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Review

# SNAREs and SNARE regulators in membrane fusion and exocytosis

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**Abstract.** Eukaryotes have a remarkably well-conserved apparatus for the trafficking of proteins between intracellular compartments and delivery to their target organelles. This apparatus comprises the secretory (or 'protein export') pathway, which is responsible for the proper processing and delivery of proteins and lipids, and is essential for the derivation and maintenance of those organelles. Protein transport between intracellular compartments is mediated by carrier vesicles that bud from one organelle and fuse selectively with another. Therefore, organelle-specific trafficking of vesicles requires specialized proteins that regulate vesicle transport, docking and fusion. These proteins are generically termed SNAREs and comprise evolutionarily conserved families of membrane-associated proteins (i.e. the synaptobrevin/VAMP, syntaxin and SNAP-25 families) which mediate membrane fusion. SNAREs act at all levels of the secretory pathway, but individual family members tend to be compartment-specific and, thus, are thought to contribute to the specificity of docking and fusion events. In this review, we describe the different SNARE families which function in exocytosis, as well as discuss the role of possible negative regulators (e.g. 'SNARE-masters') in mediating events leading to membrane fusion. A model to illustrate the dynamic cycling of SNAREs between fusion-incompetent and fusion-competent states, called the SNARE cycle, is presented.

**Key words.** Exocytosis; synaptic vesicle; SNARE; synaptobrevin/VAMP; syntaxin; SNAP-25; synaptotagmin; Munc18; synaptophysin; NSF; SNAP.

## Introduction

In general terms, SNAREs comprise distinct families of conserved membrane-associated proteins which facilitate membrane fusion in eukaryotes. They are found throughout the secretory pathway and participate in a number of membrane-trafficking events, including trafficking of cargo-containing carrier vesicles, compartmental organization and organelle fusion. Specificity of action by members of the different SNARE families contributes to the mechanism by which these diverse intracellular trafficking events occur with a high degree of fidelity.

Historically speaking, the term SNARE was coined over 5 years ago by J. Rothman and colleagues to describe entities which could participate in the binding of soluble factors (e.g. NSF—*N*-ethylmaleimide-sensitive fusion protein and its membrane-attachment proteins, SNAPs) to membranes derived from bovine brain homogenates [1, 2]. These factors had been described previously as agents which could mediate membrane fusion events in an in vitro assay system, based upon protein transfer between donor and acceptor compartments. The surprise was that these so-called SNAP receptors (hence the name, SNAREs) turned out to be proteins previously identified and characterized, at least

Table 1. SNARES participating in exocytosis in yeast and mammals.

| Mammals | Trafficking event/localization | Yeast equivalent (site of action) |
|---|---|---|
| *v-SNARES* | | |
| VAMP1/Synaptobrevin I | regulated exocytosis/synaptic vesicles | Snc1 and Snc2 (Golgi to plasma membrane) |
| VAMP2/Synaptobrevin II | regulated/synaptic vesicles secretory granules | same as above |
| VAMP3/Cellubrevin | regulated and constitutive/secretory granules, secretory vesicles | none (functionally redundant with Snc1 and Snc2) |
| VAMP5 | constitutive/secretory vesicles, myotubes, tubulovesicular structures | none |
| VAMP7/TI-VAMP | constitutive and regulated?/apical membrane, secretory granules, endosomes | none |
| VAMP8/Endobrevin | endocytosis?/early endosomes | none |
| Synaptotagmin I, II, III, V, X | regulated/synaptic vesicles, secretory granules | none |
| *t-SNAREs* | | |
| Syntaxin IA and IB | regulated/plasma membrane | Sso1 and Sso2 (Golgi to plasma membrane) |
| Syntaxin 2 | constitutive/apical and basolateral membrane | none (probably redundant with Sso1 and Sso2) |
| Syntaxin 3 | regulated and constitutive?/apical membrane | none |
| Syntaxin 4 | regulated and constitutive?/basolateral membrane | none |
| SNAP25 A and B | regulated/plasma membrane | Sec9 (Golgi to plasma membrane) |
| SNAP23/Syndet | regulated and constitutive/plasma membrane | none (probably redundant with Sec9) |

in part, by groups studying neuronal transmission or protein trafficking in the yeast, *Saccharomyces cerevisiae*. Like characters from Pirandello's play 'Six characters in search of an author' these proteins, which were in search of a clear and directed function, were christened as founding members of the three major SNARE families: the syntaxin, SNAP-25 and synaptobrevin/VAMP families. They were particularly notable in that structurally conserved homologs had already been identified in lower eukaryotes, such as *Caenorhaboitis elegans* and yeast, allowing for genetic dissection of their physiological roles. Subsequent studies have yielded a more precise determination of the chain of events which lead to membrane fusion, as well as defining new proteins which act upon the process. Many of these proteins fulfill regulatory roles, some of which we have defined as SNARE-masters due to their apparent ability to control the direct entry of SNAREs into binary and ternary SNARE complexes. In this review, we will examine the different families of SNAREs, the putative SNARE-masters and other possible regulators of vesicle docking and fusion. A hypothesis called the 'SNARE cycle' is put forth to illustrate that SNAREs are engaged in a dynamic cycle of assembly and disassembly, which is regulated by NSF/SNAP and rab proteins.

## SNAREs and their brethren

Two distinct categories of SNAREs have been described (see table 1 for individual listings). SNAREs present on the vesicle (or donor) compartment are known as v-SNAREs, while those on target (or acceptor) compartment are known as t-SNAREs. In the original study by Sollner et al. [1], the v-SNARE identified was a synaptic vesicle-associated membrane protein known either as VAMP or synaptobrevin. At the level of the presynaptic membrane in neurons, physical association of VAMP with t-SNAREs of the plasma membrane, SNAP-25 (no relation to $\alpha$- and $\beta$-SNAP etc.), and syntaxin, led to the formation of a ternary (or 'core') SNARE complex of 7S [3]. This core complex binds to $\alpha$-SNAP and NSF in vitro to form a particle of 20S, which could be disassembled upon adenosine triphosphate (ATP) hydrolysis [1, 3]. The 20S complex was designated as the putative fusion particle which could then effect bilayer fusion, upon ATP hydrolysis by NSF. However, later works have proposed an entirely different function for both NSF and SNAPs (discussed below).

Structural information regarding the ternary SNARE complex has been lacking until recently. Earlier works suggested that SNAREs associate within the complex by forming coiled coils, heptad repeats of considerable $\alpha$-helicity which coil around one another. However, this conception was based primarily upon the use of computer-generated algorithms and lacked the structural evidence necessary for confirmation. More recent studies revealed that the v- and t-SNAREs are aligned in parallel within the core complex [4–6]. Moreover, the individual SNAREs were found to undergo a significant

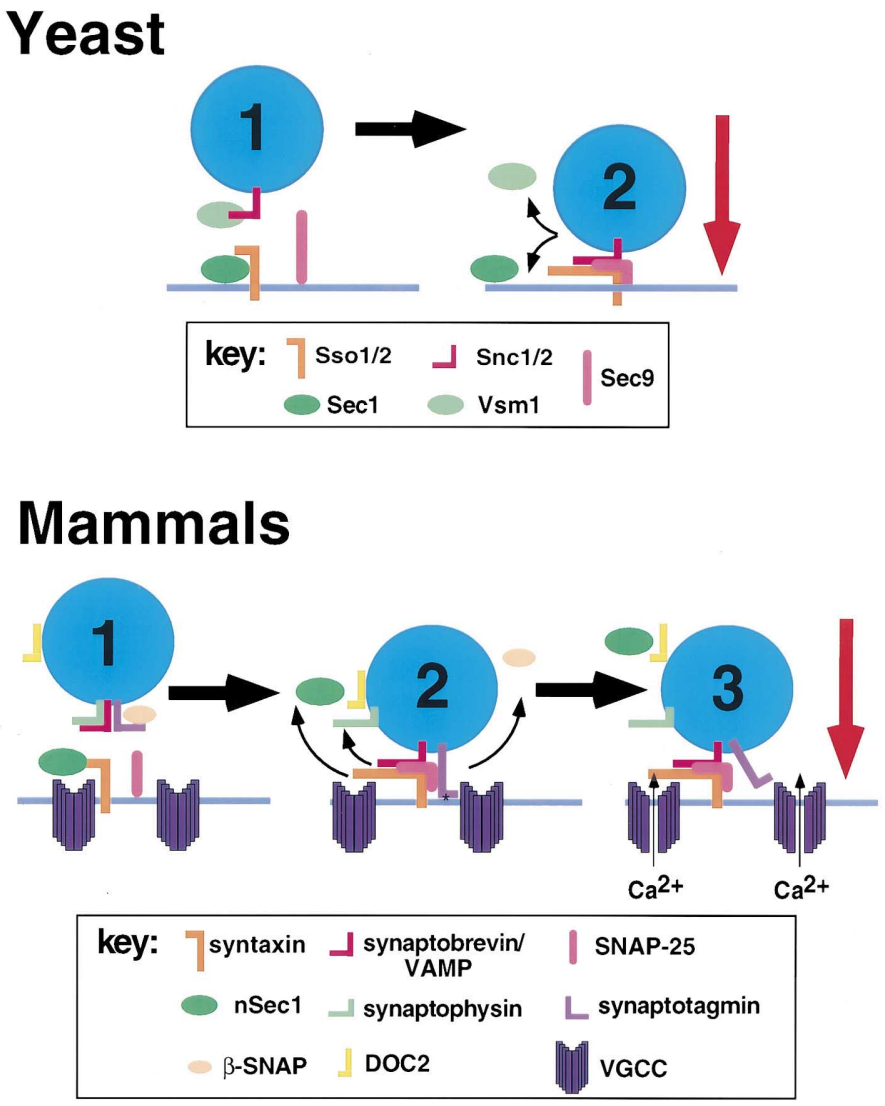


Figure 1. Exocytosis in yeast and mammals. Docking and fusion of exocytic vesicles is conserved between simple eukaryotes, such as yeast, and mammals. Yeast: in yeast, secretory vesicles dock and fuse with the plasma membrane in a constitutive fashion via formation of a Snc-Sso-Sec9 SNARE complex. (1) Prior to docking and fusion, putative SNARE-masters (i.e. Sec1 and Vsm1) are bound to the Sso t-SNARE and Snc v-SNAREs, respectively. These may serve to restrict docking and fusion until a specific site for docking and fusion is established. This is likely to involve components of the exocyst complex and, perhaps, the Sec4 GTPase, that act upstream to membrane fusion (not shown). (2) Dissociation of the putative SNARE-masters is presumed to occur after establishment of the secretion landmark (at the site of budding) allowing for vesicle encroachment, SNARE assembly and fusion to occur. The mechanism by which Sec1 and Vsm1 are dissociated is not yet resolved but may involve a signal resulting from Sec4 GTPase function at the bud site. The red arrow indicates the membrane fusion event. Mammals: in mammalian systems, as well as with other higher eukaryotes (i.e. *Drosophila* and *C. elegans*), neurotransmitter release from presynaptic nerve terminals is mediated in a regulated calcium-dependent fashion. (1) Prior to docking and fusion, the putative SNARE-masters (i.e. nSec1/Munc18 and synaptophysin) are bound to the syntaxin t-SNARE and synaptobrevin/VAMP v-SNARE, respectively. (2) Vesicles may undergo docking and remain in the docked state, by which a stable VAMP-syntaxin-SNAP-25 complex is assembled between opposing membranes. Dissociation of the SNARE-masters (i.e. nSec1 and synaptophysin) occurs prior to SNARE assembly via an unknown mechanism which may involve the rab3a GTPase (not shown). At this stage, synaptotagmin is likely to function as a component of the SNARE complex and act as a calcium ($Ca^{2+}$)-sensitive clamp. The asterisk (*) denotes protein-lipid interactions between synaptotagmin and the presynaptic membrane. Voltage-gated $Ca^{2+}$ channels (VGCCs) are also expected to be associated with syntaxins at this stage, effectively maintaining the channel in its inactive state. (3) Upon stimulus, nerve cell depolarization allows for the influx of calcium via VGCCs, resulting in the dissociation of synaptotagmin and allowing for membrane fusion to proceed in a SNARE-dependent fashion. The red arrow indicates the membrane fusion event.

increase in helicity upon association with their SNARE partners [7–9], suggesting that formation of the complex is energetically favorable. This lends credence to the idea that SNARE assembly overcomes the electrostatic forces which prevent membrane fusion from occurring.Far greater knowledge of the core complex at the molecular level has very recently become available. Studies on the structural arrangement of the exocytic SNARE complex have revealed that the $\alpha$-helical regions of the v- and t-SNAREs assemble into a structure composed of four parallel helical domains [10–12]. More important, crystallographic resolution of the complex, as determined by Brunger et al., reveals that it forms a tight four-helix bundle possessing both a packed charged and hydrophobic core, and grooved outer surface [12]. The $\alpha$-helices are held together by hydrophobic interactions, as well as side chain-mediated hydrogen bonding and ionic interactions. Moreover, it was found that leucine zipper-like layers present within the complex stabilize the bundle and shield the ionic and hydrophobic core regions from interactions with the solvent [12]. Although bearing a resemblance to leucine zippers found in molecules such as the Gcn4 transcription factor, the geometry of the helical domains of the fusion complex deviates from classical zippers, as well as from the standard coiled-coil domains. This suggests a unique structure which differs markedly from known viral fusion proteins, such as hemagglutinin. The shallow surface grooves, formed by helix association, are likely to allow for the binding of specific regulatory factors, such as SNAPs, NSF and so on, which have been proposed to mediate complex disassembly (see below). The exact mechanism by which membrane fusion occurs can still be regarded as speculative; however, studies of the mechanism of viral entry have proven quite informative [13–18]. Models to explain how membranes bridge and fusion pores form have already been put forth [16, 19–21]. Nevertheless, recent pioneering work has demonstrated that the SNAREs themselves may possess the information necessary and sufficient to confer bilayer fusion. This was shown, albeit at low efficiency, using a liposome-based in vitro fusion assay [22]. With this in mind, our review will focus upon the SNAREs and their accessory proteins that appear to play intimate roles in mediating the fusion event. Several schematics to illustrate the events leading to bilayer fusion. For example, figure 1 illustrates the events and protein-protein interactions which lead to vesicle docking and exocytosis in constitutive (yeast) and regulated (neurons) secretory systems. Figure 2 illustrates the putative protein-protein interactions mediated by the different types of SNAREs and their regulators. Finally, a model for the dynamic cycling of SNAREs, as they transit from from fusion-incompetent to fusion-competent states, and back, is presented (fig. 3).

**The VAMP/synaptobrevin family of v-SNAREs**

Synaptobrevin/VAMPs were first identified as components of synaptic vesicles [23, 24] and only later as elements which participate in the binding of SNAP proteins in vitro [1]. No less important was their identification [25] as substrates for clostridial neurotoxins (i.e. botulinum, BoNT, and tetanus, TeTx, toxins) which proteolyze specific targets at the presynaptic membrane and inhibit synaptic transmission [26–28]. These studies provided convincing evidence for the involvement of SNAREs in stimulus-coupled exocytosis

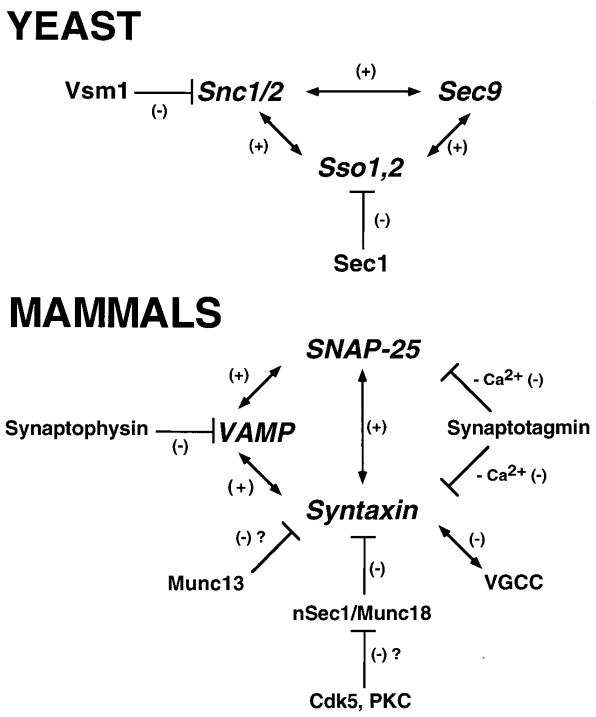


Figure 2. Interactions between SNAREs and SNARE-masters The proposed interactions between SNAREs and SNARE-masters in yeast and mammalian systems are shown. Positive interactions (+) are designated as those protein-protein interactions which lead to SNARE assembly and membrane fusion. Negative interactions (−) are designated as those which restrict protein function and inhibit membrane fusion. Possible negative interactions are represented as '(−)?'. Negative interactions illustrated with two-way arrows indicate that each protein involved may exert an inhibitory constraint upon the other. In the case of mammalian Sec1/munc18, '(−)?' indicates possible negative regulation by PKC and cyclin-dependent kinase (Cdk5) via phosphorylation. Voltage-gated calcium channels are represented as VGCC.

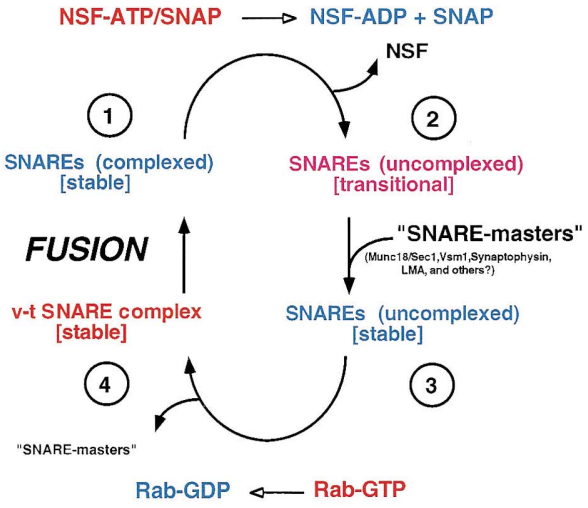


Figure 3. The SNARE cycle. Membrane fusion in eukaryotes is controlled by a hierarchy of factors which act upon SNAREs to regulate their assembly, leading to vesicle docking and fusion. In this model, we conjecture that SNAREs exist in four distinct phases (or states) during the SNARE cycle which leads to fusion. SNAREs are initially found in a stable complex on the same membrane (e.g. arranged in cis) (phase 1). This is likely to occur on SNAREs present both on vesicles and the plasma membrane. A cue which is mediated presumably by vesicle encroachment to the acceptor membrane allows NSF to bind ATP and associate (via SNAP) with ternary SNARE complexes, forming the 20S particle. Subsequent hydrolysis of ATP by NSF, results in dissociation of the particle and leaves SNAREs in an unfolded transitional state (phase 2). This now allows for the association with proteins that stabilize SNAREs in an uncomplexed (relative to other SNAREs) and activated state (phase 3). These proteins include the SNARE-masters, that is Sec1/Munc18 (yeast and mammals), synaptophysin (mammals), Vsm1 (yeast), LMA (yeast) and, possibly, others. Next, rab proteins in their activated GTP-bound state associate with SNAREs in their stable, SNARE-master-bound, uncomplexed state. This might occur directly or via their putative effectors (i.e. rabphilin, RIM etc.). Presumably, it occurs on both donor and acceptor membranes in order to the ready v- and t-SNAREs on their respective membranes. Hydrolysis of GTP by rab is expected to lead to dissociation of the SNARE-masters, allowing for formation of a stable ternary SNARE complex between opposing membranes (phase 4). Alternatively, GDP-GTP exchange mediated by rab GEFs might be sufficient to confer such an event, based upon recent studies (see text). SNARE-mediated membrane fusion may occur immediately in constitutive secretory systems, but requires the influx of calcium in regulated secretory systems. Following fusion, SNAREs return to their stable cis conformation (phase 1). v-SNAREs are expected to cycle back to donor membranes via retrograde sorting mechanisms, whereas t-SNAREs may remain associated with the acceptor membrane.

from neurons. Additionally, the use of neurotoxins which have defined substrate specificities has proven invaluable for dissecting the involvement of individual SNARE components in given exocytic events. Specifically, light chains belonging to the TeTx and BoNT/B, D, F and G toxins were found to cleave certain synaptobrevin/VAMP molecules, whereas those of BoNT/A and E cleave SNAP-25, and that of BoNT/C acts upon syntaxins [26–28]. Although each toxin recognizes a specific peptide bond (the cleavage site), a second determinant, consisting of a common $\alpha$-helical motif that comprises both hydrophobic and negatively charged residues, may be necessary for toxin function [29].

**Synaptobrevin/VAMP forms in mammals.** Currently, there are at least four synaptobrevin/VAMP isoforms (excluding alternative splicing) which have been identified as functioning upon secretory granule, dense core vesicle (DCV) and synaptic vesicle release in mammalian systems (see table 1). For reviews regarding the biogenesis and nature of these different secretory organelles, see [30–34]. Synaptobrevin/VAMP family members are small type II membrane proteins of about 120 amino acids. Structurally, they consist of a variable region of 25–35 amino acids located at the amino terminus, followed by either one extended or two short amphipathic $\alpha$-helical segments predicted to form coiled-coil structures, and have a transmembrane domain located at their carboxyl terminus. The putative $\alpha$-helical regions, originally designated as Helix 1 (or H1) and Helix 2 or (H2) [35–37], are required for these SNAREs to mediate their protein-protein interactions, as shown by studies employing in vitro binding experiments [35, 36, 38, 39], as well as in vivo exocytosis assays, using both yeast [37] and mammalian cells [35, 36]. Targeting to synaptic-like vesicles is mediated by the H1, as shown using PC12 cells [35], whereas both helices participate in binding to the syntaxin and SNAP-25 t-SNAREs [35, 36, 38–40]. Synaptobrevin/VAMP binding to these t-SNAREs in vitro has been shown to occur at regions of their carboxyl terminals which have the potential to form coiled coils [38, 41–44]. Interestingly, bacterially synthesized synaptobrevin/VAMP lacking the membrane-spanning domain has been proposed to be largely unstructured in monomeric form [7, 9]. Yet both yeast and mammalian isoforms were found to assume an $\alpha$-helical conformation upon SNARE assembly, based upon circular dichroism studies [7, 9, 45]. This supports the idea that complex thermostability achieved by the ternary SNARE assemblages could provide the driving force for bringing opposing membranes into juxtaposition. To this end, it has been shown that SNAREs on liposomes can, by themselves, confer docking and fusion reactions, although at low efficiency [22].

The use of neurotoxins and specific antibodies has shown that toxin-sensitive isoforms, like VAMP2, are involved in stimulus-coupled secretion in a variety of cell types, including neurotransmitter release from neurons [23, 24]; insulin release from pancreatic $\beta$-cells [46]; zymogen granule release from pancreatic acinar cells

[47]; catecholamine release from adrenal chromaffin cells [48]; and insulin-stimulated GLUT4 translocation in adipocytes [49]. As predicted, VAMP2 localizes to secretory organelles from these cell types, including synaptic vesicles in neurons [24, 50]; secretory granules in adipocytes [51], pancreatic $\beta$-cells [46] and adrenal chromaffin cells [48]; tubulovesicles in parietal cells [52]; and water channel-containing vesicles in kidney medullar cells [53]. Its closest relative, VAMP1, is insensitive to neurotoxins and is widely expressed in nervous tissue. It is also expressed in some nonneuronal tissues [54], but was shown specifically not to localize to GLUT4-containing vesicles in adipocytes [55], water channel-containing vesicles in medullar cells [53] or secretory granules in pancreatic acinar cells [47], and is not detected in membranes derived from PC12 cells [56]. Thus, unlike VAMP2, the functioning of this isoform seems limited to certain cell types (i.e. neurons). VAMP1 has been shown to exist in two alternatively spliced forms (VAMPs 1A and 1B) which may be tissue- and organelle-specific [57, 58]. Interestingly, the VAMP1B spliceoform has been shown to localize to mitochondria in endothelial cells, suggesting that it may be involved either in mitochondrial membrane fusion or, perhaps, the tethering of mitochondria to the cell membrane [58].

While VAMP1A and VAMP2 are discretely expressed and appear involved in stimulus-coupled secretory granule, DCV and synaptic vesicle release, other VAMP isoforms, including cellubrevin [59] and toxin-insensitive VAMP (TI-VAMP) [60], are more widely expressed and may act upon constitutive secretion. Cellubrevin (VAMP3) is a ubiquitously expressed VAMP isoform which may confer both [59]. In principle, this is due in part to its tissue-wide expression, coupled with its described localization to numerous types of secretory organelles, including GLUT4-containing vesicles in adipocytes [55, 61, 62]; secretory granules in pancreatic acinar cells and $\beta$-cells [63]; water channel-containing vesicles in kidney medullar cells [64]; synaptic-like microvesicles in PC12 cells [65]; and transferrin receptor-containing vesicles in Chinese hamster ovary (CHO) cells [66]. TI-VAMP (VAMP7), on the other hand, localizes to the apical membrane in polarized CaCo-2 epithelial cells and, like cellubrevin, was proposed to act upon constitutive exocytosis in collaboration with other ubiquitously expressed SNARE elements, including SNAP-23 and syntaxin 3 [67] (discussed below). It may also participate in stimulus-coupled secretion and confer membrane fusion in exocytic events which have been shown experimentally to be only partially sensitive to toxin treatment [46, 47, 55, 68]. TI-VAMP/VAMP7 might also be involved in endosome-trafficking events, which would explain its localization to a compartment in normal rat kidney fibroblasts (NRK) cells that contains the lgp120 lysosomal marker [69]. Another ubiquitously expressed VAMP homolog, known as endobrevin (VAMP8), was shown to localize with the transferrin receptor to a late recycling compartment of early endosomes, suggesting that it may act at the level of endosomal sorting [69, 70]. Finally, other vertebrate synaptobrevin/VAMP homologs (i.e. VAMPs 4–6) are still in the process of being characterized and no doubt will serve to highlight the role of v-SNARE family members in other trafficking events. For example, VAMP5 has been very recently shown to reside at the level of the plasma membrane and may be implicitly involved in myotube formation during myogenesis [71].

**Synaptobrevin/VAMP forms in lower eukaryotes.** VAMP/synaptobrevin isoforms have been described in several lower eukaryotes and have lent themselves to genetic analysis in vivo. For example, two synaptobrevin/VAMP homologs are found in the yeast *S. cerevisiae*. These proteins, Snc1 and Snc2 [72, 73], are components of the two types of secretory vesicles in yeast [73–75] that deliver new lipids, secreted enzymes and membrane proteins to the plasma membrane in a constitutive fashion. Like their neuronal counterparts, these v-SNAREs interact physically to form a ternary complex in vitro with t-SNAREs of the plasma membrane [76], which include the SNAP-25 homolog, Sec9 [77], and the syntaxin homologs Sso1 and Sso2 [78]. Genetic studies also support the idea that these SNAREs act together in vivo to confer vesicle docking and fusion [37, 79]. Deletion of both the *SNC1* and *SNC2* genes results in an accumulation of secretory vesicles and inhibited exocytosis, leading to conditional lethality [73]. Thus, yeast maintain some level of secretion competence, despite the lack of these exocytic v-SNAREs. Interestingly, these mutations are suppressed by the inactivation of genes involved in ceramide and sphingolipid synthesis [75], suggesting that sphingolipid metabolism may play an important role in SNARE functioning and membrane fusion in yeast (see below).

Similar to synaptotagmin and the SNAP-25 t-SNARE in mammals (see below), Snc v-SNAREs were found to undergo palmitoylation [80]. This posttranslational modification occurs on unique cysteine residues lying adjacent to the transmembrane domain in the two homologs [80]. The role of this palmitoylation is not fully known, but it is important for Snc protein stability. It may also prove relevant to protein-protein interactions with other components of the secretory pathway [81]. Palmitoylation of synaptobrevin/VAMPs in higher organisms has not yet been demonstrated, but remains a possibility due to the presence of conserved cysteines at the same position. Structural analysis of the Snc v-SNAREs, using in vivo expression studies, has revealed

that the requirements for exocytosis in yeast [37] are identical to those described for VAMP2 [36]. The latter study examined the requirements necessary for conferring insulin secretion from permeabilized HIT-T15 cells, using toxin-insensitive forms of the VAMP expressed in toxin-treated cells [36]. Thus, members of the synaptobrevin/VAMP family are not only structurally conserved in evolution, but functionally conserved as well [37, 73, 82, 83].

This idea is equally borne out by studies on other lower eukaryotes. A gene encoding a synaptobrevin/VAMP homolog (*snb-1*) was identified in the nematode *C. elegans*, and its inactivation was found to result in embryonic lethality, whereas hypomorphic mutants show defects in synaptic transmission [84]. Interestingly, these mutations tended to fall in, or near, the conserved heptad repeats which comprise the putative $\alpha$-helices required for exocytosis in yeast [37] and mammals [35, 36]. One mutation was found to terminate the transmembrane domain prematurely [84], and it would be interesting to determine whether palmitoylation of this mutant v-SNARE helps confer sorting of this protein to vesicles. In *Drosophila*, targeted expression of TeTx results in the loss of evoked neurotransmitter release, presumably resulting from the inactivation of one of the synaptobrevin isoforms (*n-syb*) [85]. The presence in these embryos of *syb* [86], a ubiquitously expressed form of synaptobrevin/VAMP, as well as synaptotagmin, another synaptic vesicle protein which has some v-SNARE-like qualities [87] and [88] (see below) may prevent defects in embryogenesis from occurring. More recently, null mutations in *n-syb* were shown to result in a loss of evoked neurotransmitter release at neuromuscular junctions, leading to paralysis [89]. In contrast, spontaneous release of neurotransmitter, which may result from the fusion of individual synaptic vesicles at the presynaptic membrane, was not blocked, suggesting that another synaptobrevin/VAMP molecule may be involved in synaptic transmission in *Drosophila*.

## The syntaxin family of t-SNAREs

In addition to synaptobrevin/VAMP, which was proposed to act as the v-SNARE in membrane fusion, two other types of proteins are present in the detergent-resistant and thermostabile complex that binds to $\alpha$-SNAP and NSF in vitro [1, 3]. These include members of the syntaxin and SNAP-25 families of proteins, that are proposed to constitute the t-SNAREs which participate in membrane fusion.

**Syntaxins in mammals.** Like synaptobrevin/VAMP, the first syntaxin family members were first identified as components of presynaptic nerve terminals [90, 91] and only later as elements that participate in the binding of the SNAP proteins in vitro [1]. Currently, there are 16 described members of the syntaxin family (excluding alternative spliceoforms) in mammalian cells, making it by far the largest SNARE family. The discrepancy between the number of syntaxin and VAMP or SNAP-25 isoforms is unclear, but does suggest some promiscuity in the protein-protein interactions of the latter two families. Currently, at least four syntaxins have been shown to localize to the plasma membrane and are thought to participate in exocytosis. These include syntaxins 1A, B and, possibly, C, as well as syntaxins 2 through 4 [90–93]. Syntaxins 1A and B were the first syntaxin family members identified as SNAREs [1] and are widely expressed in the nervous system [92]. Syntaxin 1A, which is sensitive to the BoNT/C, was also suggested to be present on secretory granules and to mediate insulin release from pancreatic $\beta$-cells [94]. Syntaxins 2 and 3 have been localized to the apical membranes of polarized epithelial cells [95–97], whereas syntaxin 4 has been proposed to localize to the apical membrane in inner medullar cells [98] and the basolateral membrane in pancretic acinar and Madin-Darby kidney cells [97, 99, 100], and is found on GLUT4-containing vesicles in adipocytes [49, 93, 101]. It is not clear yet whether different syntaxins are necessary to mediate DCV and synaptic release from the same neurons, although such a possibility should not be ruled out. The numerous other syntaxin family members are likely to function at earlier steps along the secretory pathway, including endoplasmic reticulum (ER)-Golgi, intra-Golgi, Golgi-lysosome and endosomal transport.

Syntaxin open reading frames typically encode proteins of around 300 amino acids which bear at least three putative coiled-coil forming regions at the amino and carboxyl ends of the protein (designated H1, H2 and H3). Like synaptobrevin/VAMP, syntaxins are membrane proteins which possess a single transmembrane domain [90, 92]. The carboxyl terminal H3 coiled-coil region (typically, residues 190–270) was found to mediate SNARE-SNARE interactions, binding to both synaptobrevin [38, 42–44] and SNAP-25 [38, 41, 44]. The H1 and H2 regions may also inhibit the association of SNAREs with the H3 region, suggesting possible intramolecular regulation of SNARE assembly [42, 102]. Interactions with other components of the secretory pathway are also mediated by the putative coiled-coil domains, yet precise structural information has been lacking until recently. Circular dichroism studies performed on bacterially synthesized protein indicate that, unlike synaptobrevin/VAMP and SNAP-25, syntaxins possess a high degree of $\alpha$-helicity present in the proposed SNARE binding domain [8, 45, 103]. Interactions with $\alpha$-SNAP and, perhaps, other SNAP molecules are also mediated via this region [43, 104] and are strengthened by the core complex of syntaxin and

synaptobrevin [105], and the ternary SNARE complex [43, 105].

Unlike synaptobrevin/VAMP, syntaxins are involved in a number of other protein-protein interactions. Syntaxins have been shown to interact with voltage-gated calcium chennel (VGCC) via the carboxyl coiled-coil domain, although residues embedded in the membrane may also participate in this interaction [106, 107]. Electrophysiological studies in *Xenopus* oocytes revealed that full-length syntaxin stabilizes L- and N-type channels in an inactive state, whereas a carboxyl terminal truncated form was ineffective in doing so [108]. In addition, syntaxin 1A was also found to interact with, and to regulate, a nucleotide-gated chloride channel encoded by the cystic fibrosis gene [109]. Thus, syntaxins appear to play an important role in channel conductance and influence their control over the fusion machinery. Syntaxins were also found to bind synaptotagmin, a potential v-SNARE (see below), in a calcium-dependent fashion [44, 110, 111], as well as to a ubiquitously expressed form of SNAP-25, named SNAP-23/Syndet [112, 113]. The latter interaction implicates a role for syntaxins in constitutive exocytic events.

A function for the putative coiled-coil domain at the N terminus of syntaxin may be to bind to mammalian homologs of the yeast Sec1 protein [114, 115], a regulator of SNARE-SNARE interactions (see below). This interaction appears to be inhibited by phosphorylation of the Sec1 homolog, suggesting a role for protein modification in directly regulating vesicle docking and fusion [116]. Another function for the N terminus is to bind to the other C2 domain-containing proteins which interact with calcium and phospholipids, such as the mammalian unc-13 homologs [117] and syncollin [118] (see below).

**Syntaxin homologs in lower organisms.** As with synaptobrevin/VAMP, genetic studies performed in lower organisms have shed light on the functioning of syntaxin-like molecules in protein trafficking. In yeast, there are two syntaxin homologs, Sso1 and Sso2, which were discovered as multicopy suppressors for mutations in the yeast *SEC1* gene and essential for cell viability [78]. Deletion of either *SSO* gene has no effect in yeast; however, the knockout of both genes results in an accumulation of secretory vesicles and blocked secretion. Thus, these syntaxin-like proteins confer an essential function. Sso proteins were also shown to form ternary SNARE complexes with Snc1 and Snc2, along with the yeast SNAP-25 homolog, Sec9 [76, 77]. Therefore, the neuronal and yeast SNARE complexes are analogous. Like other syntaxins, recombinant Sso proteins possess a considerable $\alpha$-helical structure which increases upon forming binary complexes with the SNAP-25 domain of Sec9 and ternary complexes with Snc2 [9, 102]. This is essentially the same as that shown for the mammalian syntaxins in mammals [7, 8, 45] and illustrates a conserved link between folding and complex formation.

In *C. elegans*, the *unc-64* locus encodes a syntaxin 1 homolog which is expressed in neuronal and secretory tissues [119, 120]. The null mutant is paralyzed and is arrested in development, whereas hypomorphic alleles that have mutations in the carboxyl coiled-coil domain result in altered neurotransmitter release and behavioral defects. In *Drosophila*, a syntaxin 1 homolog, *syx-1A* [121, 122], is expressed in the nervous system and localizes to axons and synapses. Loss-of-function mutations result in embryonic lethality, whereas electrophysiological studies performed on these embryos indicated that a complete absence of spontaneous, and near absence of evoked, synaptic transmission had occurred [121]. Null mutations in *syx-1A* abolish neurotransmitter release entirely, with synaptic vesicles remaining docked at the plasma membrane [123]. Additional studies revealed that *syx-1A* is required for cellularization of the syncytial blastoderm and is present in the newly forming cleavage furrows [124]. This suggests that membrane fusion events play a pivotal role in the creation of surface membrane and may be mediated by the recruitment of vesicles to the site of membrane invagination. Thus, syntaxin-like proteins in lower eukaryotes play important roles in synaptic transmission, as well as development.

## The SNAP-25 family of t-SNAREs

The third SNARE family represented in the detergent-resistant 7S complex that binds $\alpha$-SNAP and NSF in vitro [1, 3] is the synaptosomal protein, SNAP-25 [125]. Like synaptobrevin/VAMP and syntaxins, this SNARE family includes mammalian forms thought to act upon both regulated and constitutive secretion, and homologs in lower eukaryotes which have lent themselves to genetic analysis.

**SNAP-25 proteins in mammals.** SNAP-25 was originally identified as a synaptosomal protein of 25 kDa which is expressed in nervous tissue [125] and has been shown to exist in two variants (SNAP-25 A and B) [126]. SNAP-25 is also expressed in adrenal chromaffin cells, insulin-responsive tissues (i.e. adipose and skeletal muscle) and clonal pituitary cells, suggesting that it may participate in stimulus-coupled secretion from nonneuronal cells [127–130]. As with other SNAREs, SNAP-25 possesses heptad repeats that have a high probability of forming $\alpha$-helices and coiled coils. A putative coiled-coil region located at the carboxyl terminus of SNAP-25 has been shown to mediate association with synaptobrevin/VAMP and syntaxin [38, 41]. Despite the probability of forming coiled coils (based upon al-

gorithmic analyses), the $\alpha$-helical composition of recombinant SNAP-25 was found to be only marginal, as measured by circular dichroism [7, 45]. Yet $\alpha$-helicity was found to increase dramatically upon binding to a soluble domain of syntaxin, indicating that the secondary (and tertiary) structures are induced by ternary complex formation [7, 45]. Again, this suggests a direct link between protein folding and complex assembly. One small note of caution might be in order here, as it is not entirely clear whether SNAREs expressed in bacteria undergo proper folding. This may be due to the lack of specific chaperones which might play a role in SNARE folding in eukaryotes. While the recombinant proteins are apparently able to undergo assembly and disassembly in vitro, their secondary structures may not be identical to those of proteins expressed in eukaryotic cells. Since recombinant synaptobrevin/VAMP and SNAP-25 are largely unstructured with respect to helical content, one begins to suspect that SNARE chaperones may be needed to prevent spontaneous SNARE assembly, perhaps, upon protein insertion into the ER.

Like synaptobrevin/VAMP and syntaxin, SNAP-25 is sensitive to neurotoxins (e.g. BoNT/A and E) [128, 131, 132]. Toxin treatment and cleavage, which result in truncation of the carboxyl terminus, weakens the interaction between SNAP-25 and VAMP in vitro [41, 43] and inhibits exocytosis from nerve endings, permeabilized PC12 and chromaffin cells [132, 133]. Microinjection of peptides or antisense oligonucleotides was found to mimic the effects of neurotoxins and inhibited both axonal growth [134] and catecholamine release from chromaffin cells [135, 136]. Thus, inhibition of SNAP-25 functioning has a strong impact upon secretion competence, as described for synaptobrevin/VAMP and syntaxin.

Unlike synaptobrevin/VAMP and syntaxins, SNAP-25 does not bear a membrane-spanning domain, although it is membrane-associated [125]. This association is likely to be mediated by palmitoylation, as mutants lacking in specific cysteine residues which precede the coiled-coil domain are found in the cytosolic fraction [137, 138]. The association of SNAP-25 with membranes may also be mediated by interactions with syntaxins, which complex together with SNAP-25 in axons [139] and recycling synaptic vesicles [140].

In addition to interactions with v- and t-SNAREs, SNAP-25 interacts with the proposed calcium sensor in exocytosis, synaptotagmin (see below), even after toxin treatment [88]. In addition, SNAP-25 has been suggested to bind to, and regulate, calcium channels [141–143]. These and earlier findings imply an additional regulatory role for SNAP-25 in the calcium-activated steps which lead to membrane fusion. Correspondingly, SNAP-25 has also been found to undergo phosphorylation in response to glucose or a glucagon analog in an insulinoma cell line [144] and under in vitro conditions [145, 146]. This suggests that that posttranslational modification may be directly involved in its regulation. Finally, a constitutively expressed form of SNAP-25, SNAP-23 [112, 113], is present in nonneuronal tissues and is likely to participate in both constitutive and stimulus-coupled exocytic event [147–151]. This form is sensitive to cleavage by the BoNT/E, but not BoNT/A, neurotoxin [152] and appears able to replace SNAP-25 in insulin secretion from toxin-treated hamster pancreatic beta cell line (HIT) cells [147, 148]. Thus, members of this t-SNARE family are expected to act upon constitutive secretion, along with cellubrevin/TI-VAMP and different syntaxin family members.

**SNAP-25 homologs in lower eukaryotes.** A protein of the late exocytic pathway in yeast, Sec9, has been shown to bear some homology to SNAP-25 at its carboxyl terminus and to confer similar functions in secretion. The *SEC9* gene was identified as a multicopy suppressor of mutations in the Sec4 guanine nucleotide triphosphatase (GTP)ase (see below) and encodes a component of the yeast exocytic SNARE complex [76, 77]. This complex consists of the Snc v-SNARE components and Sso t-SNARE components, described above. Sec9 is an essential component of yeast, and disruption of the *SEC9* gene results in a block in exocytosis and lethality [77, 153]. Like its mammalian homologs Sec9 is found associated with plasma membrane, although it bears no cysteine residues which could be sites for palmitoylation and membrane association. The mechanism by which Sec9 adheres to membranes is unknown, but is likely to be mediated, in part, by its association with the Sso t-SNAREs. Interestingly, two independent temperature-sensitive alleles of *SEC9* have been identified, one (*sec9-4*) which cannot undergo ternary complex formation in vitro at restrictive temperatures, whereas the other (*sec9-7*) is able to, though it cannot confer constitutive exocytosis in vivo [76]. These two mutations map to different areas of the protein and imply a role for the carboxyl terminus in membrane fusion. Other homologs from lower eukaryotes, such as *Drosophila*, have been identified [154] and should prove useful in identifying the actions of SNAP-25 in development.

## The synaptotagmin family of proteins: SNAREs, SNARE-masters, or both?

Synaptotagmins are an extremely interesting and somewhat enigmatic class of molecules, based upon their actions and possible functions. At least a dozen synaptotagmin family members have been identified thus far. Several of them have been demonstrated to undergo alternative splicing, and are expressed in neurons and neuroendocrine tissues [155–158], while at least one

form is expressed in nonneuronal tissues [159]. Synaptotagmins were first identified as synaptic vesicle-associated membrane proteins that possess two calcium-binding motifs, called C2 domains (C2A and B), which are preceded by an $\alpha$-helical coiled-coil domain [155, 160]. The C2A domain was shown to bind negatively charged phospholipids in a calcium-dependent fashion [161], whereas the C2B domain has been proposed to bind to inositol polyphosphates independent of calcium [162, 163]. More recently, the C2B domain has been shown to bind to phosphatidyl-inositol-3,4,5-triphosphate ($PIP_3$) in the absence of calcium and $PIP_2$ in its presence, suggesting that a lipid-interaction switch might occur during depolarization [164].

Interestingly, calcium binding also alters synaptotagmin's protein-protein interactions. Calcium binding to the C2A domain increases its affinity for syntaxin [165], whereas binding to the C2B domain appears to confer dimerization [166, 167] and binding to the SV2 synaptic vesicle protein [168]. Synaptotagmin was also found to associate with voltage-gated calcium channels (VGCCs) [169–172], like syntaxins, suggesting a similar role in channel regulation. Finally, synaptotagmin was found to bind stably to the SNAP-25 t-SNARE via the C2B domain, even after neurotoxin treatment [88], and was proposed therein to act as a possible calcium-regulated v-SNARE in exocytosis.

The potential to bind calcium implicated synaptotagmin as a possible calcium sensor which could confer the calcium-mediated signal in exocytic release [173–176]. Genetic studies using clonal variants of PC12 cells which lack synaptotagmins initially suggested that depolarization-induced acetylcholine release could be calcium-independent and that the protein might not be essential for secretion [177]. However, studies using knockout mice revealed that homozygous knockouts died shortly after birth and had defects in evoked neurotransmitter release and synaptic transmission [175]. These defects in mice are similar to those described above for null mutants of *n-syb* in *Drosophila* [89]. Mutations in *Drosophila* synaptotagmin also resulted in a substantial decrease of the evoked calcium-triggered response, coupled with a corresponding increase in frequency of spontaneous release [178–181]. These works suggest that the evoked and spontaneous releases of neurotransmitter are likely to be mediated by separate pathways, one of which being devoid of synaptotagmin functioning. In *C. elegans*, mutations in the *snt-1* gene resulted in deficiencies in synaptic transmission leading to behavioral and motor defects [182]. Results from these genetic studies are supported by microinjection experiments using peptides corresponding to, or antibodies directed against, the synaptotagmin protein [183–185]. Thus synaptotagmin plays an essential role in evoked synaptic transmission.

Because null mutations tend to increase the frequency of spontaneous neurotransmitter release, it has been suggested that synaptotagmin acts as a calcium-dependent clamp (or sensor) in exocytosis [173–176, 186] (see fig. 1). This idea is supported by studies using *Aplysia*, whereby genetic manipulations of synaptotagmin expression were consistent with an inhibitory function. One possible explanation is that synaptotagmin participates in clamping by acting as a regulator of syntaxin-SNAP-25 and syntaxin-VGCC interactions (i.e. as a SNARE regulator). Synaptotagmin has also been proposed to act directly as a v-SNARE in stimulus-coupled secretion [88]. Although not isolated in the original SNAP binding assay [1], a second SNAP isoform ($\beta$-SNAP) was found to bind to synaptotagmin in vitro and to recruit NSF [3, 87]. Additionally, this group noted that synaptic vesicles remain docked in neurotoxin-treated cells and that the synaptotagmin-SNAP-25 interaction is unperturbed [88]. Thus, synaptotagmin was proposed to act as a negative regulator for the binding of $\alpha$-SNAP in $\alpha$-SNAP-mediated fusion mechanisms and a positive regulator in $\beta$-SNAP-mediated fusion mechanisms [3, 87].

In contrast, others found that there is no difference in the extent of calcium-regulated exocytosis from permeabilized cells, conferred using either $\alpha$- or $\beta$-SNAP, and concluded that these proteins may not necessarily play distinct roles in secretion [187]. Recent work (discussed below) suggests that the SNAP and NSF components are more likely to act prior to SNARE assembly [188] and are involved in the priming step which dissociates preformed complexes in the same membrane [189–191]. Thus the issue of whether or not synaptotagmin is a calcium-regulated SNARE or SNARE regulator remains unresolved. Clearly, it has SNARE properties since it acts as a SNAP receptor in vitro, undergoes protein-protein interactions similar to synaptobrevin/VAMP and localizes to vesicles. Yet, since it appears to clamp the ternary complex in a calcium-modulated fusion-inhibited state, synaptotagmin is also a regulator of SNARE function. While largely a semantic issue, it might be put to rest by determining whether synaptotagmin can substitute for synaptobrevin/VAMP in the in vitro, SNARE-dependent, liposome fusion assay [22]. Likewise, extensive mutational analysis of the C2 domains of synaptotagmin might yield molecules which are attenuated as clamps and could, potentially, confer fusion constitutively in in vitro and in vivo assay systems.

## SNARE regulators in exocytosis: masters or slaves to the system?

SNARE assembly culminating in membrane fusion is expected to occur only at the appropriate target mem-

Table 2. SNARE regulators participating in exocytosis in yeast and mammals.

| Mammals | Secretion event/localization | Target | Yeast equivalent (site target of action) |
|---|---|---|---|
| *Putative v-SNARE regulators* | | | |
| Synaptophysin I | regulated/synaptic vesicles, secretory granules | VAMP | none |
| Synaptophysin II | regulated/synaptic vesicles, secretory granules | VAMP | none |
| Pantophysin | constitutive/secretory vesicles | cellubrevin | none |
| mVsm1 (not yet identified) | | VAMP? | Vsm1 (secretory vesicles) |
| mVAP33 | | VAMP? | Scs2 (endoplasmic reticulum) |
| *Putative t-SNARE regulators* | | | |
| nSec1/Munc18 | regulated/plasma membrane | syntaxin | Sec1 (plasma membrane/ Sso1,2) |
| mLMA1 (not yet identified) | | syntaxin | LMA1 (vacuoles, ER-Golgi?/Vam3) |
| *Other possible SNARE regulators* | | | |
| Synaptotagmin I, II, III, V, X | regulated/synaptic vesicles, secretory granules? | syntaxin, SNAP-25 | none |
| rab3 (rab3A-D) | regulated/synaptic vesicles, secretory granules | rabphilin RIM syntaxin? VAMP? | Sec4 (vesicles/Sec9-Sso, exocyst) |
| NSF | regulated and constitutive/cytoplasm | SNAP | Sec18 (non-specific/Sec17) |
| $\alpha$-SNAP | regulated and constitutive/cytoplasm and SNAREs | syntaxin, VAMP, SNAP-25 | Sec17 (non-specific/ SNAREs) |
| $\beta$-SNAP | regulated/cytoplasm and SNAREs | synaptotagmin | none (probably redundant with Sec17) |
| Munc13 | regulated/plasma membrane | syntaxin DOC2 | none |
| DOC2A and B | regulated/synaptic vesicles | nSec/Munc18, Munc13 | none |
| Syncollin | regulated/plasma membrane | syntaxin | none |
| SV2 | regulated/synaptic vesicles | synaptotagmin | none |
| Hrs-2 | regulated/plasma membrane | SNAP-25 | none |

brane, in order to confer specificity to protein transport. Yet v- and t-SNAREs involved in late trafficking events (i.e. post-Golgi) passage together through the secretory pathway and probably reside on identical ER- and Golgi-derived transport vesicles. Thus, some mechanism(s) must prevent nonproductive SNARE interactions from taking place earlier in the pathway. Likewise, other cellular mechanisms may mediate specific v-t SNARE interactions in order to confer both temporal and spatial regulation of membrane docking and fusion. Thus, cells have probably evolved mechanisms to prevent nonproductive SNARE partnering early in the pathway and ready the SNAREs for assembly when they reach the appropriate target membrane. Therefore, in the hierarchy of events which lead to membrane fusion, an activation step to remove these constraints might be a prerequisite for docking and fusion to proceed in vivo. This step might include dissociation of preformed SNARE complexes, as proposed by Ungermann et al. [190], as well as the removal of other inhibitory constraints placed upon SNAREs. Such constraints include potential negatively acting SNARE regulatory proteins, which we have designated as SNARE-masters [74, 192]. By definition, these regulators are expected to bind directly to specific v- or t-SNARE partners and downregulate trafficking functions by modulating their entry into SNARE complexes. Thus, dissociation of SNARE regulators from SNAREs, or their inactivation, is expected to precede complex assembly and membrane fusion. That in mind, it should be noted that regulation of SNARE-master function will also be an important step leading to exocytosis. This establishes a hierarchy in which a SNARE-master might find itself being more of a slave than an actual master. A list of proteins with possible SNARE regulatory functions is given in table 2.

## Putative t-SNARE regulators in exocytosis

**Sec1 proteins in lower and higher eukaryotes.** One family of SNARE regulators which act upon both constitutive and regulated exocytosis is that of the yeast Sec1

protein and its homologs (i.e. n-Sec1, rbSec1, Munc18, unc-18) found in higher organisms [114, 193–197]. *SEC1* was identified in the original *sec* mutant screen of Novick et al. [153] and encodes a soluble protein that interacts with members of the Sso/syntaxin family of t-SNAREs [78, 114, 115, 197, 198]. Like other *SEC* genes, *SEC1* is essential for growth, and inactivating mutations lead to the accumulation of post-Golgi secretory vesicles [153]. Genetic studies reveal that strains bearing temperature-sensitive mutations in *SEC1* and another late-acting *SEC* gene (i.e. *SEC4*) result in lethality under conditions permissive for the individual mutations. Thus, the Sec1 protein in yeast is likely to confer exocytosis in a positive-acting fashion.

Despite this, studies of the mammalian Sec1 homologs reveal that they are not components of the SNARE complex, but do bind directly to the syntaxin t-SNAREs [114, 115, 195]. Moreover, recombinant mammalian Sec1 was found to inhibit SNARE assembly from occurring in vitro and to dissociate from syntaxin after assembly had occurred [114, 115, 195]. This suggests that Sec1 homologs may restrict SNARE partnering by preventing the t-SNAREs (syntaxin and SNAP-25) from assembling into binary complexes. Thus, these proteins may exhibit negative regulatory functions. This idea is supported by studies performed in vivo which demonstrate that the overexpression of ROP, a *Drosophila* homolog of Sec1[199], in larvae can inhibit neurotransmitter release at the neuromuscular junction [200]. More recent work has shown that lowered levels of ROP may also inhibit both spontaneous and evoked neurotransmitter release [201]. Likewise, the *unc-18* gene from *C. elegans* was shown to be important for neurotransmitter release, as hypomorphic mutations result in paralysis, due to defects in axonal transport [194].

On the basis of the in vitro binding experiments, Sec1 and its homologs appear to act as SNARE-masters for the Sso/syntaxin family members by virtue of their ability to bind to these t-SNAREs and prevent them from forming productive interactions with the SNAP-25 components. On the other hand, in vivo experiments do not rule out effects which can be construed as positive. Thus, their interactions with syntaxin may mediate more than one function in conferring the fusion event. Interestingly, mammalian Sec1 is phosphorylated by protein kinase C in vitro [116] and by Cdk5 [202], a neuronal cyclin-dependent kinase. This modification was shown to inhibit interaction with syntaxin in vitro, suggesting a possible role for protein phosphorylation in the regulation of vesicle docking and fusion by Sec1 isoforms.

## Putative v-SNARE regulators in exocytosis

**Synaptophysins.** A candidate SNARE-master for v-SNAREs involved in regulated exocytosis is synaptophysin, a membrane protein from synaptic vesicles [203, 204]. Synaptophysin was found to complex directly with members of the synaptobrevin/VAMP family [205–207]. Moreover, such complexes were devoid of the known t-SNARE partners, when antibodies were used to precipitate synaptophysin from detergent extracts [206–208]. This suggests that the synaptophysin-synaptobrevin interaction prevents synaptobrevin from undergoing assembly with its partner t-SNAREs (see fig. 1). However, the mechanism by which this protein disassociates to allow for formation of the fusion complex remains unknown. No synaptophysin homologs are present in the yeast genome, and analysis of homologs in other organisms is incomplete.

**VSM1 in yeast.** The yeast Vsm1 protein was identified recently as an interacting partner for the Snc v-SNAREs [74]. *VSM1* encodes a soluble protein that is membrane-associated and localizes to the plasma membrane, identical to the Sso and Sec9 t-SNAREs. Genetic studies reveal that the deletion of the *VSM1* gene results in increased secretion of proteins into the medium, whereas its overexpression has the opposite effect. In yeast possessing a temperature-sensitive mutation in the Sec9 t-SNARE, overexpression of Vsm1 results in the accumulation of low-density secretory vesicles and a reduction in cell growth. Based upon genetic studies [74] and preliminary in vitro binding experiments [209], Vsm1 may regulate the entry of Snc proteins into the yeast exocytic SNARE complex and, thus, is a candidate SNARE-master (see fig. 1). No mammalian homologs of Vsm1 have been identified, thus far, although expressed sequence tags (ESTs) with some homology have been noted.

## Other probable regulators of SNAREs

**NSF/SNAP and LMA.** A general class of SNARE regulators includes the NSF/Sec18 and SNAP/Sec17 protein families which function upon membrane transport at various stages of the secretory pathway [210–212]. These proteins were originally proposed to act as agents which mediate the ATP-dependent step in membrane fusion [210, 211, 213, 214] and, later, to disassemble the ternary SNARE complex [1, 3, 215]. More recently, NSF/SNAP homologs in yeast have also been suggested to prime the docking step in SNARE assembly by dissociating preformed assemblages and allowing the syntaxin-like component to assume an 'activated' state [190, 216]. Based upon these and other studies, NSF is now beginning to

look more like a molecular chaperone and SNARE regulator, rather than a component involved directly in the fusion event.

NSF and its membrane attachment proteins (SNAPs) were first identified as factors which confer the transport of proteins in vitro from donor Golgi membranes. This assay is based upon protein modification (i.e. glycosylation) of cargo marker, usually a heterogenously expressed viral protein, as it transits from donor to acceptor compartments [210, 211, 217–219]. This assay has proven extraordinarily fruitful in identifying soluble components of vesicle transport machinery, particularly those involved in ARF-mediated retrograde events, including components of the COPI coat (coatomer), NSF and SNAPs [210, 211]. Moreover, the identification of SNAPs [214, 220, 221] as proteins required for the binding of NSF to Golgi membranes led directly to the discovery of SNAP receptors [1, 3, 222]. NSF was first presumed to confer the ATP-dependent step in membrane fusion, due to the presence of two ATP binding and hydrolysis domains, and given its participation in the in vitro transport assay [211, 213, 215, 223]. Yet early studies also revealed that NSF action was not required for the steps of vesicle attachment and fusion, suggesting that it was more likely to be required for vesicle formation or early thereafter [224]. This became more apparent using in vitro assays to measure the homotypic fusion of vacuoles isolated from yeast [188]. The important point is that ATP and the yeast NSF component, Sec18, were found to be needed only at an early stage of the reaction, and did not require the presence of the acceptor membrane [188]. This resulted in the release of Sec17 (the yeast SNAP component), which was found to precede the rab-dependent step in vacuole docking and fusion [188, 190]. Interestingly, a recent structural analysis has demonstrated that NSF is a hexamer in solution and forms a hexagonal cylinder that is reminiscent of the ATPases known to act as molecular chaperones [225]. Thus, NSF regulation of the priming steps in vesicle docking may be based upon conferring a chaperone-like effect of protein unfolding and refolding in an ATP-dependent fashion, as proposed by Burgoyne et al. [226]. Other studies showed that LMA1 [227, 228], a heterodimer consisting of thioredoxin and the $I^B{}_2$ protease B inhibitor, cooperates with and may be initially bound to Sec18 to release Sec17. This appears to occur on SNARE complexes residing in a cis conformation, that is on assemblages formed in the same membrane. The binding of LMA1 to the syntaxin-like component on vacuolar membranes, Vam3, appears to stabilize the t-SNARE in an activated state [216]. Thus, NSF, SNAP and LMA act together as regulators to ready SNAREs for forming associations in trans (i.e. between donor and acceptor compartments). NSF/SNAP may act as general SNARE regulators to disassemble preformed v-t-SNARE complexes present in the same membrane and later to regulate the association/dissociation of other, more specific, SNARE regulators, that is the SNARE-masters. In this light, LMA1 fits the criteria for being a SNARE-master by virtue of its ability to maintain the t-SNARE in an uncomplexed (with respect to other SNAREs) and activated prefusion state. Interestingly, LMA1, like Sec17/Sec18, may act upon different levels of the secretory pathway, suggesting it could be a general regulator in membrane fusion in yeast [229]. However, this does not rule out the possibility that other, more specific, possible LMA-like factors (i.e. P16) act upon different transport events [230, 231]. Identification of a mammalian LMA factor would be quite useful in order to determine whether this function is also conserved in evolution.

These pioneering studies in vacuolar fusion are supported in part by experiments in higher eukaryotes which also suggest a priming, rather than a triggering, mode for NSF in calcium-mediated exocytosis. This includes studies describing the release of catecholamine from chromaffin cells [232–234] and neurotransmitter release from squid giant axons [235]. Furthermore, these new roles prescribed for the NSF and SNAP proteins in no way contradict with the circumstances in which they were originally described.

**Rabs and their effectors.** A large class of potential SNARE regulators is that of the rab family GTPases, which act upstream of SNARE complex assembly. Rabs have been suggested to regulate the specificity in membrane-trafficking steps due to their distinct subcellular localization and interaction with SNARE components [186, 236–238]. In the hierarchy of events leading to fusion, rabs are implicated at the level of SNARE activation, and act downstream of NSF and SNAP [188, 239]. For example, in the case of ER-Golgi transport in yeast, the Ypt1 GTPase has been proposed to bind directly to the Sed5 t-SNARE and to displace the Sec1 homolog, Sly 1 [240]. Meanwhile, other genetic studies performed in yeast have demonstrated that overexpression of v- and t-SNAREs can suppress the temperature sensitivity of mutant rab proteins that are specific to the transport step on which the SNAREs function [77, 241, 242]. Thus, rab proteins appear not only to regulate the fidelity of docking and fusion, but could serve to dissociate proteins which act as negative regulators for SNARE complex formation (i.e. SNARE-masters).

At the level of exocytosis, rab3 in higher eukaryotes and Sec4 in yeast are the relevant GTPases. Rab3, which is anchored to membranes by fatty acylation [243], was shown early on to associate with secretory vesicles [244], although it can be removed from membranes in its inactive GDP-bound form by a soluble dissociation factor, GDI [245]. The fact that rab proteins associate with membranes in their GTP-bound state and are removed after GTP hydrolysis, suggests that they exist

in a dynamic cycle of attachment and removal. This cycle has been proposed to be essential for the functioning of rabs in exocytosis and is supported by localization studies showing rab3 release from membranes after evoked stimulation [246, 247]. The dynamic cycling of rabs is strongly supported by studies on the yeast GTPase, Sec4, and its regulatory factors, which act to confer constitutive exocytosis [245, 248–251].

Ironically, studies on rab3A ablation in mice revealed only minor behavioral changes, instead of catastrophic defects in synaptic transmission [252]. This is supported by studies in *C. elegans*, in which null mutants for the rab3 homolog were also affected in a minor fashion [253]. This may result from compensation by other rab3 isoforms in nerve terminals, although in the case of mice, some synapses were shown to be free of rab3. Despite this, an enhancement in evoked transmitter release was observed in certain synapses, resulting apparently from an increased number of vesicles undergoing fusion, as evidenced by paired-pulse experiments [254].

The mechanism by which rabs function remains unclear, but appears likely to be at the level of vesicle docking, as evidenced from studies in yeast. Several putative effectors of the rab response have been identified, including rabphilin [255–257], RIM [258] and rabin [259]. Common to rabphilin and RIM are two calcium-dependent phospholipid-binding C2 domains and a zinc finger motif located at the amino terminus. The latter functions as the rab-binding motif [257]. Rabphilin is a vesicle-associated protein which is recruited by rab3 and dissociates after GTP hydrolysis, and in the presence of GDI [260]. Microinjection of rabphilin or rabphilin fragments was shown to inhibit neurotransmitter release in squid giant axons and cortical granule release from mouse eggs [261, 262], but both native and a membrane-anchored form of rabphilin induced insulin secretion from HIT-T15 cells [263]. RIM, on the other hand, localizes to the presynaptic active zones in synapses, but like rabphilin associates only with the GTP-bound form of rab3 [258]. Both proteins possess C2 domains that are likely to be relevant to phospholipid binding and to mediate calcium-dependent exocytosis. Thus, rabphilin and RIM and are proposed to act directly as rab3 effectors. Nevertheless, their direct target(s) and mechanism of action remain obscure.

Studies in lower eukaryotes also suggest a dynamic cycle of recruitment of rab3-like proteins to vesicles is essential for vesicle docking and fusion [245, 249–251]. Sec4 in yeast is an essential protein, and temperature-sensitive mutations result in blocked exocytosis and an accumulation of secretory vesicles at restrictive temperatures [248]. In addition, expression of an activated form of Sec4 ($Sec4^{q79l}$) is lethal to yeast and results in vesicle accumulation [250]. A GDI homolog, Sec19, is also essential for viability, and its depletion results in a decrease in the soluble pool of Sec4 and inhibited transport [245]. This suggests that Sec4 recruitment to vesicles is essential for docking and fusion, although the mechanism of Sec4 action on SNARE assembly remains unclear. Possibly, it acts like yeast Ypt1, a GTPase involved in ER-Golgi transport that appears to dissociate the Sec1-like protein, Sly1, and mediate SNARE assembly [240]. This is supported by the finding that the Sec9 t-SNARE can rescue defects associated with Sec4 proteins mutated in their putative effector domain [77], although Sec4 has not been demonstrated to bind directly to the t-SNAREs involved in constitutive exocytosis in yeast. In *Aplysia*, neurotoxin treatment inhibits the release of acetylcholine, yet the presence of an activated form of rab3 delays the onset of inhibition [264]. This suggests that the toxin-sensitive components (i.e. the v- and t-SNAREs) are probably in an assembled, toxin-insensitive state which is mediated by rab3 [264]. Thus, strong circumstantial evidence points to a role for rabs in mediating SNARE assembly into ternary complexes, presumably after priming steps(s) mediated by NSF and SNAP.

**DOC2.** DOC2 proteins are another family of C2 domain-containing proteins, like synaptotagmin and Munc-13, and as such are thought to play a role in calcium-regulated secretion. DOC2 was first isolated as protein expressed in nervous tissue which localizes to synaptic vesicle-enriched fractions [265–267] and enhances dense core vesicle release from PC12 cells, based upon overexpression studies [268]. More recent work has shown that DOC2 binds to Munc13 [269] via phorbal ester binding to the C1 domain of Munc13, suggesting a dual role for diacylglycerol regulation of both proteins. In addition, other work has shown that Munc18/nSec1 also binds to DOC2 and that the proteins are in competition for interactions with syntaxin [267]. Thus, DOC2 was proposed to act as a modulator of the syntaxin-Munc18 interaction and might function as part of an exchange mechanism to relieve the inhibition conferred upon the syntaxin t-SNARE by the Sec1 homolog [267]. That both Munc13 and Munc18/nSec1 interact with DOC2 suggests that a mutual relationship between these proteins is important for calcium-dependent exocytosis.

**Munc-13.** Protein kinase C (PKC), a calcium-dependent and diacylglycerol-binding serine/threonine kinase, has been implicated in modulating synaptic transmission. This stems in part from studies which show that neurotransmitter release from presynaptic terminals and various other exocytic events are modulated by exposure to phorbal esters [270, 271], which are known PKC agonists. Moreover, PKC has been shown to phospho-

rylate substrates involved in vesicle fusion (i.e. Munc18/nSec1) [116]. Nevertheless, some phorbal ester-mediated effects upon membrane trafficking were shown to be PKC-independent, suggesting an alternative mechanism could be operant [272–274]. This is supported by the finding that the unc-13 class of proteins binds diacylglycerol and phorbal esters via a C1 domain [275–277]. The unc-13 class of proteins was identified, along with unc-18, in screens for *C. elegans* mutants which were defective in acetylcholine transport and release [278, 279]. Recently, a mammalian homolog, Munc13, was shown to associate with the plasma membrane in a phorbal ester-dependent fashion and to modulate evoked and spontaneous neurotransmitter release in overexpression studies [277]. Thus, unc-13 proteins are likely to modulate synaptic transmission by acting as a mediary of diacylglycerol-activated signaling events. Since unc-13 was also shown to bind directly to syntaxin [117] and DOC2 [269], these effects are likely to be elicited at the level of membrane fusion, although the exact mechanism has not yet been resolved.

**Companions and competitors: syncollin, Hrs-2, complexins, SV2, CAPS and VAP33.** Numerous other proteins have been suggested to play roles in mediating calcium-regulated exocytosis, although the timing and extent of their involvement is still being characterized. One factor is syncollin, a small protein which binds to the syntaxin t-SNARE at low levels of calcium [118]. Addition of recombinant syncollin was also found to inhibit zymogen granule release in pancreatic cells, implying a possible regulatory role in exocytosis. Hrs-2 is an ATPase which binds to SNAP-25 in the absence of calcium and inhibits calcium-evoked release from PC12 cells [280]. Small soluble factors, called complexins, were found to compete with $\alpha$-SNAP for binding to syntaxin and the core SNARE complex. Because they compete with SNAP, and not with synaptotagmin, and colocalize with the t-SNAREs, it has been suggested that they may regulate SNAP-SNARE interactions in a sequential fashion [281]. SV2 is a synaptic vesicle-associated membrane protein [282, 283] expressed as two isoforms, SV2A and SV2B [284], of which the SV2A form interacts with the C2B domain of synaptotagmin in the absence of calcium [168]. SV2 appears to be a transporter protein, due to its two sets of six membrane-spanning domains and homology to sugar transporters from bacteria and neurotransmitter transporters from presynaptic membranes [282, 283]. Like the possible regulation of VGCCs by syntaxin, SV2 could be also influenced by the actions of synaptotagmin and vice versa.

CAPS was identified using an assay for the reconstitution of DCV release from semiintact cells [285]. CAPS is a calcium- and phospholipid-binding protein which appears to be required late in exocytosis, after docking of the DCV has occurred [286–289]. Although a cytosolic protein, CAPS was shown recently to associate with DCVs, but not with synaptic vesicles, suggesting a specific role in DCV release of norepinephrine from neurons [290, 291]. These studies also imply that the physiology of DCV and synaptic vesicle release may utilize both common and distinct determinants, CAPS belonging to the latter. While both processes are calcium-regulated, differences in the site of exocytosis and dependence upon the level of intracellular calcium are strongly apparent [292–295].

VAP33 was identified as a protein which associates with *Aplysia* VAMP, and in vivo studies revealed that microinjection of antibodies into the presynaptic nerve terminal resulted in inhibition of neurotransmitter release [296, 297]. This strongly suggested that VAP33 is potential SNARE regulator for synaptobrevin/VAMP. However, studies from yeast do not support this idea, but suggest, perhaps, an alternate function. A probable yeast homolog, Scs2 [298], was found to be an ER-resident protein that is involved in conferring inositol metabolism [299]. Mutations in *SCS2* were found to result in inositol auxotrophy [299], whereas overexpression suppresses loss of the gene which encodes inositol 1-phosphate synthase activity (*INO1*) [298]. Given that yeast Scs2 is ER-localized and that mammalian VAP33 may also be ER- and Golgi-localized [231], it may be more likely that this protein is involved in protein sorting from the ER to Golgi, possibly via a phosphoinositide- or sphingolipid-dependent mechanism. Where the physical interaction with synaptobrevin/VAMP occurs and how it is important for SNARE function remain unclear. More work will be required to adequately address this point.

## SNAREs and their environment

**Protein phosphorylation and regulation of SNAREs.** Numerous components involved in calcium-regulated exocytosis have been demonstrated to undergo phosphorylation in vitro. This ever-growing list of phosphorylated components includes synaptobrevin/VAMP [36, 145], synaptotagmin [169], Munc18/nSec1 [116], synapsins [300, 301], CAPS [302], synaptogyrin/cellugyrin [303] and other proteins which have been proposed to act at some level upon exocytosis, including annexins I and II [304–306], 14-3-3 protein [307], GAP-43 [308] and the myristoylated alanine-rich C kinase substrate (MARCKS) [309]. Other lines of evidence, including in vivo studies, suggest that phosphorylation modifies these proteins and, consequently, alters exocytosis and synaptic transmission [300, 310–312]. Protein phosphorylation is likely to directly affect protein-protein interactions by altering the affinity of the interaction.

In vivo studies involved the use of activators (i.e. phorbal esters) or inhibitors (i.e. staurosporine) of certain protein kinases to demonstrate their effects upon synaptic transmission. These studies showed that PKC is likely to play a regulatory role in modulating secretion in a quantitative fashion [270, 271]. Activators of G proteins (i.e. aluminum fluoride) and phospholipase C, which function upstream of PKC, also support this line of reasoning. PKC itself is a calcium- and diacylglycerol-activated serine/threonine kinase that is activated as a consequence of receptor-mediated stimulation of phospholipaseC [271, 313]. Amongst many studies, PKC activity was shown to phosphorylate CAPS and to enhance DCV release in semiintact PC12 cells [302]; to phosphorylate Munc18/nSec1 and inhibit the interaction with syntaxin in vitro [116]; and to phosphorylate SNAP-25 and enhance catecholamine release from intact PC12 cells [146]. Direct regulation of these and other components by PKC is likely to modulate synaptic transmission in a manner which is likely to be calcium-dependent in part. Alternatively, since PKC also appears to modulate lipid-modifying enzymes (i.e. phospholipase D), there is reason to believe that some aspects of these effects are lipid-mediated (see below).

Aside from SNAP-25, other SNAREs are targets for phosphorylation. For example, synaptobrevin/VAMP was shown to undergo phosphorylation in vitro, suggesting a possible role for protein phosphorylation in directly modulating SNARE function [145]. However, site-directed mutation of the putative phosphorylation sites in VAMP was later shown not to have a deleterious effect upon its ability to confer insulin secretion from permeabilized HIT cells [36]. In other in vitro experiments, syntaxin 4 was shown to be phosphorylated by casein kinase II (CKII) and protein kinase A (PKA), and weakly by protein kinaseC [314]. Interestingly, the interaction between syntaxin 4 and SNAP23 was strongly affected by phosphorylation by PKA, suggesting a possible role for cAMP-mediated signaling in constitutive exocytosis. In contrast, syntaxin 1, which is known to be involved in regulated secretion, was not phosphorylated by PKA [314]. Synaptotagmin was shown to be phosphorylated by CKII adjacent to the C2 domains [315], as well as by calcium-calmodulin kinase II (CaM kinase II) [316]. Other proteins known as synapsins were initially identified as major phosphoproteins of the nerve terminal. Synapsins are also phosphorylated by CaM kinase II, which may serve to modify their binding to calmodulin [317, 318]. Synapsins are thought to play a role in vesicle-actin filament interactions by promoting actin polymerization and bundling [319]. These activities are inhibited by phosphorylation by CaM kinase II, implying its role in anchoring vesicles to the cytoskeleton and making them available for exocytosis. Correspondingly, inhibitors of CaM kinase II were shown to inhibit DCV release in PC12 cells [320]. Some evidence also exists for the role of tyrosine phosphorylation in exocytic events, but no clear-cut mechanism of regulation has been defined. Nevertheless, the evidence weighs in heavily for a role of protein kinases and protein kinase activation in modulating synaptic transmission, presumably at the level of SNARE function itself.

**Lipids as possible SNARE regulators.** Biochemical and genetic analyses led to the discovery of SNAREs and identified the protein-protein interactions which lead to membrane fusion. However, less attention has been paid to the role of membrane lipids in the regulation of SNAREs. Given the wealth of accumulating evidence, it would be shortsighted not to consider some possibilities.

The cytoplasmic leaflet of cellular membranes is characterized by acidic phospholipids, like phosphatidylserine and phosphoinositide (PI). In contrast, the inner leaflet of organelles of the secretory pathway, especially vesicles and secretory granules, along with the outer leaflet of the plasma membrane are rich in glycosphingolipid (GSL) moieties. Abundant evidence already implicates the phosphorylated products of PI in the regulation of membrane trafficking [321, 322]. This has been nicely illustrated for DCV release from PC12 cells, wherein factors other than NSF were shown to be necessary for the ATP-dependent steps in calcium-stimulated exocytosis. These were shown to include phosphatidylinositol transfer protein (Sec14/PITP/PEP3/CAST) and phosphatidylinositol-4-phosphate 5-kinase (PIP5K) [321–324]. These late ATP-requiring or 'priming' steps reveal an important role for 5-PIs in stimulus-coupled exocytosis [323, 325]). The nature of this requirement is not entirely clear, but is supported by studies which show potential roles for $PIP_2$ and $PIP_3$ in membrane fusion. For example, antibodies directed against $PIP_2$ were shown to inhibit calcium-stimulated release [323]. Other studies have shown that $PIP_3$ levels rise upon stimulation in insulin-secreting cells [326] and that a granule-associated PI 4-kinase activity is necessary for catecholamine secretion from permeabilized adrenal cells [327]. More directly, CAPS associates to membranes characterized by acidic phospholipids, particularly PIP2 [289], whereas synaptotagmin was shown to bind to $PIP_3$ in the absence of calcium and to $PIP_2$ in its presence [164]. These studies suggest that an alteration in lipid binding might occur during depolarization and might reflect changes in attachment sites during fusion [164].

Other evidence strengthens the relationship between PI metabolism and exocytosis. For example, many exocytic and endocytic processes are sensitive to wortmannin, a specific inhibitor of PI 3-kinase. Morever, an inositol 5-phosphatase, synaptojanin, has been shown

to be modified directly by phosphorylation and is required for synaptic vesicle endocytosis [328]. Additionally, the AP-2 clathrin adaptor molecule is an inositol phosphate- and PI-binding protein, suggesting that PI modulates the assembly of clathrin coats [321, 329, 330]. Thus, involvement of these different PI-modifying and -binding factors in membrane trafficking suggests an important role for PI metabolism therein. While the precise roles are still not fully understood, the fact that the charge of PI can be modified dramatically by phosphorylation and dephosphorylation does make it an attractive candidate for a membranal component that can mediate protein interactions with bilayers, as well as influencing protein-protein interactions occurring within bilayers. Structural roles for these lipids in vesicle biogenesis and membrane fusion have been suggested [31, 321, 331]. There are a number of possible roles for PI and its metabolites, including modulation of the activity of actin-binding proteins [332], regulating PLD activity [331, 333] and, as described above, modulation of proteins which mediate membrane fusion.

While a regulatory role for PI and PI kinases in membrane trafficking has become clear, that of GSLs is still in its infancy. The main role proposed, thus far, has been in the modulation of protein sorting, in particular that of glycophosphatidylinositol (GPI)-anchored protein sorting to apical aspects of polarized epithelial cells [334]. In polarized cells, GSLs are targeted to the apical surface [335, 336], where GPI-anchored proteins were also shown to be targeted [337]. This, coupled with the fact that GPI-anchored proteins do not have cytoplasmic sorting signals, suggested that GSLs actively aid in the sorting of these proteins [337, 338], possibly via hydrogen bonding of the GPI anchor and the GSL [339]. In addition, these same molecules are present in cholesterol-rich detergent-insoluble microdomains or 'rafts' [334, 340–342]. Studies in yeast have supported the idea that inositol sphingolipids (SLs) are required for the transport of GPI-anchored proteins to the cell surface [343, 344]. Studies with GSL synthesis inhibitors also have revealed a role for these GSLs in axonal growth [345].

Up until now, no strong evidence for a role for SLs in membrane fusion and exocytosis has been found. Nevertheless, our recent work on the exocytic v-SNAREs in yeast, Snc1 and Snc2 may illustrate an important linkage. As described above, yeast lacking their exocytic v-SNAREs accumulate secretory vesicles and have a conditional lethal phenotype [73]. Yet spontaneous recessive (inactivating) mutations in either of two genes which encode ER-localized enzymes involved in long-chain fatty acid elongation and SL synthesis (*ELO3*/*SUR4*/*VBM1* and *ELO2*/*GNS1*/*VBM2*) were found to rescue *snc* cells. Gene inactivation was found to fully rescue their growth deficiencies and to restore vesicle trafficking of at least one branch of the yeast exocytic pathway. The results suggest that reduced levels of ceramide and SL synthesis restore the trafficking of these vesicles, but in a manner which remains t-SNARE-dependent. The mechanism is still unclear, but could involve altering the fusion competence of the vesicle by decreasing membrane rigidity, modulating t-SNARE-t-SNARE interactions, altering protein sorting to vesicles or a combination thereof [75]. Thus, a potential role for ceramides and GSLs in exocytosis should not be excluded.

**The SNARE cycle.** Cumulative evidence suggests that SNAREs assemble into higher-order structures that are thermostable and can be found in the same membrane or created between opposing membranes [1, 3–5, 22, 189, 191]. The later assemblage constitutes the putative fusion engine which brings together bilayers in apposition. On top of this, general factors like NSF/SNAP are capable of disassembling preformed SNARE complexes and maintaining them in a state which can be construed as being activated or 'primed' [3, 188–190, 234, 346, 347]. By definition, SNARE activation would be a step in which a SNARE is deprived physically and energetically from its association with other SNAREs. Since at some point both v- and t-SNAREs are present in the same membrane [74, 139, 140, 189, 190, 348], this activated state is presumably necessary to allow for the future association between partner SNAREs held on the opposing membranes (i.e. between donor and acceptor membranes). Nevertheless, the evidence for priming suggests that other factors are required to maintain and stabilize this state, presumably until tethering and/or docking between membranes can occur. These factors are likely to include some of those described above as SNARE-masters (i.e. nSec1/Munc18, LMA1, synaptophysin, Vsm1 etc.). The evidence for LMA1 as a stabilizer of the primed state in vacuolar fusion is probably the most conclusive, although it is likely that other components, perhaps the SNARE-masters, will fulfill a similar role in heterotypic fusion.

Because of the tendency of SNAREs to self-assemble, there appears to be a requirement for a transition phase between the complexed (with partner SNAREs) and uncomplexed states of SNAREs. This transition phase should include a state whereby the SNARE undergoes an altered conformation to allow entry of the SNARE-master in an NSF- and ATP-dependent manner. Strong evidence for conformational changes in SNAREs exists [7, 8, 10, 45, 104], although the mechanisms by which assembly occurs are unclear. This would allow the SNARE to maintain its primed state while waiting for the chance to meet an appropriate and available partner. Naturally, partner availability depends upon two factors: (i) the primed state of the partner and (ii) proximity to that partner. While SNARE regulators

determine the former, discrete physical constraints will determine the latter. These constraints include (i) SNARE specificity, in terms of defining the protein-protein interaction; (ii) the actual area wherein fusion can occur (i.e. the 'active zone'); and (iii) the cytoskeletal dynamics that underly vesicle encroachment and tethering to the acceptor membrane.

Passage of a SNARE from its complexed state to a SNARE that is, for the sake of argument, bound to a SNARE-master also predicts a transition phase. This second transition phase allows for dissociation of the regulator and association with a partner SNARE to invoke reassembly into the ternary complex. Again, the mechanism is still unclear, but a role for rab proteins in mediating this transition would seem likely, given the wealth of information connecting them to SNARE assembly. After assembly, membrane fusion may proceed presumably without constraint in the case of constitutive exocytosis and as a consequence of calcium influx in the case of regulated exocytosis. After fusion, SNARE assemblages are likely to be disassembled to eventually allow for a new cycle of sorting to vesicles and participation in fusion reactions. Again, NSF/SNAP are expected to play a significant role in this process [189–191, 347, 349].

A hypothetical model illustrating the physical states of SNAREs is presented in figure 3. Therein, we conjecture that SNAREs exist in four distinct states (or phases) during the 'SNARE cycle' which leads to fusion. Briefly, SNAREs are initially found in a stable complex on the same membrane (e.g. arranged in cis) (phase 1). A cue mediated presumably by vesicle encroachment or tethering to acceptor membranes allows NSF to bind ATP and associate (via SNAP) with ternary SNARE complexes to form the 20S particle. The nature of this cue is yet to be determined but is likely to involve the cytoskeletal components which bring vesicles into juxtaposition with their acceptor membrane. Subsequent hydrolysis of ATP by NSF results in dissociation of the particle and leaves SNAREs in a transitional state, which may include partial unfolding (phase 2). This now allows for the association with proteins that stabilize SNAREs in an activated uncomplexed state (relative to other SNAREs) (phase 3). These proteins include the SNARE-masters, that is Sec1/Munc18 (yeast and mammals), synaptophysin (mammals), Vsm1 (yeast), LMA (yeast) and possibly others. Next, rab proteins in their activated GTP-bound state associate with SNAREs in their stable SNARE-master-bound, uncomplexed state. This interaction might occur directly or via the putative rab effectors (e.g. rabphilin, RIM etc.). Presumably, it occurs on both donor and acceptor membranes in order to the ready v- and t-SNAREs on their respective membranes. Hydrolysis of GTP by rab is expected to lead to a second transition state by which dissociation of the SNARE-masters occurs. It is not clear at this point whether rab guanine nucleotide-exchange factor (GEF) function and GDP-GTP exchange alone would be sufficient to confer such a step, although it remains a distinct possibility, based upon recent studies of the Ypt1 GTPase in yeast [350, 351]. Nevertheless, we presume that rab functioning allows for the association of primed partner SNAREs and formation of a stable ternary complex between opposing membranes (phase 4). SNARE-mediated membrane fusion occurs immediately in constitutive secretory systems, but requires the influx of calcium in regulated secretory systems. Proteins described above, like synaptotagmin, DOC2, Munc13 and CAPS, presumably play an important role in release of the calcium-regulated clamp, although the ordering of these events is poorly understood. Following fusion, SNAREs return to their stable cis conformation (phase 1). In particular, v-SNAREs are expected to cycle back to donor membranes via retrograde sorting mechanisms, whereas the t-SNAREs might remain associated with the acceptor membrane.

## Future perspectives

Clearly, several aspects of SNAREs and their functions have eluded a comprehensive understanding. While models abound, we lack sufficient knowledge regarding how SNARE assembly comes about, even under in vitro conditions. In addition, we are still in the dark regarding how and where SNARE regulators associate with SNAREs and, likewise, how rabs promote the process of SNARE assembly. These open questions are likely to be solved in part by precise structural information obtained through X-ray crystallography. With the recent availability of this information, it is now possible to predict how intermolecular assembly of the binary and ternary complexes occurs. Based upon this knowledge, we may be able to better order the events occurring both upstream and downstream of SNARE assembly. This extrapolation should allow us to predict how SNARE-interacting proteins associate and dissociate from SNAREs and, possibly, how formation of the ternary complex brings membranes into juxtaposition. These additional issues are far from trivial, given the likely involvement of highly polar lipids in mediating both the protein-lipid interactions and, presumably, lipid-lipid interactions occurring between bilayers. Moreover, in evoked secretory systems an additional hierarchy of proteins appears responsible for mediating the calcium-dependent steps that lead to unclamping of the ternary complex and vesicle fusion. Moreover, the analysis is further complicated by the fact that additional components which act upon docking and fusion are identified on a yearly basis.

Resolution of the events leading to docking and fusion is likely to come from the same disciplines that brought it to fruition thus far. Yet it seems clear that the usage of model organisms, coupled with the continued development of in vitro and in vivo fusion assays, will allow for a precise ordering of these events. Simple organisms such as yeast should prove effective for understanding constitutive secretion, whereas the use of *C. elegans* and *Drosophila* will be useful to understand regulated secretory systems. The use of mouse models should also prove invaluable, in particular towards understanding the molecular genetic basis underlying neurosecretory disorders. In short, while we are still in the 'clone and categorize' phase, there is sufficient room for optimism regarding the development of an all-encompassing hypothesis to explain the events which lead to protein export.

*Note added in proof*. Papers relevant to several issues discussed herein were published after acceptance of this manuscript. These include demonstrations that rab GTPase family members may act to 'tether' donor and acceptor membranes prior to SNARE assembly [Ungermann et al. (1998) Nature **396:** 543–548 and Cao et al. (1998) EMBO J. **17:** 2156–2165]; this tethering event may involve pre-requisite Sec18/NSF-mediated priming in the case of vacuole fusion in vitro [Ungermann et al. (1998) Nature **396:** 543–548].

In the latter paper, it was also suggested that disassembly of the Vam3-Nyv1 t-v SNARE complex (formed between *vam3* and *nyv1* mutant vacuoles) is catalyzed by Sec18/NSF, without blocking vacuolar fusion. The authors propose that SNARE pairing may not be the final event leading to membrane fusion and that other factors may be involved. At face value this is an important finding, but it cannot be considered as definitive proof. This is particularly true since the authors did not monitor the status of other v- and t-SNAREs involved in endosomal protein sorting. These include the Pep12 t-SNARE and Vti1 v-SNARE, which could confer fusion of mutant vacuoles by forming distinct SNARE complexes (i.e., Pep12-Nyv1, Pep12-Pep12, Vam3-Pep12, Vti1-Vam3, etc.). Furthermore, these complexes might be resistant to the activity of Sec18/NSF in vitro.

Other published works containing structural information about SNAREs suggested that: a) the N-terminal region of Sso1 (syntaxin) may regulate SNARE assembly [Nicholson et al. (1998) Nature Struct. Biol. **5:** 793–802] and b) the parallel arrangement of helicies within the yeast exocytic SNARE complex is highly similar to that of neuronal SNARE complex [Katz et al. (1998) EMBO J. **17:** 6200–6209].

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