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A Protein Assembly–Disassembly Pathway In Vitro That May Correspond to Sequential Steps of Synaptic Vesicle Docking, Activation, and Fusion

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Summary

The SNARE hypothesis holds that a transport vesicle chooses its target for fusion when a soluble NSF attachment protein (SNAP) receptor on the vesicle (v-SNARE) pairs with its cognate t-SNARE at the target membrane. Three synaptosomal membrane proteins have previously been identified: syntaxin, SNAP-25 (t-SNAREs), and vesicle-associated membrane protein (VAMP) (v-SNARE); all assemble with SNAPs and NSF into 20S fusion particles. We now report that in the absence of SNAP and NSF, these three SNAREs form a stable complex that can also bind synaptotagmin. Synaptotagmin is displaced by α-SNAP, suggesting that these two proteins share binding sites on the SNARE complex and implying that synaptotagmin operates as a "clamp" to prevent fusion from proceeding in the absence of a signal. The α-SNAP–SNARE complex can bind NSF, and NSF-dependent hydrolysis of ATP dissociates the complex, separating syntaxin, SNAP-25, and VAMP. ATP hydrolysis by NSF may provide motion to initiate bilayer fusion.

Introduction

Fusion between cellular membranes can occur constitutively, as seen when a transport vesicle from the endoplasmic reticulum fuses with the Golgi, or fusion can be closely regulated, as when a secretory storage vesicle fuses with the surface membrane upon receipt of a signal, a process termed exocytosis. A concrete relationship between constitutive fusion and triggered exocytosis was implied when it was discovered (Söllner et al., 1993) that three membrane proteins from the synapse—synaptic vesicle-associated membrane protein (VAMP/synaptobrevin) (Trimble et al., 1988; Baumert et al., 1989), syntaxin (Bennett et al., 1992b), and synaptosome-associated protein 25 (SNAP-25) (Oyler et al., 1989)—are receptors (SNAREs) for the general fusion proteins α- and γ-SNAP (Clary et al., 1990; Whiteheart et al., 1993) and that they assemble with SNAPs and the general N-ethylmaleimide-sensitive factor (NSF) fusion protein into 20S fusion particles (Wilson et al., 1992; Whiteheart et al., 1992). This was also suggested by sequence homologies between certain secretion genes in yeast and synaptic proteins (Bennett and Scheller, 1993). The use of a constitutively operating mechanism in triggered exocytosis implied the existence of "clamps" that prevent fusion, until the appropriate signal was received (Bennett and Scheller, 1993; Söllner et al., 1993).

No form of exocytosis is more exquisitely controlled or intensively studied than the regulated release of neurotransmitters from the presynaptic nerve terminal, the basis of synaptic transmission (Kelly, 1993). Here, the local entry of calcium ion triggers the fusion of neurotransmitter-containing synaptic vesicles with the presynaptic plasma membrane at the terminal membrane. Consequently, much attention has been given to the identification of proteins that are localized to the synaptic vesicle or the terminal membrane. Among these many proteins are the SNAREs syntaxin, VAMP, and SNAP-25, as well as synaptotagmin (Matthew et al., 1981), a synaptic vesicle membrane protein likely to have a negative regulatory role (Elfrink et al., 1993; Bommert et al., 1993; DiAntonio et al., 1993; Nonet et al., 1993; Popov and Poo, 1993; Littleton et al., 1993; DeBello et al., 1993).

Because VAMP resides in the synaptic vesicle and syntaxin resides in the presynaptic terminal membrane, it was proposed (Söllner et al., 1993) that a 20S fusion particle containing both SNAREs would dock the synaptic vesicle to the plasma membrane for fusion. However, whether a single 20S particle can simultaneously bind both VAMP and syntaxin (and possibly also SNAP-25) was not actually known, and it was difficult to assign a role to SNAP-25, because this protein had not been localized within the synapse. The *SNAP-25* gene encodes an intrinsically hydrophilic protein that is most likely bound to membranes via covalently attached fatty acids (Hess et al., 1992). A role for syntaxin in vesicle docking had previously been suggested (Bennett et al., 1992b) because syntaxin (from the presynaptic plasma membrane) can be coimmunoprecipitated with antibodies to the synaptic vesicle membrane protein synaptotagmin. However, since synaptotagmin was not found along with the three SNAREs in the the 20S particle (Söllner et al., 1993), synaptotagmin could not be involved in vesicle docking at this stage.

The SNARE hypothesis (Söllner et al., 1993) was proposed to generalize this specific model for vesicle docking at the synapse to explain the specificity inherent in the many kinds of membrane interactions that occur in cells: it postulates that each transport vesicle has its own specific v-SNARE that pairs up in a unique match with a cognate t-SNARE found only at the intended target membrane. The major line of evidence supporting this generalization is that genes homologous to VAMP and syntaxin are required at distinct steps in protein secretion in yeast (Hardwick and Pelham, 1992; Bennett and Scheller, 1993). Recently, nonneuronal mammalian homologs of syntaxin (Bennett et al., 1993) and VAMP (Cain et al., 1992; McMahon et al., 1993) have been described that are localized in the plasma membrane and derived compartments, and a homolog of syntaxin closely related to the yeast *SED5*

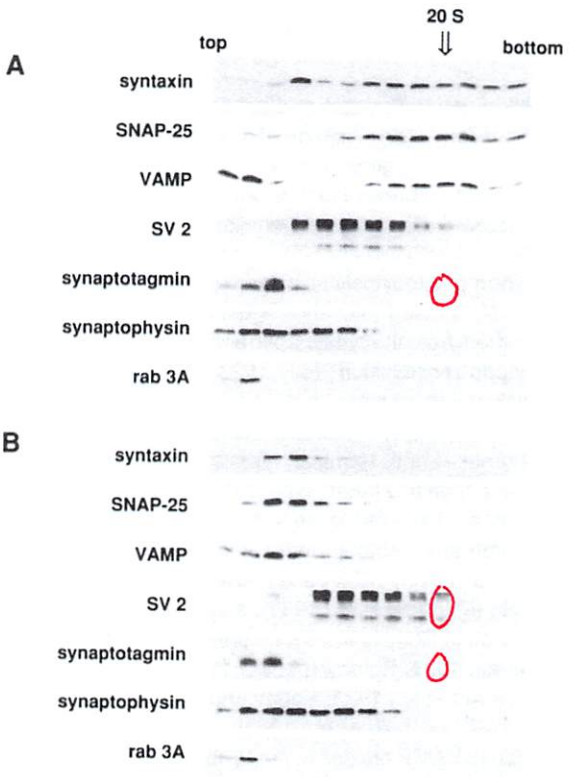


Figure 1. Incorporation of Synaptosomal Proteins into the 20S Fusion Particle

(A) Conditions favoring the formation of the 20S particle. Detergent extracts (Söllner et al., 1993) of bovine brain membranes (200 μg of protein) were incubated for 30 min at 4°C with His_6–NSF–Myc (15 μg of protein) and His_6–α-SNAP (15 μg of protein) in the presence of 0.5 mM ATP and 2 mM EDTA in incubation buffer (20 mM HEPES–KOH [pH 7.0], 100 mM KCl, 1 mM dithiothreitol, and 0.5% [v/v] Triton X-100) in a final volume of 300 μl. The reaction was layered on top of a 10%–35% (w/v) glycerol gradient containing the same buffer as above and subjected to centrifugation for 18 hr in an SW41 rotor (Beckman) at 40,000 rpm at 4°C. Fractions were collected, and the proteins were precipitated by trichloroacetic acid, separated by SDS–PAGE, electrotransferred to nitrocellulose, and immunodecorated with the indicated antibodies: anti-syntaxin polyclonal affinity-purified antibody (Bennett et al., 1992b), anti-His_6–SNAP-25 polyclonal affinity-purified antibody, anti-VAMP polyclonal affinity-purified antibody (Trimble et al., 1990), anti-SV2 monoclonal 10H3 antibody (Buckley and Kelly, 1985), anti-synaptotagmin polyclonal affinity-purified antibody (R. H. S., unpublished data), anti-synaptophysin monoclonal antibody SY38 (Wiedenmann and Franke, 1985), and anti-Rab3A polyclonal affinity-purified antibody (R. H. S., unpublished data). Bound antibodies were visualized using the enhanced chemiluminescence (ECL) system from Amersham.
(B) Conditions in which 20S particles do not form. Incubations and analysis were performed as described in (A), but NSF and α-SNAP were omitted from the particle formation reaction. (The 20S particle forms only when a membrane detergent extract, α-SNAP, and NSF are incubated together.)

gene has been found to reside in the intermediate compartment or cis-Golgi network (Bennett et al., 1993). The possibility that SNAREs encode specificity does not exclude that other proteins, e.g., small GTP-binding proteins (Zerial and Stenmark, 1993), also contribute to specificity.

Based on the observation of a pathway of assembly and disassembly of a series of complexes of synaptic proteins in a cell-free system, we now propose a series of steps in the process by which a vesicle docks, by which it is activated for fusion when a specific clamp (synaptotagmin) is released, and by which fusion is initiated as the SNAREs are rearranged in a reaction driven by the ATPase activity of NSF.

Results

Composition of 20S Fusion Particles

When NSF, α-SNAP, and (optionally but optimally) γ-SNAP are added to Triton X-100 extracts of a total brain membrane fraction containing SNAREs, 20S particles that contain all of these components form (Söllner et al., 1993). The particles assemble in the presence of magnesium–ATPγS or in the presence of ATP in the absence of magnesium (i.e., in the presence of EDTA), but disassemble when ATP can be hydrolyzed (e.g., in the presence of magnesium–ATP [Wilson et al., 1992]).

The 20S particles were assembled in the presence of ATP and EDTA and separated from unassembled proteins by sedimentation through a glycerol velocity gradient. Fractions were analyzed by Western blot analysis with antibodies to known synaptic membrane proteins. VAMP, syntaxin, and SNAP-25 assembled into 20S particles (Figure 1A), while the other synaptic membrane proteins tested (synaptotagmin, synaptophysin, SV2, and Rab3A) were not incorporated into the 20S complex. This demonstrates the specificity and efficiency of the association of VAMP, syntaxin, and SNAP-25 with NSF and α-SNAP. It is not clear why only 60% of VAMP and 70% of syntaxin assemble into 20S particles, even when excess NSF and SNAPs are added. A fraction of these molecules might be inactivated in a biologically interesting way or, trivially, may have denatured, or endogenous SNAP-25 might be the limiting component. It is also possible that only SNAREs preassembled into complexes extracted from vesicles docked in vivo (see below) are competent for particle assembly. Figure 1B shows a control in which α-SNAP and NSF were omitted from the reaction. The same result is obtained in another control (data not shown), in which 20S particles were disassembled in the presence of magnesium–ATP: all of the SNAREs now sediment at the top of the glycerol gradient.

To test whether a given 20S particle contains all three SNAREs, particles were assembled in the presence of magnesium–ATPγS and then immunoprecipitated with anti-syntaxin antibodies or with anti-SNAP-25 antibodies. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie blue staining (Figure 2A) and Western blot analysis (data not shown) revealed that the anti-syntaxin antibody efficiently coprecipitated VAMP and SNAP-25, along with syntaxin, NSF, and α-SNAP (Figure 2A, lane 3). Most of the VAMP and SNAP-25 were removed from the supernatant (data not shown). The complexes thereby isolated, corresponding to 20S particles, consist principally of NSF, α-SNAP, SNAP-25, syntaxin A plus B polypeptides, and VAMP. None of these polypeptides were recovered in a mock immunoprecipitate (Figure

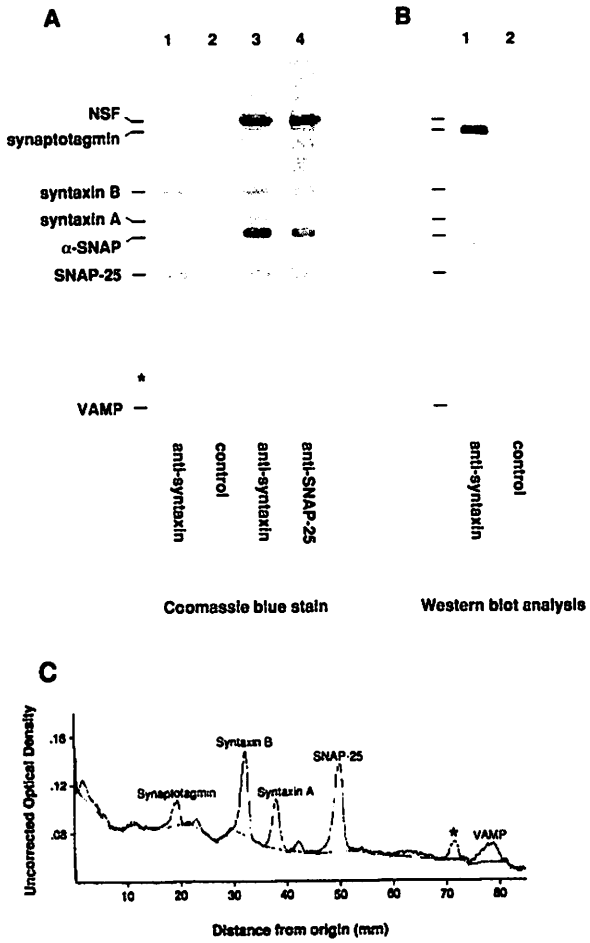


Figure 2. A Complex of VAMP, SNAP-25, and Syntaxin in the Absence and Presence of α-SNAP and NSF

(A) Detergent extract of bovine brain membranes (1.2 mg of protein) was incubated without (lanes 1 and 2) or with His_6–NSF–Myc (20 μg of protein) and His_6–α-SNAP (15 μg of protein) (lanes 3 and 4) in the presence of 0.5 mM ATPγS and 2 mM $MgCl_2$ in a final volume of 1.4 ml. Then, protein G–Sepharose 4 fast flow beads without antibodies (lane 2; control) or with covalently attached antibodies directed against syntaxin (monoclonal HPC1; Inoue et al., 1992) (lanes 1 and 3) or His_6–SNAP-25 (lane 4) were added and further processed as described in Experimental Procedures. The immunoprecipitations were analyzed after trichloroacetic acid precipitation and SDS–PAGE by Coomassie blue R-250 staining. The band indicated by an asterisk is variable among experiments and was found by amino acid sequencing to be the myelin basic protein.

(B) Western blot analysis of the immunoprecipitates with anti-synaptotagmin antibodies. Aliquots (10%) of reactions 1 and 2 of the immunoprecipitates from (A) were electrotransferred after SDS–PAGE to nitrocellulose, proteins were immunodecorated with anti-synaptotagmin antibodies, and the bound antibodies were visualized by the ECL system.

(C) Superimposed densitometer traces of lane 1 (showing peaks) and an empty lane (for background determination). Peaks representing synaptotagmin, syntaxins B and A, SNAP-25, myelin basic protein (indicated by an asterisk), and VAMP2 are labeled. Peaks were integrated above the indicated background, and the peak areas were normalized to that of SNAP-25 in each of several determinations from independent samples. Ratios: synaptotagmin, 0.26 ± 0.06 (two determinations); syntaxin B, 0.81 ± 0.04 (four determinations); syntaxin A, 0.39 ± 0.06 (four determinations); myelin basic protein, 0.19 ± 0.05 (four determinations); VAMP2, 0.33 ± 0.07 (four determinations). Assuming that staining intensity is proportional to the total mass of each polypeptide present, these ratios can be converted to mole ratios to SNAP-25 by dividing by the ratio of the molecular mass of the component in question into that of SNAP-25. Doing so yields these mean mole ratios: synaptotagmin to SNAP-25, 0.10; syntaxin B to SNAP-25, 0.58; syntaxin A to SNAP-25, 0.28; VAMP2 to SNAP-25, 0.63.

2A, lane 2). When anti-SNAP-25 antibody was used in the immunoprecipitation, VAMP and syntaxins likewise coprecipitated with SNAP-25, NSF, and α-SNAP (Figure 2A, lane 4).

Because all three SNAREs as well as NSF and α-SNAP are efficiently coprecipitated with antibodies against each of two different SNAREs, we conclude that a single 20S particle contains VAMP, syntaxin, and SNAP-25, consistent with its proposed role (Söllner et al., 1993) in vesicle docking.

A Complex of SNAREs Containing Synaptotagmin but Not NSF or SNAP

When analyzing immunoprecipitations from control experiments in which α-SNAP and NSF were not added, it became apparent that the SNAREs specifically associate with each other in the detergent extract of brain membranes even in the absence of NSF and SNAP. When the detergent extract is immunoprecipitated with anti-syntaxin antibody, VAMP and SNAP-25 still coprecipitate with syntaxin (Figure 2A, lane 1) and do so with the same efficiency as when NSF and α-SNAP are present (Figure 2A, lane 3; see also Western blots in Figure 4, lanes 7 and 1). The VAMP bands in Figure 2 are stained weakly owing to the low molecular mass of VAMP relative to other components present and thus do not reproduce well. Therefore, a scan of the original Coomassie blue–stained SDS gel is shown in Figure 2C. Integration of the peaks and correction for molecular masses (see the legend to Figure 2 for details) reveals that the complex thus isolated by immunoprecipitation contains stoichiometric amounts of VAMP, SNAP-25, and syntaxins and appears free of other stoichiometric components: there are 0.86 and 0.63 mol of syntaxins (A plus B) and VAMP per mole of SNAP-25, respectively (see the legend to Figure 2C for details). The most abundant nonstoichiometric band (just above VAMP and marked by an asterisk in Figure 2A) was microsequenced and found to be a major protein from myelin, called myelin basic protein (data not shown). We presume this to be a contaminant. In addition, a nonstoichiometric (0.10 mol per mole of SNAP-25) band at 65 kd, identified by Western blotting (Figure 2B) as synaptotagmin, is also observed. None of the aforementioned bands was observed in mock immunoprecipitation controls (Figures 2A and 2B, lanes 2). The efficiency of the coimmunoprecipitation with syntaxin (pellet divided by total input, with syntaxin normalized to 100%) estimated from Western blot analysis of pellets and supernatant is as follows for each component: synaptotagmin (5%–10%), SNAP-25 (55%–65%), and VAMP (50%–60%).

The same complex can be demonstrated by coprecipitation with anti-SNAP-25 or anti-synaptotagmin antibodies (data not shown). The synaptotagmin–SNARE complex can also be revealed by velocity sedimentation of deter-

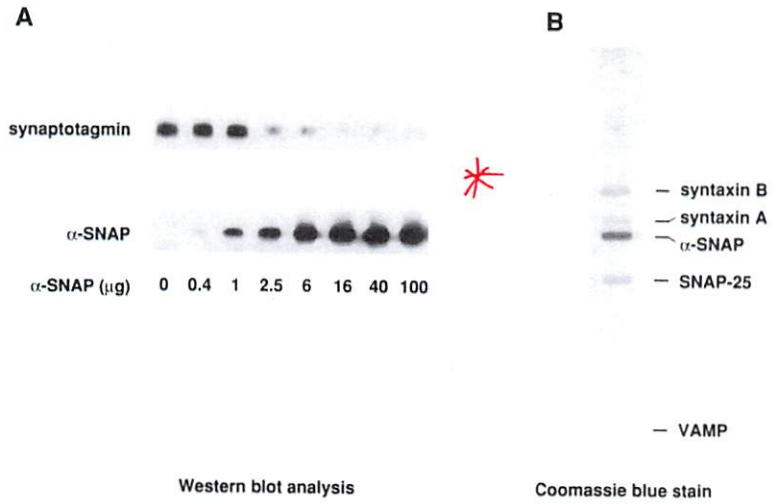


Figure 3. α-SNAP Displaces Synaptotagmin from the SNARE Complex

(A) Triton X-100 extracts of bovine brain membranes (200 μg of protein) were incubated with increasing amounts of His_6–α-SNAP in a final volume of 500 μl, and samples were precipitated with monoclonal anti-syntaxin antibodies as described in Experimental Procedures. Samples were analyzed after immunoprecipitation by SDS–PAGE, transferred to nitrocellulose, and immunodecorated with anti-α-SNAP and anti-synaptotagmin antibodies. Bound antibodies were visualized by the ECL system.

(B) Coomassie blue–stained gel of the SNARE complex containing α-SNAP. α-SNAP (15 μg of protein) was incubated together with the detergent extract of bovine brain membranes (1.2 mg of protein) in a final volume of 1.4 ml, and the SNARE complex was isolated with anti-syntaxin antibodies. The protein pattern was resolved by high Tris–urea SDS–PAGE (Söllner et al., 1993), and the proteins were stained with Coomassie blue R-250.

gent extracts of a partially purified synaptic vesicle fraction, LP2 (Huttner et al., 1983), in a glycerol gradient in the presence of Triton X-100 (Bennett et al., 1992a). The complex of synaptotagmin, syntaxins, SNAP-25, and VAMP cosediments as a distinct, symmetrical peak at about 7S that can be revealed by coprecipitation of these components with either anti-synaptotagmin or anti-syntaxin antibodies (data not shown).

We conclude that the three SNAREs (VAMP, syntaxin, and SNAP-25) can form a specific and approximately equimolar complex. The SNARE complex must be stable, as its stochiometry implies that it resists dissociation during repeated washing in the course of immunoprecipitation, and the complex must be specific because of its simple and defined polypeptide chain composition with no stoichiometric components apparent other than the SNAREs themselves. Thus, the existence of this complex implies that a v-SNARE can bind directly to a t-SNARE, a fundamental tenet of the SNARE hypothesis. Whether the SNARE complex forms after detergent extraction or exists in docked vesicles prior to extraction is not yet clear. However, the SNARE complex represents the major form of these constituents, based on the high efficiency of coimmunoprecipitation.

Synaptotagmin can bind to the SNARE complex, although only a small fraction of the immunoprecipitated SNARE complex contains synaptotagmin (Figure 2A). This suggests that the SNARE complex can exist either freely or as a synaptotagmin–SNARE complex. The low abundance of synaptotagmin may be explained by the fact that we washed the membranes with 1 M KCl before detergent extraction; this concentration of salt is known to dissociate synaptotagmin from syntaxin-containing complexes (Bennett et al., 1992b).

α-SNAP Displaces Synaptotagmin from the SNARE Complex

Because synaptotagmin is present in the SNARE complex but is not present in the 20S particle containing SNAREs, NSF, and SNAPs (see Figure 1), synaptotagmin must be specifically bound so as to dissociate at some stage during the assembly of 20S particles. To determine at what stage this occurs, detergent extract containing the synaptotagmin–SNARE complex was mixed with increasing amounts of pure α-SNAP, and the SNARE complex was then immunoprecipitated with anti-syntaxin antibody (Figure 3). Synaptotagmin, but no other subunit (Figure 3A; see also Figure 4, lane 3 versus lane 1), was released as α-SNAP bound to the SNARE complex. At saturation, about 6 mol of α-SNAP monomer was bound per mole of SNAP-25 monomer in the SNARE complex, based on scanning Coomassie blue–stained SDS gels, in the presence of ATP or ATPγS (Figure 4, lanes 3 and 4). To displace completely synaptotagmin from the SNAREs, a 25-fold excess of exogenous α-SNAP over endogenous SNAP-25 was necessary (data not shown). NSF does not displace synaptotagmin, nor does NSF bind to SNAREs (see Figure 4, lanes 5 and 6) in the absence of α-SNAP.

The specific displacement of synaptotagmin by α-SNAP provides compelling evidence that the binding of synaptotagmin to SNAREs is meaningful and implies that synaptotagmin competes with the general fusion protein α-SNAP for binding to a common site on the SNARE complex. Assuming that this result extends to a synapse, it would appear that synaptotagmin is intrinsically programmed to stay "on" the SNAREs to keep fusion "off" by preventing α-SNAP from binding. We imagine that an excess of α-SNAP is needed to displace synaptotagmin by mass action in the artificial conditions we employed in the cell-free system because key signaling molecules are either absent or not activated. In neurons, where regulatory components can function, we envision that an appropriate signal triggers the efficient release of synaptotagmin, enabling the efficient binding of α-SNAP.

Based on these results with isolated proteins, we suggest that synaptotagmin illustrates the principle postulated (Bennett and Scheller, 1993; Söllner et al., 1993) for a fusion clamp needed to keep the constitutive fusion ma-

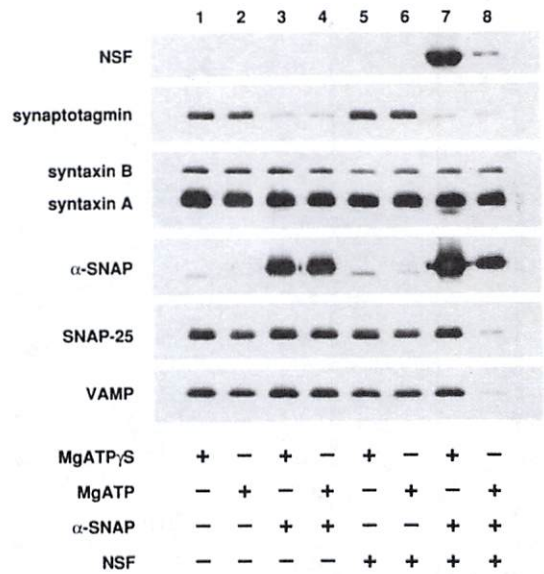
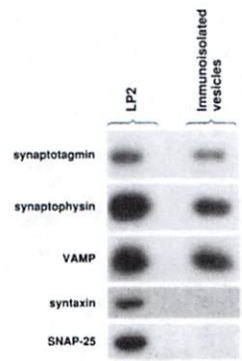


Figure 4. Assembly and ATP Hydrolysis-Dependent Disassembly of the 20S Fusion Particle

Bovine brain membrane extracts (200 μg of protein) were either incubated in the presence of magnesium–ATPγS or magnesium–ATP, without or with His_6–α-SNAP (6 μg of protein), without or with His_6–NSF–Myc (15 μg of protein), and with His_6–α-SNAP and His_6–NSF–Myc together in a final volume of 0.5 ml, as described in Experimental Procedures. The samples were precipitated with anti-syntaxin antibodies, and the proteins were resolved by high Tris–urea SDS–PAGE, transferred to nitrocellulose, and immunodecorated with antibodies directed against synaptotagmin, syntaxin, His_6–α-SNAP, His_6–SNAP-25, and VAMP as described above. (The anti-His_6–α-SNAP and the anti-His_6–SNAP-25 antibodies also react with the His_6 epitope of His_6–NSF–Myc.)



Meh.

Figure 5. SNAP-25 Is Absent from Synaptic Vesicles

Intact synaptic vesicles were immunoprecipitated from a rat brain synaptic vesicle-enriched fraction, LP2 (Huttner et al., 1983), and suspended in HKA buffer (10 mM HEPES–KOH [pH 7.5], 140 mM potassium acetate, 1 mM $MgCl_2$, 0.1 mM EGTA) supplemented with 0.5 M NaCl. LP2 (1 mg) was mixed with anti-p65 (synaptotagmin) monoclonal antibody M48 (Matthew et al., 1981) prebound to protein A–Sepharose, and the immune complex was recovered and washed as previously described (Bennett et al., 1992a). The immunoprecipitated sample, as well as 50 μg of starting LP2, was resolved by SDS–PAGE, transferred to nitrocellulose, and probed with the following antibodies: anti-p65 monoclonal antibody M48, anti-synaptophysin monoclonal antibody SY38, anti-VAMP polyclonal antibody, anti-syntaxin polyclonal antibody, and anti-SNAP-25 polyclonal antibody. Note that syntaxin (from presynaptic plasma membrane) is absent from this immunopurified synaptic vesicle fraction, but is present in the LP2 fraction.

chinery off prior to receipt of a signal for exocytosis. Accumulating genetic and physiological evidence leads to the same general conclusion (Popov and Poo, 1993; DeBello et al., 1993; Littleton et al., 1993), for which we offer this simple and specific molecular mechanism: synaptotagmin covers an α-SNAP-binding site on the SNAREs that prevents this general fusion component from gaining access, thereby keeping fusion off until a signal releases this inhibition, allowing 20S particles to form and fusion to proceed.

Synaptotagmin did not dissociate from SNAREs in the presence of 1 mM $CaCl_2$ in the absence of α-SNAP (data not shown). This suggests that even though synaptotagmin is a calcium-binding protein (Brose et al., 1992) and may be a calcium sensor (DeBello et al., 1993), its dissociation may not be directly triggered by calcium ions alone. Perhaps an additional sensor is required for dissociation (DeBello et al., 1993) to reduce error rates, or additional protein factors or other components are needed to effect the dissociation of synaptotagmin when calcium is bound, or, more trivially, this aspect of synaptotagmin function may have become denatured in our system.

Hydrolysis of ATP Requiring NSF Disrupts the SNARE Complex

When NSF is added along with α-SNAP to the detergent extract in the presence of magnesium–ATPγS, NSF joins the α-SNAP–SNARE complex and is coimmunoprecipitated by anti-syntaxin antibodies along with α-SNAP, SNAP-25, and VAMP (Figure 4, lane 7), corresponding to the formation of 20S particles (see Figure 1A). NSF does not bind in significant amounts to the SNARE complex in the absence of exogenous α-SNAP (Figure 4, lanes 5 and 6). Based on scanning Coomassie blue–stained SDS gels, there are approximately 0.5 mol of NSF tetramer per mole of α-SNAP monomer bound to the SNARE complex. Thus, as expected (Whiteheart et al., 1992), α-SNAP can bind to SNAREs in the absence of NSF, but NSF cannot bind until SNAP has bound to SNAREs. Suprisingly, about 3-fold additional α-SNAP binds when NSF is added (Figure 4, lane 7 versus lane 3), even though saturable binding of α-SNAP had occurred in the absence of NSF (see Figure 3A) under the conditions used. The significance of this apparent polymerization of NSF–SNAP complexes on a SNARE complex remains to be investigated, but may be important for forming an array or "collar" needed for fusion (White, 1992).

When NSF is added along with α-SNAP in the presence of magnesium–ATP (rather than magnesium–ATPγS) to allow ATP hydrolysis, NSF no longer coimmunoprecipitates with anti-syntaxin antibodies (Figure 4, lane 8 versus lane 7). This is expected, because NSF is known to dissociate from 20S particles upon ATP hydrolysis (Wilson et al., 1992). However, the fate of α-SNAP and the SNAREs during particle disassembly had not been determined. Suprisingly, almost all of the SNAP-25 and VAMP are released from syntaxin. However, some α-SNAP remains bound to syntaxin, corresponding to about the half of the amount that binds (in the absence of NSF) to the entire

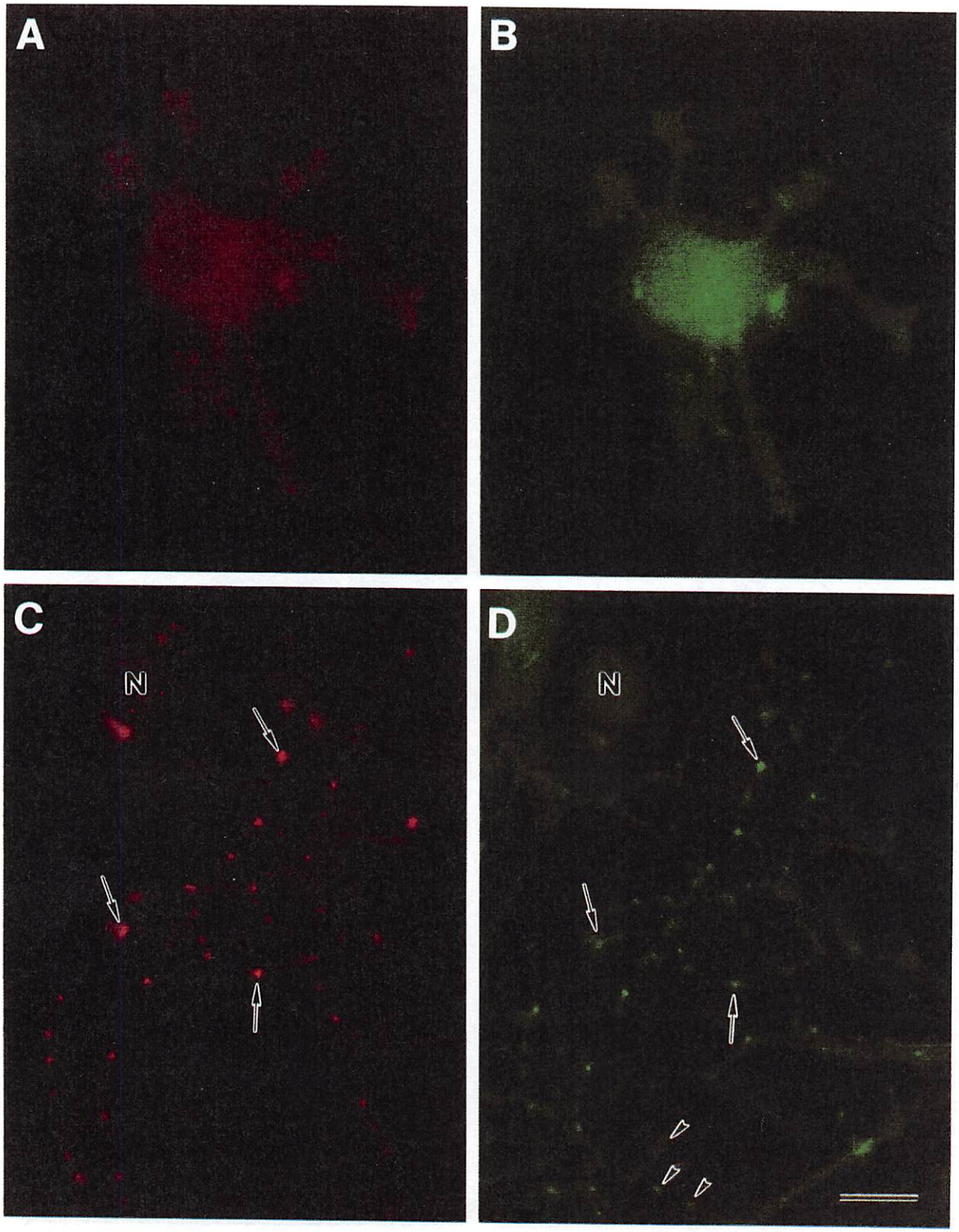


Figure 6. Syntaxin and SNAP-25 Are Colocalized on the Plasma Membrane In Vivo

Double immunofluorescence localization of syntaxin and SNAP-25 was performed on cultures of NGF-induced PC12 cells (A and B) and dissociated hippocampal neurons (C and D) as described in Experimental Procedures. SNAP-25 immunoreactivity was detected with a rhodamine-conjugated secondary antibody (A and C), while syntaxin immunoreactivity was detected with a fluorescein-conjugated secondary antibody (B and D). Arrows in (C) and (D) indicate examples of neuronal varicosities that are colabeled with both syntaxin and SNAP-25 antibodies, while arrowheads in (D) indicate syntaxin-positive varicosities that are not labeled with anti-SNAP-25. The position of a pyramidal cell nucleus (N) is indicated in (C) and (D). Scale bar, 11 μm.

SNARE complex (Figure 4, lane 8 versus lane 3). (Quantitative data are based on Western blot analysis using [^{125}I]protein A detection in the linear range.)

The dissociation of SNAP-25, VAMP, and α-SNAP from syntaxin requires both NSF and ATP (Figure 4). Almost certainly this is due to ATP hydrolysis by NSF itself, as NSF is known to be an ATP-binding protein (Block et al., 1988) and an ATPase (Tagaya et al., 1993) while neither the SNAPs nor the SNAREs are predicted to hydrolyze ATP based on their sequences; however, this remains to be formally proven.

We conclude that in the case of the synapse, NSF-dependent ATP hydrolysis can dissociate syntaxin from SNAP-25 and VAMP. Generalizing, we suggest that NSF action dissociates v-SNAREs from the t-SNAREs, releasing NSF in the process. As NSF is known to be required during a single round of vesicle transport prior to bilayer fusion (Orci et al., 1989; Kaiser and Schekman, 1990; Rexach and Schekman, 1991; Ostermann et al., 1993), the NSF- and ATP-dependent dissociation of v- from t-SNAREs must be an essential step in the process culminating in fusion, rather than part of a recycling operation after bilayer fusion has been completed.

Localization of SNAP-25 Protein

To establish better the topological relationship of SNAP-25 in the SNARE complex, its subcellular localization must be determined. Previously, it had been localized generally to the synapse and shown to behave like an integral membrane protein (Oyler et al., 1989). Subcellular fractionation revealed that SNAP-25 is absent from synaptic vesicles (Figure 5), and immunofluorescence confirmed this by showing (Figure 6) that syntaxin and SNAP-25 are both localized to the plasma membrane in cultures of NGF-induced PC12 cells (Figures 6A and 6B) and dissociated hippocampal neurons (Figures 6C and 6D).

Discussion

The composition and sequential nature of the protein complexes we have identified lead us in a natural and direct way to propose a corresponding series of steps to explain how vesicles dock, are activated, and begin to fuse in living cells and especially in neuronal synapses. In doing so, we assume that the cell-free system we employed faithfully represents the situation in vivo. The physiological relevance of the proposed docking and fusion particles is suggested by the facts that VAMP, SNAP-25, and syntaxin are each the target of particular neurotoxins that block transmitter release (Schiavo et al., 1992; Schiavo et al., submitted; Blasi et al., 1993a, 1993b; Huttner, 1993), that domains of syntaxin and Fab fragments of antibodies directed against syntaxin block exocytosis (Bennett et al., 1993), and that genes encoding NSF (Wilson et al., 1989) and α-SNAP (Clary et al., 1990) are essential for fusion in yeast (Kaiser and Schekman, 1990).

The SNARE Hypothesis

The simple view that a vesicle docks to its target membrane via partnering of cognate SNAREs (Söllner et al., 1993) is strongly supported by our isolation in a highly purified form of a stable and specific complex composed of essentially equimolar quantities of VAMP, syntaxin, and SNAP-25. In addition to the SNAREs themselves, this complex can bind either synaptotagmin or α-SNAP. Evidently, the principal interactions holding these complexes together are due to the SNAREs themselves. We note that syntaxins and VAMPs each have polymorphic sequences but structurally conserved domains predicted to form α-helical coiled coils (Newman et al., 1992; Hardwick and Pelham, 1992; Bennett and Scheller, 1993) and that the same is also true of α-SNAP, NSF, and SNAP-25 (unpublished data). Pairing of coiled-coil domains (as in leucine zippers [O'Shea et al., 1989; Baxevanis and Vinson, 1993]) would provide an attractive mechanism for the specific matching of v- with t-SNARES and for the binding and possible rearrangement (see below) of fusion proteins. An important role for coiled coils in viral fusion has been proposed by Carr and Kim (1993). Large-scale conformational rearrangements of this kind can reasonably be expected in fusion reactions that must move lipids tens of angstroms. The existence of gene families related to VAMP and syntaxin (Cain et al., 1992; Newman et al., 1992; Hardwick and Pelham, 1992; Bennett and Scheller, 1993; McMahon et al., 1993; Bennett et al., 1993) provides ample possibility for numerous specific pairings and conformational rearrangements of v-SNAREs and t-SNAREs.

The colocalization of syntaxin and SNAP-25 to the plasma membrane and in the SNARE complex implies that in the case of the synapse, the active form of the target membrane–contributed SNARE (t-SNARE) has two distinct subunits. Perhaps t-SNAREs generally are heterodimers containing synataxin and a SNAP-25-related chain. Whether possible SNAP-25-related chains of nonneuronal t-SNAREs are as polymorphic as the syntaxin chains remains to be seen.

Triggering of Exocytosis by Releasing Clamps

The use of the general (i.e., constitutively on) fusion machinery (NSF and SNAPs) in regulated exocytosis implies a mechanism to inhibit fusion until an appropriate signal for exocytosis arrives (Söllner et al., 1993; Bennett and Scheller, 1993). Our finding that synaptotagmin and α-SNAP share a common binding site on the SNARE complex affords a simple molecular mechanism and vividly illustrates how a molecular clamp can work (Figure 7), by preventing a general fusion protein (α-SNAP) from binding to an active site (in the SNARE complex).

Arguments have been advanced to suggest that synaptotagmin may be a calcium sensor that helps triggering exocytosis (DeBello et al., 1993). If synaptotagmin is such, our finding that the addition of calcium did not result in the release of synaptotagmin from the SNARES might suggest that additional components are necessary to release this clamp. These components, perhaps including other calcium sensors (DeBello et al., 1993), might be absent from or inactive in the membrane fraction we employ. Given the physiological complexity of synaptic transmission and the demonstration of multiple calcium-dependent processes in chromaffin cells (Neher and Zucker, 1993),

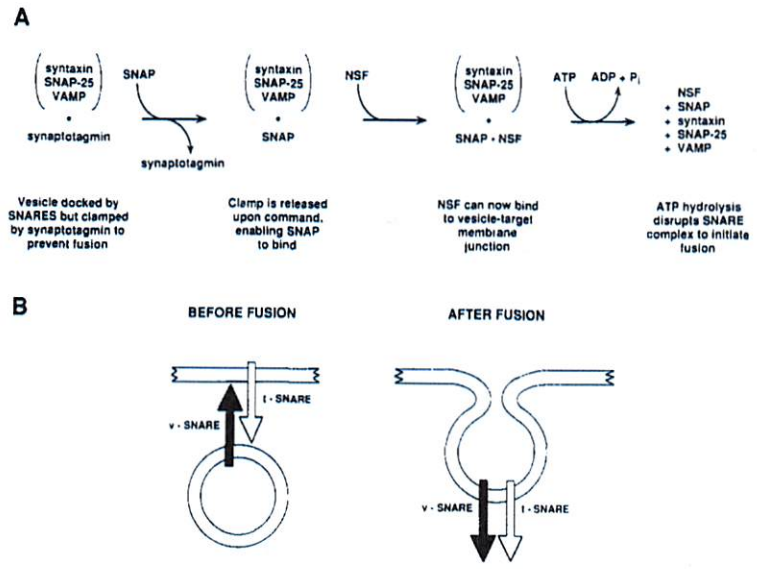


Figure 7. Interactions of SNAREs, Synaptotagmin, SNAPs, and NSF during Putative Vesicle Docking and Fusion

(A) Proposed sequential steps of vesicle docking, activation, and fusion (see below) based on experimental results (summarized above) as further described in the figure and in the text.

(B) Topological requirement for a rearrangement of SNAREs during the process of membrane fusion. v-SNAREs (closed arrows) and t-SNAREs (open arrows) are proposed to dock a vesicle to its target membrane. At this stage, the paired SNAREs reside in two distinct membranes. After fusion is complete, the v- and t-SNAREs reside in the same membrane. To accomplish this rearrangement, a 180° relative rotation is necessary at some stage during the process of fusion.

synaptotagmin would seem unlikely to be the only clamp or the only potential calcium sensor employed in the synapse. The use of multiple clamps and the means for releasing them would reduce the error rate (spontaneous discharge of neurotransmitter) of exocytosis and would explain why this error rate only doubles in the absence of synaptotagmin function (Littleton et al., 1993), rather than resulting in massive exocytosis, and why synaptotagmin may appear more essential in some systems than in others (reviewed by DeBello et al., 1993). We would predict that the many other cases of regulated exocytosis (mast cells, cytotoxic T cells, exocrine and endocrine pancreas, etc.) will employ clamps operating according to similar principles.

Our experiments documenting the sequential assembly and disassembly of complexes of synaptic and other proteins allow us to formulate a pathway of protein structures that we propose may correspond to the steps of docking, activation, and fusion of synaptic vesicles traditionally recognized by physiologists and electron microscopists studying neurosecretion (Figure 7A). We define fusion as the set of events that follows docking up to and including the mixing of the lipid bilayers of vesicle and target membranes.

In the first step (Figure 7A), we propose the synaptic vesicle docks with its intended target when VAMP (the v-SNARE) binds to its complementary t-SNARE (syntaxin–SNAP-25) at the plasma membrane. A clamp protein from the synaptic vesicle synaptotagmin binds to one or more of the SNAREs at this stage, preventing the general fusion protein SNAP from binding (in the case of nonneuronal, constitutive fusion, the simplest possibility would be that no clamp is present). When neurotransmitter release is required, the clamp dissociates, allowing SNAP to bind. This enables NSF and additional SNAP to bind. Then, NSF hydrolyzes ATP, disrupting the existing junction between vesicles and target formed by the v-SNARE and the t-SNARE and enabling fusion to proceed. The fact that synaptotagmin does not rebind to syntaxin following disruption of the SNARE complex by NSF ATPase (Figure 4, lane 8) confirms that synaptotagmin acts before, rather than after, SNAP and NSF in the pathway. It is likely that additional soluble components (Waters et al., 1992; Elazar et al., submitted) are required for fusion to be completed.

Assuming these events correspond to steps in fusion in the cell, the sequential nature of this pathway strongly suggests that the docking is done by the SNAREs and that SNAP and NSF initiate the fusion process following docking. Additional proteins (perhaps including small GTP-binding proteins) may well be involved in producing the SNARE complex, perhaps imparting an additional layer of specificity, but those do not appear to be present in stoichiometric amounts in the complexes we have studied here.

Steps in the Process of Fusion

As necessary as the initial interaction between SNAREs that are thought to dock vesicles may be, subsequent disruption of this interaction would seem from first principles to be an equally necessary part of any detailed fusion mechanism. This is because of the dramatic change in topology that inevitably must occur as two membranes coalesce (Figure 7B). While the v-SNARE and t-SNARE are antiparallel when a vesicle first docks, after fusion these SNAREs necessarily reside in the same membrane and are parallel, facing together into the cytoplasm in the same direction. This reorientation accompanying fusion amounts to a 180° relative rotation, and it is hard to imagine any specific protein–protein interface that could persist through such a drastic rearrangement. Therefore, the pairing of v-SNARE with t-SNARE, which docks the vesicle, must be broken up at some stage for fusion to occur, regardless of the mechanism of the process. Since the SNARE complex is itself a stable entity, an energy input would be needed for this rearrangement, explaining why ATP hydrolysis (by NSF) is required.

Given that a rearrangement of SNAREs during fusion is necessary topologically, it is not difficult to imagine many ways in which the relative motion of the SNAREs needed to affect this arrangement might be gainfully employed to

force the partner lipid bilayers toward a transition state for fusion. This is the case for all varieties of models, including ones with hemifusion intermediates (Monck and Fernández, 1992) and with lipid-wetting intermediates (Bentz et al., 1990). Although the SNAREs are fully dissociated by SNAP and NSF ATPases acting by themselves, this could well be an uncoupled, partial reaction due to the absence of other components of the fusion machinery (Waters et al., 1992; Elazar et al., submitted) not present in our experiments. These components might conceivably act to "capture" the dissociated SNAREs to position them in one state or in a series of different states to create protein scaffolds that might stabilize assemblies of nearby lipids in intrinsically unstable (transition state) structures, whose spontaneous resolution (Helm et al., 1989) results in fusion. In that the SNAREs are inserted into each bilayer, their motion will be coupled to that of nearby lipids, offering a means to push and pull them into and out of transition states.

The proposed steps in vesicle docking, activation, and fusion are likely to be key sites for modulating the efficacy of synaptic transmission, a process likely to underlie learning and memory. A description of the biochemical steps in this process as advanced here provides the basis for further experiments aimed at better understanding this process.

Experimental Procedures

Immunoprecipitations

Triton X-100 extracts of bovine brain membranes (Söllner et al., 1993) were incubated for 30 min at 4°C without or with His_6–α-SNAP (Whiteheart et al., 1993) and without or with His_6–NSF–Myc in immunoprecipitation buffer (20 mM HEPES–KOH [pH7.0], 100 mM KCl, 1% [w/v] polyethyleneglycol 4000, 1% [w/v] glycerol, 0.5%–0.9% [w/v] Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) supplemented with 2 mM EDTA, 0.5 mM ATP, 2 mM $MgCl_2$, 0.5 mM ATP or 2 mM $MgCl_2$, 0.5 mM ATPγS, as indicated in the figure legends. (His_6–NSF–Myc was constucted by combining the preexisting NSF–Myc clone [Wilson and Rothman, 1992] and the His_6–NSF clone [Whiteheart et al., 1993]). Then, the reactions were subjected to centrifugation for 2 min at 12,000 × g. The supernatants were incubated for 2 hr at 4°C with constant mixing with affinity-purified polyclonal antibodies (Harlow and Lane, 1988) directed against His_6–SNAP-25 or monoclonal anti-syntaxin HPC1 antibodies (Inoue et al., 1992). All antibodies were first covalently coupled to protein G–Sepharose fast flow (Söllner et al., 1993). The beads were washed five times with 10 vol of the buffer described above, but in the absence of polyethyleneglycol and glycerol. Bound antigens were eluted with 0.1 M glycine (pH 2.7).

Immunofluorescence

PC12 cells were plated on Matrigel-coated Permanox 8-chamber slides (4 × 10^4 cells/well) and differentiation induced with NGF (50 ng/ml) for 48 hr. Dissociated rat hippocampal neurons from regions CA1 and CA3 were prepared as previously described (Bennett et al., 1992b) and maintained in culture for 6 days. The cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 min, washed three times with phosphate-buffered saline containing 0.1 M glycine, and permeabilized with blocking buffer (phosphate-buffered saline containing 3% normal goat serum and 0.3% saponin) for 10 min. The cells were then incubated with primary antibodies (anti-syntaxin monoclonal HPC1 at 1:500 and anti-SNAP-25 polyclonal at 1:25) in blocking buffer for 2 hr, washed three times, and then incubated with secondary antibodies (fluorescein-conjugated goat anti-mouse immunoglobulin G at 1:100 and rhodamine-conjugated donkey anti-rabbit immunoglobulin G at 1:100) in blocking buffer for 1 hr. After washing three times, the slides were mounted with Citifluor and observed with a Zeiss Axiophot fluorescence microscope.

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