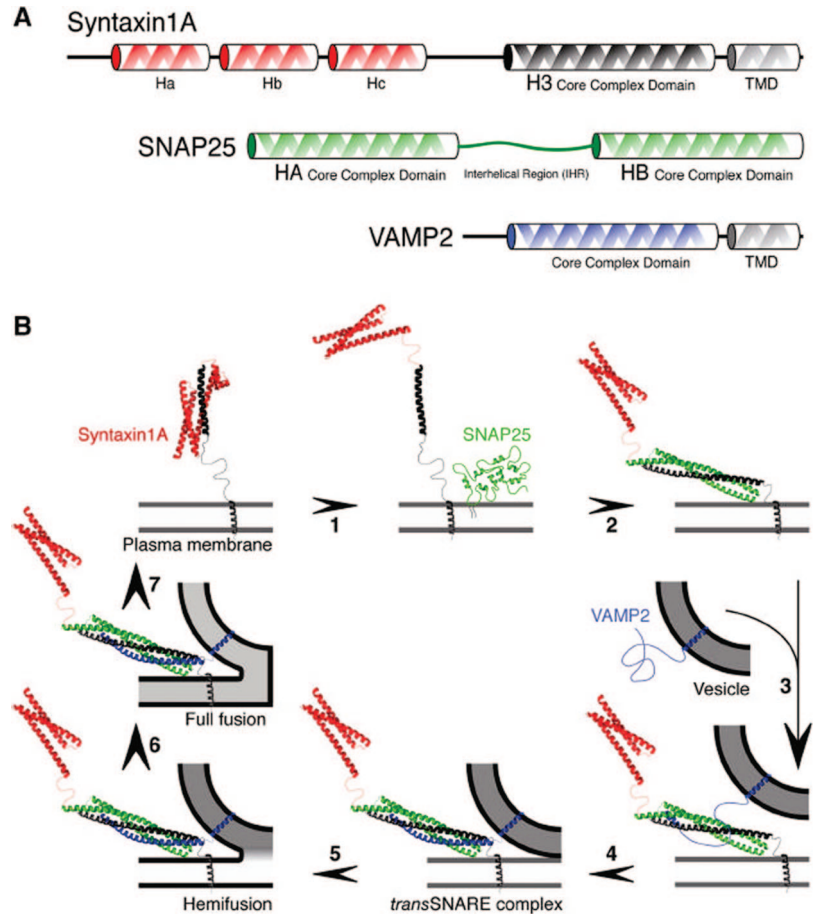

Figure 2. Domain architecture of plasma membrane SNAREs and their proposed assembly pathway. (A) Domain structure. The coiled-coil core complex regions found in the four helix bundle structure are indicated, as well as the autonomously folding N-terminal regulatory domain and transmembrane domain of syntaxin. The helices labeled "core complex domain" indicate the region of these SNARE found in the crystal structure and represent the "SNARE motif". (B) SNARE complex assembly. See text for details. The large-scale movements of the syntaxin N-terminal regulatory domain (NRD, red) are exaggerated for clarity. It is possible that subtle movement within the NRD is sufficient (see Figure 3). The color scheme set in panel A is followed in panel B. The structures depicted in this model were derived from the atomic coordinates 1SFC and 1FLO rendered in MacPymol (http://pymol.sourceforge.net/) and assembled in Adobe Illustrator.

in the relevant location. In addition, the recently solved X-ray structure of a mammalian early endosome SNARE complex composed of Syntaxin 6, Vti1b, Syntaxin 13, and VAMP4 demonstrated that other residues are allowed in the ionic layer. Vti1b contains an aspartic acid (D) residue in this location.[46] Similarly, the v−t-SNARE designation is difficult to apply universally as well because yeast Sec22p, which would have been historically considered the v-SNARE given its homology to mammalian VAMP2, functions as a component of a t-SNARE complex at the *cis* Golgi. However, it is likely that Sec22p does function as a v-SNARE in a different complex involved in retrograde traffic back to the ER.[47] These complexities make a unifying nomenclature difficult.

3.3. Domain Architecture

Syntaxin1A is an ~35 kDa protein with a single transmembrane domain (TMD) located at its extreme carboxy terminus and several regions predicted to form coiled coils (Figure 2A). VAMP2, an ~18 kDa protein, has a similar domain structure. It also contains a C-terminal TMD and a region strongly predicted to form coiled coils. SNAP25 also has two regions predicted to form coiled coils but possesses no hydrophobic segments capable of spanning a bilayer. SNAP25 is attached to membranes via fatty acylation (palmitoylation) of residues within the interhelical region.[48] Extensive sequence comparisons have defined the SNARE motif[41,43] as a 60−70 amino acid region corresponding to the segments of the SNAREs necessary for fusion. The SNARE motif is indicated as the core complex domain in Figure 2A.

Neurosecretion mediated by the neuronal SNARE proteins is the most studied vesicular transport reaction. This transport step has been studied anatomically, electrophysiologically, genetically, ultrastructurally, and biochemically. The SNARE proteins involved in synaptic vesicle fusion were the first to be completely reconstituted in synthetic membranes[29] and were the first SNARE complex characterized at atomic resolution.[42]

3.4. Structural Studies of Exocytic SNAREs

Much is known about the structures of the individual SNARE components. Free Syntaxin1A is almost entirely α-helical,[49] while isolated SNAP25 and VAMP2 are unstructured in solution. Secondary structure is induced in SNAP25 during association with Syntaxin1A and t-SNARE complex formation.[50] Similarly, α-helical structure is induced in VAMP2 as it enters the ternary complex.[50] Analogous experiments with the yeast homologues of these neuronal SNAREs demonstrate comparable properties.[51–53] A critical piece of understanding was revealed by the high-resolution crystal structure of the ternary "core" SNARE complex, a

stable proteolytic fragment of the full complex.[42] The assembled ternary "core" complex was shown to be a parallel ~12 nm long four-stranded helical bundle.[42,54] One of the four helices was contributed by a C-terminal ~80 amino acid segment of Syntaxin1A (also known as the SNARE motif), one from VAMP2 containing about three-quarters of the protein, and two helices from the SNAP25 component of the t-SNARE complex. A striking feature of this structure was that both transmembrane domains from VAMP2 and syntaxin emerged from the same end of the bundle, confirming a parallel orientation of the assembled coils. Structural characterization of other SNARE complexes[46,55–57] has shown a high degree of similarity in structure suggesting that the four-helix bundle originally seen in the neuronal SNARE complex is likely to be the general paradigm.

The energy released by SNARE complex assembly overcomes the repulsive forces between lipid bilayers and drives membrane fusion. For this reason, a thorough understanding of the molecular properties of this complex and its assembly is imperative. Biophysical characterization of both the yeast and neuronal exocytic SNAREs has outlined an assembly process that is supported by both in vitro and in vivo studies. The ordered process begins with the binary association of the syntaxin component and the SNAP25 isoform to form the t-SNARE complex (Figure 2B). In the case of the yeast homologues, t-SNARE complex formation is rate-limiting in the overall process of SNARE complex assembly because binary complex formation is 3 orders of magnitude slower than ternary complex formation.[58]

Syntaxins are clearly the most conformationally dynamic of the SNARE proteins and are known to populate many conformations. Several high-resolution structures have been solved for the syntaxin protein in different contexts. The structure of the N-terminal regulatory domain (NRD, Habc domain), not found in the core structure, was solved by NMR spectroscopy[59] and by X-ray crystallography.[49] Another snapshot of Syntaxin1A was revealed when the structure of a complex between Syntaxin1A and a regulatory protein called Munc18a (n-Sec1) was produced.[60] In addition to the neuronal proteins, a high-resolution crystal structure of the yeast homologue Sso1p has been obtained.[61] When the H3 core domain of Syntaxin1A (Figure 2A) was examined in isolation, homotetramers were identified with two pairs of α-helices in parallel and two antiparallel.[62] The physiological relevance of the structure remains to be determined. While these structures provide valuable information in and of themselves, they also afford a wealth of useful information for structure−function analysis.

3.5. Proteomics and Database Mining

While neurosecretion is a highly specialized process, the same general principles appear to be recapitulated for all intracellular fusion reactions with species and organelle-specific modifications.[13,63,64] SNARE proteins have been identified in all eukaryotic organisms examined.[43] Syntaxin family members are the easiest to identify by primary sequence analysis. While VAMP2 and SNAP25 homologues are readily identifiable, they require more detailed analysis.[41] The yeast *Saccharomyces cerevisiae* contains 24 SNARE proteins, 8 syntaxin family members, and 16 nonsyntaxins, including 13 VAMP-like proteins and 3 SNAP25 family members.[41,47,65–67] Similar bioinformatic analysis has compiled lists of SNARE proteins from all completed eukaryotic genomes including *Arabidopsis thaliana*,[68] *Caenorhabditis elegans*,[69] *Drosophila melanogaster*,[70,71] and *Homo sapiens*.[69] All known SNARE complexes contain four 70−80 amino acid long helices that likely form the same four-helix bundle structure seen in the neuronal SNARE complex. Recent bioinformatics studies have compiled extensive collections of SNARE proteins from many of the fully and partially sequenced genomes.[43,72–75]

4. Model for SNARE Complex Assembly

4.1. Conformational Flexibility of Syntaxin Family Members

Detailed structural analysis of Syntaxin1A and Sso1p reveals that the free proteins, while only 26% identical in primary sequence, adopt an essentially identical fold.[49,60,61] However, this striking similarity in structure is somewhat deceptive. The competing conformations of syntaxin have important functional consequences for t-SNARE complex formation. Kinetic analysis has revealed that yeast Sso1p is closed the vast majority of its lifetime and rarely adopts an open conformation uncatalyzed. The binary rate constant for t-SNARE complex formation (Figure 2B, steps 1 and 2) with full-length cytoplasmic domains is ~3 M^{-1} s^{-1} for Sso1p−Sec9p. When the N-terminal regulatory domain is removed, the t-SNARE complex formation rate is accelerated ~2000-fold to ~6000 M^{-1} s^{-1}, which is very similar to the rate of ternary SNARE complex formation (t-SNARE complex binding to Snc2p).[58] While removal of the NRD of Sso1p resulted in improved t-SNARE complex formation in vitro, expression of this fragment in yeast produced a nonfunctional SNARE protein.[61,76] The primary role of the NRD appears to be the regulated assembly of a functional t-SNARE complex. When t-SNARE complex formation is made intramolecular using a chimeric tandem t-SNARE, the required function of the NRD could be circumvented.[76] The identity of the protein catalyst for Sso1p-opening in vivo still remains an open question. Conversely, t-SNARE complex formation for Syntaxin1A−SNAP25 is only increased by 7-fold in the absence of the NRD.[77] Single-molecule fluorescence experiments have also determined that Syntaxin1A is mostly open (70−85%) and is infrequently in the closed conformation (15−30%) at equilibrium.[77] These data demonstrate that the residence time in the open conformation is very low for Sso1p but is considerable for Syntaxin1A. This relative instability of closed Syntaxin1A is likely used by neurons as an additional point of regulation (see section 7.3.1).

4.2. t-SNARE Complex Formation

For t-SNARE complex formation to occur in the context of neuroexocytosis, the membrane-anchored SNARE Syntaxin1A undergoes a conformational change to allow SNAP25 access to the SNARE core domain of Syntaxin1A (Figure 2B, step 1). This "opening" of Syntaxin1A is presumed to be tightly regulated in vivo. Recent evidence from work in *C. elegans* suggests that the regulatory protein UNC-13 promotes the open state of syntaxin.[78] Once Syntaxin1A is open, two helical segments from SNAP25 associate with the SNARE core domain of Syntaxin1A (Figure 2, step 2). For the yeast syntaxin Sso1p, NMR evidence suggests that the C-terminal region of the H3 core domain remains partially unstructured in the binary Sso1p/Sec9p t-SNARE complex.[52] This possibility is further supported by the structure of

Syntaxin1A in the Syntaxin1A/Munc18a complex.[60] The t-SNARE light chain SNAP25 (and its yeast homologues Sec9p and Spo20p) is mostly unstructured in solution and nucleates α-helix formation when it binds to Syntaxin1A.[50,51] Recent evidence suggests that the calcium sensor synaptotagmin may also play a role in t-SNARE complex formation.[79]

While the binary t-SNARE complex composed of the soluble domains of the yeast plasma membrane SNAREs Sso1p and Sec9p is a 1:1 complex,[58] the homologous neuronal t-SNARE complex forms a mixture of 1:1 and 2:1 complexes where two syntaxin soluble domains form a four-helix bundle with SNAP25.[50,80] This 2:1 Syntaxin1A:SNAP25 complex likely represents a dead end intermediate;[81] although, it has been the source of some confusion regarding the interpretation of in vitro experiments.[82,83] It should also be noted that this observation might be relevant to the requirement for coexpression of full-length Syntaxin1A and SNAP25 for fusion competent t-SNARE complex in vitro (see section 5.3).

The primary evidence that the functionally relevant plasma membrane SNARE complex is the Syntaxin1A and SNAP25 heterodimer is largely derived from in vitro studies examining the interactions of recombinant SNAREs.[82] For example, the only stable complex formed by any of the three neuronal SNARE proteins is the Syntaxin1A/SNAP25 t-SNARE complex.[50] In addition, the folding and unfolding kinetics of these SNAREs suggests that Syntaxin1A/SNAP25 is a binary receptor for VAMP2.[84]

4.3. Ternary SNARE Complex Formation

The assembled t-SNARE complex serves as a platform to receive the incoming vesicle bearing SNARE (v-SNARE), specifically, VAMP2 in the neuron (Figure 2B, step 3). Similar to the t-SNARE light chains, the v-SNARE is largely unstructured in solution[50] and nucleates α-helix formation upon binding to the t-SNARE complex. It is likely that assembly begins at the distal tip (N-terminus) of the SNARE domains and "zippers" toward the C-terminus.[85–87] However, some EPR data suggest that there is little directionality during SNARE assembly.[88] The metastable, fully zippered *trans*-SNARE complex (Figure 2B, step 4) exerts force on the phospholipid bilayer to drive membrane fusion through a hemifusion intermediate (Figure 2B, step 5 and 6). An alternative view is that SNAREs may not always directly provide energy input, rather that they promote a permissive environment for lipid mixing and fusion to occur.[89,90] The *trans*-SNARE complex is converted to a *cis*-SNARE complex following bilayer mixing, when both transmembrane domains reside in the same membrane. The tremendously stable *cis*-SNARE complex is resolved into individual subunits, and the system is reset with the energy investment of ATP hydrolysis by the AAA-ATPase NSF and the cochaperone α-SNAP (Figure 2B step 7).

5. Evidence that SNAREs are the Fusogens for Intracellular Transport

While most agree that the SNARE proteins themselves are the catalysts doing the mechanical work of membrane fusion, this view is not yet universally accepted.[91–99] The body of evidence supporting a direct role for SNARE protein in membrane fusion is substantial and continues to grow.

5.1. Genetic Studies

The earliest evidence supporting a role for SNAREs in membrane fusion were derived from mutant studies in lower eukaryotes including yeast, worms, and flies. Temperature sensitive or other loss of function alleles of SNARE proteins have been identified and characterized phenotypically, biochemically, morphologically, and electrophysiologically. In addition to gene deletions, many other loss-of-function alleles have also been examined. Some of these include temperature-sensitive alleles generated by random mutagenesis, as well as directed point mutations designed to disrupt specific protein−protein interactions.[100–103]

In general, loss of t-SNARE components seems to be more severe than v-SNARE proteins, perhaps because of amplified functional redundancy. For example, double knockouts of the functional redundant plasma membrane syntaxins in yeast *SSO1/SSO2* are lethal.[104] Similarly, loss of the SNAP25 homologue necessary for vegetative growth, *SEC9,* is also lethal.[105] Surprisingly, loss of the functionally redundant VAMP2 homologues *SNC1/SNC2* results in a conditional lethal phenotype.[106] Yeast lacking *SNC1/SNC2* massively accumulate 80 nm post-Golgi secretory vesicles (as do deletions of the other SNAREs) illustrating a strong defect in secretion, yet this strain is viable on minimal media at reduced temperature. One plausible explanation for this observation is that another v-SNARE is capable of providing sufficient v-SNARE function to maintain viability. This possibility is supported by the finding that mutation of lipid-modifying enzymes improved the survivability of the *SNC1*Δ/*SNC2*Δ strain.[107] Specifically, mutations of enzymes involved in the production of long chain fatty acids were identified in a second-site suppressor screen of the conditional lethal phenotype. Membranes of the secretory pathway compartments become progressively thicker as they approach the plasma membrane and SNARE localization may be determined, in part, by the physical thickness of the membrane read out by the length of the transmembrane domain.[108–110] The "thinning" of the membrane resulting from mutations in fatty acid elongation enzymes could improve the mislocalization of another v-SNARE to post-Golgi vesicles. Two potential candidates are Sec22p and Nyv1p, both of which are capable of driving fusion with the Sso1p/Sec9p t-SNARE complex in vitro.[11] While appealing, this hypothesis remains untested.

SNARE gene disruptions and mutations have also been examined in multicellular organisms. In addition to simple life or death questions, these organisms provide the ability to more closely examine other phenotypic consequences, as well as specifically monitor exocytic events by electrophysiology. One of the first reported SNARE deletions in a multicellular animal was in the fruit fly *D. melanogaster*. Complete loss of the plasma membrane syntaxin syx1a in *D. melanogaster* was found to be embryonic lethal.[112] The null embryos were immobile, failed to secrete cuticle, and showed altered gut morphology, as well as other abnormalities. Many of these phenotypes can be explained by abnormal secretion, although other secretory activity such as the salivary gland appeared to be intact. In addition to the overall developmental defects in the embryo, electrophysiological examination of neuromuscular junctions (NMJ) in animals that lack syx1a demonstrated that all neurotransmitter release is abolished. This included both stimulus-evoked release as well as spontaneous miniature synaptic potentials (minis). The severity of the syx1a deletion in flies, contrary to other

organisms discussed below, may be because *D. melanogaster* only expresses two plasma membrane syntaxins, syx1a and syx4. Unlike mammalian neuronal Syntaxin1A, *D. melanogaster* syx1a appears to be required in many cellular locations other than the synapse including epidermal cells as evidenced by abnormal cuticle secretion.

Deletions in the other plasma membrane t-SNARE component SNAP25 displayed very different effects than syx1a. The SNAP25 null was viable through embryogenesis and larval stages with relatively normal neurotransmission at NMJs in third instar larvae, yet the flies died as pharate adults.[113] This result was somewhat surprising given that a previously characterized temperature-sensitive allele of SNAP25 produced a much more severe phenotype.[114] The SNAP25ts allele was paralyzed at elevated temperatures and showed reduced synaptic transmission at 37 °C compared to 22 °C, indicating an important role for SNAP25 in synaptic vesicle fusion. These seemingly contradictory results were also the product of genetic redundancy. A SNAP25 paralog, SNAP24,[115] provided t-SNARE function in the case of the SNAP25 null through larval development but fails to do so acutely in the case of the temperature-sensitive allele.[113]

Lastly, deletion of the synaptic vesicle SNARE n-syb was also embryonic lethal, but neuronal development and NMJs appeared normal.[116] n-syb null flies also were paralyzed, and no evoked release was detected at the NMJ; however, unlike the syx1a deletion, spontaneous release (minis) persisted albeit at a reduced (75%) rate. This observation suggested that a different mechanism for evoked and spontaneous neurotransmitter release might exist. *D. melanogaster* expresses two plasma membrane synatobrevins, one expressed ubiquitously (syb or c-syb)[117] and one restricted to neurons (n-syb).[118] Elegant experiments examining the selective loss of syb or n-syb in eye cells of *D. melanogaster* illustrated that loss of syb was cell lethal and is likely required for general secretion, while n-syb is specifically involved in regulated neurosecretion.[119] This work also showed that *D. melanogaster* syb, mouse VAMP2, or mouse VAMP3 could also functionally substitute for n-syb complementing neurotransmission. Some have argued that this data indicates that SNAREs lack specificity or are promiscuous.[120] Others have more openly ridiculed the entire premise of SNARE specificity.[121] More likely, the observation that syb (VAMP3) can functionally replace n-syb (VAMP2) reflects the compartmental nature of membrane fusion. First, given the high degree of identity among all exocytotic VAMPs, this result is not particularly surprising. In addition, homologous SNAREs from different species readily drive fusion in vitro.[122,123] Unquestionably, other proteins (and lipids) are important for fusion and targeting specificity; however, a clear distinction should be made about compartmental specificity with regard to SNARE specificity. In essence, the fusion of a constitutive secretory vesicle or a synaptic vesicle with the plasma membrane is a compartmentally analogous event. This is fundamentally different from the fusion of an intra-Golgi transport vesicle with the next Golgi cisterna, for example. The discrimination of a constitutive post-Golgi secretory vesicle and a synaptic vesicle may, in part, lie in SNARE interactions but are more likely to be governed by the regulatory machinery controlling SNARE assembly (see section 5). The distinction of an intra Golgi transport vesicle from an ER-derived transport vesicle or a post-Golgi secretory vesicle by their target membrane is primarily determined by SNARE interactions.[111]

Similar genetic studies quickly followed in *C. elegans*. Neuronal synaptobrevin (snb-1) in *C. elegans* was essential for viability.[124] Although embryos developed normally, including normal cuticle secretion and properly formed synaptic connections, the dying L1 larva only had a limited capacity to move, were very uncoordinated, and could not feed; however, they were not completely paralyzed. These animals also had severe synaptic transmission defects as measured by electrical activity in the pharynx. The worm neuronal plasma membrane syntaxin (unc-64) deletion also dies as L1 larva.[125] Similar to the snb-1 deletion, these embryos developed normally and synaptic connectivity was intact. In this case, the dying larvae are completely paralyzed and show critical synaptic transmission defects. While it has been mentioned that loss of SNAP25 (ric-4) is also lethal,[126] no detailed analysis of a deletion ric-4 has been reported. As was the case in yeast, v-SNARE deletions are somewhat less severe than mutations in plasma membrane syntaxins.

Gene deletion studies in mice have largely focused on the SNAREs and regulatory proteins involved in synaptic transmission. Transgenic mice with single-gene deletions in virtually all synaptic vesicle proteins have been reported.[127] These include plasma membrane v-SNAREs VAMP2,[128] VAMP3 (cellubrevin),[129] and the t-SNARE components Syntaxin1A[130] and SNAP25.[131] VAMP2 homozygous null animals die shortly after birth with relatively minor developmental defects, while SNAP25 deficient mice were embryonic lethal. The SNAP25 null animals exhibited several developmental abnormalities, but the nervous system developed normally. Syntaxin1A and VAMP3 deletions are viable. Electrophysiological analysis of the VAMP2 and SNAP25 nulls showed a selective loss of stimulus evoked synaptic vesicle release, while minis were observed at reduced rates, very similar to the *C. elegans* and *D. melanogaster* synaptobrevin loss of function mutations. Recent work has also demonstrated functional redundancy between VAMP2 and VAMP3 in mice,[132,133] very similar to experiments performed in *D. melanogaster*.[119]

The consensus view is that deletion of nonredundant SNARE proteins is lethal, although the manner of death and severity of neurotransmission defects are somewhat variable from electrically silent to profound effects on stimulus evoked release.

5.2. Bacterial Neurotoxins

Some of the earliest evidence that SNARE proteins were important for secretion came from analysis of the mechanism of action of certain bacterial neurotoxins, namely, the toxins from the species *Clostridium tetani* and *botulinum* (Clostridial neurotoxins, CNTs). These organisms express zinc endoproteases that are exquisitely specific for a single protein.[134–136] Extensive analysis revealed that the targets of these proteases were different SNARE proteins. Tetanus toxin (TeNT)[137] and the B, D, F and G serotypes of the *C. botulinum* neurotoxins (BoNT/B,[138] BoNT/D BoNT/F,[139] and BoNT/G[140]) specifically cleave the v-SNARE VAMP2 at different locations. BoNT/A[141] and BoNT/E[142,143] cleave the t-SNARE SNAP25, while BoNT/C[144,145] cuts both t-SNAREs Syntaxin1A and SNAP25.

Neuronal intoxication with these toxins causes a specific loss of neuronal exocytosis measured by a number of techniques. Actions that reverse toxin activity are all related to SNARE proteins.[135] For example, coinjection of a peptide

spanning the toxin cleavage site[146] or an antibody to VAMP2[147] both prevent toxin activity and restore exocytosis.

In addition to their pathophysiological roles, the CNTs have been used cell biologically to more precisely define the role of SNARE proteins in exocytosis. Prior to the genetic analysis of the n-syb deletion in *D. melanogaster*, transgenic expression of tetanus toxin in fly neurons produced a phenotype strikingly similar to n-syb null.[148] Microinjection or addition of toxin to permeabilized cells has also been used to examine SNARE function.[87,89,149,150] The addition of BoNT/E to permeabilized PC12 cells strongly inhibited neuroepinephrine secretion, which could be almost completely restored by the addition of a C-terminal peptide of SNAP25 equivalent to the cleavage product of BoNT/E.[149] This "cracked cell" system has been used very extensively to conduct structure/function analysis of specific residues in the SNAP25 C-terminal helix.[89,149] Microinjection of different toxins has also been used to indirectly examine the N- to C-terminal zippering of the SNARE complex because only free SNARE, not SNAREs in complex, are susceptible to toxin cleavage.[87]

5.3. Functional Reconstitution of SNARE-Mediated Membrane Fusion

All of the data presented thus far strongly shows that SNARE proteins have an important role in secretion very proximal to the event of membrane fusion; however, secretion is a multistep process, and no in vivo experiments can rule out all possible indirect effects. To show a direct role in membrane fusion, biochemical reconstitution experiments with purified proteins and synthetic lipids were used. The neuronal t-SNARE complex containing Syntaxin1A and SNAP25 were coexpressed in bacteria and were reconstituted in proteoliposomes, and the v-SNARE protein VAMP2 was incorporated into a different proteoliposome population. Coexpression of full length Syntaxin1A and SNAP25 was required for a fusion-active t-SNARE complex.[29] This requirement may be derived from the tendency for free Syntaxin1A to self-associate[62,81] in ways that prevent subsequent binding of SNAP25. Coexpression is not required for fusion when the SNARE motif of Syntaxin1A is used[26] or the yeast plasma membrane t-SNARE complex, which forms fully functional t-SNARE complex in detergent solution, albeit very slowly.[58,111,122] The difference in stability of the closed conformation (see section 4.1) may also be responsible for this disparity. This technical hurdle has yet to be circumvented for the neuronal SNARE complex and has generated results that are difficult to interpret without this realization.[151]

A FRET-based lipid mixing assay was used to demonstrate that SNAREs are the minimal protein machinery for membrane fusion.[29] Since the original description of SNARE-mediated fusion, in vitro fusion assays have been used to examine the contribution of SNAREs to the specificity of membrane fusion,[111] address mechanistic questions, and structure/function studies,[12,26,86,152–155] as well as identify new functional SNARE complexes.[38,155–159] More recently, this technique has been used to examine the role of fusion regulators such as synaptotagmin,[79,160–164] SM proteins,[123,165] and complexin.[166] Modified versions of this liposome assay have now been used by at least eleven independent groups, some autonomously developed, to identify the formation of hemifusion intermediates during fusion,[12,26] increased liposome size following fusion,[167] and fusion of SNARE-containing liposomes to native membranes[168] and to explore the consequences of SNARE reconstitution approaches[92] or SNARE regulatory proteins on the fusion reaction.[79,161–166,169]

More recently, microscopic, rather than spectroscopic, techniques have been applied to in vitro membrane fusion. The fusion of proteoliposomes with recombinant SNAREs reconstituted into planar bilayers has been examined by total internal reflection microscopy (TIRF).[170,171] This technique allows the analysis of single vesicle fusion events, which have been used to determine the rate of SNARE-mediated fusion. While the fusion measured by FRET-based solution fusion assay occurs on the time scale of minutes, TIRF experiments have shown that recombinant SNARE can fuse vesicle with 10−25 ms rates.[170,171] This apparently large discrepancy in kinetics can be readily understood when the different methods are carefully considered. By definition, the TIRF experiments measure single vesicle fusion events, while slower ensemble solution assay measures the fusion of a large ($>10^{12}$) population of liposomes.

5.4. Arguments for Non-SNARE Fusion

Paradoxically, some of the most compelling data supporting a direct role for SNAREs in membrane fusion and one of the first suggestions that SNAREs are not the fusogen are derived from similar studies of homotypic yeast vacuolar fusion. Deletion of the relevant SNAREs Vam3p or Nyv1p strongly suggested that SNARE engagement was critical for vacuolar fusion in vitro.[172] However, these data were later reinterpreted as a simple docking requirement in light of new data examining SNARE complex formation.[173] This latter work suggested that SNARE complexes bridging vacuoles could be separated prior to membrane fusion by the SNARE chaperones Sec17p (α-SNAP) and Sec18p (NSF), yet fusion proceeded unabated.[173] The identity of these SNARE complexes as *trans*SNAREs (i.e., **between** vacuolar membranes) rather than *cis*SNARE complexes (within the same membrane, a normal product of the fusion reaction, Figure 2B) relied on the "fusion inhibitor" microcystin LR (MCLR). These data were taken to mean that fusion persists in the complete absence of assembled SNAREs.

The possibility that SNAREs were not the molecules driving membrane fusion launched a series of studies by this group and others to identify the protein(s) responsible for membrane merger.[174–180] This journey began by the suggestion that calcium and, subsequently, calmodulin were required for vacuolar fusion.[177] The requirement for calmodulin was then traced to an interaction with the vacuolar ATPase in the vacuolar membrane, specifically the V0 sector, which was then suggested to be the relevant membrane fusion protein.[176] Further support for this model was provided by mutants in *D. melanogaster* that also indicated that V0 played a role in membrane fusion in the eye.[180] While many counterarguments were made to interpret the V0 results without invoking a direct role in fusion,[13,181] there was enthusiasm for this non-SNARE model.[182]

Recently, several of the foundational discoveries suggesting that the V0 ATPase is responsible for membrane fusion have been reevaluated. First, the so-called fusion inhibitor MCLR used to discern the role of *trans*SNARE complexes[173] has been shown to directly inhibit the enzyme alkaline phosphatase (ALP), the assay readout for vacuolar fusion. In the process of developing a new assay for vacuolar fusion, Wickner's group has found that MCLR (as well as the other fusion inhibitor GTPγS) inhibits ALP enzymatic activity

rather than membrane fusion per se.[183] Hence, membrane fusion proceeds normally in the presence of MCLR and GTPγS, but measurement of membrane fusion by activation of ALP is impaired. This observation readily explains the ability of Sec17p/Sec18p to resolve *cis*SNARE complexes in ongoing fusion reactions that were previously thought to be *trans*SNARE complexes.[173] Next, the calcium requirement for vacuolar fusion that led to calmodulin involvement and eventually V0-ATPase has also been reassessed. Vacuolar fusion was found to be sensitive to the calcium chelator BAPTA, implying a calcium requirement.[184,185] In addition, vacuoles, a known calcium storage organelle, were shown to efflux calcium following SNARE assembly.[184] However, BAPTA inhibition of fusion was determined to be caused by reasons other than calcium chelation. While BAPTA inhibition can be overcome by the reintroduction of calcium, it can also be entirely circumvented by the addition of the soluble SNARE Vam7p.[186] These and other observations make it difficult to maintain the argument that the V0-ATPase plays a direct role in membrane fusion.

5.5. Cell−cell Fusion by "Flipped" SNAREs

Finally, one of the most recent and compelling demonstrations of SNARE sufficiency for fusion was their ability to promote cell−cell fusion when ectopically expressed on the surface of tissue culture cells.[187] In this study, the neuronal exocytotic SNARE protein Syntaxin1A, SNAP25, and VAMP2 were engineered to be expressed in the cell surface, topologically opposite to their normal orientation in the membrane. When cells expressing the t-SNARE complex were cocultured with cells expressing VAMP2, cell−cell fusion occurred. This fusion was sensitive to normal inhibitors of SNARE function, such as soluble SNAREs.

These results, in addition to the tremendous weight of the experimental genetic, biochemical, cell biological, morphological, and electrophysiological evidence presented above, overwhelmingly argue that SNAREs are the mechanical machine that does the work of membrane fusion.[187]

6. Non-neuronal Plasma Membrane and Internal Membrane SNARE Complexes

6.1. Examples of Non-neuronal Plasma Membrane SNARE Complex Functions

While neuronal plasma membrane SNAREs have received the most attention, other regulated post-Golgi fusion events are beginning to be better understood with regard to SNARE function. It is clear that all cells need to deliver vesicle-enclosed material to the plasma membrane constitutively; however, specialized fusion events are more prevalent than just neurotransmission. One example that has received recent attention is the regulated translocation of glucose transporter (GLUT4) to the plasma membrane in insulin responsive cells.[188–192] Adipocytes and myocytes use a highly specialized facilitative glucose transporter-4 (GLUT4) for the insulin-stimulated uptake of glucose.[193] In the absence of extracellular cues, GLUT4 is mostly sequestered inside intracellular organelles, including trans-Golgi network (TGN), recycling endosomes, and tubulo-vesicular structures.[194] This exclusion of GLUT4 from plasma membrane is the result of reduced exocytosis and rapid endocytosis.[195,196] Insulin triggers these GLUT4 containing vesicles to be rapidly transported to plasma membrane. The insulin-induced fusion between GLUT4-storage vesicles (GSV) and the plasma membrane is mediated by the plasma membrane t-SNAREs Syntaxin4, a paralog of neuronal Syntaxin1A, and SNAP-23, a SNAP25 paralog. The v-SNARE present in the GSV is the same VAMP2 present in synaptic vesicle. Botulinum neurotoxin D (BoNT/D), which specifically cleaves both VAMP2 and VAMP3, completely abolished insulin-stimulated translocation of GLUT4 to plasma membrane in permeabilized 3T3-L1 adipocytes.[197] Similar results were observed when permeabilized adipocytes were treated with soluble VAMP2 or soluble Syntaxin4.[197] Additional evidence for the involvement of Syntaxin4 was provided by the observation that the mobilization of GLUT4 was unaffected by BoNT/C, which cleaves Syntaxin1A/1B, Syntaxin2, and Syntaxin3 (but not Syntaxin4).[197] SNAP25 is not expressed in adipocytes; however, its paraolog SNAP23 is abundantly expressed. SNAP-23 is primarily localized to the plasma membrane and is able to interact with Syntaxin4 and VAMP2 to form a functional SNARE complex.[156,198,199] The insulin-induced GLUT4 translocation in 3T3-L1 adipocytes can be inhibited by microinjection of anti-SNAP23 antibody or a synthetic SNAP23 C-terminal peptide[199] or by overexpression of C-terminally truncated SNAP23.[198] These studies provide strong evidence that Syntaxin4, SNAP23, and VAMP2 have physiological roles in mediating the fusion between GSV and the plasma membrane in response to insulin.

Other examples of regulated secretion driven by SNAREs includes translocation of the gastric proton pump, a H,K-ATPase, in parietal cells.[200] The parietal cell appears to be the only epithelial cell type known to express all four of the plasma membrane syntaxins, Syntaxins1, 2, 3, and 4.[201,202] RNA interference (siRNA) mediated knockdown of Syntaxin3 (but not Syntaxin 2), inhibited histamine stimulated acid secretion in parietal cells.[203] In addition, the introduction of the recombinant cytoplasmic domain of Syntaxin3 into streptolysin O (SLO) permeabilized rabbit gastric glands inhibited acid secretion.[204] These data suggest that Syntaxin3, SNAP25, and VAMP2 are responsible for the regulated deposition of the gastric H,K-ATPase in the plasma membrane of parietal cells.

Finally, continuing studies are defining the SNARE participants involved in regulated secretion in several cell types including platelet secretion of dense granules (Syntaxin2/SNAP23-VAMP3) and α-granules.[205] The assignment of SNARE function in some regulated secretory systems is complicated by the ability of these cell types to undergo compound exocytosis, which includes vesicle-vesicle fusion as well as vesicle-plasma membrane fusion.[206] These cell types include mast cell degranulation where Syntaxin4/SNAP23 and VAMP8 appear to be operational[207] and zymogen granule secretion from pancreatic acinar cells,[208,209] where Syntaxin3 functions in granule−granule fusion[210] and Syntaxin2, SNAP23, and VAMP8 drive fusion with the plasma membrane.[210–212] More work will be necessary to define precisely the role of all SNARE contributors.

6.2. Subunit Composition and Methods of Membrane Attachment

While the major focus of this review is exocytosis, SNARE proteins are involved in every membrane fusion reaction in the entire secretory pathway. SNAREs are differentially distributed throughout the secretory endomembrane system and come together in many different combinations to provide

Table 1. SNARE Regulator Proteins

class	regulator	interaction partner	ref
SM proteins	Munc18a	Syntaxin 1A, SNAP25, VAMP2, t-SNARE complex, ternary SNARE complex	60, 123, 276
	Sec1p	Sso1p/Sec9c t-SNARE complex, ternary SNARE complex	165, 240
complexins	CpxI-IV	Ternary SNARE complex	293, 294, 296
synaptotagmins	Synaptotagmin I	Syntaxin 1A, SNAP25, t-SNARE complex, ternary SNARE complex	34, 330, 332, 333
Munc13	Munc13-1	Syntaxin 1A	287
ion channels	N-type Ca^{2+} channel	Syntaxin 1A	339
	voltage-gated K-channel	Syntaxin 1A, SNAP25	340, 341
botulinum toxins	BoNT/A,E	SNAP25	135
	BoNT/B,D,F,G and tetanus	VAMP2	135
	BoNT/C	Syntaxin 1A, SNAP25	135
SNAPs	α,β,γ	free SNAREs, ternary SNARE complex	28, 342
Lgl family	Mlgl	Syntaxin 4	343
	Tomosyn	Syntaxin 1A, t-SNARE complex	344, 345
	Sro7	Sec9p	346, 347
Tethering proteins	p115	Syntaxin 5, GOS28	232
	GARP/VFT	Tlg1p	229, 230
	Dsl1	Ufe1p	231
coat proteins	Sec23/24	Bet1p, Sed5p, Sec22p	223, 224
coat regulators	ARFGAP	Free SNAREs?	225, 226
miscellaneous	amisyn	Syntaxin 1A, Syntaxin 4	348
	rabphilin	SNAP25	349−351
	synaptophysin	VAMP2	352−354
	$G\beta\gamma$	Syntaxin 1A, SNAP25, VAMP2, t-SNARE complex, ternary SNARE complex	355
	synip	Syntaxin 4	356
	snapin	SNAP25	357, 358
	HRS2	SNAP25	359
	syncollin	Syntaxin 1A	360
	calmodulin	VAMP2	361

specificity to membrane fusion and compartmental identity. In some ways, the neuronal SNARE complex is more the exception than the rule. SNARE complexes that drive fusion on internal membranes are almost exclusively composed of four separate proteins, each contributing a single SNARE domain helix to the overall four helix bundle.[38,157] In addition, many methods of membrane attachment are employed, ranging from all four SNAREs containing transmembrane domains to one or more SNAREs containing sites for isoprenylation (Ykt6p),[213] fatty acylation (Ykt6p, SNAP25, SNAP23, Sso1/2p, Tlg1p, Snc1/2p),[214–218] or specific lipid binding (Vam7p, Spo20p),[219,220] as well as soluble SNAREs (Sec9p).[105]

6.3. Combinatorial SNARE Complexes on Internal Membranes

SNARE complexes that drive fusion within the secretory pathway are much more combinatorial in nature than the plasma membrane SNAREs. For example, many, if not all SNARE complexes within the Golgi contain the t-SNARE Sed5. One yeast t-SNARE complex located on the *cis* face of the Golgi is composed of Sed5p bound to Bos1p and Sec22p. This t-SNARE complex fuses with vesicles bearing Bet1p.[155] While this topological arrangement appears likely in yeast, arguments have been made that these four proteins are required in mammals, but mammalian Sec22 may be the vesicle SNARE.[221] Another SNARE complex likely participating in transport within the Golgi contains Sed5p, Gos1p, and Ykt6p as a t-SNARE complex with Sft1p providing v-SNARE function.[157] A similar combinatorial code is probably operational within the endosomal/vacuolar system as well. For instance, a t-SNARE complex on late endosomes composed of Pep12p, Tlg1p, and Vti1p[222] shares subunits with two other t-SNARE complexes: Vam3p, Vam7p, and Vti1p on the vacuole[38] and Tlg2p, Tlg1p, and Vti1p on early endosomes.[158]

7. Regulation of SNARE Complex Formation

7.1. SNARE Interacting Proteins

While it is clear that SNAREs provide the mechanical force required for membrane fusion, it is also clear that they do not work alone in the cell. More than twenty regulatory proteins have been identified that interact with individual SNAREs and those that interact with the assembled SNARE complex. Two protein families have emerged as the primary players at all fusion steps involving SNARE proteins. These include the SM (Sec1/Munc18) family of regulatory proteins and the Rab family of small GTP-binding proteins. Additional regulatory factors have been identified that are either organism-specific or compartment-specific, such as those that have evolved specifically to regulate the speed and efficacy of synaptic transmission. The complex interactions between the SNARE proteins and SNARE regulators serve to ensure that SNARE assembly occurs in the correct temporal and spatial pattern. A complete list of proteins known to bind to SNAREs is shown in Table 1.

7.2. Rabs, Coats, and Tethers

Coat proteins, tethering proteins, and Rab proteins play critically important roles in the process of vesicle docking and attachment. Increasing evidence suggests that coat components play a more active role in SNARE function than previously appreciated. It is clear that coat proteins have a defined role in recruiting and including SNAREs into transport vesicles. Biochemical and structural studies have shown that selective SNARE packaging and exit from the

ER into COPII coated vesicles is directed by the COPII subcomplex Sec23p/24p.[223,224] However, new studies imply that regulators of coat initiation such as the ARFGAPs may directly bind and catalytically influence SNARE conformations.[225,226]

Vesicle tethers are important for providing spatial control. These activities can be attributed to single fibrous proteins such as p115, giantin, and the golgins,[227] which are important within the Golgi complex, as well as large mega-Dalton complexes such as the Exocyst, TRAPP, and others.[228] SNAREs have also been shown to interact with the tethering machinery.[229–231] In some cases, these interactions actively promote the formation of *trans*SNARE complexes.[232]

Rab proteins are still a bit enigmatic, although absolutely required for vesicle docking and fusion.[233,234] An emerging view is that Rabs communicate between the vesicle tethering apparatus and the SNARE machinery.[235] This process may also aid in determination of the specificity of vesicle trafficking, one of the original models for Rab function.[236,237]

7.3. Direct SNARE Regulators

The best-characterized SNARE regulators are those that directly interact with and regulate the SNARE assembly cycle (Figure 2B). These include the SM protein family, the calcium sensor synaptotagmin, and the fusion clamp complexin.

7.3.1. SM proteins

SM (Sec1p/Munc18) proteins are a class of relatively large (~68−80 kDa) cytosolic proteins known to be essential regulators of SNARE protein function. The first SM gene was discovered in 1974 in a *C. elegans* mutant screen to map genes of the nervous system by EMS mutagenesis.[238] Several phenotypes were observed in these mutants, including an uncoordinated (unc) phenotype that deviated from the pattern of motion observed in wild-type worms. The mutation termed unc-18, which was later classified as an SM gene, resulted in total paralysis.[238] The yeast unc-18 ortholog *SEC1* was identified in 1979 in a screen for temperature-sensitive secretion-deficient strains of *S. cerevisiae* that accumulated post-Golgi secretory vesicles at the restrictive temperature.[239] Thus, SM proteins were functionally implicated in secretion from their initial identification.

SM protein isoforms function at all trafficking steps of the secretory pathway. Most species contain between four and seven SM genes functioning at different transport steps. *S. cerevisiae* expresses four SM proteins: Sec1p functions at the plasma membrane,[165,240,241] Sly1p regulates ER to Golgi transport,[242,243] Vps33 controls vacuolar traffic,[244,245] and Vps45p operates in the TGN/endosomal system.[246–248] While Vps33p can be readily identified as an SM protein in BLAST searches, functional evidence that it is a true SM protein is somewhat lacking. In addition, Vps33p has features not described for other SM proteins, such as its ability to bind ATP[249] and its residence in a complex with three other proteins Vps11p, Vps16p, and Vps18, the so-called Class C VPS complex.[250] Like Sec1p, Sly1p, Vps33p, and Vps45p are conserved evolutionarily, pointing to a universal role for SM proteins in the secretory pathway. Three isoforms of the plasma membrane SM proteins, Munc18a (Munc18-1), Munc18b (Munc18-2), and Munc18c (Munc18-3) are expressed in mammals in a tissue-specific manner. Munc18a is localized primarily to brain tissue,[251] while Munc18b and Munc18c are expressed ubiquitously; however, platelets express all three isoforms.[252] Munc18a also plays a role in insulin secretion from pancreatic β-cells.[253,254] Munc18b has been implicated in secretion of saliva,[255] mucous in lung tissue,[256] translocation of H^+-K^+-ATPase in gastric parietal cells[203] and in blood platelets.[257]

Although the precise function of SM proteins is still debated, they perform an apparently essential role in secretion because they are required for life. Deletion of the yeast *SEC1* gene or ROP (Ras opposite) in *D. melanogaster* results in death.[258] Surprisingly, the unc-18 knockout in *C. elegans* is viable, but the worms display severely lowered levels of neurotransmission and accumulate the neurotransmitter acetylcholine.[259] Deletion of the Munc18a gene in mice completely eliminates neurotransmitter secretion, which results in complete paralysis and suffocation soon after birth, although the brain develops normally.[260] Primary chromaffin cells in culture from Munc18a knockout mouse show reduced Ca^{2+}-dependent exocytosis of large dense core vesicles by a factor of 10.[261]

The history of SM protein function has been confused by paradoxical in vitro and in vivo data.[262–265] Most evidence suggests that SM proteins act through SNARE proteins, although SM proteins also interact with non-SNARE proteins.[266–268] Munc18a was originally identified by its association with Syntaxin1A,[251,269,270] and the biochemical interaction of Munc18a and Syntaxin1A has been extensively studied culminating in an atomic model of the binary complex solved by X-ray crystallography.[60] Many in vitro studies have suggested that Munc18a binds to the closed conformation of Syntaxin1A, thereby stabilizing the "off" conformation of Syntaxin1A.[271,272] As illustrated in Figure 2B, the N-terminal domain of Syntaxin1A must move to allow SNAP25 access to the H3 SNARE core domain of syntaxin. This movement could be prevented by Munc18a, essentially disallowing the formation of a t-SNARE complex with SNAP25 by Syntaxin1A. The extensive characterization of the Munc18a/Syntaxin1A interaction has led to a putative inhibitory role for Munc18a, despite its apparently positive role shown in vivo.

Analysis of the Munc18 homologue ROP in *D. melanogaster* led to additional conflicting results.[103,273] Overexpression of ROP reduced neurotransmission that could be compensated by coordinate overexpression of fly Syntaxin1A (syx1A), indicating that the ratio of syntaxin and ROP are important. However, this observation may not be general for all SM proteins since overexpression studies in other organisms have shown different results.[165,261,274] Phenotypic characterization of four ROP point mutants in flies also revealed contradictory behavior.[273] Two mutations, P254S and R50C, both of which are homozygous viable at room temperature, displayed improved neurotransmitter release, while two others, H302Y and D45N, which are homozygous lethal, inhibited synaptic transmission at the larval neuromuscular junction (NMJ). These results suggest that ROP executes two functions that can be genetically separated by these mutations. Subsequent studies analyzing a specific mutation in fly syx1A(I236A) also suggested that ROP interaction with syntaxin was inhibitory for neurotransmission.[103] Electrophysiological examination of NMJs in the syx1A(I236A) mutant showed enhanced synaptic transmission. This increase in synaptic transmission was interpreted to be caused by the inability of syx1a(I236A) to bind to ROP. However, this interpretation must be carefully considered because other work has suggested that the interaction

between ROP and syx1A is unaffected by the I236A mutation or the homologous I233A in mammalian Syntaxin1A.[275] This observation raises a larger issue of interpretation of in vivo results on the basis of the negative in vitro binding data that is discussed below.

Recent work suggests that SM protein binding is not limited to free syntaxin proteins. The first indication that SM proteins bind other SNARE conformations was derived from immunoprecipitation experiments in *S. cerevisiae* examining Sec1p.[240] This work showed that Sec1p associated with the fully assembled ternary SNARE complex, although it was unclear if this interaction was meaningful. Next, it was shown that a functionally relevant mode of Sec1p interaction was through the assembled t-SNARE complex (Sso1p/Sec9c) and this interaction stimulated in vitro fusion.[165] Ternary SNARE complex binding was also documented, although this is likely the end result of the functional bridging of t-SNARE complex and v-SNARE. Ensuing studies with mammalian proteins have confirmed and extended these observations in the yeast system. Munc18a has now been shown to also bind to the assembled t-SNARE complex (Syntaxin1A/SNAP25) and stimulate in vitro fusion with the neuronal SNARE proteins.[123] In addition, a complex containing Munc18a, Syntaxin1A, and SNAP25 has been detected by NMR.[276] Furthermore, SM protein interaction with v-SNARE proteins has also been documented in other systems. While a direct interaction between Sec1p and Snc2p was not detected in yeast exocytosis,[165] a similar interaction between the endosome SM protein Vps45p and Snc2p has been demonstrated.[277] SM protein binding to the t-SNARE complex likely exists in native membrane as well since SNAP25 is able to bind to a Munc18a/Syntaxin1A heterodimer in exposed plasma membrane sheets.[278] This mechanism of action is probably general since other SM proteins like the non-neuronal plasma membrane SM protein Munc18c also interact with the assembled Syntaxin4/SNAP-23/VAMP2 *cis*SNARE complex.[279]

The fact that Syntaxin1A binds tightly to Munc18a is unquestioned. Examination of the crystal structure of this dimer suggests that there are many interactions between Munc18a and Syntaxin1A that include the Habc domain, as well as H3 core domain. However, another mode of SM protein interaction with a free syntaxin family member has also been seen structurally and biochemically. This association mechanism involves extreme N-terminal residues from the syntaxin interacting with the SM protein in different manner than that seen in the Munc18a-Syntaxin1A structure.[242,280] The apparently different mode of interaction within the same type of protein dimer has made understanding the family as a whole somewhat challenging. However, it is possible to reconcile these seemingly disparate interaction modes with a simple extrapolation. It is possible that Syntaxin1A also interacts with Munc18a via N-terminal residues. Perhaps this interaction is less stable for Syntaxin1A and Munc18a than other interactions allowing it to be disordered in the crystal and not resolved. The generality of N-terminal binding is quite appealing and is supported by functional evidence. Mutation of a single residue, L8A, in Syntaxin1A eliminates the ability of Munc18a to stimulate in vitro fusion. This residue was chosen on the basis of the structure of Sly1p bound to a Sed5p peptide and was confirmed by the structure of Munc18c bound to the N-terminal peptide of Syntaxin 4. This leucine is conserved in most plasma membrane syntaxin including *D. melanogaster* and *C. elegans*; however, it is notably absent in Sso1/2p from yeast, which has a minimal ability to form a binary complex with Sec1p, the SM protein involved in exocytosis in yeast. In addition, removal of the entire N-terminal regulatory domain (NRD) also prevents stimulation of in vitro fusion by Munc18a.[123] Recent NMR evidence supports the hypothesis that the extreme N-terminus of Syntaxin1A interacts with Munc18a in a manner analogous to Syntaxin4-Munc18c or Sed5p and Sly1p.[276] This study also found that Munc18a interacts with the fully assembled SNARE complex.

This hypothesis has profound implications for in vitro binding experiments between Syntaxin1A and Munc18a, as well as the interpretation of in vivo experiments derived from the expression of mutants whose binding properties were based on in vitro binding. Wildtype interactions between Syntaxin1A and Munc18a can be defined by three general regions including the possibility of multiple contacts at any given site: (1) extreme N-terminus, (2) the Habc three-helix bundle, and (3) the H3 SNARE domain. In vitro binding may require at least two of these contact points for a stable interaction. The vast majority of in vitro binding studies uti lize GST-Syntaxin1A as a binding partner.[203,268,271,275,281–286] When Syntaxin1A is tagged with N-terminal GST, one potential interaction is unknowingly lost. Mutations in Munc18 that disturb interactions with the Habc three-helix bundle (for example Munc18a-E59K), H3 interactions (R59C), or mutations in Syntaxin1A ("open" mutants L265A/E166A) now completely lose their ability to interact. However, if the extreme N-terminal mode of interaction remains intact, as it would be in vivo (in most cases) Syntaxin1A would still bind to free Munc18a and perhaps t-SNARE complex and ternary SNARE complex. These possibilities are currently being examined.

Invoking a Syntaxin1A N-terminal interaction with Munc18a also allows for new interpretations of Munc18a t-SNARE complex binding. The interaction of Munc18a with N-terminal amino acids of Syntaxin1A may allow t-SNARE complex formation between Syntaxin1A and SNAP25 without dissociation of Munc18a. A regulatory factor, perhaps Munc13,[78,287] interacts with the Syntaxin1A/Munc18 binary complex and alters the association of the Habc domain without changing the extreme N-terminal interaction, keeping Munc18a bound but liberating the Syntaxin1A-H3 SNARE domain. SNAP25 then is free to bind to the available H3 domain of Syntaxin1A while still bound to Munc18a. This likely changes the contacts of the H3 domain with Munc18a as well. The NRD effectively "rolls" out of the way to allow SNAP25 binding. One possible structural scenario is outlined in Figure 3. Panel A shows the structure of the Syntaxin1A/Munc18a heterodimer.[60] A potential mechanism for the movement of the NRD is shown in Panels B and C. First, helix c (cyan) pivots approximately 18° with the N-terminus of this helix being the pivot point. This movement is followed by a rotation of helix a and b (orange and magenta) of about 45° out of the plane of the page. This change stretches out the loop-helix-loop (red) that connects Hc with H3 in Syntaxin1A and effectively moves the NRD (Habc) away from the H3 core domain allowing access of SNAP25. This theoretical structure would be Munc18a bound to the t-SNARE complex (Figure 3D). The location of SNAP25 precludes contact between SNAP25 and Munc18a but allows VAMP2 to access to the N-terminal region of the core bundle to allow SNARE zippering (Figure 3E). The extreme N-terminus of VAMP2 would also be capable of initial

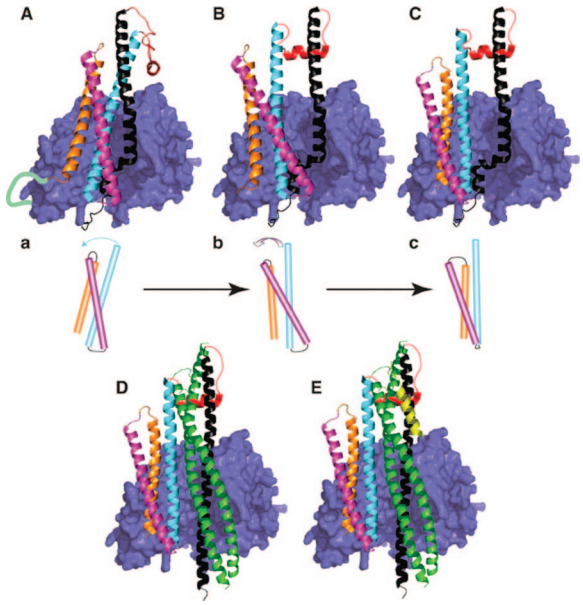

Figure 3. Model for Munc18a-stimulated membrane fusion. (A) The structure of Munc18a bound to Syntaxin1A (1DN1) is shown where Munc18a is colored blue and shown as a surface representation. The colors of the N-terminal regulatory domain (NRD, Habc) of Syntaxin1A have been altered for clarity. The most N-terminal helix (helix a) is orange; helix b is magenta, and helix c is cyan. The short loop-helix-loop connecting helix c with the H3 core domain is red, and the H3 core itself remains black. The extreme N-terminal residues of Syntaxin1A, which likely interact with Munc18a, are shown as a thick pale green line. (B−D) Hypothetical structures illustrating movements that would allow SNAP25 binding to Syntaxin1A bound to Munc18a. (B) First, the entire NRD pivots about 18° with the fixed pivot at the N-terminus of helix c. (C) Next, helices a and b rotate approximately 45° out of the plane of the page. (a−c) Cylindrical cartoons of the NRD selectively illustrating the motions within and among the NRD helices. (D) The C-terminal region of Syntaxin1A straightens as SNAP25 binds to Syntaxin1A, forming a t-SNARE complex bound to Munc18. SNAP25 makes no contact with Munc18a. This model of t-SNARE complex was derived from 1SFC removing the VAMP2 helix. (E) The positioning of the N-terminal region of the t-SNARE three-helix bundle bound to Munc18a exposes the groove occupied by VAMP2 allowing binding. A short piece (residues 25−45) of VAMP2 (yellow) is shown to illustrate initiation of *trans*SNARE complex formation. As the VAMP2 helix twists into the t-SNARE complex during zippering, C-terminal regions of the VAMP2 helix interact with Munc18a.

associations with Munc18a prior to or coordinated with the initiation of SNARE core zippering. Given that VAMP2 twists as it assembles into the helical bundle structure, the C-terminal regions of the VAMP2 core helix may also interact with Munc18a. This final step may help to explain the effects of point mutations located in the C-terminus in VAMP2 that abolish Munc18a stimulation in vitro.[123] While the models are speculative, they suggest several avenues of experimental testing.

7.3.2. Complexin

The complexins (also known as synaphins) are a family of small (14−20 kDa) proteins found in all multicellular eukaryotes. Complexins are primarily restricted to the nervous system; however, they have been identified in testis,[288,289] pancreatic beta cells,[290] and other cells that perform regulated secretion such as mast cells.[291]

Complexins were originally identified by their interaction with the neuronal SNARE core complex.[292,293] Much is known about the biophysical characteristics of complexin and its interaction with the SNAREs. A central ~58 amino acid α-helical segment of complexin I binds in an antiparallel orientation to the neuronal SNARE core complex.[294–296] The complexin helix occupies the groove formed by Syntaxin1A and VAMP2 in the four-helix bundle and stabilizes the overall SNARE complex.[56,296] The specificity of the complexin interaction suggests that both VAMP and syntaxin contribute to complexin binding,[294] as would be expected on the basis of its location in the crystal structure. While complexin binding stabilized the ternary SNARE complex,[296] it did not accelerate the rate of SNARE complex formation.[295]

Early studies examining the role of complexins in regulated secretion yielded mixed conclusions. Complexin proposed functions ranged from competition with the SNARE chaperone α-SNAP[293] to facilitation of oligomerization of SNARE complexes.[297] Overexpression of complexin in chromaffin cells,[298] PC12 cells,[299] and microinjection into *Aplysia* neurons[300] has shown that increasing the amount of complexin reduces exocytosis, suggesting that complexin may inhibit fusion. Similarly, recombinant CpxII inhibits acrosomal exocytosis in streptolysin O permeabilized human sperm.[289] Mice lacking Cpx II are phenotypically normal, while the single CpxI deletion is more severe. CpxIΔ mice exhibit severe ataxia and seizures and die with 2−4 months. In addition, acrosomal exocytosis is inhibited in sperm from CpxI knockout mice, supporting a required role for Cpx in membrane fusion.[288] The CpxI/II double knockout mice die within a few hours of birth.[301] However, analysis of primary hippocampal neurons from complexin I/complexin II (CpxI/CpxII) deficient mice suggested that complexins have a positive role. Electrophysiological studies in cultured autaptic synapses showed that exocytosis is somewhat impaired in the absence of CpxI and CpxII; however, this reduction in exocytosis could be corrected by increasing external calcium,[301] although the complexins do not bind calcium ions. Unfortunately, analysis of the CpxI/CpxII double knockout has been complicated by the discovery of two additional complexin isoforms (CpxIII and CpxIV).[302] CpxIV appears to be specifically expressed in the retina, while CpxIII is expressed in the retina, as well as many parts of the brain including the hippocampus.[302] While CpxI and CpxII are soluble proteins, CpxIII and CpxIV contain a CaaX box specifying posttranslational modification by isoprenylation for membrane attachment.[303] The functional consequences of complexin membrane attachment remain to be explored.

The first analysis of a complete complexin null was very recently reported in *D. melanogaster*.[304] The *D. melanogaster* genome contains only one complexin gene,[70,71] and it encodes a protein that is most homologous to human CpxI (40% identical, 49% similar); however, it also contains a CaaX box similar to CpxIII and CpxIV. *D. melanogaster* is predicted to express six transcripts of the Cpx gene,[305] and two lack the exon encoding the CaaX sequence; therefore, both membrane-anchored and soluble versions of Cpx likely exist. The complete loss of complexin function resulted in a semilethal phenotype and dramatic neurosecretory defects. Null animals exhibited a substantial increase (>20-fold) in spontaneous fusion (minis), while evoked response was reduced in the presence of calcium. In addition, the complexin nulls had neuronal developments defects that manifested as a profound increase in the total number of synapses. The data provides a compelling argument that complexin is

normally a negative regulator whose absence results in improperly regulated spontaneous fusion.

Recent biochemical work has begun to clarify the molecular role of complexin. Three studies in very different experimental systems arrived at the conclusion that complexin acts as a fusion clamp.[166,306,307] Complexin was found to inhibit SNARE-mediated fusion in vitro and the fusion was inhibited at hemifusion.[166] Similarly, ectopic expression of complexin on the surface of tissue culture cells inhibited cell-cell fusion by flipped SNAREs.[187,306] In this model, cellular fusion induced by cell surface SNAREs was significantly inhibited with recombinant complexin I added to culture medium and essentially abolished when CpxI was also attached to the exoplasmic face of the plasma membrane by the addition of a glycosylphosphatidyl inositide (GPI) anchor. Another approach used a chimeric protein that fused complexin I to the N-terminus of VAMP2. When this chimera was expressed in virally infected primary dissociated cortical neurons from mice, a significant (~80%) reduction in neurotransmitter release was observed.[307] Inhibition was derived from the complexin portion of the chimeras since a similar fusion protein containing mutations that disrupt complexin interaction with SNAREs was without effect.

Recently, the function of mouse complexin I mutants was examined using lentiviral infected primary neurons from the CpxI/CpxII double knockout mice.[308] This study found that the central α-helix of complexin that binds to the SNARE complex[56,296] was necessary but not sufficient for complexin function. In addition to SNARE binding, an N-terminal "accessory α-helix" was required for function and the effect of its inclusion was inhibition of fast synaptic transmission. Again, these data must be interpreted with caution because residual complexin activity is likely provided by CpxIII and CpxIV, given the definitive results of the *D. melanogaster* null phenotype.

These biochemical and structural results, taken together with the phenotypic analysis of complexin null flies, suggest very strongly that the molecular function of complex is to stop or "clamp" the fusion process at the very last step of vesicle fusion to prevent vesicle release until calcium influx (see below).

7.3.3. Synaptotagmin

The synaptic vesicle resident protein called synaptotagmin has emerged as the calcium sensor for fast synaptic transmission.[309] Synaptotagmin (Syt) was originally identified as a 65 kDa (p65) component of rat synaptic vesicles that was recognized by a specific monoclonal antibody.[310] It was later cloned,[311] named synaptotagmin, and its primary sequence characterized as containing region of homology to protein kinase C (C2 domains).[312] The domain structure of synaptotagmins consists of a short N-terminal sequence that is within the lumen of the synaptic vesicle, a single transmembrane domain, and a cytoplasmic sequence that contains two C2 domains, C2A and C2B, from N- to C-terminus. Synaptotagmin I (SytI, p65) was the founding member of a growing family of synaptotagmin isoforms. Currently, there are thirteen isoforms identified and perhaps six more from partial sequences.[313]

SytI is, by far, the best characterized isoform due to its intimate involvement in fast calcium stimulated neurotransmission.[314] It is a fairly abundant protein representing ~7% of total SV protein,[315] which corresponds to about fifteen copies per synaptic vesicle.[316] The atomic structure of the C2A[317] and C2B[318] subdomains has been solved, as has the entire cytoplasmic domain of SytIII.[319] Recently, the complete soluble domain of SytI was also solved.[320] C2 domains are calcium binding motifs and six isoforms of synaptotagmins are known to bind calcium. The C2A domain of SytI binds three calcium ions,[321] while the C2B domain binds two[318] for a total of five calcium binding sites. SytI also binds avidly to membranes, specifically negatively charged phospholipids including phosphatidylserine and polyphosphatidyl inositides.[322,323] Lipid binding improves the apparent affinity for calcium,[324] and the presence of bound calcium significantly improves lipid binding.[322]

In addition to calcium- and lipid-binding activities, SytI also interacts with a variety or protein partners. Synaptotagmins interact with themselves to form both hetero- and homooligomers.[325] Calcium-independent homomultimers require the sequences in the N-terminus[326] of the protein, while calcium-dependent oligomerization requires the cytoplasmic domain.[327,328] The observation that native synaptotagmins oligomerize[328] and that calcium-binding mutants that disrupt oligomerization reduce secretion in vivo suggests that this property is functionally relevant.[329] Synaptotagmins also interact directly with the SNARE fusion machinery. SytI binds to the C-terminal SNARE domain of Syntaxin1A,[34,330] the extreme C-terminus of SNAP25,[331,332] the binary Syntaxin1A/SNAP25 t-SNARE complex, and the fully assembled ternary SNARE complex.[333] SytI is also capable of binding both the assembled SNARE complex and membranes simultaneously.[333] SytI-SNARE interactions are also regulated by calcium.[330,332] A very recent study has defined specific point mutations within C2A and C2B that specifically disrupt calcium-dependent SNARE interactions but that do not affect lipid binding.[161] These mutants successfully demonstrated that calcium-mediated SNARE interactions are an important function of synaptotagmin in vitro and in vivo.

Genetic studies in a variety of organisms support the proposal that SytI functions to couple calcium influx with synaptic vesicle fusion.[334–336] Deletion of the SytI gene in mice is lethal.[334] Electrophysiological analysis of primary hippocampal neurons from knockout mice showed that fast synchronous synaptic transmission was severely impaired. Similar observations were made for large dense core vesicle (LDCV) exocytosis in chromaffin cells isolated from the SytI null.[337] Further support for a calcium sensor function for SytI was derived from the introduction of mutant forms of SytI in these same chromaffin cells lacking endogenous SytI. While the wildtype protein was capable of restoring calcium sensitivity to LDCV secretion, mutants that eliminated calcium binding did not, even in the presence of photoinducible calcium release effectively rule out upstream events involved in the generation of calcium transients.[337]

A calcium-dependent stimulatory role for synaptotagmins has also been provided by in vitro reconstitution studies. In vitro fusion driven by the neuronal SNARE protein, but not their yeast counterparts, is significantly stimulated in a concentration dependent manner by the addition of SytI in the presence of calcium[162,164] In the absence of calcium, SytI is slightly inhibitory.[162,169] Importantly, stimulation required the presence of phosphatidylserine in the liposome, emphasizing the importance of lipid binding to SytI function.[162] However, lipid binding is not sufficient for calcium dependent stimulation of fusion as some have suggested.[338] SytI mutants that have lost calcium-dependent interaction with

SNARE but retain full lipid binding are unable to simulate in vitro fusion.[161] Subsequent studies have shown that other synaptotagmin isoforms also display this property, specifically SytVII and SytIX. In fact, the different Syt isoforms exhibit a very large difference in response to divalent cations. SytVII showed maximal stimulation at a half-maximal calcium concentration of 0.3 μM, compared to 116 μM for SytI in this assay.[163] Finally, recent work has shown that SytI cooperates intimately with complexin to regulate membrane fusion. As previously discussed, complexin inhibits SNARE-mediated fusion[306] at hemifusion.[166] SytI, in the presence of calcium, is able to overcome this inhibition[166,306] and allows the SNAREs to progress to full fusion. How complexin arrests SNAREs at the stage and the mechanism of SytI release are actively being investigated.

8. Conclusions

The process of exocytosis continues to fascinate and captivate generations of researchers. With a relatively complete "parts list" in hand, we are now challenged with the task of fitting together the many pieces of this extraordinarily complex puzzle. Improved technology and novel approaches will paint an increasingly detailed picture of the molecular interactions necessary to deliver lipids and proteins to the extracellular environment. The SNARE proteins, now 15 years old as a family, will complete their teenage years with continued scrutiny. Much remains to be discovered. It is now clear that the SNAREs are the mechanical nanomachine that executes the task of membrane fusion. However, this "brawn" must be controlled, and we are only beginning to scratch the surface with regard to how this occurs. An increasingly complicated network of regulatory proteins closely inspects and manages the activity of the SNARE proteins. These regulatory "brains" receive input from multiple pathways ultimately coordinating the fusion process and exocytosis.

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