The EMBO Journal vol.14 no.10 pp.2317–2325, 1995

Disassembly of the reconstituted synaptic vesicle membrane fusion complex *in vitro*

Tetsuya Hayashi, Shinji Yamasaki, Sonja Nauenburg, Thomas Binz and Heiner Niemann[1]

Department of Microbiology, Federal Research Center for Virus Diseases of Animals, Paul Ehrlich Strasse 28, D72076 Tübingen, Germany

[1]Corresponding author

Communicated by E.Neher

The interaction of the presynaptic membrane proteins SNAP-25 and syntaxin with the synaptic vesicle protein synaptobrevin (VAMP) plays a key role in the regulated exocytosis of neurotransmitters. Clostridial neurotoxins, which proteolyze these polypeptides, are potent inhibitors of neurotransmission. The cytoplasmic domains of the three membrane proteins join into a tight SDS-resistant complex (Hayashi *et al.*, 1994). Here, we show that this reconstituted complex, as well as heterodimers composed of syntaxin and SNAP-25, can be disassembled by the concerted action of the *N*-ethylmaleimide-sensitive factor, NSF, and the soluble NSF attachment protein, α-SNAP. α-SNAP binds to predicted α-helical coiled-coil regions of syntaxin and SNAP-25, shown previously to be engaged in their direct interaction. Synaptobrevin, although incapable of binding α-SNAP individually, induced a third α-SNAP binding site when associated with syntaxin and SNAP-25 into heterotrimers. NSF released prebound α-SNAP from full-length syntaxin but not from a syntaxin derivative truncated at the N-terminus. Disassembly of complexes containing this syntaxin mutant was impaired, indicating a critical role for the N-terminal domain in the α-SNAP/NSF-mediated dissociation process. Complexes containing C-terminally deleted SNAP-25 derivatives, as generated by botulinal toxins type A and E, were dissociated more efficiently. In contrast, the N-terminal fragment generated from synaptobrevin by botulinal toxin type F produced an SDS-sensitive complex that was poorly dissociated.

Key words: clostridial neurotoxins/membrane fusion/NSF/SNARE complex/α-SNAP

Introduction

In the eukaryotic cell, membrane-associated transport between intracellular compartments involves sequestering of cargo substances into carrier vesicles that bud from one compartment and fuse with another in a highly organized manner. The machinery that controls vesicle–target membrane fusion is structurally and functionally highly conserved in all eukaryotic cells from yeast to mammalian neurons (Ferro-Novick and Jahn, 1994). Rothman and colleagues first identified distinct classes of cytosolic proteins that were essential in an assay system designed to study intracisternal Golgi transport *in vitro* (for review see Rothman and Warren, 1994). In this system, transport was abolished by the alkylating agent *N*-ethylmaleimide (NEM) (Malhotra *et al.*, 1988), a property that led to isolation of an NEM-sensitive factor (NSF) (Glick and Rothman, 1987; Block *et al.*, 1988). NSF was shown to be essential in several intracellular fusion events such as the homotypic fusion of endosomes (Diaz *et al.*, 1989) and the transport between the rough endoplasmic reticulum and Golgi complex (Beckers *et al.*, 1989) and from the Golgi to the plasma membrane (Graham and Emr, 1991).

Membrane association and induction of fusion by NSF requires the presence of accessory proteins, termed α-, β- and γ-SNAP (for soluble NSF attachment proteins) (Clary *et al.*, 1990). Whereas β-SNAP constitutes a brain-specific isoform of α-SNAP, γ-SNAP cannot functionally replace α-SNAP but acts synergistically with it (Whiteheart *et al.*, 1993). α-, β- and γ-SNAPs bind to specific SNAP receptors (SNAREs) present in the vesicle (v-SNARE) and target membrane (t-SNARE) (Söllner *et al.*, 1993a,b). According to the SNARE hypothesis (Rothman and Warren, 1994), each transport vesicle has its specific v-SNARE that partners a cognate t-SNARE present exclusively in the intended target membrane, thereby contributing to transport specificity. Using an affinity approach with recombinant α- and γ-SNAPs together with NSF, three SNAP receptors were recently identified from bovine brain (Söllner *et al.*, 1993a). Synaptobrevin, also termed VAMP (for vesicle-associated membrane protein) (Trimble *et al.*, 1988), is anchored in the synaptic vesicle via a C-terminal transmembrane anchor and exposes most of its N-terminal sequence into the cytosol (Trimble *et al.*, 1988; Baumert *et al.*, 1989). Synaptobrevin interacts with two t-SNAREs, syntaxin and SNAP-25, a synaptosome-associated protein of 25 kDa (which is completely unrelated to α-, β-, γ-SNAPs). Whereas syntaxin contains a C-terminal transmembrane anchor domain and thus resembles synaptobrevin in its membrane topology (Bennett *et al.*, 1992), SNAP-25 is anchored in the membrane by fatty acid acylation of one or more cysteine residues present in the middle of the molecule (Hess *et al.*, 1992).

Independent evidence for the importance of synaptobrevin, SNAP-25 and syntaxin for exocytosis came from the finding that each of these membrane proteins is the molecular target of one of the eight clostridial neurotoxins, tetanus toxin (TeTx) and botulinal toxins A to G (BoNT/A to BoNT/G). These toxins block neurotransmission by selectively proteolyzing a single peptide bond in their respective substrates (Niemann *et al.*, 1994). The interaction between the SNAREs takes place in the absence

of SNAPs and NSF (Söllner *et al.*, 1993b) and involves particular regions with heptad symmetry characterized by a high probability of forming coiled-coil helices (Chapman *et al.*, 1994; Hayashi *et al.*, 1994). Indeed, such ternary complexes consisting of the cytoplasmically exposed domains of synaptobrevin, syntaxin and SNAP-25 are not dissociated by SDS-containing buffers at 65°C (Hayashi *et al.*, 1994). *In vitro*, proteolysis of SNAP-25 by BoNT/A and BoNT/E, or of synaptobrevin by BoNT/D and BoNT/F, does not interfere with the assembly of the corresponding cleavage products into ternary complexes. However, such complexes are rapidly dissociated by SDS, indicating that the SDS-resistant phenotype constitutes a biologically important intermediate in the cascade of events from vesicle docking to fusion (Hayashi *et al.*, 1994).

To gain further insights into the molecular principles underlying vesicle fusion, we have now analyzed the binding of α-SNAP to synaptobrevin, SNAP-25 and syntaxin *in vitro* using exclusively recombinant proteins. We mapped the minimal essential regions required for binding. We show that SNAP-25 and syntaxin can each bind 1 mol of α-SNAP and that binding involves predicted α-helical regions characterized previously to be essential for the direct interaction between the two t-SNAREs. In contrast, binding of synaptobrevin to α-, β- or γ-SNAP was not detetctable. Heterodimers consisting of syntaxin and SNAP-25 can bind 2 mol of α-SNAP, and are efficiently dissociated by NSF. Association of synaptobrevin with syntaxin and SNAP-25 into ternary complexes generates a third binding site for α-SNAP. The effects of specific deletions or toxin cleavage on the disassembly process were also analyzed.

Results

α-SNAP does not bind synaptobrevin but associates with syntaxin and SNAP-25

The cytoplasmic domains of syntaxin, SNAP-25 and synaptobrevin assemble spontaneously into a tight complex, which is thought to play an essential role in the fusion of synaptic vesicles with the presynaptic membrane (Söllner *et al.*, 1993a,b; Hayashi *et al.*, 1994; Pevsner *et al.*, 1994b). In the course of fusion, this complex is dissociated through the concerted action of SNAPs and NSF (Söllner *et al.*, 1993b). However, sequences that interact with SNAPs and NSF and the contribution of the individual SNAREs to binding have not been identified.

In a first set of experiments, we studied the α-SNAP binding properties of synaptobrevin. A fusion protein consisting of an N-terminal glutathione-*S*-transferase (GST) coupled to rat synaptobrevin 2 (residues 1–96 plus a C-terminal His_6-tag) was attached to glutathione (GT)–agarose beads and used in an *in vitro* binding assay with recombinant α-SNAP. No binding of α-SNAP was observed, even when the latter was applied at a high molar excess (Figure 2, open diamonds). We then pursued the opposite approach with GST–α-SNAP bound to the GT-beads and a synthetic 93mer synaptobrevin peptide as a ligand. Again, no binding was detectable (data not shown). Experiments performed with β- and γ-SNAPs were also unsuccessful, suggesting that synaptobrevin alone has no SNARE function in this assay system.

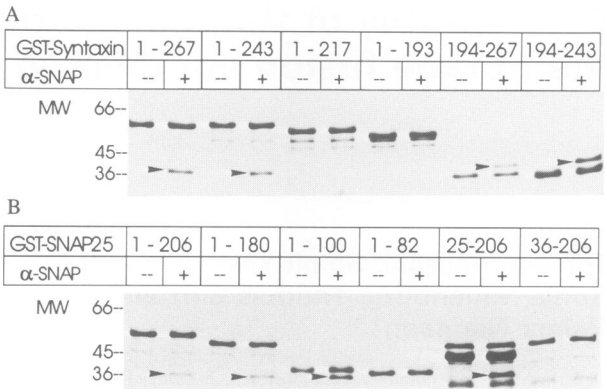

Fig. 1. Binding of α-SNAP to SNAP-25 and syntaxin, and identification of sequences involved in binding. (**A**) Binding of α-SNAP to GST–syntaxin derivatives (0.1 nmol each) immobilized on glutathione (GT)–agarose beads. GT-beads preloaded with various GST–syntaxin fusion proteins (numbers specify amino acid residues of syntaxin 1a) were incubated in the presence (+) or absence (–) of recombinant α-SNAP (0.8 nmol). Bound material was analyzed by SDS–PAGE and visualized by Coomassie Blue staining. Binding of α-SNAP involves residues 194–243 of syntaxin. (**B**) Binding of α-SNAP to GST–SNAP-25 deletion mutants. Residues 26–100 of SNAP-25 are essential for binding. Arrowheads designate bound α-SNAP.

In contrast, a GST fusion protein containing the entire cytoplasmic domain of syntaxin (residues 1–267) did bind α-SNAP (Figure 1A). Mapping of the minimal essential domains of syntaxin indicated that no binding was obtained with fragments containing the N-terminal 193 or 217 residues. Efficient binding of α-SNAP required the sequence of residues 194–243 (Figure 1A). β-SNAP had a somewhat altered binding site and required residues 194–267 for optimal binding, whereas γ-SNAP did not bind at all (data not shown). We conclude that the α-SNAP binding domain of syntaxin is identical with the domain that was previously shown to interact with SNAP-25 and synaptobrevin (Hayashi *et al.*, 1994).

Using a similar experimental approach, we then identified sequences in SNAP-25 that were essential for α-SNAP binding. Figure 1B demonstrates that the 24 N-terminal residues and the entire C-terminal portion beyond amino acid residue 101 could be deleted without destroying the α-SNAP binding function. From these data it is clear that residues 25–100 of SNAP-25, which are known to be engaged in the interaction with syntaxin (Chapman *et al.*, 1994; Hayashi *et al.*, 1994), are also responsible for α-SNAP binding. β-SNAP also bound to the same N-terminal region of SNAP-25 (data not shown).

Synaptobrevin creates an additional α-SNAP binding site

To determine whether binding of α-SNAP to syntaxin or to SNAP-25 is saturable, we incubated GST-fusion proteins with increasing amounts of the appropriate ligand (Figure 2). α-SNAP bound to GST–syntaxin (open squares) in a dose-dependent, saturable manner, yielding a GST–syntaxin to α-SNAP ratio of 1.00:0.82 at a 32-fold molar excess of α-SNAP. A similar ratio (1.00:0.89 ± 0.03) was obtained in experiments with immobilized GST–α-SNAP and soluble syntaxin (data not shown).

Binding of SNAP-25 to GST–α-SNAP (filled squares) showed a similar dose dependency to that observed for

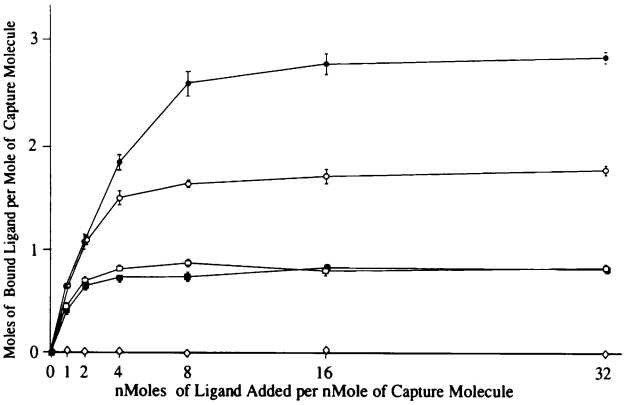

Fig. 2. Quantification of binding of α-SNAP to SNAREs and SNARE complexes. GT-beads charged with 0.10 nmol of GST–synaptobrevin (◇), GST–syntaxin (□), GST–syntaxin/SNAP-25 heterodimers (○) or GST–syntaxin/SNAP-25/synaptobrevin (1–93) heterotrimers (●) were incubated in the presence of increasing amounts of α-SNAP as indicated. (■) GST–α-SNAP immobilized on GT-beads was used as a matrix for binding of recombinant SNAP-25–His_6. The amounts of GST fusion protein and bound ligand were determined by SDS–PAGE and Coomassie staining, and densitometer scanning. Values represent mean values ± standard deviations of three independent experiments.

GST–syntaxin and α-SNAP. At saturation, a SNAP-25 to α-SNAP ratio of 1.00:0.80 was obtained. The opposite approach with immobilized GST–SNAP-25 and soluble α-SNAP yielded a ratio of 1.00:0.50 at the highest α-SNAP concentration (data not shown). We ascribe this reduced binding to steric hindrance imposed by the fusion of the glutathione-*S*-transferase sequence to the N-terminus of SNAP-25.

We demonstrated previously that the two t-SNAREs form a tight complex, suggesting that they are normally present as heterodimers in the presynaptic membrane (Hayashi *et al.*, 1994). We therefore wanted to examine the effects of this association on the subsequent interaction with α-SNAP. Figure 2 (open circles) shows that formation of this heterodimer did not impair α-SNAP binding. Binding was clearly additive, yielding 1.78 mol of α-SNAP per mole of heterodimer at high α-SNAP concentrations. Interestingly, although synaptobrevin alone did not interact with α-SNAP (open diamonds), its addition to syntaxin/SNAP-25 heterodimers further enhanced binding of α-SNAP (filled circles), yielding 2.87 mol per mole of heterotrimer.

Domains and specificity of α-SNAP binding to SNAREs

All binding studies reported here involved immobilized recombinant capture molecules and ligands added in a defined stepwise manner, and relatively high α-SNAP concentrations. These conditions do not necessarily reflect the *in vivo* situation, where the sequence of interactions and the local concentrations may differ significantly. To gain further insights into the α-SNAP/SNARE interaction, we first generated a set of α-SNAP mutants in which predicted α-helices were deleted. As shown in Figure 3A, a deletion of the 28 N-terminal residues [α-SNAP (29–295)] reduced binding to the heterotrimeric complex by ~75%. The removal of 63 N-terminal or 37 C-terminal residues from α-SNAP abrogated its binding to syntaxin, SNAP-25, syntaxin/SNAP-25 heterodimers (data not shown) or the ternary complex (Figure 3A). Interestingly, bound α-SNAP (29–295) retained the function to attract and activate NSF: the degree of NSF-catalyzed disassembly of complexes containing the mutant α-SNAP was diminished by about the same factor as was the binding of α-SNAP (T.Binz, T. Hayashi and H.Niemann, unpublished observation).

To corroborate that the observed interaction between α-SNAP and the ternary SNARE complex was indeed specific, we studied binding of α-SNAP from a buffer solution containing 10% fetal calf serum. As shown in Figure 3B, the presence of serum proteins had no effect on binding of α-SNAP. Densitometer scanning revealed that 2.60 mol of α-SNAP were bound per mole of GST–syntaxin. The exclusive presence of bovine serum albumin in the supernatant (S) fraction underscores the specificity of the protein interactions. We then tested whether α-SNAP binding was sensitive to high salt concentrations (Clary and Rothman, 1990). α-SNAP eluted quantitatively from matrices containing GST–syntaxin, GST–SNAP-25 or GST–syntaxin/SNAP-25 heterodimers when 1 M KCl was included in the washing buffer (data not shown). The same treatment caused dissociation of >90% of α-SNAP from the ternary complex without affecting the GST–syntaxin:SNAP-25:synaptobrevin ratio (Figure 3B). In addition, α-SNAP binding was barely detectable when 1 M KCl was included in the binding buffer. These findings support the idea that α-SNAP binds specifically both *in vivo* and in our *in vitro* system mainly by means of ionic interactions requiring most of the α-SNAP molecule.

Munc-18 prevents association of α-SNAP with syntaxin

As shown above, binding of α-SNAP to syntaxin and SNAP-25 involves precisely those regions that are essential for the direct interaction of the two t-SNAREs. We further showed that this interaction does not block binding sites for α-SNAP. Munc-18 (also termed n-Sec1) is thought to play a regulatory role in the assembly of SNARE complexes because its association with syntaxin 1a prevents binding of SNAP-25 and synaptobrevin (Hata *et al.*, 1993; Garcia *et al.*, 1994; Pevsner *et al.*, 1994a). Munc-18 binding involved two regions of syntaxin, encompassing residues 1–76 and residues 194–267 (Hata *et al.*, 1993; Pevsner *et al.*, 1994a). Since α-SNAP binds to the latter region, we wanted to determine whether Munc-18 influenced the association between α-SNAP and syntaxin. To this end, we preincubated GST–syntaxin with increasing concentrations of Munc-18 and subsequently with α-SNAP (Figure 4). No interaction was observed between Munc-18 and α-SNAP or with control GST beads (Figure 4 and data not shown). The syntaxin/Munc-18 heterodimer failed to bind α-SNAP. In a separate experiment, the incubation of GST–syntaxin–α-SNAP complexes with free Munc-18 led to a significant replacement of α-SNAP, providing evidence that the affinity between Munc-18 and syntaxin is higher than that between syntaxin and α-SNAP.

Disassembly of reconstituted heterotrimers and heterodimers

Our previous experiments indicated that the cytoplasmic domains of syntaxin and SNAP-25 can bind 1 mol of

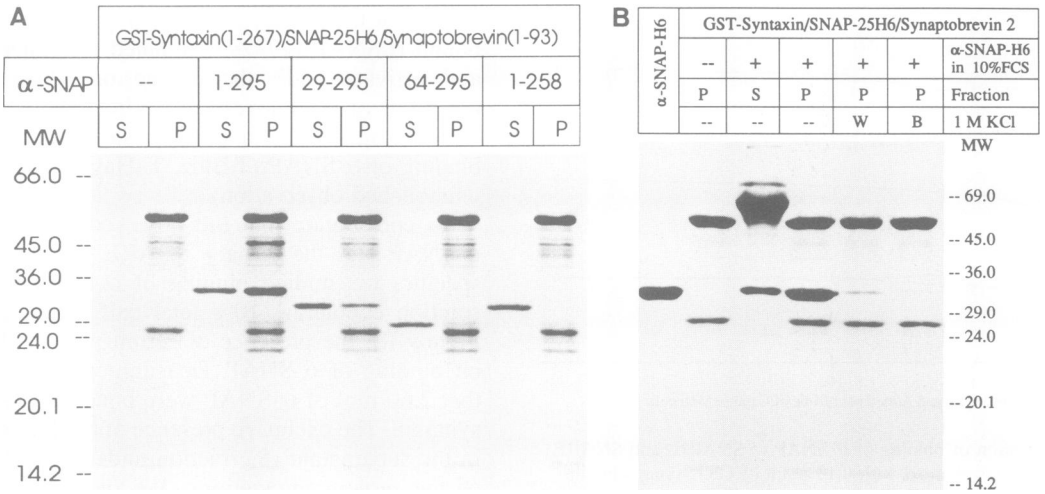

Fig. 3. Specificity of α-SNAP binding. (**A**) Determination of α-SNAP sequences involved in binding. Heterotrimeric complexes were assembled on GT-beads as described in Materials and methods. After washing, the individual mutant α-SNAP protein (1 nmol each, numbers specifiy amino acids) was added, and incubation was continued for 16 h at 4°C. Unbound (S) and bound materials (P) were analyzed by SDS–PAGE. (**B**) Effects of serum proteins and high salt on α-SNAP binding. Ternary complexes bound to agarose were incubated with α-SNAP as above in binding buffer containing 10% (v/v) fetal calf serum (FCS). Note that bovine serum albumin stays in the supernatant (S) fraction. Sensitivity of α-SNAP binding to presence of high salt was demonstrated by including 1 M KCl in the washing buffer (W) or in the binding buffer (B).

α-SNAP each, that binding is additive when the two polypeptides join into heterodimers and that addition of synaptobrevin causes binding of a third mole of α-SNAP. We next wanted to see whether this heterotrimer, precharged with α-SNAP, could serve as a substrate for recombinant NSF. To measure disassembly, we used an experimental approach in which >90% of radiolabeled synaptobrevin was incorporated into two SDS-resistant ternary complexes of 114 and 230 kDa (Hayashi *et al.*, 1994). Figure 5 shows that both complexes could be disrupted by NSF and that this process strictly depended upon the presence of Mg^{2+}, ATP and α-SNAP. β-SNAP displayed a similar activity to that of α-SNAP, whereas γ-SNAP alone was inactive. At lower α-SNAP concentrations (1 μg per assay) the efficiency of disassembly was nearly doubled by the addition of γ-SNAP (1 μg) (data not shown), thus supporting the reported synergistic effect of γ-SNAP (Whiteheart *et al.*, 1993). We may thus conclude (i) that the dissociation of the reconstituted heterotrimer follows precisely those criteria that were previously established for SNARE complexes derived *in vivo* (Söllner *et al.*, 1993b) and (ii) that dissociation of the SNARE complex does not require the presence of transmembrane anchor domains of the individual SNAREs.

Since syntaxin/SNAP-25 heterodimers were also capable of binding α-SNAP, we then determined whether they were also dissociated by NSF. We therefore established the α-SNAP and NSF concentrations required for optimal disassembly. To measure disassembly of syntaxin/SNAP-25 heterodimers, we used GST–syntaxin matrices preloaded with radiolabeled SNAP-25 prior to addition of various amounts of recombinant α-SNAP and NSF. For disruption of the ternary SNARE complex, we used radiolabeled synaptobrevin as a marker. After the incubation step, residual radioactivity was determined in the beads' fractions to calculate the degree of disassembly. These experiments and the results presented in Figure 6 allow three conclusions to be drawn. First, the syntaxin/

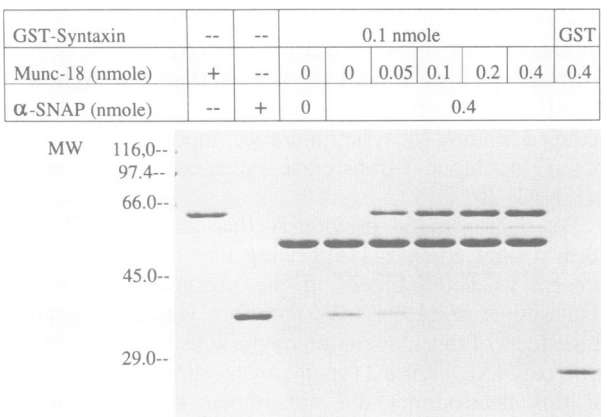

Fig. 4. Munc-18 prevents binding of α-SNAP to GST–syntaxin. GT-beads preloaded with 0.1 nmol of GST–syntaxin (1–267) were first incubated for 1 h at 4°C with various concentrations of recombinant Munc-18 and then for 1.5 h in the absence or presence of α-SNAP (0.4 nmol). Bound material was analyzed together with control proteins by SDS–PAGE using 12.5% gels. Gels were stained with Coomassie Blue.

SNAP-25 heterodimer is indeed dissociated. As observed with the heterotrimer, dissociation strictly depended upon the presence of ATP and Mg^{2+} and disassembly was enhanced by γ-SNAP (data not shown). Second, optimal dissociation of both the heterodimer and the heterotrimer (each containing 50 pmol of GST–syntaxin) was achieved with 6 μg (25 pmol) of homotrimeric NSF (Figure 6A) and 8 μg (230 pmol) of α-SNAP (Figure 6B). Third, at low α-SNAP and NSF concentrations (as presumably found in the *in vivo* situation), dissociations of the heterodimer and the heterotrimer were about equally efficient (Figure 6B). This could imply that syntaxin/SNAP-25 heterodimers are relevant targets for α-SNAP and NSF unless they are protected by other cellular proteins.

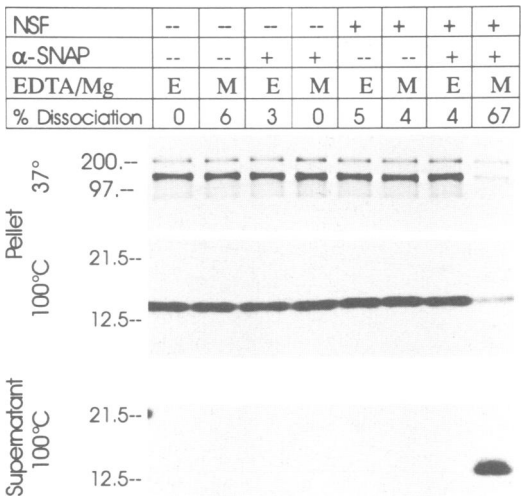

Fig. 5. Disassembly of the SDS-resistant SNARE complex by α-SNAP and NSF. Ternary complexes consisting of GST–syntaxin, SNAP-25–His_6 and radiolabeled synaptobrevin 2 (residues 2–96) were assembled on GT-beads. About 95% of the radiolabel was incorporated into the SDS-resistant complex under these conditions. Washed beads were resuspended in dissociation buffer supplemented with 2 mM EDTA, or 2 mM $MgCl_2$, α-SNAP (32 μg = 0.9 nmol) and NSF (12 μg = 0.3 nmol) as indicated. After incubation for 1 h at 4°C, bound and released materials were separated and incubated in sample buffer at the temperatures indicated prior to SDS–PAGE and analysis by autoradiography.

A role for the N-terminal domain of syntaxin in the disassembly reaction

The next step was to identify domains within syntaxin, SNAP-25 and synaptobrevin that played a discrete role in the α-SNAP/NSF-catalyzed disassembly reaction. We therefore first determined whether α-SNAP could also bind to SNAP-25 and syntaxin when NSF and ATP were included in the binding assay. Whereas the presence of NSF had no effect on α-SNAP binding to SNAP-25 (Figure 7, right panel), α-SNAP was not detected in samples containing full-length syntaxin (residues 1–267) (Figure 7, left panel). In a separate experiment, a quantitative dissociation of α-SNAP from precharged full-length GST–syntaxin was observed, when NSF and ATP were added after binding of α-SNAP. No such dissociation was observed in the presence of ATPγS indicating that the dissociation of α-SNAP depended upon NSF-specific hydrolysis of ATP (data not shown). In contrast, NSF was not able to dissociate α-SNAP from the N-terminally truncated syntaxin mutant (residues 194–267) (Figure 7). In this instance, α-SNAP and NSF remained affixed to the matrix.

To further our understanding of the role of this N-terminal domain of syntaxin, we compared the efficiency of disassembly of heterotrimers containing SNAP-25, radiolabeled synaptobrevin and either full-length syntaxin or its N-terminally truncated variant (Figure 8B). Determination of the α-SNAP content of ternary complexes containing truncated syntaxin revealed no significant differences from complexes containing full-length syntaxin (data not shown). Furthermore, both types of complex were resistant to SDS at 37°C. There was, however, a marked discrepancy with respect to their susceptibility to NSF: whereas a complex pre-assembled around a full-length GST–syntaxin molecule was efficiently dissociated by α-SNAP and NSF, disruption was not observed with complexes in which the syntaxin lacked the N-terminal 193 residues (Figure 8B). A similar inhibition of disassembly was found using ternary complexes that were immobilized on agarose beads via GST–synaptobrevin or GST–SNAP-25 and contained the truncated syntaxin derivative in addition to full-length SNAP-25 or synaptobrevin (data not shown). From these results, we conclude that the N-terminal domain of syntaxin is required neither for the formation of ternary SDS-resistant complexes nor for binding of α-SNAP, and that dissociation of the SNARE complex by α-SNAP/NSF relies upon the presence of an intact N-terminal domain of syntaxin.

Using a similar experimental approach, we studied whether C-terminal deletions of SNAP-25 affected the disassembly reaction. Such deletions do not prevent the assembly of ternary complexes which, however, are dissociated in SDS-containing buffers (Hayashi *et al.*, 1994). As shown in Figure 8A, C-terminal deletions generated by BoNT/A [SNAP-25 (1–197)] or BoNT/E [SNAP-25 (1–180)] either had no effect or even enhanced the disruption process of the complex. The same observation was made with a SNAP-25 deletion mutant that lacked the entire C-terminal portion, including the central cysteine cluster. These data support our finding above that the N-terminal portion of SNAP-25 is sufficient for binding of α-SNAP and for NSF-catalyzed disassembly. Furthermore, they strengthen our hypothesis that the C-terminal domain of SNAP-25 interacts with synaptobrevin for the acquisition of the SDS-resistant phenotype of the complex.

Distinct properties of the N- and the C-terminal portions of synaptobrevin in the disassembly reaction

In a final set of experiments, we wanted to study the effects of cleavage products derived from synaptobrevin on the disassembly reaction. We previously showed that the N-terminal fragment released by TeTx (cleaving the Gln76–Phe77 bond) continued to bind to GST–syntaxin/SNAP-25 heterodimers. BoNT/F (cleaving the Gln58–Lys59 bond) produces two fragments that can be distinguished by gel electrophoresis, whereby the N-terminal fragment has a reduced mobility (labeled with an arrow head in Figure 9). Both fragments bind tightly to syntaxin/SNAP-25 heterodimers but fail to induce SDS-resistance (Hayashi *et al.*, 1994). IgA protease from *Neisseria gonorrheae* (that cleaves the peptide bonds Pro10–Ala11 and Pro20–Ala21) is a potent inhibitor of exocytosis from bovine chromaffin cells (Binschek *et al.*, 1995). Figure 9 shows that all reconstituted complexes, with the exception of those containing the BoNT/F-specific N-terminal fragment, were efficiently dissociated by α-SNAP/NSF. Under our experimental conditions, the radiolabeled fragments were applied at a much lower concentration than were GST–syntaxin/SNAP-25 heterodimers (calculated ratio of ~1:1000). It is, therefore, highly unlikely that the two fragments generated by BoNT/F were trapped together into the same complex. About 50% of the N-terminal fragment remained in the bound fraction, indicating that complexes containing this fragment largely resisted treatment with α-SNAP and NSF.

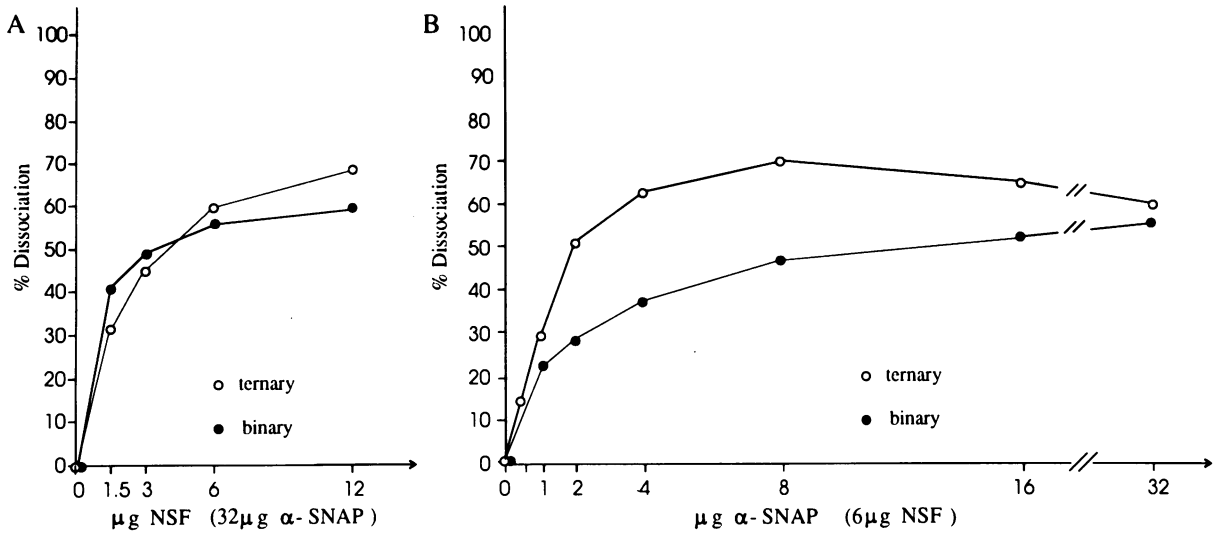

Fig. 6. Determination of NSF- and α-SNAP-concentrations required for optimal disassembly. Complexes containing 0.05 nmol GST–syntaxin and radiolabeled SNAP-25 (binary), or SNAP-25–His_6 and radiolabeled synaptobrevin 2 (ternary) were assembled on GT-beads as detailed in Materials and methods. (**A**) NSF dose dependency of disassembly. Beads were incubated for 1 h in the presence of 32 μg α-SNAP and increasing amounts of NSF as indicated. (**B**) α-SNAP dose dependency. Reaction mixtures contained increasing amounts of α-SNAP and 6 μg of NSF. Control samples (0% dissociation) were incubated in the absence of α-SNAP and NSF. Radiolabeled material in the pellet fractions was quantified by liquid scintillation counting to calculate the percentage dissociation. Values represent mean values of two independent experiments.

Discussion

SNAP-25, syntaxin and synaptobrevin form the core of a protein complex that ensures docking and fusion of synaptic vesicles with the presynaptic membrane. Isoform subsets of these three proteins participate in the intercompartmental traffic in all eukaryotic cells (Ferro-Novick and Jahn, 1994; Rothman and Warren, 1994). According to the SNARE hypothesis, synaptobrevin, SNAP-25 and syntaxin act as vesicular or target membrane receptors for the cytoplasmic proteins α-SNAP and the homotrimeric ATPase, NSF, which induce disassembly of the complex and membrane fusion (Rothman and Warren, 1994). However, the α-SNAP binding properties and the interactive domains of the individual proteins that participate in this latter mechanism have not been resolved. Here, we have applied various recombinant peptides representing the cytoplasmic domains of synaptobrevin, SNAP-25 and syntaxin to study their ability to bind α-SNAP and to allow subsequent NSF-mediated dissociation of reconstituted binary or ternary complexes. Six major observations were made (Figure 10). (i) We show that SNAP-25 and syntaxin bind ~1 mol of α-SNAP each, whereas a binding of α-SNAP to synaptobrevin is not detectable. We mapped the minimal essential domains for this binding to those regions of syntaxin and SNAP-25 that are involved in their direct interaction, i.e. domains that have a high probability to form coiled-coils. (ii) The association of Munc-18 (n-Sec1) with syntaxin prevents binding of α-SNAP to the latter. (iii) We report that almost the entire α-SNAP molecule is required for binding. A derivative lacking the first 28 amino acid residues demonstrates reduced binding, but unaltered complex-dissociating activity. (iv) We show that α-SNAP binding is additive when SNAP-25 and syntaxin associate into heterodimers, and that association of synaptobrevin induces a third binding site for α-SNAP. (v) We show that the syntaxin/SNAP-25 heterodimer is

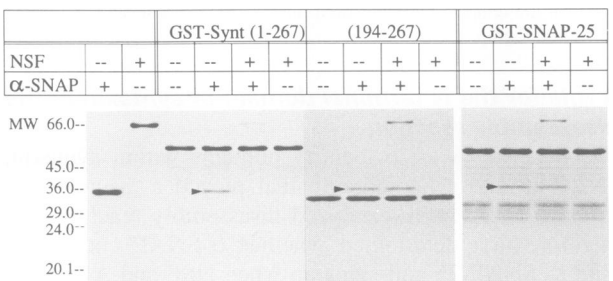

Fig. 7. NSF prevents binding of α-SNAP to full-length syntaxin. GT-beads preloaded with various GST–syntaxin fusion proteins (numbers specify amino acid residues of syntaxin) or with GST–SNAP-25 were incubated with α-SNAP (32 μg per assay) and/or NSF (6 μg), as indicated. Binding of α-SNAP occurs in the absence of NSF to all three capture molecules, whereas it is specifically inhibited with full-length syntaxin when NSF is included.

as susceptible to α-SNAP/NSF-catalyzed dissociation as the heterotrimer. (vi) We demonstrate that disassembly of complexes does not require the presence of the transmembrane anchor regions of the SNARE proteins. Dissociation is impaired, however, when the N-terminus of syntaxin or the C-terminal portion of synaptobrevin are deleted. In contrast, C-terminal deletions of SNAP-25 enhance disassembly of the ternary complex.

Several lines of evidence suggest that the reconstituted system does in fact behave in the manner to be expected from reports involving material derived *in vivo*. First, binding of α-SNAP to all combinations of SNAREs was reversed by treatment with 1 M KCl, and occurred in the presence of large amounts of unrelated proteins. Second, α-SNAP and β-SNAP were reported to share the same binding site on salt-extracted Golgi membranes (Wilson *et al.*, 1992). Here, we report that α-SNAP and β-SNAP bind to the same subdomains of syntaxin and SNAP-25. Third, the disassembly was in all instances strictly

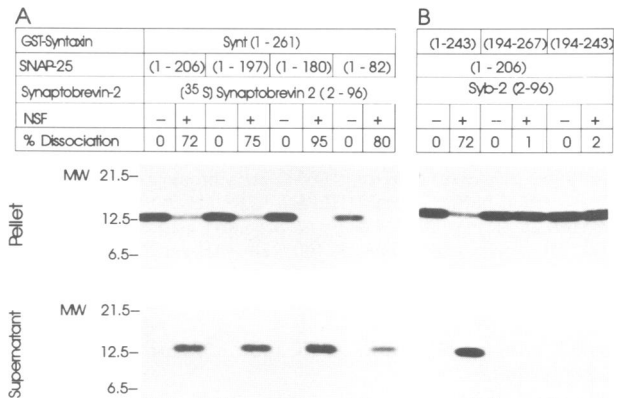

Fig. 8. Identification of sequences of SNAP-25 and syntaxin that enhance or inhibit α-SNAP/NSF-specific disruption of ternary complexes. (**A**) Ternary complexes consisting of GST–syntaxin, radiolabeled synaptobrevin and various recombinant SNAP-25 peptides as indicated were incubated in dissociation buffer containing 8 μg of α-SNAP and no (–) or 6 μg of NSF. C-terminal deletions enhance the disassembly process. (**B**) Similar approach to that in (A), however, GST–syntaxin proteins of various length and full-sized SNAP-25 were used. A deletion of residues 1–193 of syntaxin renders ternary complexes that resist treatment with α-SNAP and NSF. The degree of dissociation was determined as above.

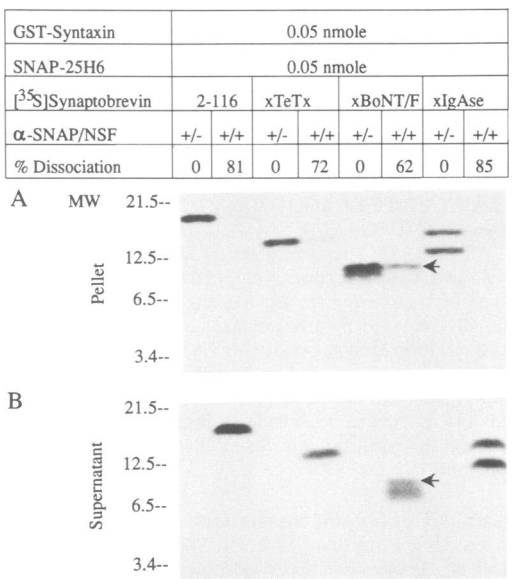

Fig. 9. Identification of disassembly-inhibiting sequences in synaptobrevin. Ternary complexes containing GST–syntaxin, SNAP-25–His_6 and various radiolabeled fragments from synaptobrevin were incubated in dissociation buffer containing α-SNAP (8 μg) with or without NSF (6 μg). BoNT/F generates two synaptobrevin fragments, both of which bind to GST–syntaxin/SNAP-25 heterodimers but fail to bind to monomeric GST–syntaxin (Hayashi *et al.*, 1994). The arrowheads specify the N-terminal fragment generated by BoNT/F. Note that the N-terminal synaptobrevin fragment generates complexes that are largely resistant to α-SNAP/NSF-specific dissociation. In contrast, cleavage of synaptobrevin at other sites (see text for details) has no effect on the dissociation. Note that for technical reasons we were unable to scan the degree of dissociation for the individual fragments, and thus the percentage of dissociation refers to both the N- and the C-terminal fractions, the latter being dissociated quantitatively and enriched in the supernatant fraction.

dependent upon Mg^{2+}, ATP and α-SNAP as reported for complexes derived from brain homogenate (Söllner *et al.*, 1993b). Fourth, γ-SNAP was reported to have low activity

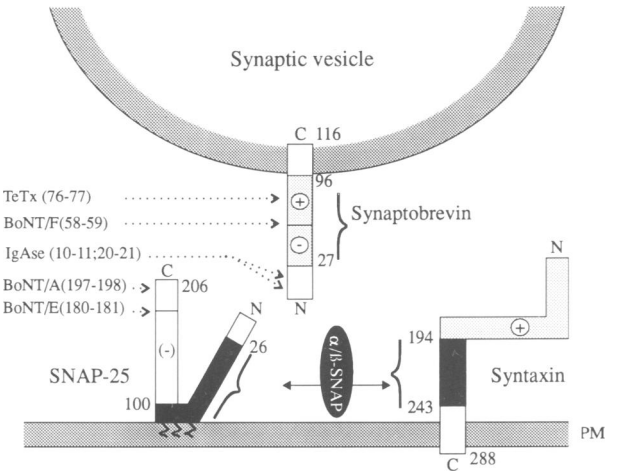

Fig. 10. Schematic drawing of synaptobrevin, SNAP-25 and syntaxin. Identification of domains that are required for binding of α-SNAP (black) or that modulate the SNAP/NSF-catalyzed disassembly reaction (shaded). The entire conserved core portion of synaptobrevin is required for formation of the SDS-resistant ternary complex and for simultaneous generation of a third α-SNAP binding site. Domains required for efficient disassembly (+) or having an inhibitory effect (–) on disassembly are marked. The effect of the C-terminal domain of SNAP-25 (residues 101–180) is marginal. Cleavage sites of IgA protease and toxins used in this study are indicated.

in the absence of α-SNAP, but to act synergistically at low α-SNAP concentrations (Whiteheart *et al.*, 1993). The same observations were made with the reconstituted system. Fifth, it was reported that Munc-18 (n-Sec1) binding to syntaxin prevents association of the latter with SNAP-25 (Pevsner *et al.*, 1994b). We show here that the Munc-18–syntaxin complex does not bind α-SNAP.

What are the structural features that are recognized by α-SNAP? We have mapped its binding to regions of syntaxin and SNAP-25, previously proposed to interact with each other via coiled-coil formation. Binding of α-SNAP increases when the two t-SNAREs are assembled into heterodimers, and synaptobrevin, although it does not appear to bind α-SNAP alone, creates an additional binding site when incorporated into the heterotrimer. It is intriguing to speculate that binding of synaptobrevin is paralleled by a structural rearrangement that becomes evident by the establishment of SDS-resistance, and may involve the formation of a third coiled-coil. Taking into account that α-SNAP binding is salt sensitive, these data suggest that α-SNAP specifically recognizes an as-yet undefined pattern of polar residues exposed on the surface of the coiled coils.

What is the biological significance of the observed α-SNAP/NSF-specific dissociation of syntaxin/SNAP-25 heterodimers? After release of Munc-18 (n-Sec1), syntaxin can bind SNAP-25 with high affinity (Pevsner *et al.*, 1994b), suggesting that the two t-SNAREs exist in the presynaptic membrane as heterodimers, thus providing high-affinity binding sites for synaptobrevin (Hayashi *et al.*, 1994; Pevsner *et al.*, 1994b). The finding that syntaxin/SNAP-25 heterodimers are dissociated at low α-SNAP and NSF concentrations as efficiently as the heterotrimer suggests a second regulatory mechanism in addition to the blockade of syntaxin by Munc-18, resulting in the removal of potential vesicle attachment and fusion

sites from areas outside the active zones. At the release sites, decoration of syntaxin/SNAP-25 dimers with synaptotagmin or other members of the Ca^{2+}-sensing machinery may prevent dissociation of the heterodimer in the absence of the Ca^{2+} signal.

The reconstituted system further allowed us to identify subdomains in syntaxin and synaptobrevin that assisted or inhibited the disassembly reaction. The interpretation of these data relies, of course, on the assumption that the individual deletions did not cause long-range conformational changes to the rest of the molecules. The N-terminal domain of syntaxin (residues 1–193) was not essential for binding of synaptobrevin and SNAP-25, for generation of an SDS-resistant complex, for binding of α-SNAP or, finally, for binding of NSF. All these assembly steps were mediated by the C-terminal portion (residues 194–267) of syntaxin. NSF-catalyzed disassembly of the various complexes, however, clearly required the presence of the N-terminal portion of syntaxin. According to the algorithm of Lupas *et al.* (1991), this domain contains two helices with coiled-coil forming potential (residues 30–64 and 68–112). We suggest that these helices are essential to partner coiled-coils of the assembled complex in order to facilitate its disruption. This would require an interaction between the N- and the C-terminal domains of syntaxin, as has indeed been proposed previously (Calakos *et al.*, 1994).

The second sequence acting in a disassembly-inhibiting manner was identified within the BoNT/F-specific N-terminal cleavage product of synaptobrevin (residues 1–58). The region between Arg30 and Arg56 is likely to fold into a helix with relatively low coiled-coil propensity (Chapman *et al.*, 1994; Hayashi *et al.*, 1994). Binding of this fragment to syntaxin/SNAP-25 heterodimers failed to generate SDS-resistant ternary complexes (Hayashi *et al.*, 1994), and this binding did not enhance the α-SNAP binding capacity of the complex. We show here that, in addition, such complexes display an enhanced resistance against α-SNAP and NSF. It remains to be shown whether full-length synaptobrevin can also bind in a similar manner, perhaps *in vivo* at a stage when the vesicle is docked to the presynaptic membrane. This would then imply a two-step mechanism for the binding of synaptobrevin: in a first step, only the N-terminal helix of synaptobrevin makes contact with the syntaxin/SNAP-25 heterodimer, thus generating SDS-sensitive complexes that are not easily disassembled through bound α-SNAP and NSF. In a second step, the C-terminal domain of synaptobrevin could join into the complex in a process that is also likely to involve the C-terminal region of SNAP-25 (Hayashi *et al.*, 1994). This rearrangement should then lead to SDS-resistance by the formation of another coiled-coil, as evidenced by the enhanced α-SNAP binding capacity. As a consequence, disassembly of the structurally altered ternary complex could be facilitated. In its active form, NSF is a homotrimer in which each subunit has to be functional as an ATPase for NSF to be active in fusion reactions (Whiteheart *et al.*, 1994). It remains to be shown whether each of these subunits acts independently on one of the putative coiled-coils, requiring 1 mol each of bound α-SNAP for efficient disassembly of the vesicle fusion complex.

Materials and methods

Buffers

PBS: 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4. Binding buffer: 20 mM HEPES–KOH pH 7.0, 100 mM KCl, 2 mM EDTA, 1 mM DTT, 0.5% Triton X-100. Sample buffer: 60 mM Tris–HCl pH 6.75, 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 10% (w/v) glycerol, 0.007% (w/v) bromophenol blue.

Dissociation buffer: 20 mM HEPES–KOH pH 7.0, 100 mM KCl, 1 mM DTT, 1 mM ATP, 1% (w/v) polyethyleneglycol 4000, 1.6% (w/v) glycerol, 0.5% Triton X-100 (all substances from Merck, Darmstadt, Germany), 0.1% bovine serum albumin (BSA; Boehringer Mannheim, Germany).

Plasmid constructions and purification of recombinant fusion proteins

GST–synaptobrevin 2-His_6, subcloned into pGEX-2T (Pharmacia Biotech GmbH, Freiburg, Germany) and GST–syntaxin Ia (residues 1–261 or 194–267) subcloned in pGEX-KG (Guan and Dixon, 1991) were described previously (Hayashi *et al.*, 1994). GST–syntaxin Ia (1–243), (1–217), (1–193) and (194–243) were constructed by PCR using the upstream primers 5′-CTCTGAATTCTAATGAAGGACCGAACCC-AGG-3′ (1–243), (1–217), (1–193) and 5′-CTCTGAATTCTAATGGAG-ATCGAGACCAGGCACAG-3′ (194–243), and the downstream primers 5′-CTCTAAGCTTCTAGTAGTCCACAGCGTGTTC-CAC-3′ (1–243; 194–243), 5′-CTCTAAGCTTCTACATGAACATATCGTGTAGCTC-3′ (1–217) and 5′-CTCTAAGCTTCTAACTGAGGGCCTGCTTCGAG-3′ (1–193), respectively.

GST–SNAP-25 mutants were produced by subcloning individual coding regions from the corresponding *in vitro* transcription clones (Hayashi *et al.*, 1994) into pGEX-2T. For GST–SNAP-25 (1–100) the primers 5′-CTCTGGATCCATGGCCGAAGACGCG-3′ and 5′-CTCTG-AATTCTCAAGCATCACTTGATTTAAGCTTG-3′ were used. GST–α-SNAP deletion mutants were generated by PCR using the following upstream primers: 5′-CTCTGGATCCATGGACAACTCCGGGAA-GGA-3′ (1–295 and 1–258); 5′-CTCTGGATCCATGTCGGGCCTCTT-CGGAG-GCTC-3′ (29–295); 5′-CTCTGGATCCATGGCTTTCTGCC-AGGCGGCCCA-3′ (64–295). As downstream primers we used 5′-CTCTGAATTCTTAGCGCAGGTCTTCCTCGT-3′ (1–295; 29–295; 64–295) and 5′-CTCTGAATTCTTAGCTGTCCACGTTCTGCTCCT-3′ (1–258). All PCR products were digested with *Bam*HI and *Eco*RI and ligated with pGEX-2T (Pharmacia) digested accordingly. GST–β-SNAP was created by transferring the *Bam*HI–*Sac*I fragment from pQE30-β-SNAP (kindly provided by J.E.Rothman) to pGEX-KG. Recombinant proteins were either affinity-purified as GST fusion proteins on glutathione (GT)–agarose or released by thrombin cleavage after binding to GT–agarose (Guan and Dixon, 1991). His_6–α-SNAP, His_6–γ-SNAP and His_6–NSF were isolated as described (Block and Rothman, 1992; Söllner *et al.*, 1993a). The purification of SNAP-25–His_6 was described earlier (Binz *et al.*, 1994).

In vitro transcription and translation

Plasmids encoding synaptobrevin 2 and SNAP-25 (Binz *et al.*, 1994; Hayashi *et al.*, 1994) were linearized downstream from their coding regions. mRNA was synthesized using SP6 RNA polymerase (Boehringer Mannheim, Germany) and translated in rabbit reticulocyte lysate (Promega, Madison, USA) in the presence of [^{35}S]methionine (Mayer *et al.*, 1988).

Toxin treatment

GST–SNAP-25–His_6 and *in vitro* translated SNAP-25 were digested with BoNT/A or BoNT/E as detailed in Hayashi *et al.* (1994) and Binz *et al.* (1994). Radiolabeled synaptobrevin 2 was cleaved with TeTx, BoNT/F and IgA protease, respectively, as described in Hayashi *et al.* (1994).

In vitro protein binding studies

In a typical binding assay, 0.1 nmol of the GST fusion protein was coupled to 10 μl slurry of GT–agarose beads (Pharmacia) suspended in 200 μl of PBS supplemented with 2 mM EDTA and 0.1% (v/v) Nonidet P-40 (Boehringer Mannheim, Germany). After 2 h at 4°C, > 98% of the GST fusion protein was trapped on the beads. The beads were washed five times with binding buffer and resuspended in 200 μl binding buffer containing the appropriate amount of ligand protein and rotated (head over head) for 16 h at 4°C. Beads in the pellet fraction were washed four times with 400 μl of binding buffer and bound proteins

were eluted by incubation for 30 min at 37°C in 30 µl sample buffer (Hayashi *et al.*, 1994).

For the preparation of GST–syntaxin/SNAP-25 heterodimers or GST–syntaxin/SNAP-25/synaptobrevin heterotrimers, 0.1 nmol GST–syntaxin was coupled to GT–agarose beads as described above and the washed beads were then incubated with 0.8 nmol SNAP-25–His_6 or the appropriate amount of the SNAP-25 deletion mutant or toxin cleavage product. For ternary complexes, 0.8 nmol of synaptobrevin (1–93) or the same amount of proteolytic cleavage products was included. After incubation for 16 h at 4°C, wild-type or mutant α-SNAP was added at the concentrations specified in the text.

In vitro dissociation studies

For dissociation studies, binary or ternary SNARE complexes were assembled as above, except that 50 pmol of GST fusion protein and radiolabeled SNAP-25 or synaptobrevin, generated by *in vitro* transcription/translation, were used (Hayashi *et al.*, 1984).

Agarose beads bearing binary or ternary complexes were incubated in the presence or absence of His_6–SNAPs and His_6–NSF in 600 µl of dissociation buffer supplemented with 2 mM $MgCl_2$ or 2 mM EDTA as indicated. α-SNAP and/or NSF were added as indicated and samples were incubated by gentle mixing for 1 h at 4°C. Beads were collected by centrifugation. Unbound material in the supernatant was recovered by precipitation with trichloroacetic acid and resuspended in 60 µl of sample buffer (S fraction). Beads were washed five times with 400 µl dissociation buffer in the presence of 2 mM $MgCl_2$ or 2 mM EDTA omitting polyethyleneglycol, glycerol and BSA. Bound proteins were eluted by incubation for 30 min at 37°C in 60 µl sample buffer (P fraction). For quantification, 10% of the pellet and supernatant fractions were subjected to liquid scintillation counting. Equal amounts of the P and S fractions were either incubated at 37°C for 30 min or boiled for 3 min prior to SDS–PAGE.

SDS–PAGE

SDS–PAGE was carried out as described by Laemmli (1970) using 12.5 or 15% gels. Gels were stained with Coomassie Blue and bands were quantified with a Sharp JX-325 high resolution scanner supplied with an ImageMaster TM 1-D program version 1.10 (Pharmacia).

Acknowledgements

We thank Drs Sidney Whiteheart, Thomas Söllner and James Rothman (Sloan-Kettering Institute, New York) for plasmids encoding α-/β-/γ-SNAPs and NSF, Dr Richard Scheller (Stanford University, Stanford) for clones encoding syntaxin isoforms. We thank Dr Thomas Südhof (Dallas) for clones encoding SNAP-25 and syntaxin. We also thank Agathe Authaler and Yumiko Hayashi for help with purification of recombinant proteins. This work was supported by the Deutsche Forschungsgemeinschaft. T.H. and S.Y. were supported by fellowships from the Alexander-von-Humboldt Foundation.

References

Baumert,M., Maycox,P.R., Navone,F., DeCamilli,P. and Jahn,R. (1989) *EMBO J.*, **8**, 379–384.

Beckers,C.J.M., Block,M.R., Glick,B.S., Rothman,J.E. and Balch,W.E. (1989) *Nature*, **339**, 397–398.

Bennett,M.K., Calakos,N. and Scheller, R.H. (1992) *Science*, **257**, 255–259.

Binschek,T., Bartels,F., Bergel,H., Bigalke, H. Yamasaki,S., Hayashi,T., Niemann,H., and Pohlner,J. (1995) *J. Biol. Chem.*, **270**, 1730–1734.

Binz,T., Blasi,J., Yamasaki,S., Baumeister,A., Link,E., Südhof,T.C., Jahn,R. and Niemann,H. (1994) *J. Biol. Chem.*, **269**, 1617–1620.

Block,M.R. and Rothman,J.E. (1992) *Methods Enzymol.*, **219**, 300–309.

Block,M.R., Glick,B.S., Wilcox,C.A., Wieland,F.T. and Rothman,J.E. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 7852–7856.

Calakos,N., Bennett,M.K., Peterson,K.E. and Scheller,R.H. (1994) *Science*, **263**, 1146–1149.

Chapman,E.R., An,S., Barton,N. and Jahn,R. (1994) *J. Biol. Chem.*, **269**, 27427–27432.

Clary,D.O. and Rothman,J.E. (1990) *J. Biol. Chem.*, **265**, 10109–10117.

Clary,D.O., Griff,I.C. and Rothman,J.E. (1990) *Cell*, **61**, 709–721.

Diaz,R., Mayorga,L.S, Weidman,P.J., Rothman,J.E. and Stahl,P.S. (1989) *Nature*, **339**, 398–400.

Ferro-Novick,S. and Jahn,R. (1994) *Nature*, **370**, 191–193.

Garcia,E.P., Gatti,E., Butler,M., Burton,J. and DeCamilli,P. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 2003–2007.

Glick,B.S. and Rothman,J.E. (1987) *Nature*, **326**, 309–312.

Graham,T.R. and Emr,S.D. (1991) *J. Cell Biol.*, **114**, 207–218.

Guan,K.L. and Dixon,J.E. (1991) *Anal. Biochem.*, **192**, 262–267.

Hata,Y., Slaughter,C.A. and Südhof, T.C. (1993) *Nature*, **366**, 347–351.

Hayashi,T., McMahon,H., Yamasaki,S., Binz,T., Hata,Y., Südhof,T.C. and Niemann,H. (1994) *EMBO J.*, **13**, 5051–5061.

Hess,D.T., Slater,T.M., Wilson,M.C. and Skene,J.H.P. (1992) *J. Neurosci.*, **12**, 4634–4641.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Lupas,A., VanDyke,M. and Stock,J. (1991) *Science*, **252**, 1162–1164.

Malhotra,V., Orci,L., Glick,S., Block,M.R. and Rothman,J.E. (1988) *Cell*, **54**, 221–227.

Mayer,T., Tamura,T., Falk,M. and Niemann,H. (1988) *J. Biol. Chem.*, **263**, 14956–14963.

Niemann,H., Blasi,J. and Jahn,R. (1994) *Trends Cell Biol.*, **4**, 179–185.

Pevsner,J., Hsu,S.-C. and Scheller,R.H. (1994a) *Proc. Natl Acad. Sci. USA*, **91**, 1445–1449.

Pevsner,J., Hsu,S.-C., Braun,J.E.A., Calakos,N., Ting,A.E., Bennett,M.K. and Scheller,R.H. (1994b) *Neuron*, **13**, 353–361.

Rothman,J.E. and Warren,G. (1994) *Curr. Biol.*, **4**, 220–233.

Söllner,T., Whiteheart,S.W., Brunner,M., Erdjument-Bromage,H., Geromanos,S., Tempst,P. and Rothman,J.E. (1993a) *Nature*, **362**, 318–324.

Söllner,T., Bennett,M.K., Whiteheart,S.W., Scheller,R.H. and Rothman, J.E. (1993b) *Cell*, **75**, 409–418.

Trimble,W.S., Cowan,D.M. and Scheller,R.H. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 4538–4542.

Whiteheart,S.W., Griff, I.C., Brunner, M., Clary,D.O., Mayer,T., Buhrow,S.A. and Rothman,J.E. (1993) *Nature*, **362**, 353–355.

Whiteheart,S.W., Rossnagel,K., Buhrow,S.A., Brunner,M., Jaenicke,R. and Rothman,J.E. (1994) *J. Cell Biol.*, **126**, 945–954.

Wilson,D.W., Whiteheart, S.W., Wiedmann,M., Brunner,M. and Rothman,J.E. (1992) *J. Cell Biol.*, **117**, 531–538.

Received on January 5, 1995; revised on February 22, 1995

Note added in proof

While this work was in the reviewing stage, H.T.McMahon and T.C.Südhof (1995) *J. Biol. Chem.*, **270**, 2213–2217, also reported that synaptobrevin fails to bind α-SNAP and that binding of the latter to syntaxin involves the C-terminal portion of syntaxin.