Review

SNARE regulators: matchmakers and matchbreakers

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Abstract

SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) are membrane-associated proteins that participate in the fusion of internal membranes in eukaryotic cells. SNAREs comprise three distinct and well-conserved families of molecules that act directly as membrane fusogens or, at the least, as elements that bring membranes into close apposition and allow for subsequent fusion events to occur. While the molecular events leading to fusion are still under debate, it is clear that a number of additional factors are required to bring about SNARE-mediated membrane fusion in vivo. Many of these factors, which collectively can be called SNARE regulators (e.g. Sec1/Munc18, synaptotagmin, GATE-16, LMA1, Munc13/UNC-13, syntophysin, tomosyn, Vsm1, etc.), bind directly to SNAREs and are involved in the regulation of SNARE assembly as well as the ability of SNAREs to participate in trafficking events. In addition, recent studies have suggested a role for posttranslational modification (e.g., phosphorylation) in the regulation of SNARE functions. In this review the possible role of SNARE regulators in SNARE assembly and the involvement of SNARE phosphorylation in the regulation of intracellular membrane trafficking will be discussed.

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Keywords: Arf-GAP; Complexin; Apg8/GATE-16; LMA1; Munc13; Munc18/Sec1; Phosphorylation; SNARE; Synaptophysin; Synaptotagmin; Tomosyn; Vsm1/Ddi1

1. Introduction

1.1. SNARES and membrane fusion

Intracellular membrane trafficking in eukaryotic cells utilizes transport vesicles and tubulovesicular structures to deliver cargo proteins and lipids from one internal compartment to the next [1,2]. An ever-widening variety and number of factors are required to mediate the controlled transport of cargo along the secretory pathway. Factors necessary to confer fusion between donor and acceptor compartments (or organelles) include the Rab GTPases, tethering complexes, AAA-type ATPases, and SNAREs [3–5]. These evolutionarily conserved factors populate the sites of membrane fusion and confer an ordered chain of events that ultimately leads to bilayer fusion. Multiple homologs of these factors are found in eukaryotic cells, though individual family members tend to show compartmental specificity [3,4]. Thus, the highly specific nature of transport between compartments (e.g. endoplasmic reticulum (ER) to Golgi, Golgi to lysosome/vacuole, Golgi to plasma membrane, etc.) appears to necessitate the use of distinct combinations of mutually recognizable factors in order confer targeted membrane fusion. The combinatorial involvement of the different elements presumably ensures that cargo molecules are transported only to the correct target compartment.

SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) comprise three conserved families of membrane-associated proteins (e.g., the synaptobrevin/VAMP, syntaxin, and SNAP-25/light chain families) that act late in the events leading to bilayer fusion [6,7]. Acting downstream of both tethering factors and Rabs (which confer loose membrane attachment) [3,4], SNAREs confer the tight docking and, probably, subsequent fusion of membrane bilayers. Originally designated as v- (vesicle) and t- (target) SNAREs, and then later as either R- or Q-SNAREs (depending upon the presence of arginine or glutamine residues in their core binding domains), members of these three families assemble into a complex in trans that bridges membranes [6,8,9] (see Fig. 1). The basis for this model was the discovery that all SNAREs bear at least one α-helical domain and arrange themselves in parallel to form...
a coiled-coil four-helix bundle [10–13]. Formation of the SNARE complex is thought to involve zipperring up of the helices of individual SNAREs from their membrane distal to membrane proximal ends [14,15]. This results in the four-helix bundle and allows for the close apposition of donor and acceptor membranes.

Formation of the trans-SNARE complex may be sufficient for membrane fusion, as it results in liposome fusion in vitro and as substitution of the transmembrane domains of SNAREs with lipid anchors blocks fusion, but not membrane docking [16,17]. The hypothesis that SNAREs are the principal fusogens of the secretory pathway is supported by additional findings. First, there is a strong structural similarity between viral fusion proteins and SNAREs [18]. Second, genetic screening and analyses of yeast secretion mutants for over 20 years have not revealed any universal elements which act downstream of the SNAREs and which might obviate their requirement in membrane fusion. Third, kinetic resolu-
tion studies using secretory chromaffin or PC12 cells support a direct correlation between complex formation and exocytic events [19,20]. Together, this has led investigators to propose a proximity model as the basis of SNARE-mediated membrane fusion, i.e., that the zipperping of SNAREs into the fully assembled trans complexes forces the membranes into tight apposition [21,22]. This is expected to result in local dehydration at the site of membrane attachment, hemifusion between the outer leaflets of the bilayers, and subsequent fusion of the inner leaflets. This model necessitates a direct role for the transmembrane domain of the SNAREs in bilayer fusion [6,17,18,21,22].

Yet, these issues are still under debate, particularly as studies on homotypic fusion using purified yeast vacuoles suggest that formation of a proteinaceous pore plays an important role in membrane fusion [22,23]. The role for a membrane-integral pore complex comes from studies showing that V0 sector components of the vacuolar H+-ATPase form trans complexes between docked vacuoles and that their absence affects fusion [23]. Additionally, other studies have suggested that AAA-type ATPase-mediated dissociation of trans-SNARE complexes precedes the fusion of cortical vesicles in sea urchin eggs [24]. Thus, a role for proteolipids, like those of the V0 sector, in the formation of a proteinaceous pore that leads to fusion should be considered.

1.2. Regulation of SNARE assembly

While SNAREs play a central role in membrane fusion, the steps leading to SNARE assembly are not entirely understood. Many SNARE-interacting proteins have been identified and play critical roles in the ordered assembly of SNAREs into functional trans complexes (for a partial list see Table 1). Before v- and t-SNAREs can assemble together, t-SNAREs on acceptor membranes must first assemble into a (all-Q) three-helix SNARE complex. This may result from the assembly of monomeric SNAREs as they are transported along the secretory route or from post-fusion disassembly of the four-helix cis-SNARE complexes by AAA-type ATPases [5]. The ATPase may also be necessary for the activation of individual SNAREs in vivo [25,26]. In either case, formation of the t-SNARE complex (by either two or three molecules) precedes v–t SNARE assembly into trans complexes. Support for this comes from studies showing that v- and t-SNAREs interact poorly in pairwise combinations in vitro and that membrane fusion in vitro is blocked when only one type of t-SNARE is present on the acceptor membranes [16,27,28].

Yet, little is known about the events that regulate formation of the t-SNARE complex in vivo and whether this occurs prior to, or only after, tethering. Studies on homotypic vacuolar fusion in vitro have revealed that AAA-type ATPase (e.g. Sec18) priming activity is necessary before tethering to allow for the dissociation of cis SNARE complexes and subsequent trans complex formation [29,30]. But whether the t-SNARE complex fully disassembles after ATPase action is not clear. This question is especially relevant to exocytotic systems, wherein both syntaxin and SNAP-25 family members are widely distributed over the plasma membrane in yeast and neurons, yet, only a small percentage are actually present in binary complexes [31,32]. Thus, t-SNARE complex formation may be tightly regulated in vivo. On the other hand, it has been shown that the endogenous t-SNARE pool in membranes can directly bind recombinant SNAREs in vitro [33].

Crystallographic resolution of the neuronal SNARE complex (VAMP2–syntaxin 1a–SNAP-25) revealed that the four-helix bundle has a packed hydrophobic core and grooved outer surface [11]. The grooves may allow for the binding of factors that mediate complex disassembly, such as SNAP and the NSF AAA ATPase (Sec17 and -18 in yeast), which are essential for membrane fusion. In addition, other factors that modulate the ability of SNAREs to form complexes, such as SM proteins, synaptotagmin, LMA1, etc., might interface with these surfaces. These and others comprise an ever-growing repertoire of potential regulatory components that order SNARE assembly.

Table 1

<table>
<thead>
<tr>
<th>SNARE Interacting Proteins</th>
<th>Trafficking step</th>
<th>Target</th>
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<tbody>
<tr>
<td>Matchmakers</td>
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<tr>
<td>Sec1/Munc18 (SM proteins)</td>
<td>all</td>
<td>syntaxin homologs</td>
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<td>Synaptogamin</td>
<td>regulated exocytosis</td>
<td>syntaxin, SNAP-25, SNARE complex</td>
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<td>Munc13</td>
<td>regulated exocytosis</td>
<td>syntaxin, SNAP-25, SNARE complex</td>
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<tr>
<td>GATE-16/Apg8 (UFT proteins)</td>
<td>Golgi transport, Golgi reassembly, autophagy</td>
<td>SNAP-25</td>
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<td>LMA1</td>
<td>vacuolar membrane fusion (yeast)</td>
<td>Vam3 (syntaxin homolog)</td>
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<td>Complexins</td>
<td>regulated exocytosis</td>
<td>syntaxin, SNAP-25, SNARE complex</td>
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<td>Snapin</td>
<td>regulated exocytosis</td>
<td>SNAP-25</td>
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<td>Matchbreakers</td>
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<td>Synaptophysin</td>
<td>regulated exocytosis</td>
<td>VAMP syntaxin, SNAP-25 ortholog (yeast)</td>
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<td>AmiIyn/Tomosyn/</td>
<td>constitutive and regulated exocytosis</td>
<td>VAMP and syntaxin orthologs</td>
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<td>Sro proteins</td>
<td>constitutive exocytosis (yeast)</td>
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One is tempted to ask why such regulation is necessary and why so many regulators? One reason may relate to geography. For example, SNARE regulation in the early part of the secretory pathway may restrict promiscuous SNARE pairing upon protein translocation into the ER or prevent trans SNARE partnering between cognate SNAREs before they reach their appropriate compartments. This, for example, might prevent post-Golgi SNAREs from operating early in the pathway. A second reason may relate to site of action. As SNAREs tend to be widely dispersed over their resident compartments, only those molecules adjacent (or recruited) to the site of fusion between apposed membranes need to be activated. Thus, some factors (i.e. SM proteins)
ensure that only apposed cognate SNAREs form functional trans-SNARE complexes, while other factors (i.e. synaptophysin, Vsm1, phosphorylation, etc.) may restrict the activity of SNAREs distal to the site of fusion. A third reason for regulation is directly related to the catalysis of SNARE assembly. This includes factors that switch SNAREs from inactive to active structural conformations. This process, known as priming, consists of the ATPase-mediated disassembly of cis SNARE complexes and association of factors that maintain SNAREs in their active conformations (i.e. LMA1, Apg8/GATE-16, tomosyn) [34–36]. Afterwards, additional factors may “hand” activated SNAREs over to each other to form functional trans complexes between apposed membranes. In regulated secretory systems, this includes elements (i.e. synaptotagmin, Munc13, complexins) that maintain the docked state of membranes, but prevent fusion from occurring (see Fig. 1).

1.3. SNARE activation—structure equals function

Numerous studies have demonstrated that SNARE structure is greatly influenced by its interactions with other proteins. Some syntaxins adopt a “closed” inactive conformation, but this is altered by the opening of an intramolecular four-helix bundle to allow for SNAP-25 association [37–40]. Removal of the NH2-autoinhibitory domain allows for direct assembly with other SNAREs and enhances fusion in vitro [41]. VAMP proteins are unstructured in vitro but show greater helicity upon assembly into v–t SNARE complexes [39,42].

Recent works have revealed that v–t SNARE complexes in the docked state are only partly zippered and, thus, are not fully assembled. This was first demonstrated in kinetic studies of exocytosis using antibodies or neurotoxin-mediated proteolysis [19,20]. More recent work has shown that synaptotagmin [43] or COOH-terminal peptides derived from the VAMP v-SNARE [44,45] impose structure upon the partly zippered membrane-proximal domains of SNAREs in the trans complex. This leads us to the hypothesis that SNARE regulators stabilize the metastable docked state of SNAREs (Fig. 1). When removed, perhaps upon stimulation by known factors involved in membrane fusion (i.e. Ca2+ in regulated exocytotic systems and PP1 phosphatase activity in vacuolar membrane fusion), full zippering of the trans complex can occur, which leads to membrane fusion (Fig. 1).

It is easy then to think of SNARE regulators as switches that maintain SNAREs in conformations that are either well- or poorly disposed to assembly. Some regulators can be variable in function and, thus, have both positive and negative activities in regards to SNARE assembly and fusion (i.e. SM proteins and synaptotagmin). Since a wide number of compartment- and organism-specific SNARE regulators have been discovered, we introduced the term “SNARE-master” to describe these proteins [46]. Nevertheless, it is necessary to introduce a little more phraseology to help simplify the task of categorization. Thus, in this review we will refer to SNARE regulators that facilitate the assembly of cognate SNAREs as “Matchmakers”, while those that keep SNAREs in a conformation-inactive state shall be referred to as “Matchbreakers”.

2. SNARE regulators—the matchmakers

2.1. SM proteins—Sec1/Munc18

One of the more controversial proteins known to regulate SNARE function are members of the conserved Sec1/Munc18 (SM) family that bind to the syntaxin class of t-SNAREs. The controversy that has arisen regarding SM proteins has not centered on their importance, which is not in doubt, but rather their precise function. In particular, data from studies using the neuronal proteins have conflicted, in part, with that obtained using the yeast orthologs.

Both the yeast and mammalian syntaxins that function in exocytosis can adopt a “closed” conformational state, in which the α-helical NH2-terminal region of molecule folds back upon the COOH-terminal SNARE binding domain to form an intramolecular four-helix bundle [37–40]. In the case of mammalian syntaxin, this “closed” conformation of the t-SNARE is unable to participate in SNARE assembly, but interacts preferentially with a Sec1 ortholog, Munc18-1 [40,47,48]. Moreover, results from a variety of experimental approaches suggest that some syntaxins switch between the “closed” conformation, which binds to SM proteins, and an “open” conformation that can form the core complex [38–40]. At the very least, the interaction of SM proteins with syntaxins is predicted to exert significant regulatory constraints upon membrane fusion events.

Studies using *Saccharomyces cerevisiae* imply that the yeast SM family members (e.g. Sec1—Golgi to plasma membrane; Vps45—endosome to Golgi, cytoplasm to vacuole; Sly1—ER to Golgi, etc.) positively regulate the formation of SNARE complexes [49–51]. Unlike mammalian nSec1/Munc18, yeast Sec1 does not interact with the soluble form of Sso (a syntaxin ortholog) in vitro [52], but binds to preassembled exocytic SNARE complexes containing Sso, Sec9 (a SNAP-25 ortholog), and Snc1 (a VAMP ortholog) [49]. As *SEC1* was originally isolated as a gene essential for exocytosis [53], this work suggested a probable role for Sec1 in mediating SNARE assembly and fusion in vivo. Recent studies on other yeast Sec1 homologs have, in fact, strengthened this idea. For example, the functional counterpart of Sec1 in ER–Golgi transport, Sly1, binds to preassembled SNARE complexes containing the Sed5 t-SNARE and the Bet1, Sec22, and Bos1 SNAREs [51]. More importantly, formation of the Sed5–Sly1 dimer in vitro confers specificity to SNARE assembly by preventing the formation of non-physiological SNARE complexes that assemble promiscuously in vitro [51]. Likewise, Vps45 association with the Tlg2 endocytic t-SNARE facilitated assembly with the Tlg1 and
Vti1 t-SNAREs in vivo, but which was blocked in its absence [50]. These results are consistent with the idea that SM proteins, even if functionally distinct, act positively to enhance SNARE assembly perhaps by allowing for the transition of t-SNAREs from their inactive to active conformations. In cases where the yeast t-SNAREs do not necessarily adopt “closed” conformations (i.e., Tlg2, Pep12, Vam3 and Sed5) [54,55], SM proteins utilize an NH2-terminal peptide to elicit SNARE receptivity [54,56].

In contrast, earlier studies with the neuronal SM proteins suggested a different function. The binding of nSec1 to a soluble form of syntaxin 1A was found to block subsequent association with SNAP-25 and VAMP, and to dissociate from syntaxin after assembly had occurred [57]. This suggested that nSec1 might restrict SNARE partnering. Support for this notion came from in vivo experiments involving Drosophila and C. elegans. Overexpression of ROP, the Drosophila ortholog of Sec1, inhibited neurotransmitter release in larvae [57,58], while hypomorphic mutations in C. elegans unc-18 resulted in paralysis [57,59]. Other studies implied a direct negative role in stimulus-coupled exocytosis in mammalian cells [60], while structural work showed that Munc18 stabilizes the inactive “closed” conformation of syntaxin [40]. Yet recent studies have implicated roles more compatible with that of the yeast data. For example, mice lacking neuron-specific Munc18-1 do not show neurotransmitter release [61]. Likewise, large dense-core vesicle-mediated exocytosis from chromaffin cells was inhibited in the absence of Munc18 and stimulated by its overexpression [62].

So can the data from these different studies be fully reconciled? In vivo studies, principally involving the yeast SM proteins, suggest a positive role in allowing for the transition of syntaxins from their inactive to active conformations in order to facilitate SNARE assembly. Structural data, which present the SNARE–SNARE and SNARE–SM protein interactions in their most stable low-energy state, suggest a role for SM proteins in maintaining syntaxins in their inactive conformation. The latter, however, does not preclude a positive role for SM proteins in assembly. Rather it suggests that the “closed” form of some syntaxins represents an intermediate of the process at, presumably, an early stage [57]. If SM interactions with syntaxins are temporal and restricted to the sites of membrane fusion [49], this lends credence for an assembly or matchmaker function, rather than an inhibitory one. Finally, structural insights obtained from X-ray crystallography suggest that syntaxins per se tend to form homo-oligomers in vitro [63] and that SM binding to the NH2 terminus could be preventative, thus allowing for the assembly of physiologically relevant SNARE complexes. Thus, a common theme is developing by which SM proteins appear to aid in SNARE assembly by regulating t-SNARE receptivity. Clearly, more experimentation is necessary, particularly towards resolving whether other proteins involved in regulating SNARE assembly (i.e. Munc13) or disassembly (i.e. NSF) interact with the NH2-terminal domain of syntaxins and are controlled by SM functions. It is possible that SM proteins impose spatial and temporal regulation upon the actions of these regulators, hence its essential role in SNARE-mediated membrane fusion.

2.2. Synaptotagmins

A family of SNARE regulators found exclusively in higher eukaryotes are the synaptotagmins, which have been proposed to mediate stimulus-coupled exocytosis [64,65]. Unlike the SM proteins, synaptotagmins are membranal proteins that localize to synaptic and large dense-core vesicles. Typified by a single transmembrane domain and two membrane-distal C2 domains (C2A and B), which act as specialized Ca2+ binding sites, synaptotagmin has been proposed to act as a calcium sensor that modulates stimulus-coupled secretion [64,65]. In addition, both C2 domains interact with lipids: C2A preferentially with anionic lipids; and C2B with phosphatidylinositol 4,5-bisphosphate [64,65]. Thus, these C2 domain–lipid interactions may enhance both calcium as well as membrane binding. This has been suggested to have major implications upon synaptotagmin functioning, as an increased membrane-binding potential (in response to Ca2+ triggering) could potentiate the binding of C2A to SNAREs and C2B to the plasma membrane, leading to membrane fusion [64,65]. On the other hand, the C2 domains may act cooperatively to regulate synaptotagmin–SNARE interactions [66,67].

Better studied than the other C2 domain-bearing synaptic proteins, synaptotagmin family members were shown to interact directly with SNAREs in a Ca2+-dependent and stoichiometric fashion [64,65,68,69]. Synaptotagmin binds syntaxin and SNAP-25 alone or in complexes, thus providing the potential connection between calcium influx and the regulation of SNARE assembly leading to exocytosis. As several studies link assembly with calcium-mediated fusion events, one interpretation is that synaptotagmin allows for the zipper-up of partially assembled complexes after the influx of calcium into the cell [64,65]. In the formation of ternary SNARE complexes, SNARE assembly proceeds from the membrane-distal portion of the SNARE-binding domains to the membrane-proximal portion. However, full assembly is likely to be impeded not only by repulsion of the lipid bilayers, but also by stresses exerted by the membrane-proximal and transmembrane domains of the SNAREs. According to this model, synaptotagmin binding to t-SNAREs blocks complete SNARE assembly and inhibits non-evoked fusion events in the absence of calcium [43,64–66,69]. Upon calcium influx and binding, synaptotagmin facilitates assembly leading to membrane fusion. Thus, synaptotagmin modulates the metastable primed state of the SNARE complex in a calcium-sensitive manner. Moreover, in this bimodal model synaptotagmin acts both as a negative and positive regulator of fusion, as evidenced by numerous in vivo and in vitro studies [64,65].
Interestingly, recent studies have questioned the role of calcium in regulating synaptotagmin functions. Mutations in the C2A domain of Drosophila synaptotagmin I that inhibit Ca\(^{2+}\)-binding were found to not affect calcium-dependent synaptic transmission [70], while those in the C2B domain did [71]. This corresponds with earlier studies showing that the knockout of synaptotagmin in nematodes, fruit flies, and mice does not block transmission per se, but only impairs the evoked release [64,65]. Interestingly, synaptotagmin was found to enhance SNARE-mediated fusion events in vitro even in the absence of calcium [43]. Together, it would appear that the primary function of synaptotagmins is in the promotion of fusion and, probably, in cooperation with other calcium sensors.

2.3. Munc13/UNC-13

Another family of SNARE regulators involved in stimulus-coupled exocytosis are the Munc13/UNC-13 proteins. Notably, they contain a conserved CI domain, which binds diacylglycerol and makes neurons sensitive to regulation by this lipid messenger [72,73]. Munc13/UNC-13 proteins are thought to act at a vesicle “priming” stage that leads to the evoked release of synaptic vesicles present in active zones [73–76] and modulates synaptic plasticity [77]. Interestingly, gene knockout experiments performed in nematodes, flies, and mice reveal that only the ready-releasable pool of synaptic vesicles is strongly affected in these neurons [72–76]. Thus, Munc13/UNC-13 has been proposed to confer some aspect of fusion competence to these partially docked vesicles [73–76,78]. This is likely to relate its role as a SNARE regulator, as Munc13 interacts with the NH\(_2\)-terminal autoinhibitory domain of syntaxin [79] and may promote a conformational switch in the t-SNARE from its “closed” to “open” state. Several studies are supportive of this idea. First, UNC-13 was found to displace UNC-18 bound to the “closed” form of syntaxin in C. elegans [80]. Second, expression of an “open” mutant of syntaxin rescues worms bearing an unc-13 mutation [81]. These studies suggest that Munc13/UNC-13 proteins facilitate SNARE assembly in conjunction with SM protein function.

2.4. The UFT proteins—GATE16/GABARAP/MAP-LC3/APG8

Independently identified as a component that facilitates the fusion of Golgi membranes in vitro, a microtubule-binding protein that clusters postsynaptic GABA receptors, and as a mediator of autophagy in yeast [82–84], members of this well-conserved family of ubiquitin-fold (UF) proteins share many functions in common with SNARE regulators. Crystallography data for the UF proteins involved in transport (UFTs) reveal that they bear a three-dimensional structure that is strikingly similar to ubiquitin, a covalently attached modifier that is attached to lysine groups of proteins via an isopeptide bond [85]. This attachment regulates the function of the substrate proteins and may alter their intracellular localization. Unlike ubiquitin, however, UFTs do not undergo covalent attachment to their interacting partners, but undergo an activation step that allows for membrane attachment. Studies from yeast reveal that Apg8/Aut7 is conjugated to phosphatidylethanolamine (PE) during starvation conditions, which leads to its attachment to newly forming autophagic membranes [84,86]. This linkage alters the properties of Apg8/Aut7 such that it can participate in elongation of the autophagosome, though the mechanism remains unclear. Thus, like ubiquitin, which undergoes a three-step activation process prior to its ligation to a substrate, some UFT proteins become lipid-modified at their COOH terminus via a distinct conjugation pathway.

In addition to their direct attachment to membranes, both yeast and mammalian UFT proteins mediate intracellular transport. In particular, mammalian GATE-16 was demonstrated to interact with the NSF AAA-type ATPase (which acts to disassemble cis SNARE complexes) and to increase its ATPase activity [82]. Moreover, the yeast and mammalian UFT proteins were both found to interact directly with SNAREs involved in ER-to-Golgi and vacuolar transport (i.e., Bet1, Sec22, and Nyv1 in yeast and GOS-28 in mammals) [82,87]. Thus, there exists a strong link between UFT protein association with SNAREs, NSF activation, and subsequent SNARE complex disassembly.

It is generally known that the switching of NSF between its ATP- and ADP-bound forms, via ATP hydrolysis, is a prerequisite for SNARE disassembly. This is typified by the Drosophila NSF mutant, comatose, which shows a reduction in neurotransmitter release and an accumulation of synaptic vesicles and SNARE complexes at restrictive temperatures [88]. In contrast, NSF has been shown to have an ATPase-independent function which allows for the fusion of post-mitotic Golgi elements [89]. In a recent work it was found that GATE-16 is recruited to uncomplexed GOS-28 (a Golgi SNARE) and prevents association with syntaxin-5 [36]. Thus, the ATPase-independent function of NSF may be to catalyze UFT assembly with SNAREs in order to prevent the re-formation of cis SNARE complexes. If so, UFT proteins provide a matchmaking role in the regulation of Golgi reassembly. How UFTs function in other trafficking events is less well understood, but a general role in regulating SNARE receptivity would parallel that of the SM proteins or LMA1 (see below). Unlike the SM proteins, however, UFTs are not essential (e.g. deletion of AUT7/ APG8 in yeast is without phenotype during vegetative growth) [87] and, thus, may have evolved to yield specific functions, such as the regulation of membrane receptor localization [83]. Interestingly, a recent finding has shown that GABARAP can switch from a monomeric form to a linear polymeric form [90]. Whether this phenomenon is common to the other UFT family members is not clear, but it might allow them to form coat-like structures on membranes, in addition to their SNARE regulatory functions. As autophagosome elongation in yeast is dependent upon lipid-
2.5. LMA1

One of the first proteins suggested to modulate SNARE functions, LMA1 binding and release from the Vam3 t-SNARE catalyzes vacuolar fusion in yeast [30,34]. In actuality a dimer composed of thioredoxin and the I2 protease inhibitor, LMA1 (low M, activity 1), acts in the priming step that occurs before Vam3 can assemble into trans complexes with its fusion partners. In isolated vacuoles, Vam3 is normally present in cis SNARE complexes along with several SNARE partners, including the Nyy1 v-SNARE and the Vam7, Vti1 or Ykt6 t-SNAREs [91]. In the priming reaction hydrolysis of ATP by Sec18 (a yeast NSF ortholog) leads to disassembly of cis SNARE complexes and the transfer of LMA1 from Sec18 to Vam3 [30,34]. In this state LMA1 now acts to mask Vam3, preventing re-formation of cis SNARE complexes. Thus, the transfer of LMA1 (from Sec18 to Vam3) is highly reminiscent of the way UFTs are thought to modulate the fusion of Golgi membranes (see above). Once primed, vacuoles can then undergo the second step of tethering and docking, the latter being dependent upon the formation of trans SNARE complexes. In the final steps leading to membrane fusion, both calcium (which is released from the vacuole) and calmodulin are necessary to initiate an event leading to LMA1 release. Although not resolved in its entirety, this includes the pairing of V0 sectors between apposing vacuoles as well as the activation of a micro-cystin LR-sensitive PP1 phosphatase (e.g. Glc7) [92]. After phosphatase activation, LMA1 is released from Vam3, leading to fusion of the bilayers. Thus, the role LMA1 plays as a t-SNARE regulator is central to the mechanism of vacuolar fusion.

2.6. Complexins

Complexins are neuronal-specific proteins implicated in regulated exocytic events [93]. Though their functions are consistent with a positive-acting role upon exocytosis, their exact mechanism of action is not fully known. Complexins have been shown to bind to assembled SNARE complexes, including those containing synaptotagmin [94]. Complexins bind to the synaptobrevin—syntaxin groove of the SNARE complex in an anti-parallel fashion, which may stabilize the complex and reduce membrane-repulsive forces [95,96]. In addition, complexins have been suggested to promote oligomerization of the SNARE complexes, by partnering one of the two SNAP-25 helices with the syntaxin of an adjacent SNARE complex [97]. This is proposed to form a ring of mutually cross-linked complexes, which could mediate fusion along the lines of the proximity model for membrane fusion [21,22]. However, this view is not without controversy, as another study found no evidence for the induction of complex oligomerization and suggested a post-assembly role for complexin [96]. In vivo experiments have shown that the microinjection of a peptide from the syntaxin-binding domain of complexin, as well as its overexpression, lead to an inhibition of neurotransmitter release [97,98].

As complexins appear to interact with assembled SNARE complexes, their role as an initiator of SNARE assembly is less substantiated. Nevertheless, their intrinsic ability to interact with individual SNAREs within the assembled complex may allow for control of the metastable primed state of the complex [94–96]. This, in turn, would control the release rate of docked vesicles.

3. SNARE regulators—the matchbreakers

3.1. Synaptophysin

A family of potential negative regulators of SNARE assembly is that of synaptophysin and its related isoform, synaptoporin. Found only in higher eukaryotes, these integral membrane proteins are major components of synaptic vesicles and have been shown to interact with VAMP/synaptobrevin [99–101]. Interestingly, the complex formed between synaptophysin and VAMP impacts directly upon SNARE function, as it prevents the v-SNARE from entering into SNARE complexes [100,101]. Thus, synaptophysin may be a bona fide controlling factor of SNARE assembly. Yet, the loss of synaptophysin function has little effect in neurons as revealed by knockout experiments using mice [102]. This suggests that its functions are either redundant with another SNARE regulator, perhaps synaptoporin, or that its role in synaptic transmission is minor. As synaptophysin overexpression can stimulate neurotransmitter secretion [103], it is also possible that it acts as a escort factor to present the v-SNARE to available (and, presumably, activated) t-SNARE complexes. Thus, the mutually exclusive complex formed between VAMP and synaptophysin may relate to an ability to facilitate SNARE complex formation. Clearly, more study of this family is necessary to determine its precise role in synaptic transmission. Interestingly, synaptophysin may also play developmental role in synaptogenesis, as its ability to interact with VAMP appears to be dependent upon neuronal aging, as well as posttranslational modifications [104].

3.2. Amisyn—tomosyn

Several syntaxin-binding proteins have been shown to possess a VAMP-like coiled-coil domain [9] that allows for association with the t-SNAREs [105,106]. These proteins include tomosyn [105] and its yeast homologs, Sro7 and Sro77 [107], and amisyn [106]. Tomosyn was found to bind to syntaxin in a manner which results in the displacement of Munc18 [105], as was shown for UNC-13 function [80]. The tomosyn–syntaxin complex was found to include SNAP-25
and synaptotagmin, but notably lacked VAMP [105]. Amisyn
is similar to tomosyn in that it binds both syntaxin 1a and
SNAP-25, but prevents their association with VAMP [106].
Furthermore, the overexpression of either tomosyn or the
amisyn coil domain was found to inhibit secretion from
cultured cells [105,106]. This suggests that the coiled-coil
region of amisyn~tomosyn mimics the VAMP v-SNARE and
fulfills a matchbreaker role which prevents v–t SNARE
assembly. Whether amisyn~tomosyn hold the t-SNAREs in
an activated state, such as that described for LMA1 and
GATE-16 (see above), is not yet known.

3.3. Vsm1/Ddi1

A putative negative SNARE regulator that acts upon
exocytosis in yeast is Vsm1/Ddi1 [108]. This protein has
interesting connections with ubiquitin, possessing both a
ubiquitin-interacting UBA domain and a ubiquitin-like UBL
domain [109]. Originally identified as a gene up-regulated
in response to DNA damage, Vsm1/Ddi1 can be either soluble
or membrane-associated and appears to have two
separate sets of function. One function is to down-regulate a
checkpoint factor in a ubiquitin-dependent fashion [110]. A
second function, related to protein export, is its ability to
bind to the exocytic v-SNAREs in yeast, Snc1 and Snc2,
and to inhibit exocytosis to some degree [108]. Recent work
from our laboratory has shown that Vsm1/Ddi1 also binds
to the exocytic t-SNAREs, Sso1 and Sso2, but in a fashion
which is phosphorylation-dependent and ubiquitin-indepen-
dent [111]. Moreover, Vsm1/Ddi1 binding to the Sso t-
SNAREs is mutually exclusive with Sec9, the SNAP-25-
like t-SNARE partner of Sso [111]. Thus, recruitment of this
SNARE regulator to t-SNAREs is modulated by signaling
cascades involved in cellular growth control and has signifi-
cant effects upon SNARE assembly.

4. Signaling to SNARES—phosphorylation as a
regulator of SNARE interactions

The connection between cell signaling and intracellular
protein transport is well established in that a variety of signal
transduction pathways are known to control the secretion of
hormones and neurotransmitters [14,46,112]. However, the
connection between the transmitted signal and regulation of
the membrane fusion machinery is less well known. Like-
wise, the connection between the physical processes of cell
growth (i.e. cell surface expansion) and cell growth control
has yet to be elucidated.

Many SNAREs and SNARE regulatory proteins have
been shown to be phosphorylated in vitro [14,46,112]. In
some cases, SNARE phosphorylation has been shown to
promote the binding of SNARE regulators at the expense of
their SNARE partners. For example, in vitro phosphoryla-
tion of SNAP-25 by protein kinase C (PKC) results in a decrease
in affinity for syntaxin [113], while phosphorylation of
syntaxin by casein kinase II increases its affinity for synap-
totagmin [114]. SNARE regulators themselves undergo
phosphorylation. For example, phosphorylation of Munc18
by cyclin-dependent kinase 5 reduces its affinity for syntaxin
[115]. Thus, kinase input is likely to have important effects
upon SNARE function.

A wide variety of kinases are known to phosphorylate
SNAREs and SNARE regulators, including protein kinase A
(PKA), PKC, Ca2+ - and calmodulin-activated kinase II
(CaMKII), and casein kinases I and II [14,46,112]. This
indicates that a number of signaling paths feed into the
transport machinery to, presumably, modulate secretory
functions. Studies in vivo show that phosphorylation indeed
modulates secretion. For example, phosphorylation by PKA
of Snapin, a SNAP-25 binding protein and SNARE regulator,
enhances synaptotagmin association with SNARE com-
plexes and increases the number of fusion-competent vesicles
[116]. Phosphorylation of SNAP-23 by a syntaxin-associated
kinase, SNAK, modulates both SNARE stability and subse-
quent assembly [117]. In addition, another syntaxin-associ-
ated kinase, CaMKII, inhibits exocytosis from neurons and
chromaffin cells [118].

Trafficking studies using yeast have revealed both the
kinase and corresponding phosphatase that impinge upon
SNARE assembly and regulate endo- and exocytosis. PKA
phosphorylation of the NH2-terminal autoinhibitory domain
of the Sso t-SNAREs was found to inhibit binding to Sec9,
the yeast SNAP-25 ortholog, leading to an inhibition of
growth and secretion [119]. Similarly, PKA phosphorylation

Fig. 2. A general role for kinases and phosphatases in t-SNARE regulation. Evidence from yeast and mammalian systems suggests that phosphorylation implicitly alters the ability of t-SNAREs to assemble with their SNARE partners to form complexes. Syntaxin (syn) from the plasma membrane can form an intramolecular four-helix bundle composed of its NH2-terminal auto-inhibitory domain folding on its helical SNARE-binding motif. This closed and inactive conformation (“Off”) prevents association with its t-
SNARE partner, SNAP-25 (snp) to form t-SNARE complexes. In contrast, opening of the intramolecular four-helix bundle allows syntaxin to assume an open and active conformation (“On”), which can bind to SNAP-25. Kinases, like protein kinase A in yeast, phosphorylate the NH2-terminal auto-inhibitory domain of syntaxin-like proteins to stabilize the “Off” state. This may involve the recruitment of SNARE regulators with matchbreaking activities (not shown). In contrast, phosphatases, like ceramide-activated protein phosphatase in yeast, dephosphorylate the NH2-terminal domain and allow for opening of the syntaxin, stabilization of the “On” state, and
SNAP-25 binding.
of like domains in the Tlg endocytic t-SNAREs led to an inhibition of SNARE assembly and a block in endocytosis [120]. Activation of a sphingoid base- and ceramide-activated protein phosphatase (CAPP) was found to rescue yeast mutants defective in endocytosis and exocytosis, as were alanine substitutions at the relevant PKA phosphorylation sites of the t-SNAREs [119,120]. Thus, the PKA–CAPP kinase and phosphatase pair act to specifically modulate trafficking functions in yeast. Interestingly, recent work has shown PKA phosphorylation of the Sso t-SNAREs greatly enhances their association with Vsm1/Ddi1 at the expense of Sec9 [111]. Therefore, phosphorylation is likely to regulate SNARE receptivity and ability to participate in complex assembly (see Fig. 2) by altering interactions between SNAREs and their regulators. How phosphorylation specifically regulates the NH2 regulatory domains of syntaxin family t-SNAREs is unclear at present, but presumably it imposes structural alterations that allow for better association with SNARE regulators.

5. Arf regulatory factors—GAPs in our knowledge of SNARE regulation

Members of the Arf family of GTPases are involved in the biogenesis of transport vesicles and confer retrograde protein transport from the Golgi to the ER via coatamer protein I (COPI)-coated vesicles [121]. Mechanistically, they have been shown to recruit COPI to membranes in a GTP-dependent manner and drive COPI vesicle formation in vitro [122,123]. Thus, they are critical factors that nucleate vesicle formation by recruiting the protein coat necessary to induce membrane deformation. Interestingly, recent work has shown that SNAREs involved in ER-Golgi transport in yeast (e.g. Bet1, Bos1, and Sec22) interact in vitro with COPI coat components (e.g. Arf-GTP and coatamer) in a catalytic manner requiring Arf GTPase-activating protein (Arf-GAP) [124]. Moreover, this interaction requires only the transient presence of Arf-GAP, which appears to impose a change in structure upon the SNAREs themselves. This conformational change suggests that Arf-GAPs regulate SNARE function and, perhaps, not only with respect to Arf binding and vesicle formation. It is possible that SNAREs bound to Arf-GAP or Arf-GTP are unable to assemble into SNARE complexes or interact with other SNARE regulatory proteins. In that sense, Arf and other small GTPases may ultimately prove to be direct SNARE regulators.

6. Concluding remarks

While still much remains to be learned it is clear that SNARE assembly is the key to the fusion of intracellular compartments and secretion in eukaryotic cells. SNARE structure is tightly regulated by intramolecular constraints imposed by auto-inhibitory domains and phosphorylation, as well as inter-molecular constraints posed by SNARE regulatory proteins. Under the control and timing of Rab GTPases and tethering complexes, these regulatory factors help mold SNAREs into the ternary and quaternary folded structures that lead to bilayer fusion.

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