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Membrane fusion: SNAREs and regulation

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Abstract. SNARE (SNAP receptor) proteins drive intracellular membrane fusion and contribute specificity to membrane trafficking. The formation of SNAREpins between membranes is spatially and temporally controlled by a network of sequentially acting accessory components. These regulators add an additional layer of specificity, arrest SNAREpin intermediates, lower the energy required for fusion, and couple membrane fusion to triggering signals. The functional activity of some of these regulators determines the plasticity of regulated exocytosis. (Part of a Multi-author Review)

Keywords. Exocytosis, fusion, vesicle, tether, synaptotagmin, SM protein, Munc13, complexin.

Introduction

To transfer contents and lipids between distinct biological compartments or to generate compartments from preexisting ones, lipid bilayers have to fuse in a controlled manner. This fundamental principle of life is exploited in the simple reproduction of bacteria by cell division and the merger of gametes to generate progenies. In eukaryotes, membrane fission and fusion processes recur at numerous transport steps to move cargo between intracellular organelles, to transmit information between cells and organs, and to respond to external stimuli. Membrane fission within a single membrane produces transport vesicles or viruses, whose properties usually differ from the compartment of origin. In this process, coat-like structures shape the membrane into symmetric vesicular or tubular structures, sort cargo, and finally physically 'push' the membrane together, to create two separate compartments [see accompanying review by Anne Spang]. In contrast to membrane fission, fusion processes merge two compartments, which requires specific compartment recognition and a machinery that 'pulls' the membranes together. Membrane fusion between cells, viruses and cells, or transport vesicles and intracellular organelles employs distinct molecular machines. However, intracellular membrane fusion along the endocytic and secretory pathway and cytokinesis in eukaryotic cells is conserved and uses SNAREs to drive fusion. The only intracellular compartments that do not depend on SNAREs are chloroplasts and mitochondria, consistent with the endosymbiotic theory. In this review we will focus on the mechanistic principles that underly SNARE-mediated membrane fusion and its regulation.

SNAREs are the membrane fusion machinery

SNAREs (SNAP receptors) have been identified in a search for membrane receptors that bind SNAPs (soluble NSF attachment proteins) and NSF (N-ethylmaleimide sensitive factor) [1]. SNAREs constitute a compartment-specific protein family with 25 members in *Saccharomyces cerevisiae*, 36 members in *Homo sapiens,* and 54 members in *Arabidopsis thaliana* (for review see [2]). Most SNAREs are small type II membrane proteins. The majority of the protein is exposed in the cytoplasm, followed by a single membrane-spanning region and a few amino acids, facing either the lumen of an intracellular compartment or the extracellular side (Fig. 1). Some SNAREs lack a membrane-spanning region, but are attached to the membrane by posttranslational acyl modifications such as palmitoylation or farnesylation.

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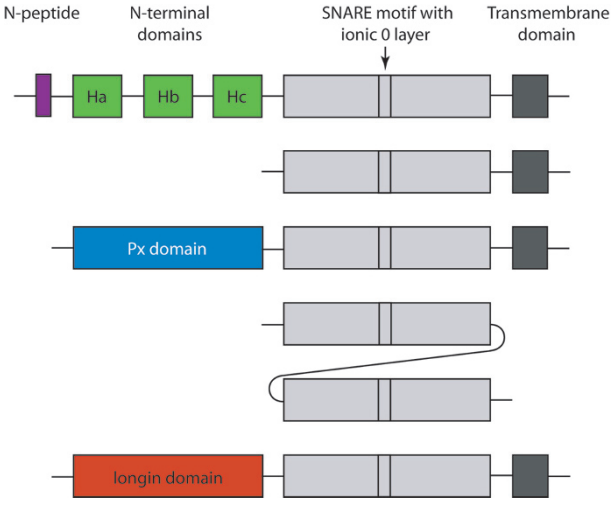

Figure 1. Domain structure of SNAREs (SNAP receptors). The various N-terminal domains are represented by colors. The SNAP (soluble NSF attachment protein)-25 homologues contain two covalently joined SNARE motifs and lack a transmembrane domain.

According to their function, SNAREs have been classified as v- and t-SNAREs, because they operate on opposing membranes, usually on a transport vesicle and a target membrane. Distinct trafficking steps employ different v-/t-SNARE complexes (for reviews see [2, 3]. Thus, the intracellular distribution of SNAREs provides a roadmap for membrane trafficking. The assembly of cognate v-/t-SNAREs between two membranes generates trans-SNARE complexes or SNAREpins, which bring the lipid bilayers in close proximity and drive membrane fusion [4]. The structural mechanism underlying SNAREpin formation is the assembly of a parallel four-helix bundle [5, 6]. The helix-forming units are so-called SNARE motifs, stretches of 60–70 amino acids containing heptad repeats with a central '0' layer. Based on the sequence and the amino acid located in the central '0' layer, SNAREs are classified into four subclasses: Qa-, Qb-, Qc-, and R-SNAREs [7]. The Q-SNAREs usually function as t-SNAREs and the R-SNAREs as v-SNAREs. (The Qa-SNARE is also called syntaxin or t-SNARE heavy chain; Qb- and Qc-SNAREs have been termed t-SNARE light chains). Functional fusogenic SNARE complexes contain one member of each subclass. The reconstitution of SNAREs into liposomes and the expression of 'flipped' SNAREs on the cell surface have shown that cognate v- and t-SNAREs are sufficient to fuse artificial lipid bilayers (liposomes) and biological membranes (entire cells) [4, 8]. Since cognate v- and t-SNAREs fuse membranes without an additional input of energy, SNARE complex formation – protein folding – provides enough energy to overcome the repulsive forces that keep membranes apart. Separate v- and t-SNAREs are largely unfolded [9]. Fully assembled SNARE complexes, which likely represent the post-fusion state, are extremely stable, and some complexes resist SDS-denaturation and thermal denaturation up to 80°C [10]. Surface force measurements have shown that the formation of a single SNAREpin provides enough energy to fuse the outer, but not the inner leaflet of a pure lipid bilayer (hemifusion) [11]. Indeed, SNAREpins involved at several transport steps can cause hemifusion at least as a reaction intermediate [12–14]. Such intermediates can be further stabilized by regulatory components as has been shown for neuronal SNAREpins *in vitro* [15]. SNAREpin assembly itself occurs in a stepwise manner starting at the amino-terminal membrane distal end of the SNARE motif and proceeding in a membrane proximal direction [16–19]. The membrane proximal regions are initially largely unstructured and are targets for regulatory components that either accelerate or delay SNAREpin assembly [9, 17, 18, 20]. The number of SNAREpins needed to completely fuse membranes *in vivo* is not known. At least three SNAREpins seem to be required to fuse secretory granules with the plasma membrane in PC12 cells [21]. The membrane curvature, the lipid/protein composition of a compartment, and the presence of lipid bilayer-pertubing regulators could significantly influence the minimal number of required SNAREpins.

For fusion to occur, the four-helix bundle assembly has to be coupled to the transmembrane region (TM) [22, 23]. The insertion of linkers between the SNARE motif and the TM reduces or abolishes fusion *in vitro* and *in vivo* [24, 25]. The replacement of the TM by lipid anchors allows vesicle docking but blocks fusion to a large degree [22]. The interaction between the membrane spanning regions of v-and t-SNAREs contributes to the transition from hemifusion to full fusion [26]. Transmembrane domains have also been implicated in lining the fusion pore and controlling its conductivity [25, 27].

SNARE reconstitution into liposomes has shown that at most transport steps SNAREs function in a topologically restricted manner. For example, SNAREs involved in yeast endoplasmic reticulum (ER)-Golgi transport (Sed5p, Sec22p, Bos1p, Bet1p) are only fusogenic when Bet1p is reconstituted into one liposome and Sed5p/Sec22p/Bos1p are present in an opposing liposome [28]. Other combinations are inactive. In other words, it matters which SNARE is localized in which membrane. Such topological restriction could significantly restrain the fusion potential of compartments and requires high accuracy in SNARE sorting. However, when the SNAREs (syn-

taxin13, syntaxin6, vti1a, VAMP4) involved in homotypic fusion of early endosomes were reconstituted into liposomes, multiple topologies were fusogenic [29]. It is unclear to which degree these differences might distinguish between homo- or heterotypic fusion events. An ordered assembly pathway could be critical for the formation of a fusogenic SNARE complex, and certain assembly intermediates might only be stable when formed within a compartment.

Although numerous SNARE complexes can form in solution, only a few complexes are able to drive membrane fusion [28, 30, 31]. Indeed, SNAREs contribute remarkable specificity to membrane trafficking: of approximately 300 different combinations of yeast SNAREs tested in *in vitro* liposome fusion assays, only 9 were fusogenic. To which degree non-fusogenic complexes are formed in the presence of appropriate regulators *in vivo* is unclear. Nevertheless, the formation of non-fusogenic complexes could have regulatory functions. For example, the binding of a so-called inhibitory i-SNARE to a subunit of a fusogenic SNAREpin results in a non-fusogenic complex and can help to generate sharper gradients of fusogenic SNARE complexes in neighboring compartments [32]. In addition, different t-SNARE complexes with distinct v-SNARE specificities can share a common t-SNARE component. For example, the syntaxin Sed5p is found in two different SNARE complexes (Sed5p/Bos1p/Sec22p/Bet1p and Sed5p/Gos1p/Ykt6/Sft1) mediating two distinct transport steps [33]. Thus, it is not necessary to encode for each transport step an entire new set of four SNAREs, and cells seem to make use of a combinatorial SNARE code. The presence of a common SNARE in different transport steps could also help to coordinate the overall membrane flow.

A prerequisite for SNAREpin assembly is the formation of a functional t-SNARE complex, which provides the template for v-SNARE binding. t-SNARE complex assembly is tightly regulated, and it has been shown that different assembly states can form *in vitro*. Such assembly states can change on a timescale of seconds as shown by single-molecule FRET measurements [34]. The presence of regulatory components steers t-SNARE assembly towards functional complexes that can form fusogenic SNAREpins. Thus, in addition to the SNARE motif, many SNAREs contain an N-terminal regulatory domain (see Fig. 1). These domains adapt SNAREs to the specific needs required at the distinct transport steps. All syntaxins contain an N-terminal domain. In the case of t-SNARE light chains, amino-terminal extensions might be present on both chains, on one chain, or lacking entirely. v-SNAREs usually lack or contain only short (up to 50 amino acids) amino-terminal domains. The following amino-terminal domains have been identified. The *Habc domain* – an amino-terminal three-helix bundle – is present in all syntaxins, Vti1b, and the t-SNARE light chain syntaxin 6 [35–38]. This domain seems to provide a binding site for SM proteins. In certain syntaxins, such as Sso1, syntaxin 1 and syntaxin 7, the Habc domains fold back on the SNARE motif, resulting in a closed conformation which blocks the binding of cognate SNARE partners. In the case of regulated exocytosis the closed conformation is further stabilized by SM proteins and becomes a critical step in regulating SNARE complex assembly and vesicle priming. The *PX domain* – a phosphatidylinositol-3-phosphate binding domain – is present in Vam7p, a component of the vacuolar t-SNARE in yeast. It contributes to the sorting of Vam7p to the yeast vacuole. Interestingly, Vam7p is one of the few SNAREs that lacks a covalent-attached membrane anchor [39]. The *Longin domain* is present in Ykt6, ERS-24/Sec22b, and VAMP7/TI-VAMP [40–42]. In the case of Ykt6 and VAMP7, the Longin domains have an autoinhibitory role [41]. The Longin domain of VAMP7 is also involved in VAMP7 sorting by its capacity to bind AP3 adaptors (for review see [43]). An *α-helical domain* of unknown function is present in the t-SNARE light chain of syntaxin 8 [38]. Thus, in addition to SNARE sorting, N-terminal extensions function as regulatory domains. The binding of an appropriate regulator can locally regulate SNARE function and membrane fusion. Furthermore, lateral SNARE segregation provides another means to control fusion [44]. For example, syntaxin 1 and 4 form distinct multimeric clusters in the plasma membrane via homophilic interactions that involve SNARE motifs [45]. However, it still remains to be shown whether these syntaxin clusters define release sites for distinct types of vesicles or have other physiological functions.

SNARE complex dissociation by NSF and SNAP

v- and t-SNAREs are 'consumed' during the fusion reaction, but are reused/recycled for repetitive rounds of transport (Fig. 2). Recycling is mediated by a molecular machinery that dissociates the extremely stable cis v-/t-SNARE complexes residing in a single lipid bilayer. SNAPs and NSF are cytosolic proteins which target cis-SNARE complexes at all transport steps [46]. Three isoforms of SNAPs (α, β, and γ) have been identified and function as adaptors between SNAREs and NSF [47]. NSF is a hexameric ATPase and a member of the AAA protein family. NSF and SNAPs in the cytosol do not interact with each other. Electron microscopy and protein-map-

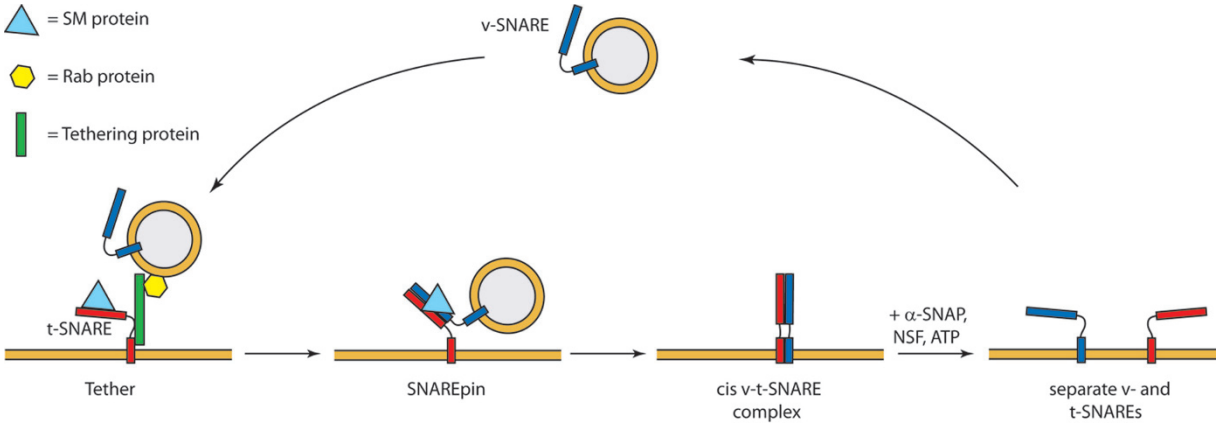

Figure 2. Basic machinery controlling membrane fusion and SNARE recycling. The initial interaction of a vesicle with its target membrane is mediated by tethering proteins and a small Rab GTPase. Subsequently, v- and t-SNARE proteins form SNAREpins. These events involve SM proteins and finally culminate in membrane fusion, which results in cis v-/t-SNARE complexes. In the presence of SNAPs, ATP-hydrolysis by NSF segregates v- and t-SNAREs for another round of fusion.

ping studies revealed that three molecules of SNAPs bind to the longitudinal surface of the four-helix SNARE bundle [48, 49]. Subsequently, the N-terminal domain of NSF binds the SNAP/SNARE complex. Then, ATP-hydrolysis by NSF dissociates the SNAP-SNARE complex and the four-helix SNARE bundle [50, 51]. The resulting individual SNAREs are largely unfolded and in an energy-rich state. ATP-hydrolysis by NSF is the only step where a net input of energy is invested. The actual fusion process is driven by the SNARE folding. The mechanisms that prevent the immediate reassembly of cis-SNARE complexes are not understood in detail. The N-terminal regulatory domains of SNAREs, regulatory proteins, or the sequestration/packing of the v-SNARE into transport vesicles could be potential mechanisms. In contrast to cis-SNARE complexes, SNAREpins are not substrates for NSF and SNAPs allowing the stable formation of SNAREpin intermediates [52]. Inactivation of NSF *in vivo* results in an accumulation of SNARE complexes and docked vesicles and ultimately leads to cell death. When a temperature-sensitive NSF mutant in *Drosophila* – *comatose* – is shifted to the restrictive temperature, the flies get paralyzed, because neurotransmitter release and the fast synaptic vesicle cycling have a high demand for NSF [53]. Whether significant levels of cis-SNARE complexes accumulate in living cells is still debated. However, it has been shown that assembled endogenous cis-SNARE complexes are not detectable in fresh cellular extracts, but accumulate over prolonged incubation time *in vitro* [54]. Thus, it is likely that cis-SNARE complexes are only transient reaction intermediates under most conditions. However, cis-SNARE complexes can accumulate *in vivo* when the SNAP and NSF levels are diminished or their activity is downregulated. Interestingly, the NSF activity is physiologically regulated by phosphorylation and nitrosylation (for review see [55]). For example, NSF nitrosylation has been shown to affect regulated exocytosis, indicating a locally restricted regulation or high demand for NSF at this transport step [56].

SM proteins are key regulators of vesicle docking and SNAREpin assembly

Sec1/Munc18 (SM) proteins were discovered in screens for secretion deficiency in *Saccharomyces cerevisiae* (sec1) and uncoordinated movement in *C. elegans* (unc18) [57, 58]. Characterized by three domains, SM proteins are arch-shaped cytosolic proteins of 60–70 kDa [59, 60]. Several SM isoforms are expressed in cells, and the individual SM proteins function at distinct intracellular transport steps. Whenever the transport step is essential for cell survival, the inactivation of the associated SM proteins results in cell death [61]. SM proteins are key partners of SNAREs and appear to be an integral part of the fusion machinery. Furthermore, SM proteins are involved in vesicle tethering, thereby linking tethering to SNAREpin assembly.

The primary effectors of SM proteins are syntaxins and SNARE complexes. Biochemical affinity purification experiments identified the mammalian SM protein Munc18-1 as a binding partner of syntaxin 1 [62, 63]. However, in contrast to syntaxins, SM proteins are exclusively found at fusion sites rather than showing a broad membrane distribution [64]. In *Saccharomyces cerevisiae*, four SM proteins are linked

Table 1. SM proteins and their syntaxin partners.

S. cerevisiae			*M. musculus*		
SM protein	Syntaxin	Pathway	SM protein	Syntaxin	Pathway
Sly1	Sed5p Ufe1p	ER-Golgi	mSly1	Syx5 Syx18	ER-Golgi
Vps45	Tlg2p	TGN-vacuole	mVps45	Syx16	TGN-endosomes?
Vps33	Vam3p	vacuole	Vps33a Vps33b	N.D.	endosomes
Sec1	Sso1/2p	exocytosis	Munc18–1 Munc18–2 Munc18-c	Syx 1, 2, 3 Syx 1, 2, 3 Syx 2, 4	reg. exocytosis const. exocytosis Glut4 exocytosis

to six syntaxins (see Table 1). Like yeast, *Drosophila* encodes four SM proteins. In mammals, concomitant with an increased number of syntaxin isoforms in the endo-/exocytic pathway, the number of SM proteins rose to a total of seven. SM proteins bind to syntaxins in essentially two discrete modes, which were correlated with two different functions.

In one mode, the SM protein binds syntaxin in a so-called closed conformation, in which the N-terminal Habc domain of syntaxin folds back on the C-terminal SNARE motif and blocks SNARE complex assembly [65]. Such an inhibitory function of SM proteins has been predominantly shown for SNAREs involved in regulated exocytosis such as syntaxin 1. The SM binding sites include an N-terminal syntaxin peptide, the Habc domain, the linker between the Habc domain and the SNARE motif, and a part of the SNARE motif [60, 66]. In the Munc18-1/syntaxin 1 complex, the linker between the Habc domain and the SNARE motif is structured [60]. In contrast, in the assembled SNARE complex the linker region is largely unstructured, indicating that the interaction of the linker with the SM protein stabilizes the closed conformation of syntaxin 1 [67]. Indeed point mutations in the linker of syntaxin 1 destabilize the 'closed' conformation, resulting in an 'open' conformation, which allows binding of its SNARE partners [65]. Remarkably, such mutations in the linker also bypass the requirement for regulatory components as outlined below. Thus, in regulated exocytosis, the binary Munc18-1/syntaxin 1 complex in its 'off' mode becomes one of the major targets for regulatory proteins. It appears that an entire cascade of regulatory components converges at this point to switch syntaxin into an 'on' mode, thereby controlling vesicle priming and regulated exocytosis.

In the second mode, the SM/syntaxin interaction apparently does not impede SNARE complex formation. The SM proteins Sly1p, Vps45p, and Munc18c interact with their syntaxins in an open conformation [68–71]. The SM binding sites include an N-terminal syntaxin peptide and a part of the SNARE motif [66, 71–75].

In addition to syntaxin, apparently all SM proteins interact with v-/t-SNARE complexes [69, 72, 75–77]. Direct SM/v-/t-SNARE complex interactions have been reported at the following transport steps: ER – Golgi transport; TGN – endosomes, regulated and constitutive exocytosis at the plasma membrane (including neurotransmitter release and glucose transporter secretion) [69, 71, 77–80]. *In vitro* liposome fusion assays demonstrated that the interaction of SM proteins with SNAREpins stimulates specific membrane fusion and adds specificity to membrane trafficking [81, 82]. For example, t-SNARE liposomes containing syntaxin 1/SNAP-25 can fuse with v-SNARE liposomes containing five different v-SNAREs *in vitro*. However, when Munc18–1 is added to such liposomes connected by SNAREpins, it selectively promotes the fusion of the t-SNARE liposomes with the cognate VAMP2 and VAMP8 (endobrevin) liposomes. Thus, the binding of Munc18-1 likely provides additional energy to augment the fusion of physiological SNARE complexes. Consistent with this model, yeast Sec1p, which controls exocytosis at the plasma membrane, and Sly1p, which is involved in ER-Golgi transport, stimulate membrane fusion and specific SNARE pairing [77, 81].

Remarkably, in mammals, the number of SNAREs in the endocytic and exocytic pathway is increased and these SNAREs show redundant interactions *in vitro*. However, *in vivo*, specific membrane trafficking is maintained likely due to the concomitant increase in the number of SM proteins acting at these transport steps. Thus, it appears that the burden to provide specific v-/t-SNARE interactions is shifted from the amino acid layers directly involved in SNARE-SNARE interaction to residues facing the outside of SNAREpins. Indeed, Munc18-1 interacts with VAMP2 residues exposed at the surface of the SNARE bundle [82]. Since most SM proteins are associated with syntaxins and contribute to SNARE pairing, SM proteins could be considered as additional t-SNARE components controlling SNAREpin formation. Thus, SNAREs encode the specificity for

membrane trafficking by using distinct interaction layers – one is provided by SM proteins.

The Munc18-1/syntaxin 1 binding sites in the SM/v-/t-SNARE complexes still need to be mapped. Recent results suggest that this interaction is restricted to the Habc domain [66]. The SM protein binding to the N-terminal syntaxin peptide apparently provides a link between SM binding to syntaxin and to the v-/t-SNARE complex and likely has key regulatory functions. For example, the binding of domain 1 of rSly1 to the N-terminal peptide of syntaxin 5 can induce conformational changes in rSly1 [83]. Binding of Vps45p to the SNARE complex depends on the presence of the N-terminal peptide of Tlg2p (a syntaxin). The N-terminal peptide of syntaxin 1 is required to stimulate liposome fusion and regulates SNARE complex assembly *in vitro* [66, 82]. In addition, injection of the free N-terminal syntaxin 1 peptide into nerve terminals blocks exocytosis *in vivo* [72]. Thus, in the simplest model, the interaction of the N-terminal peptide of syntaxin with the SM protein could change the conformation of the SM protein and possibly of syntaxin and thereby control SNARE complex assembly. However, it is not clear whether such a syntaxin N-peptide/SM interaction is a strict requirement at all transport steps.

In addition to the role in SNARE complex assembly, SM proteins have also been implicated in syntaxin stabilization, transport, and vesicle docking (for review see [84]). In yeast, the ER syntaxin Ufe1 is protected from degradation by an ERAD-like mechanism when bound to the SM protein Sly1 [85]. The inactivation of Vps45p and Munc18-1 reduces the levels of Tlg2p and syntaxin 1, respectively [86, 87]. Further, SM protein interactions have also been implicated in facilitating the transport of newly synthesized syntaxins to their final destination, possibly preventing premature t-SNARE complex formation during transport [86, 88]. A role for SM proteins in vesicle docking is supported by the observation that mouse chromaffin cells lacking Munc18-1 accumulate undocked vesicles [89]. In general, many members of the Sec1/unc18 family show genetic and biochemical interactions with tethering proteins, which in turn interact with Rab proteins – small GTP binding proteins [90, 91]. As already mentioned, Vps33p is an integral part of the HOPS tethering complex, which is recruited to the membrane by the Rab protein Ypt7p [92, 93]. Recent data point to a direct binding of SM proteins to Rab tethering complexes [94]. It will be of particular interest to reveal whether and how vesicle tethering proteins affect the binary SM/syntaxin interaction and control SNAREpin formation/assembly. In summary, Sec1/unc18 proteins seem to accompany syntaxins at several functional stages.

Tethers interact with SNAREs and locally restrict fusion

To target vesicle fusion to distinct membrane subdomains, vesicle tethering is locally restricted. Tethering proteins can directly interact with SNAREs and provide an additional layer of specificity and contribute to high-fidelity fusion (for review see [95]). Vesicle tethering precedes SNARE complex formation, and at least some tethering proteins play an active role in SNARE complex formation [96, 97]. Tethering factors have been identified in nearly all intracellular membrane transport steps and employ Rab proteins and some of their effectors [98; see also accompanying review by Fukuda].

Rabs are small compartment-specific GTPases that continuously cycle between the cytosol (in an inactive GDP-bound state) and membranes (in an active GTP-bound state). Eleven Rabs have been identified in yeast, and at least 66 isoforms are expressed in mammalian cells [99]. Membrane binding of an activated Rab protein at distinct intracellular compartments is followed by the recruitment of effectors and is temporally restricted due to GTP-hydrolysis. Rabs recruit many functional diverse effectors that operate in cargo sorting, vesicle motility, modeling of membrane subdomains, regulation of SNARE activity, or tethering. Hence, in an orchestrated manner with their effectors, Rabs appear to coordinate the sequential steps in distinct intracellular trafficking pathways. A specific Rab protein appears to regulate not only one trafficking step but seems to be required at multiple stages of the exocytic and endocytic pathway. In the presence of cognate tethering proteins, Rabs directly link membranes that subsequently undergo homo- or heterotypic fusion [95].

In contrast to highly conserved SNAREs, tethering proteins are much more heterogeneous and fall into two broad categories as long coiled-coil proteins and multisubunit complexes (for review see [100]). This suggests that tether proteins are adapted to the specific needs of the particular transport step. Both types of tethers seem to interact with t-SNARE components, and one representative even stimulates SNARE complex formation, linking vesicle tethers to the downstream fusion machinery [96] (Table 2). The homology between components of the multisubunit tethering complexes is only limited and restricted to short coiled-coil regions. One of these multiprotein complexes, the exocyst, is required for polarized exocytosis from yeast to mammals (for review see [101]). Built from eight components which are localized to sites of active exocytosis, the assembly mediates the targeting and tethering of post-Golgi secretory vesicles for subsequent fusion at the plasma

Table 2. Tether proteins and their Rab and SNARE partners.

Putative Tether	Localization	Rab	SNARE interaction	References
COG/*sec34/35*	early Golgi	Rab1/ *Ypt1*	Gos1, Sed5, Ykt6	[207, 208]
P115/*Uso1*, GM130	early Golgi	Rab 1/ *Ypt1*	syntaxin 5, GOS-28, membrin	[96, 105, 209]
TRAPP I/II	early Golgi	*Ypt1*	*Bet1, Sec22*	[210]
GARP/*VPS 51/52/53/54, VFT*	late Golgi	*Ypt6, Arl1*	*Tlg1*	[211, 212]
EEA1	endosomes	Rab 5	syntaxin 6	[213–215]
Class C VPS complex / HOPS	yeast vacuole	*Ypt7*	*Vam3, Vam7*	[91, 216, 217]
CORVET	endosomes	VPS21	n.d.	[104]
Exocyst	plasma membrane (polarized secretion)	Sec4	n.d.	[218, 219]
RIM 1	plasma membrane (active zone, neuron)	Rab3	SNAP-25	[126, 141]

membrane. Direct interactions of exocyst components with SNAREs have not been reported. Other multiprotein tethers, which all function at the Golgi, are the COG complex (conserved oligomeric Golgi complex), implicated in retrograde transport within the Golgi; the TRAPP complex (transport protein particle), which might tether ER-derived vesicles to the Golgi; and the GARP/VFT complex (Golgi associated retrograde protein), with a function in trafficking between endosomes and the late Golgi (for reviews see [102, 103]). For all three complexes interactions with SNAREs are described. SNARE interactions have also been described for another multiprotein complex, termed HOPS/class C Vps (homotypic fusion and vacuole protein sorting), which tethers vesicles to late endosomes and the vacuole [91]. The endosomal CORVET complex is a homolog of the HOPS complex, but a direct SNARE interaction has not been described so far [104].

Coiled-coil Rab effectors with functions in tethering at the Golgi and endosomes are p115, the Golgins, and early endosomal antigen 1 (EEA1). Coiled-coil tethers assemble as long, rod-like complexes bridging vesicle and target membrane. A well-characterized coiled-coil tether which functions in membrane trafficking at the early Golgi comprises the tripartite complex of p115, Giantin, and GM130 [105]. The cytosolic protein p115 bridges Giantin, present on COPI vesicles, to GM130 localized to membranes of the early Golgi.

In summary, tethers establish a physical link between transport vesicles and their specific target membranes and provide at least a kinetic advantage for subsequent SNAREpin assembly. The SNARE-independent sorting of tethers to membrane subdomains contributes an additional layer of vesicle targeting specificity. Some tethers directly bind specific t-SNARE components, thereby preselecting the cognate t-SNARE partner for a tethered vesicle, which might be important in the highly dynamic secretory and endocytic pathway, and at the plasma membrane where different SNAREs could exist in overlapping distributions. Many tethers are part of larger protein scaffolds harboring regulatory components that can direct vesicle trafficking, influence cytoskeleton dynamics, and more importantly could control SNARE assembly. In general, Rabs and their tethering effectors provide the local environment for efficient membrane fusion.

Components controlling regulated exocytosis

In contrast to constitutive transport, regulated exocytosis requires an additional level of control to couple a triggering signal to the fusion machinery. Thus, specific components that sense the signal interact either directly or indirectly with the SNAREs, and their concerted action fuses the lipid bilayer. In most cases the ultimate triggering signal is a local increase in the calcium concentration caused by the opening of calcium channels in the plasma membrane. In the following sections, neurotransmitter release will be used as a model because it is the paradigm for tight spatial and temporal regulation. Neurotransmitter release occurs in less than 1 ms after calcium influx at active zones. Active zones are restricted vesicle tethering sites in the presynaptic nerve terminal, and are directly opposed to the postsynaptic density containing the neurotransmitter receptors in the

downstream neuron. Locally confined release sites become a strict necessity because 1000 synapses can impinge on a single neuron, and information needs to be processed locally and temporally. In addition, neurons modulate their neurotransmitter release dependent on the previous release history and on inputs derived from impinging neurons. Depending on the frequency of a previous stimulation, the neurotransmitter release can either be increased or depressed, a process called synaptic plasticity. Therefore, distinct pools of vesicles are found in nerve terminals, and the pool sizes can be dynamically regulated. These pools include i) a reserve pool of vesicles, which under standard conditions does not participate in neurotransmitter release and is not in direct contact with the release zone, ii) a pool of vesicles tethered at the active zone area but not yet primed for calcium-triggered release, and iii) a readily-releasable pool which is in direct contact with the plasma membrane and can fuse within a short time frame. The active zones contain a network of proteins that tether vesicles and steer vesicle pool sizes and release probabilities. Many of the regulators are high molecular weight proteins (up to 500 kD) and contain several domains that have regulatory and networking functions. The relatively simple multicellular organism *Caenorhabditis elegans* with its 302 neurons usually contains a few isoforms of regulators. Alternative splice variants are limited, and orthologues of some of the mammalian active zone proteins such as Bassoon and Piccolo/Aczonin are completely missing. Thus, the *in vivo* analysis of regulatory proteins in simple organisms results in less complex phenotypes and usually pinpoints the basic function of a regulator. With the development of more complex organisms the complexity of regulatory steps increased significantly. In mammals the analysis of distinct regulator isoforms at different types of synapses reveals functional heterogeneities that give us a glimpse of the molecular mechanisms underlying memory and learning. For simplicity and due to a lack of knowledge of the emerging complex regulatory circuits, the discussion will be limited to the basic mechanistic principles that are observed for regulator orthologues that are found in most organisms and directly interact with SNAREs.

In comparison to other intracellular transport steps, the individual components of the fusion machinery operating in regulated exocytosis display additional properties. For example, the plasma membrane t-SNAREs consist of only two components instead of three, as the two separate non-syntaxin t-SNARE light chains (Qb- and Qc-SNAREs) are merged into a single polypeptide, the SNAP-25 homologues. In addition, SNAP-25 homologues lack a membrane-spanning region but contain posttranslational acyl modifications. The plasma membrane syntaxins adopted a 'closed' conformation, in which the amino-terminal regulatory domain folds back on the SNARE motif, blocking SNAP-25 binding. This closed conformation is further stabilized by Munc18 isoforms. To switch syntaxin from a closed into an open SNAP-25-binding competent conformation, a complex machinery emerged that regulates this specific step. Furthermore, it seems that SNAREpins can be arrested at distinct assembly steps, allowing fast submillisecond response times. Interestingly, the neuronal syntaxin 1 has an amino acid in the SNARE motif that slows down SNAREpin assembly, which likely introduces an additional regulatory step [106]. In addition, fusion pores with defined conductivities have evolved, which indicates that their architecture is determined by distinct properties of particular SNAREs or regulators. Furthermore, fusion pores have two fates: i) either complete dilation, integrating the vesicular membrane components into the plasma membrane, or ii) closure, a process called kiss and run (or kiss and stay) that maintains the integrity of the vesicle and bypasses clathrin-mediated endocytosis. It is likely that regulatory components control this process. Thus, in the following sections, the function of the major regulators (Munc13, RIM, tomosyn, synaptotagmin, and complexin) that control regulated exocytosis is outlined (Fig. 3).

Priming vesicles for fusion

Vesicle priming controls the availability of vesicles for immediate fusion. It involves the transition from a tethered vesicle pool to a readily releasable pool (RPR) that is linked to the plasma membrane by SNAREpins.

Munc13s are molecular switches controlling t-SNARE assembly in regulated exocytosis

Members of the Munc13/unc-13 family were first discovered as *C. elegans* mutants that show uncoordinated movements [58]. In *C. elegans* and *Drosophila* a single gene encodes a few major isoforms [107–110]. In vertebrates, three genes encode several Munc13 isoforms [111]. Munc13s likely activate syntaxins by switching their conformations and thereby control t-SNARE complex assembly.

Munc13/unc-13 family members are large multidomain proteins consisting of a highly conserved carboxy-terminal region called R region and a variable amino terminus called M or L region, which determines the different subfamilies (for review see

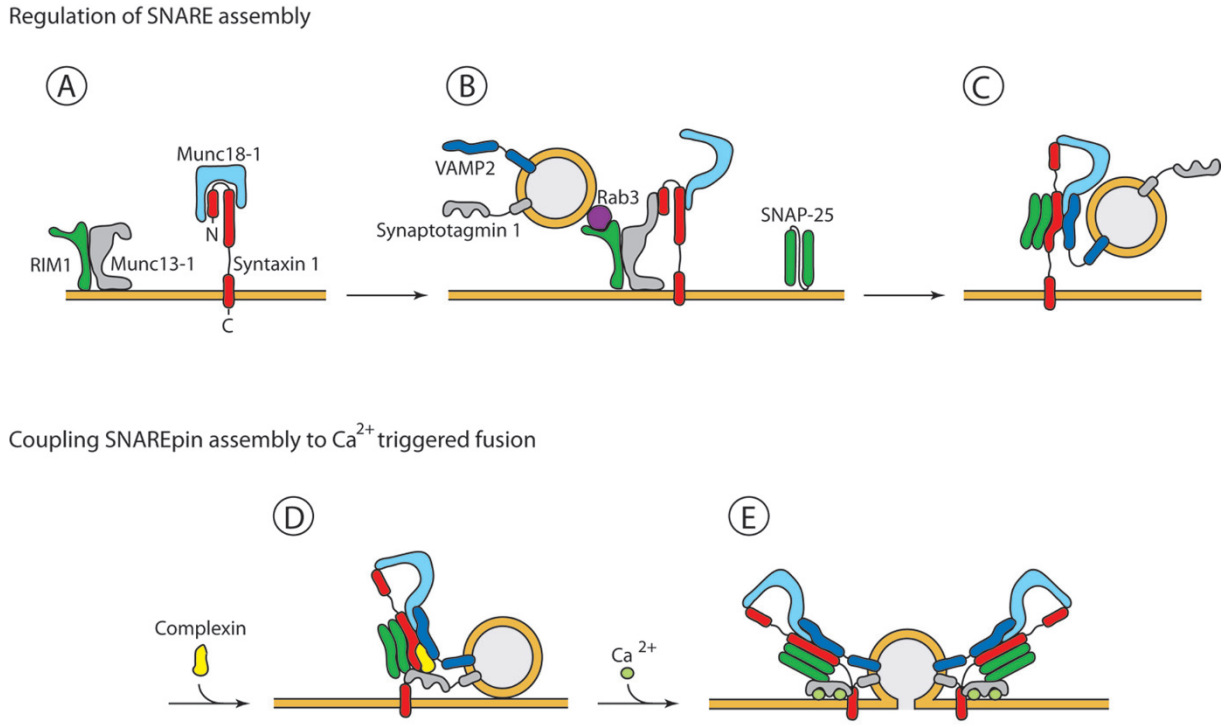

Figure 3. Model of how regulatory components control vesicle priming and calcium-triggered fusion at the neuronal synapse. Major regulatory components are depicted at their site of action. (*A*) Munc18-1 is bound to the 'closed' conformation of syntaxin 1. (*B*) Docking of a synaptic vesicle results in a tripartite tethering complex comprising Rab3, RIM1 and Munc13-1 and might catalyze the conversion of syntaxin 1 into an 'open' conformation. (*C*) SNAP-25 binds syntaxin 1 to form the t-SNARE complex which is a target for VAMP2 on synaptic vesicles, resulting in SNAREpin formation. (*D*) Complexin binds to SNAREpins and prevents the completion of SNARE complex zippering, thereby acting as a fusion clamp. (*E*) Synaptotagmin 1 releases the complexin clamp upon sensing an increase in the local Ca^{2+} concentration and triggers fusion pore opening.

[111] (Fig. 4). The inactivation of Munc13 in mice, *C. elegans*, and *Drosophila* almost completely abolishes spontaneous and evoked release [109, 112–114]. A dramatic reduction in the RRP size suggests a role for Munc13 in vesicle priming [112]. Consistent with a role in vesicle priming, Munc13 seems to affect SNARE complex formation. Munc13-1 directly binds the regulatory Habc domain of syntaxin 1 [115]. The syntaxin 1-interacting domain is located in the autonomously folded MUN domain of Munc13, which also binds the assembled t-SNARE complex [116]. Indeed, the MUN domain is sufficient to rescue synaptic transmission in Munc13-deficient organisms [117, 118]. Remarkably, Munc13 and Munc18 have overlapping binding sites in the regulatory Habc domain of syntaxin 1, and one study reported that Munc13 can displace Munc18 from syntaxin 1 [119]. Thus, in the simplest model, Munc13 binding could switch syntaxin 1 from a closed into an open conformation. Consistent with this hypothesis, unc13 in *C. elegans* becomes dispensable in the presence of an open form of syntaxin 1, which contains point mutations in the linker between the Habc domain and the SNARE motif [120].

Since syntaxin 1 is the primary target of Munc13, it is likely that the various other Munc13 domains regulate the Munc13/syntaxin interaction and/or recruit Munc13 to its site of action. Indeed, diacylglycerol, phorbol esters, and Ca^{2+}-calmodulin stimulate regulated exocytosis [117, 121]. These Munc13-binding partners also support the interaction with Doc2, a Ca^{2+}-binding protein which localizes to the cytoplasm and associates with the plasma membrane in a calcium-dependent manner [122]. Interestingly, Doc2 also interacts with Munc18 and interferes with the binding of Munc18-1 to syntaxin 1 [123]. Thus, the Munc13-1/syntaxin 1 and the Doc2/Munc18-1 interactions might cooperatively activate syntaxin 1. Another Munc13-1 partner is the Rab-binding protein RIM1, which is discussed below.

In summary, the Munc13/syntaxin interactions play a key role in vesicle priming and likely switch syntaxins from a closed into an open conformation. This switch is regulated by a network of protein-protein interactions and posttranslational modifications that fine-tune vesicle priming and pool sizes.

A functional homologue of Munc13 is the calcium-activated protein for secretion (CAPS), which regu-

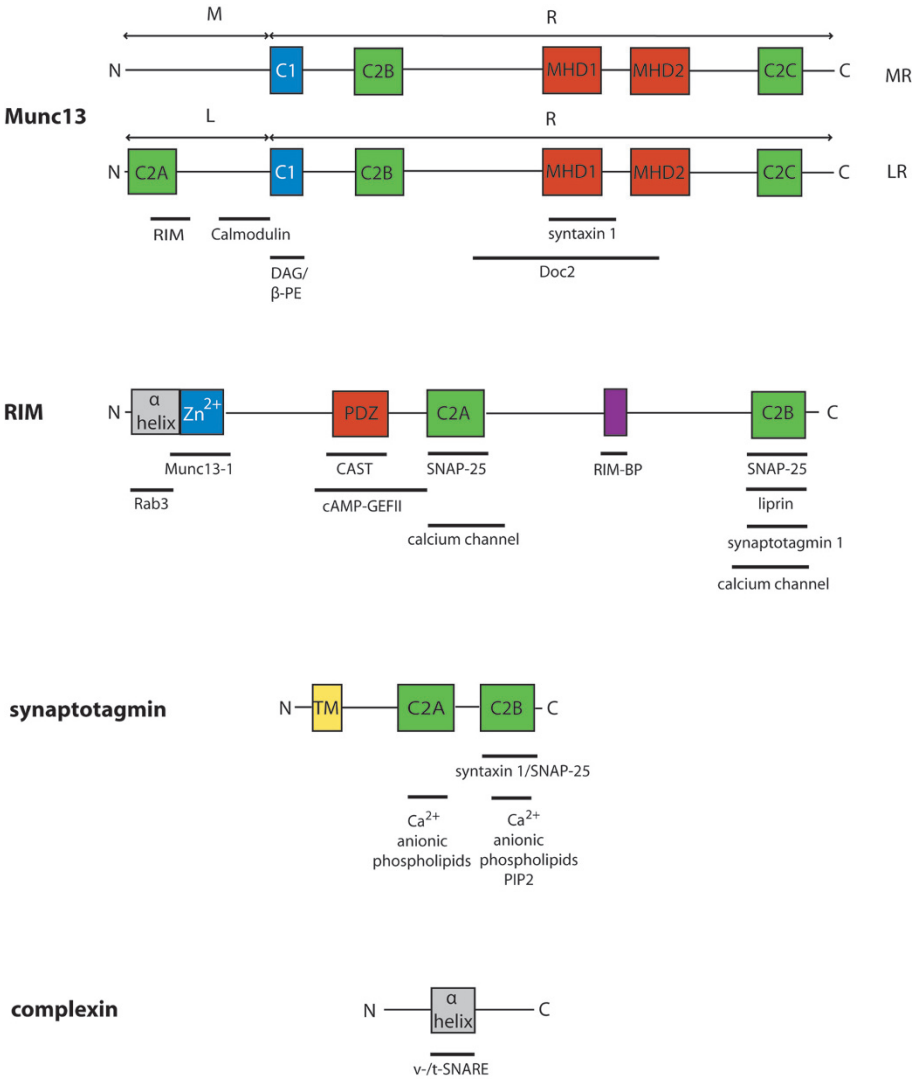

Figure 4. Domain structure of components involved in regulated exocytosis. Effectors and their binding sites are depicted by black bars.

lates the exocytosis of dense core vesicles in endocrine secretion [124]. CAPS is encoded by unc31 in *C. elegans* and can alter the conformation of syntaxin [125]. However, with the exception of the MUN domain, the domain composition of CAPS and Munc13 differs significantly, suggesting that these two regulators recognize distinct signals to prime vesicles for fusion.

RIM provides a regulatory platform

Members of the RIM protein family were originally identified as Rab3 effectors [126]. However, dependent on the RIM isoform, additional Rab proteins, such as Rab10, Rab26, Rab37, and Rab8, have been reported to be RIM-binding partners [126, 127]. In vertebrates, the RIM protein family is encoded by four genes, resulting in numerous RIM isoforms, which are predominantly expressed in neurons, insulin-secreting cells, and testis [128]. In *C. elegans*, a single gene, unc10, encodes RIM. RIMs are large proteins (approximately 180 kDa), and their multiple domains provide a binding platform for several components that control vesicle priming at the exocytosis sites [126, 129] (Fig. 4).

In *C. elegans*, unc10 mutants are viable, and the overall phenotype is less severe than knockouts of SNAREs and unc-13 [130]. The general architecture of nerve terminals is not affected, but evoked release and spontaneous fusion events are reduced. Remarkably, an open form of syntaxin 1 suppresses physiological defects of unc-10 mutants, indicating that RIM targets t-SNARE complex assembly. In mice, the knockout of the RIM1 isoform, which is an integral component of the active zone in the neuronal synapse, is viable. However, these animals are characterized by a decreased readily releasable

vesicle pool, alterations in short- and long-term synaptic plasticity, and severe impairments in learning and memory [131–133].

Consistent with a vesicle-priming function, one of the major targets of RIM1 is Munc13. Since the absence of RIM1 results in a significant reduction of Munc13 levels, RIM might stabilize Munc13 and prevent its degradation [132]. Overexpression of the amino-terminal domain of RIM1, which in addition to Munc13 also contains Rab3-binding sites, stimulates exocytosis in PC12 and chromaffin cells [126, 134]. Rab3, Munc13, and RIM can form a tripartite complex [135], but it remains to be shown how this complex controls t-SNARE complex assembly and vesicle priming at the molecular level.

RIM contains binding sites for several other proteins: i) ERC family members (CASK/ELKs, Rab6-interacting protein 2), which could localize RIM to vesicle release sites [136–138]; ii) cyclic AMP-GEFII, which provides a link to the signal transduction chain that is involved in insulin secretion in pancreatic b-cells [139, 140]; iii) SNAP-25, a component of the neuronal t-SNARE [141]; iv) synaptotagmin 1, the calcium sensor located on synaptic vesicles [141]; v) the a1B pore-forming subunit of N- and L-type calcium channels [140, 141]; vi) α-liprins, which are involved in organizing the active zone [132]; and RIM-BPs (RIM-binding proteins), which bind voltage-gated calcium channels [142].

In summary, RIM and Munc13 bind most of the factors that are required for regulated exocytosis and are central parts of a network that directly controls vesicle priming and SNARE complex formation. In other words, they seem to function like components of an integrated circuit that locally compile regulators and are dependent on input modulate membrane fusion and synaptic plasticity.

Tomosyn inhibits vesicle priming

Tomosyn was discovered as a syntaxin 1-binding protein that is capable of dissociating Munc18-1 from syntaxin [143]. In mammals two genes encode 7 tomosyn isoforms generated by differential splicing. In *C. elegans* tomosyn is encoded by a single gene, tom-1, generating three alternative spliced isoforms [144]. Tomosyns are large cytosolic proteins (130 kDa) and function as negative regulators.

Tomosyn inactivation stimulates exocytosis [145, 146]. In contrast, the overexpression of tomosyn inhibits the exocytosis of synaptic vesicles, dense-core granules, Glut4-containing vesicles, and blocks insulin secretion [143, 145, 147, 148]. Consistent with this observation, tomosyn antagonizes unc-31/CAPS and unc-13-dependent vesicle priming in *C. elegans* [146, 149]. Therefore, loss of tomosyn partially suppresses the behavioral and electrophysiological phenotypes caused by unc13 mutants [146, 150].

Tomosyns are characterized by an N-terminal domain rich in WD40 repeats and a C-terminal SNARE domain highly homologous to the SNARE motif of the v-SNARE VAMP2 (synatobrevin2). Indeed, tomosyn binds t-SNAREs, such as syntaxin 1/SNAP-25 and syntaxin 4/SNAP-23, and assembles into a four-helix bundle [151]. Since tomosyn lacks a membrane-spanning region and blocks v-/t-SNARE complex formation, it inhibits regulated exocytosis. Binding of tomosyn to the t-SNARE is differentially regulated by kinases, such as the cAMP-dependent kinase (PKA), the serin/threonin kinase (ROCK), and casein kinase II [152, 153].

In summary, tomosyn inhibits vesicle priming. Phosphorylation modulates the tomosyn/SNARE interaction, suggesting a function in neuronal plasticity.

Coupling SNAREs to the signal that triggers fusion

Membrane fusion of docked synaptic vesicles at the active zone is tightly regulated to prevent neurotransmitter release in the absence of calcium. Therefore, fusion of synaptic vesicles must be regulated both negatively (fusion arrest of docked vesicles in the absence of a calcium trigger) and positively (by a calcium-sensing machinery). Two candidate proteins involved in this process are synaptotagmin and complexin.

Synaptotagmins function as calcium sensors

Synaptotagmins (Syt) were originally identified as prominent components of synaptic vesicles and as a binding partner of syntaxin 1 [154, 155]. Synaptotagmins are type I membrane proteins with a short domain localized in the vesicle lumen, a transmembrane domain, a linker region, and two tandem C2 domains (designated C2A and C2B) in the cytosol [156] (Fig. 4). In vertebrates 16 synaptotagmin isoforms are known. Synaptotagmins are usually localized to distinct secretory vesicles and control their calcium-dependent fusion. For example, Syt VII has a broad tissue distribution and localizes to lysosomes and insulin-secreting granules of pancreatic β-cells [157–159], whereas Syt I is predominantly expressed in neuronal cells as an abundant constituent of synaptic vesicles [154, 160]. With a few exceptions, most of the C2 domains in synaptotagmins can function as calcium-sensing units that bind calcium

in the presence of anionic phospholipids [161, 162]. Based on their affinity for calcium, the synaptotagmin isoforms were classified into three distinct kinetic groups [163]. It was suggested that during evolution, synaptotagmins have diverged to sense different ranges of Ca^{2+} (from low μM to ~200 μM Ca^{2+}) to trigger the fusion of distinct organelles with target membranes dependent on the calcium concentration [164]. High calcium concentrations are thought to exist only near the pores of calcium channels, suggesting that the distance of the release machinery to the calcium channels controls membrane fusion [165]. Indeed, Syt I, which has a low calcium affinity, can directly interact with calcium channels [166, 167].

Flies and mice that lack Syt I or contain point mutations in the calcium-binding C2 domains display a defect in synchronous neurotransmitter release [168–172]. In addition, an increase of asynchronous spontaneous fusion events has been reported in synaptotagmin-deficient organisms, indicating that synaptotagmins might act as fusion clamps [173–176]. The role of Syt I in evoked calcium-dependent membrane fusion is further supported by the finding that the cytoplasmic domain of Syt I stimulates the fusion of v- and t-SNARE liposomes and cells containing 'flipped' SNAREs upon addition of calcium [15, 177, 178].

Both C2 domains of synaptotagmin functionally cooperate and penetrate membranes in response to calcium [179, 180]. More recently this interaction was found to tubulate liposomes, and a model was proposed in which the C2 domains upon calcium binding penetrate the lipid bilayer and induce positive curvature, hence lowering the energy barrier leading to membrane fusion [181]. Another lipid effector of Syt I is phosphatidylinositol-4,5-bisphosphate (PIP2), which is strongly bound by the C2B-domain in a calcium-dependent manner [182]. PIP2 is highly enriched in plasma membrane microdomains of PC12 cells at sites of exocytosis [183]. It was proposed that this interaction might help to 'steer' the membrane penetration activity of Syt I towards the target membrane, avoiding nonproductive interaction with lipids residing on the synaptic vesicle membrane [184].

To couple synaptotagmin to the fusion machinery, the C2B domain of Syt I specifically interacts with the membrane proximal regions of syntaxin 1, SNAP-25, and the t-SNARE complex in a calcium-independent manner [185]. Thus, Syt might initially bind the t-SNARE in the developing v-/t-SNARE complexes and subsequently cause calcium-dependent structural rearrangements in the SNARE complex [186–188]. In concert with its lipid-bilayer perturbing properties, synaptotagmin finally triggers fusion pore opening [189]. Another set of data indicates that Syt could also regulate the dilation of fusion pores [190, 191]. As a consequence, modulation of Syt action might directly determine the choice between kiss-and-run and full fusion [192].

Thus, most Syts function as calcium sensors for regulated exocytosis, directly coupling the triggering signal to the fusion machinery. A role of synaptotagmin as a fusion clamp in the absence of calcium is debated, but recent data suggest that other proteins – complexins – fulfill this function and prevent the release of primed vesicles.

Complexins are potential fusion clamps

Complexins (also called synaphins) are small cytoplasmic proteins which were identified as binding partners of SNARE complexes [193, 194]. Only one complexin orthologue is expressed in *Drosophila,* whereas four isoforms are found in vertebrates [195]. Together with synaptotagmins, complexins control calcium-regulated exocytosis.

The inactivation of complexin in *Drosophila* blocks regulated exocytosis and results in a dramatic increase in the spontaneous fusion of synaptic vesicles at neuromuscular junctions [196]. Mice lacking the complexin isoforms CpxI and II die at birth and are impaired in synaptic vesicle exocytosis at the Ca^{2+}-dependent step, indicating a stimulatory role for complexins [197]. However, non-synchronous release is not affected in these mice [197]. Remarkably, overexpression of CpxI and CpxII inhibits fusion in chromaffin and PC12 cells, respectively [198, 199]. Liposome fusion assays as well as a cell-cell fusion assay provide further support for an inhibitory function of complexins [15, 178, 200, 201]. Taken together, these studies argue for a dual role of complexins, exerting inhibitory as well as stimulatory functions in membrane fusion.

Complexins contain a central α-helix, which binds with low affinity to neuronal t-SNAREs (Fig. 4). High-affinity binding requires v-/t-SNARE complexes [202]. The crystal structure of complexin I together with the assembled cis v-/t-SNARE core complex shows that the central α-helix (amino acids 48–70) of CpxI binds in an anti-parallel manner to a groove in the four-helix SNARE bundle, which is formed by syntaxin 1 and VAMP2 [203]. This binding mode likely stabilizes SNAREpin intermediates and could function as a clamping mechanism as shown by liposome fusion experiments [15, 178, 200, 203]. Liposome fusion assays and studies in transformed cells also revealed that synaptotagmin in the presence of calcium can release the complexin

clamp. Since both synaptotagmin and complexin target the membrane proximal region of the SNAREpin, synaptotagmin and calcium-dependent rearrangement in this area could trigger fusion and fusion pore opening. In addition, synaptotagmin locally perturbs the lipid bilayer. However, the exact mechanism is still unclear, and the architecture of fusion pores remains elusive. Furthermore, clusters of conserved amino acids that flank the SNARE complex-binding helix apparently modulate complexin function [204].

In summary, the distinct functional domains of complexins could be important to fine-tune neurotransmitter release in the brain, allowing a reversible switch between inhibitory and stimulatory functions. In the absence of calcium, complexin and synaptotagmin likely stabilize SNAREpin intermediates. Upon binding of synaptotagmin to calcium and anionic phospholipids, the clamp is released, allowing synchronized fusion to progress.

Concluding remarks

The discovery of SNAREs and their key regulators has provided critical insights into intracellular membrane fusion and has shown that the basic mechanisms are applicable to all transport steps. We are just beginning to uncover the functional and structural aspects of the regulatory cascade that controls membrane fusion. In the case of regulated exocytosis, additional components control distinct aspects of SNAREpin formation and vesicle priming and become themselves targets for regulation – short term by posttranslational modification and long term by alterations in protein levels. Furthermore, local changes in the lipid composition recruit regulators, alter SNARE activity and membrane curvature, and thus contribute to membrane fusion (for review see [205, 206] and accompanying review by De Matteis and colleagues). Although significant progress has been made in deciphering the complex regulatory networks that control fusions, many basic questions remain open, such as the role of Rabs in SNARE complex formation, the biophysics of membrane fusion, and the functional and structural organization of fusion pores.

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