Annu. Rev. Biophys. Biomol. Struct. 2001. 30:157–71
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STRUCTURE OF PROTEINS INVOLVED IN SYNAPTIC VESICLE FUSION IN NEURONS

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Key Words exocytosis, vesicle fusion, neurotransmission, coiled coils, chaperone

■ **Abstract** The fusion of vesicles with target membranes is controlled by a complex network of protein-protein and protein-lipid interactions. Structures of the SNARE complex, synaptotagmin III, nSec1, domains of the NSF chaperone and its adaptor SNAP, and Rab3 and some of its effectors provide the framework for developing molecular models of vesicle fusion and for designing experiments to test these models.

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INTRODUCTION

Vesicular trafficking in eukaryotic cells is essential for diverse cellular processes, including maintenance of distinct subcellular compartments, protein and hormone secretion, egg fertilization, and neurotransmitter release (4, 16, 39, 73, 75, 84). The life cycle of a vesicle generally consists of three stages: endocytosis or formation of the vesicle from specific cellular membranes, exocytosis or fusion of the vesicle

1056-8700/01/0610-0157$14.00

with its target membrane, and recycling of the components of the protein machinery after exocytosis. This review focuses on structural studies of the key proteins responsible for exocytosis and recycling (Figure 1).

Vesicular exocytosis utilizes a protein machinery that is conserved from yeast to human (2, 29). SNARE (Soluble NSF Attachment protein REceptor) proteins are essential components of this machinery (39, 84). In synaptic vesicle exocytosis, three SNAREs are involved: the plasma membrane associated proteins syntaxin, SNAP-25, and the vesicular protein synaptobrevin, also referred to as VAMP (Vesicle-Associated Membrane Protein). Other conserved proteins include the ATPase NSF (N-ethylmaleimide Sensitive Factor) (95) and its adaptor SNAP (soluble NSF attachment protein) (14), the Rab class of small G proteins and their effectors (30), the synaptotagmin family (6), and the nSec1 family (67). Other factors have been characterized that appear to interact with SNAREs, such as complexin (57), VAP33 (81), and synaptophysin (96).

Initially, syntaxin is bound to nSec1 (Figure 1a). Both syntaxin and synaptobrevin are single-pass membrane proteins. At the docking stage, the syntaxin/nSec1 complex is dissociated, perhaps assisted by a Rab effector (58). Synaptobrevin then binds to syntaxin and SNAP-25 (Figure 1a). At the priming stage, the system becomes competent to undergo fusion upon an increase in Ca^{2+} concentration, possibly involving a Ca^{2+} binding protein, such as synaptotagmin. At the recycling stage, α-SNAP and NSF bind to the SNARE complex, and the SNARE complex is then dissociated upon ATP hydrolysis (Figure 1b).

Prior to docking, vesicles have to be targeted to the correct location at the appropriate time. This process is much less well understood than the later stages of vesicle fusion. However, some of the molecular components for this targeting process are beginning to emerge. Among them are the sec6/8 complex in mammalian cells (44) and the exocyst in yeast (89). These are large (>700 kDa) macromolecular complexes that could be involved in targeting processes before SNAREs become involved.

SNAREs

The SNARE complex can be isolated from neuronal cell extracts (65). It can also be assembled from recombinantly expressed and purified proteins in vitro (26, 71). The membrane anchors are not required for the assembly of the SNARE complex, so most biophysical and structural studies have been carried out on the soluble domains of the SNAREs. The SNARE complex exhibits remarkable thermal and chemical stability (26, 71). Limited proteolysis of the synaptic SNARE complex revealed a core complex with similar biophysical properties to the full-length complex (25, 69). This core complex is sufficient to promote vesicle fusion in vitro (66).

The SNARE core complex consists of a parallel four-helix bundle (87), whereas the amino-terminal domain of syntaxin consists of an antiparallel three-helix

bundle (28). The core of the four-helix bundle of the SNARE complex is composed of layers formed by interacting side chains from each of the four α-helices (87). These layers are highly conserved across the entire SNARE family. At the center of the core complex, a conserved ionic layer consisted of an arginine and three glutamine residues contributed from each of the four α-helices (87). It is interesting that this ionic layer is sealed off against solvent by adjacent hydrophobic layers.

Mutations in these and other layers reduce complex stability and cause defects in membrane traffic even in distantly related SNAREs (27). Based on the conservation of the core of the SNARE complex, SNAREs were reclassified into Q-SNAREs and R-SNAREs, and it was proposed that fusion-competent SNARE complexes generally consist of four-helix bundles of the type 3(Q-SNARE):1(R-SNARE) (27). A possible exception to the 3Q:1R rule is the homotypic vacuolar fusion system where five distinct SNAREs interact (92). However, these experiments were carried out with extracts of yeast and analyzed by immunoprecipitation, so it is not certain that all five vacuolar SNAREs interact quantitatively in a single pentameric complex.

SNAREs have at least three conformational states (20, 31, 42): first, the "closed" conformation of uncomplexed syntaxin and the unstructured or flexible conformations of synaptobrevin and SNAP-25; second, the binary complex of syntaxin and SNAP-25; and third, the ternary complex of syntaxin, SNAP-25, and the cytoplasmic domain of synaptobrevin. The closed conformation of uncomplexed syntaxin contains a four-helix bundle made up of the regulatory N-terminal $H_AH_BH_C$ domain and roughly half of the core complex domain H_{core}. The topology of this closed conformation was predicted based on NMR data (31). A similar conformation of syntaxin has recently been observed in the crystal structure of syntaxin in the syntaxin/nSec1 complex (58), suggesting that it is the closed conformation of syntaxin that binds to nSec1.

Syntaxin switches to an open state upon binding to SNAP-25 (31). In this open state, binding to the other SNAREs is mediated by the H_{core} domain. Conformational switching of the H_{core} domain, mediated by the $H_AH_BH_C$ domain, represents a regulatory mechanism for SNARE complex assembly by affecting the kinetics of ternary complex formation (60, 66). Formation of binary and ternary complexes is associated with increasing induction of α-helical structures in previously unstructured or flexible regions (20, 24, 26, 31, 42). Because the N-terminal half of the syntaxin H_{core} domain is always folded, these data suggest that SNARE complex assembly begins distal to the membrane surfaces and proceeds toward them. This "zipper" model of vesicle fusion was also suggested by experiments using fluorescence resonance energy transfer (53), electron microscopy (37, 39, 47), and electron spin polarization (70) of labeled SNARE complexes.

Role of SNAREs

While the precise function of the SNAREs is the topic of some debate, there is overwhelming evidence that they play a fundamental role in membrane fusion.

First, site-specific cleavage of SNAREs by clostridial neurotoxins inhibits neurotransmission (40, 46). Second, SNAREs represent a minimal fusion machinery: SNAREs reconstituted into artificial liposomes can induce fusion in vitro (61, 66, 94). Experiments in a permeabilized PC12 cell system also confirmed the importance of SNAREs for fusion in vivo (13). Third, the soluble domains of SNAREs spontaneously assemble into an extremely stable four-helical bundle in vitro (26, 87). The α-helical composition, high thermal and chemical stability is similar to proteins involved in viral fusion, possibly indicating a common ancestral mechanism for both fusion systems (80). Fourth, complex formation likely proceeds in a directed fashion starting at the membrane-distal end of the complex toward the membrane-proximal C-terminus (31). This directed assembly process may bring membranes into close proximity, thus overcoming the free-energy barrier for membrane fusion.

This hypothetical model assumes the existence of a partly assembled state of SNAREs docked between two membranes. Although this state has not been directly observed, there is indirect evidence for such an intermediate state. First, the cleavage sites of all clostridial neurotoxin proteases are located in the C-terminal (membrane-proximal) half of the core complex (46, 87). Because SNAREs are protected against proteolysis in the fully assembled complex (40, 46), this suggests that SNAREs must exist in partly assembled or "loose" states for significant periods of time. Recent experiments further support this hypothesis: the C-terminus of synaptobrevin is sensitive to toxins in the docked state but not the N-terminus (45). Kinetic studies of chromaffin cell exocytosis revealed a fusion-competent state that is sensitive to the attack of clostridial neurotoxins (97). Inhibition of SNARE complex assembly by antibody-binding differentially affected kinetic components of exocytosis, suggesting the existence of loose and tight SNARE complex states (98).

Analysis of polyethyleneglycol-induced (PEG) fusion of artificial liposomes suggested the existence of two intermediate stages of vesicle fusion: a stalk and a hemifusion state (49, 50). Assuming that similar stages exist during the fusion of cellular vesicles with target membranes, one can speculate that SNARE complex formation could lower the free energy barrier to reach the stalk intermediate state. In addition, SNARE complex formation could lower the free energy transition state barriers between the stalk state, the hemifusion state, and the fused state of the system. It is likely, however, that other factors (proteins or the lipid composition of synaptic vesicles) are involved in regulating these free energy barriers, especially in view of the fact that neuronal vesicle fusion is tightly Ca^{2+} regulated and proceeds at a faster time scale (milliseconds) than can be accomplished by in vitro fusion induced by SNAREs (minutes).

Other experiments have been interpreted to cast doubt on the central role of SNAREs in membrane fusion. In vitro experiments on sea urchin cortical vesicles showed a discrepancy between vesicle fusion, as assayed by turbidity measurements, and the time course of SNARE complex formation (17, 88). Homotypic

fusion between cortical vesicles is an extremely unlikely event in vivo. In these experiments, contacts between fusing vesicles therefore had to be induced by applying a centrifugal force. Thus, these experiments do not necessarily dispute the role of SNAREs in the biological context.

In vitro studies of homotypic vacuolar fusion during yeast cell division showed that SNARE complexes could be disassembled before fusion (91). This observation does not necessarily rule out a role of SNAREs for membrane fusion. Perhaps SNARE complexes could be disassembled without undocking the membranes if the system was committed for fusion at the irreversible hemifusion stage or other factors were involved downstream of SNARE function (49, 50).

SNARE Interactions Are Promiscuous

The primary sequence conservation of the structural core of the SNARE complex casts doubt on the targeting role of SNAREs for vesicle trafficking, as originally proposed by the SNARE hypothesis (73). Indeed, very similar biophysical and biochemical properties were obtained in vitro for complexes consisting of artificial combinations of SNAREs that are localized to different compartments in vivo (23, 101). Furthermore, some SNAREs can function at several different transport steps in vivo (33). Thus, SNARE pairing cannot be the sole determinant for vesicle targeting specificity. Rather, the observed SNARE localizations may be important for the interactions with other factors such as nSec1 that interact with nonconserved SNARE residues (58).

Interactions with nSec1

The partially structured, "closed" state of syntaxin interacts with nSec1 (58, 100). The conformation of syntaxin found in the crystal structure of this complex is dramatically different from that in the ternary SNARE complex (Figure 1a). C-terminal syntaxin residues that are unstructured or flexible in solution adopt a sequence of small α-helical fragments connected by short loops when complexed with nSec1. In the ternary SNARE complex, these residues form a continuous α-helix.

The flexible regions of uncomplexed syntaxin could have local structure that is related to the structure of syntaxin in the nSec1/syntaxin complex. The chemical shift dispersion of α-helices is small, and the motion produced by flexible hinges may explain the ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) nuclear magnetic resonance (NMR) spectrum that was observed for uncomplexed syntaxin (20) and its yeast homologue, Sso1 (31). It is thus likely that nSec1 acts by stabilizing one of the conformations of uncomplexed syntaxin. The conformational transition of syntaxin is a striking example of the role of conformational flexibility in biological function.

Experiments in yeast suggest an interaction between sec1 and the assembled plasma-membrane SNARE complex (7). This is in contrast with the neuronal

case where interactions between syntaxin and nSec1 and syntaxin, SNAP-25, and synaptobrevin are mutually exclusive (100). If the conclusions drawn from the experiments in yeast and neurons are correct, one could speculate that the yeast homologue of nSec1 has a different structure, that two distinct conformations exist for the sec1 family of proteins, or that a transient interaction exists between nSec1 and the partially assembled SNARE complex (58, 100).

SYNAPTOTAGMIN

Synaptotagmin is a membrane-associated protein that interacts with SNAREs, phospholipid membranes, Ca^{2+} channels, and proteins involved in endocytosis (6, 11, 52, 76, 77). In the cytosolic portion of this protein, a flexible seven amino acid linker joins two homologous C2 domains, C2A and C2B (86, Figure 1a). The C2A domain binds to anionic phospholipids (9, 10) and other accessory proteins, such as syntaxin (12), in a Ca^{2+} dependent fashion. No conformational change is observed upon Ca^{2+} binding (78) except for rotamer changes of the Ca^{2+} coordinating aspartic acid residues. The C2B domain promotes binding to other C2B domains (11), as well as accessory proteins independent of Ca^{2+}. The crystal structure of synaptotagmin III, which includes the C2A and C2B domains, exhibits differences in the shape of the Ca^{2+}-binding pocket, the electrostatic surface potential, and the stoichiometry of bound divalent cations for the two domains (86). The C2A and the C2B domains do not directly interact; synaptotagmin, therefore, covalently links two independent C2 domains, each with potentially different binding partners. It is interesting that neuronal proteins, such as rabphilin (83) and Doc2 (63), also possess multiple C2 domains similar to those of synaptotagmin. The structure of the C2B domain of rabphilin is very similar to the C2B domain of synatotagmin III (86, 90).

Synaptotagmin and the SNARE complex interact independent of Ca^{2+}, although the interaction is enhanced upon the addition of Ca^{2+} (19, 86). The Ca^{2+} binding domains are likely to interact with the plasma-membrane (10), whereas the polybasic regions could interact with the SNARE core complex (86, 87).

Rab3

Members of the Rab family of small G proteins regulate vesicular membrane traffic in all eukaryotic cells (30, 35, 68, 79, 82). Rab3A predominantly localizes to synaptic vesicles and plays an important role in the regulation of neurotransmitter release (32). Rab proteins were suspected to be determinants of vesicle targeting specificity because distinct isoforms display unique cellular localizations (79). However, studies of chimeric rab proteins suggested that Rabs can function at two distinct transport steps: the vesicular transport from the endoplasmic reticulum

to the Golgi and fusion of postGolgi secretory vesicles to the plasma membrane (5, 22), suggesting that Rabs cannot be the sole targeting determinant. Like other small G proteins, the Rab family members may function as molecular switches or timers, cycling between the inactive guanine diphosphate-bound (GDP) and active GTP-bound form, regulating their effector proteins and downstream targets accordingly.

In the cytosol, Rab proteins are kept in the inactive, GDP-bound, state by the Rab GDP-dissociation inhibitor (GDI), preventing them from nonspecific binding to membranes. Upon binding to a specific donor compartment or vesicle, GDI is displaced by a GDI-displacement factor (GDF). Exchange of GDP to guanine tri-phosphate (GTP) is then catalyzed by guanine exchange factors (GEFs), activating the Rab protein and rendering it resistant to removal from the membrane by Rab GDI. GTP is hydrolyzed by the intrinsic GTPase activity of the Rab protein. The transition-state barrier of the hydrolysis reaction is lowered by GTPase activating proteins (GAPs). Once vesicle fusion has occurred, GDI releases the GDP-bound form of Rab to the cytoplasm and the cycle begins again.

Gene knockout of Rab3A impairs regulation of neurotransmitter release (8, 35). The GTP-bound form of Rab3A interacts with at least two effector proteins, rabphilin-3A (83) and rim (93), which may interact with as yet unknown downstream targets. Activated Rab3A reversibly recruits rabphilin-3A to synaptic vesicles (83). Rim has sequence similarity to rabphilin-3A but localizes to the active zone of the presynaptic plasma membrane instead of to synaptic vesicles (93).

A relatively large number of Rab proteins and their effectors are present in eukaryotic cells. A structural basis for the specific pairing between these proteins was recently proposed based on the structure of activated Rab3A/GTP/Mg^{2+} bound to the effector domain of rabphilin-3A (64). Rabphilin-3A contacts Rab3A primarily in two distinct areas (64); few conformational changes are observed upon complex formation (21, 64). The first area involves the Rab3A switch I and switch II regions, which are sensitive to the nucleotide-binding state of Rab3A. The second area consists of a hydrophobic surface pocket in Rab3A that interacts with a SGAWFF structural element of rabphilin-3A. Based on sequence analysis, biochemical, and structural data, it was proposed that this pocket or "Rab complementarity-determining region" (RabCDR) establishes a specific interaction between each Rab protein and its effectors (64). Based on the crystal structure of the Rab3A/rabphilin-3A complex, it was proposed that small G proteins generally can have diverse surface areas for effector recognition. The recent structure of Rho complexed with the effector domain of PKN/PRK1 (55) supports this hypothesis: The variable C-terminal α-helix of Rho is involved in effector binding in a fashion similar to rabphilin.

The crystal structure of GDI has also been solved (75). GDI functions in recycling and regulating Rab proteins. Residues that are likely involved in binding to

Rab proteins have been identified, but full elucidation of these interactions has to await structure solution of a GDI/Rab complex.

NSF

According to a current model, NSF and SNAP act together to disassemble SNARE complexes before and after fusion. SNARE proteins can form both *cis* (same membrane) and *trans* (opposing membranes) complexes that are substrates for SNAPs and NSF (65, 91). As discussed above, *trans* SNARE complexes are important for membrane fusion. Fusion of opposing membranes results in the formation of *cis* SNARE complexes that are disassembled for recycling and reactivation by the joint action of SNAP and NSF. It is interesting that NSF also interacts with glutamate receptors (62). An additional function of NSF could be in glutamate receptor cycling in and out of the synaptic postsynaptic membrane through endo- and exocytosis (54), a process that may be connected to long-term potentiation and long-term depression.

NSF is a hexamer (34) and belongs to the AAA (ATPases Associated with cellular Activities) family of proteins (3). Each NSF protomer contains three domains: an N-terminal domain required for SNAP/SNARE binding and two ATPase domains, termed D1 and D2. ATP binding and hydrolysis by D1 is necessary for the SNARE disassembly reaction to occur, and ATP binding, but not hydrolysis, by D2 is necessary for hexamer formation (85, 95). SNAP and NSF bind sequentially to SNARE complexes, forming so-called 20S particles, named after the sedimentation behavior of the supercomplex. Rotationally averaged electron micrographs of 20S particles have been obtained in the presence of ATPγS (43) and allow one to speculate about the localization of the structurally known components in the 20S complex.

NSF-D2

D2 consists of a nucleotide binding subdomain and a C-terminal subdomain that is structurally unique among nucleotide binding proteins (51, 103). There are interactions between the ATP moiety and both the neighboring D2 protomer and the C-terminal subdomain, which may be important for ATP-dependent oligomerization. Of particular importance are three well-ordered and conserved lysine residues that interact with the β- and γ-phosphates, one of which emerges from a neighboring NSF protomer and likely contributes to the low hydrolytic activity of D2 (103).

NSF-N

The N-terminal domain of NSF (N) is required for SNAP/SNARE binding and disassembly (59). The structures of the N-terminal domain of NSF (56, 104), of the yeast homologue Sec18p (1), and of the homologous VAT protein of the

archaebacterium *Thermoplasma acidophilum* (15) are nearly identical, illustrating the structural conservation of the family of AAA ATPases. The N-terminal domain is composed of two subdomains: a double-ψ–ψ-barrel and an α-β roll (56, 104). The interface between the two subdomains forms a groove that is a likely site of interaction with the C-terminal portion of α-SNAP (104). Unexpectedly, both subdomains are structurally similar to domains of the transcription factor EF-Tu (104). Both proteins have an adjacent nucleotide binding domain, D1 in NSF and domain 1 in EF-Tu, and both proteins couple nucleotide hydrolysis to large conformational changes between domains.

α-SNAP

The structure of Sec17, the yeast homologue of α-SNAP, consists of a twisted sheet of α-helical hairpins and a globular C-terminal domain that is primarily composed of α-helical hairpins (72). Sec17 is structurally related to several other α/α proteins known to mediate protein-protein interactions as part of larger assemblies: tetratricopeptide repeats (TPRs) (18), 14-3-3 (99), HEAT repeats (36), and clathrin heavy chain repeats (102). The Sec17 twisted sheet has local similarity to the structure of the TPRs from protein phosphatase 5, but the overall twist of the sheet of α-helical hairpins in the two structures is very different.

Interactions between α-SNAP/Sec17 and SNAREs have been partially mapped using deletion mutagenesis and in vitro binding studies. The SNAP-interacting region of SNAREs overlaps with their core complex-forming regions (38, 41, 48). This, in conjunction with the structure of the synaptic core complex (87) and the observed promiscuity of SNAP:SNARE interactions, suggests that SNAPs recognize general surface features (shape or electrostatic charge distribution) of the parallel four-helix bundle. Indeed, the curvature of the grooves of the four-helix bundle of the SNARE complex is similar to the curvature of the twisted sheet of Sec17. Electron microscopy and mutagenesis studies of SNAP:SNARE complexes suggest that SNAP interacts with the SNARE complex along most of its length (43). Residues that are conserved in a variety of homologous SNAP sequences map predominantly to one face and to one ridge of the structure of Sec17. These surfaces are likely to interact with SNAREs and/or NSF/Sec18. SNAP may function as rigid levers, transmitting force generated by conformational changes in NSF/Sec18 to drive disassembly of SNARE complexes (72).

CONCLUSION

Significant progress has been made to elucidate structures of proteins involved in vesicular exocytosis. A striking property of the vesicular fusion machinery is the highly dynamic nature of the protein-protein interactions where binding partners frequently change and proteins undergo dramatic conformational changes. Crystal structures can only provide snapshots of the protein machinery. It remains

a challenge to connect these snapshots in order to obtain a movie of the vesicular fusion machinery and the fusion process itself.

ACKNOWLEDGMENTS

The author thanks Bryan Sutton, Mark Bowen, Reinhard Jahn, William Weis, and Richard Scheller for stimulating discussions, and Kira Misura for providing an image of the nSec1/syntaxin complex.

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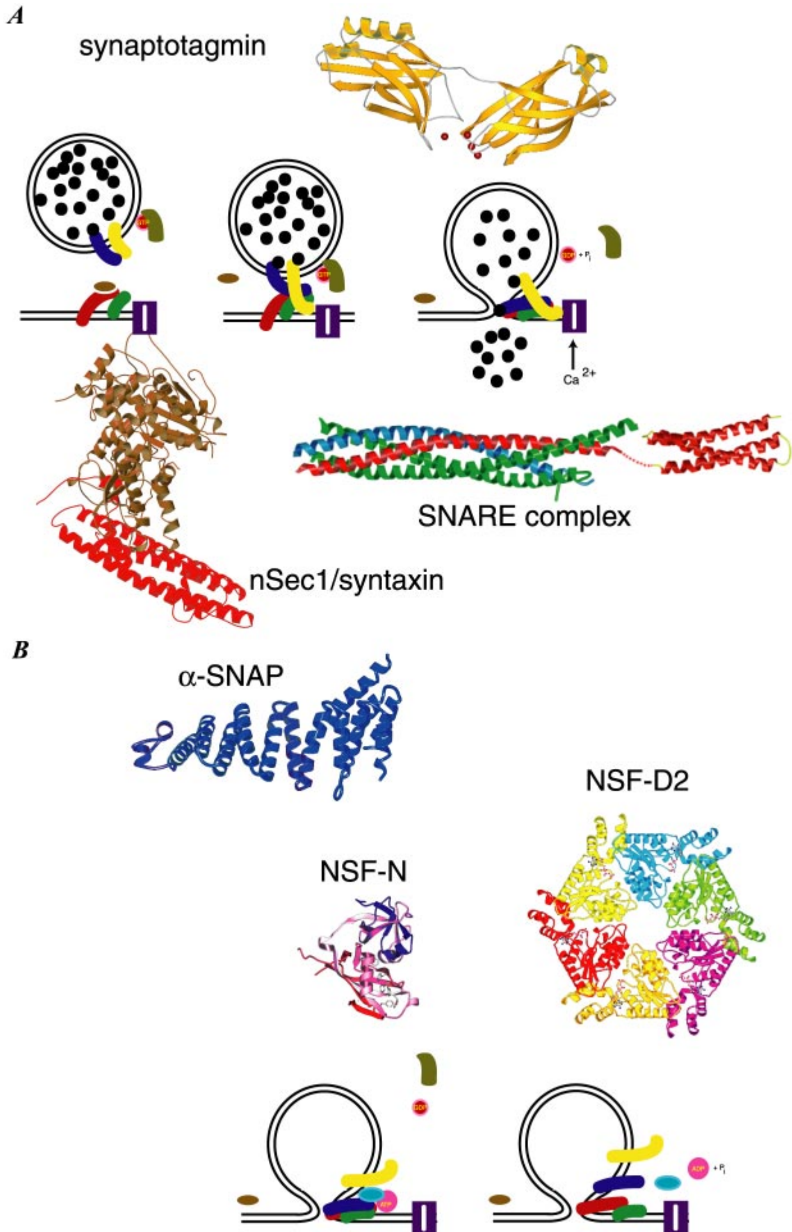

Figure 1 Summary of structures of proteins involved in synaptic vesicle exocytosis and disassembly of the SNARE complex. (*A*) SNARE complex (blue: synaptobrevin, red: syntaxin, green: SNAP-25) (87), N-terminal domain of syntaxin (28), nSec1/syntaxin complex (red: syntaxin, brown: nSec1) (58), synaptotagmin C2AB (86). (*B*) α-SNAP (Sec17) (72), (*B*) NSF-N (56, 104), NSF-D2 (51, 103).