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Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly

Tetsuya Hayashi, Harvey McMahon[1], Shinji Yamasaki, Thomas Binz, Yutaka Hata[1], Thomas C.Südhof[1] and Heiner Niemann[2]

Department of Microbiology, Federal Research Center for Virus Diseases, Paul-Ehrlich-Strasse 28, D-72076 Tübingen, Germany and [1]Department of Molecular Genetics and Howard Hughes Medical Institute, The University of Texas Southwestern Medical Center, Dallas, TX 75235, USA
[2]Corresponding author

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Clostridial neurotoxins inhibit neurotransmitter release by selective and specific intracellular proteolysis of synaptobrevin/VAMP, synaptosomal-associated protein of 25 kDa (SNAP-25) or syntaxin. Here we show that in binary reactions synaptobrevin binds weakly to both SNAP-25 and syntaxin, and SNAP-25 binds to syntaxin. In the presence of all three components, a dramatic increase in the interaction strengths occurs and a stable sodium dodecyl sulfate-resistant complex forms. Mapping of the interacting sequences reveals that complex formation correlates with the presence of predicted α-helical structures, suggesting that membrane fusion involves intermolecular interactions via coiled-coil structures. Most toxins only attack the free, and not the complexed, proteins, and proteolysis of the proteins by different clostridial neurotoxins has distinct inhibitory effects on the formation of synaptobrevin–syntaxin–SNAP-25 complexes. Our data suggest that synaptobrevin, syntaxin and SNAP-25 associate into a unique stable complex that functions in synaptic vesicle exocytosis.

Key words: membrane fusion complex/neurotransmitter release/SNAP-25/synaptobrevin/syntaxin

Introduction

In eukaryotic cells, transport between intracellular organelles is mediated by vesicles that bud from one compartment and fuse with another (Palade, 1983; Mellman and Simons, 1992; Rothman and Orci, 1992). In the last 2 years, data from different research areas have revealed that the mechanisms underlying the fusion of carrier vesicles with target membranes are conserved from yeast to mammals; the mechanisms even include highly regulated membrane fusion events such as vesicular neurotransmitter release from nerve terminals (for a review see Südhof *et al.*, 1993). Studies on the constitutive vesicular transport pathway led to the identification of the soluble cytoplasmic proteins *N*-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment proteins (α/β/γ-SNAPs) which were shown to be essential for a series of intracellular transport steps (Rothman and Orci, 1992). According to a current model, NSF and α-SNAP bind to membrane-associated SNAP receptors present in the vesicle and target membrane (designated v- and t-SNAREs, respectively). In the nerve terminal, the v- and t-SNAREs were identified as the synaptic vesicle protein synaptobrevin/VAMP and the presynaptic membrane proteins syntaxin and SNAP-25 (synaptosomal-associated protein of 25 kDa, not related to the α/β/γ-SNAPs; Söllner *et al.*, 1993a,b).

The hypothesis that synaptobrevin, syntaxin and SNAP-25 have a direct function in synaptic vesicle exocytosis has received strong support from the identification of these proteins as the targets of clostridial neurotoxins. Tetanus toxin (TeTx) and seven structurally related botulinal neurotoxins (BoNT/A–G) are potent inhibitors of neurotransmitter release and proteolyze synaptobrevin (TeTx, BoNT/B, BoNT/D, BoNT/F and BoNT/G), SNAP-25 (BoNT/A and BoNT/E) or syntaxin (BoNT/C1; reviewed in Niemann *et al.*, 1994). The neurotoxins constitute di-chain protein toxins whose heavy (H) chains control neuroselective binding, internalization, intraneuronal sorting and translocation of the light (L) chains into the cytoplasm. Once released from their H chains, the L chains proteolyze their individual intracellular substrates with unique specificity.

Although the identification of syntaxin, synaptobrevin and SNAP-25 as targets for the clostridial neurotoxins demonstrates a function in synaptic vesicle exocytosis, the mechanisms that control synaptic vesicle docking and fusion are enigmatic. TeTx-toxified squid giant nerve terminals contain more docked synaptic vesicles than non-toxified synapses, suggesting that the inhibition of synaptic vesicle exocytosis by TeTx operates at a step downstream of the docking of the vesicles at the active zone (Hunt *et al.*, 1994). Since syntaxin, synaptobrevin and SNAP-25 form a complex with each other (Söllner *et al.*, 1993b), it is likely that this complex is involved in the fusion reaction; however, the nature of the complex and the effect of the clostridial toxins on the complex are unknown. As a first step towards defining the mechanisms involved in specifying interactions between synaptobrevin and syntaxin Ia, Calakos *et al.* (1994) demonstrated that the two proteins bind to each other with relatively low affinity; however, this is an insufficient explanation for the observed efficiency of vesicular transport and fusion reactions. For this reason we have examined the interactions of synaptobrevin, syntaxin and SNAP-25 in ternary complexes and the effects of clostridial neurotoxins on these interactions. Our data reveal that all three membrane-associated proteins are capable of relatively weak binary interactions, but that in a ternary complex a dramatic increase in the stability of the complex is observed that could account for the exquisite specificity of *in vivo* fusion reactions. Furthermore, we demonstrate that clostridial

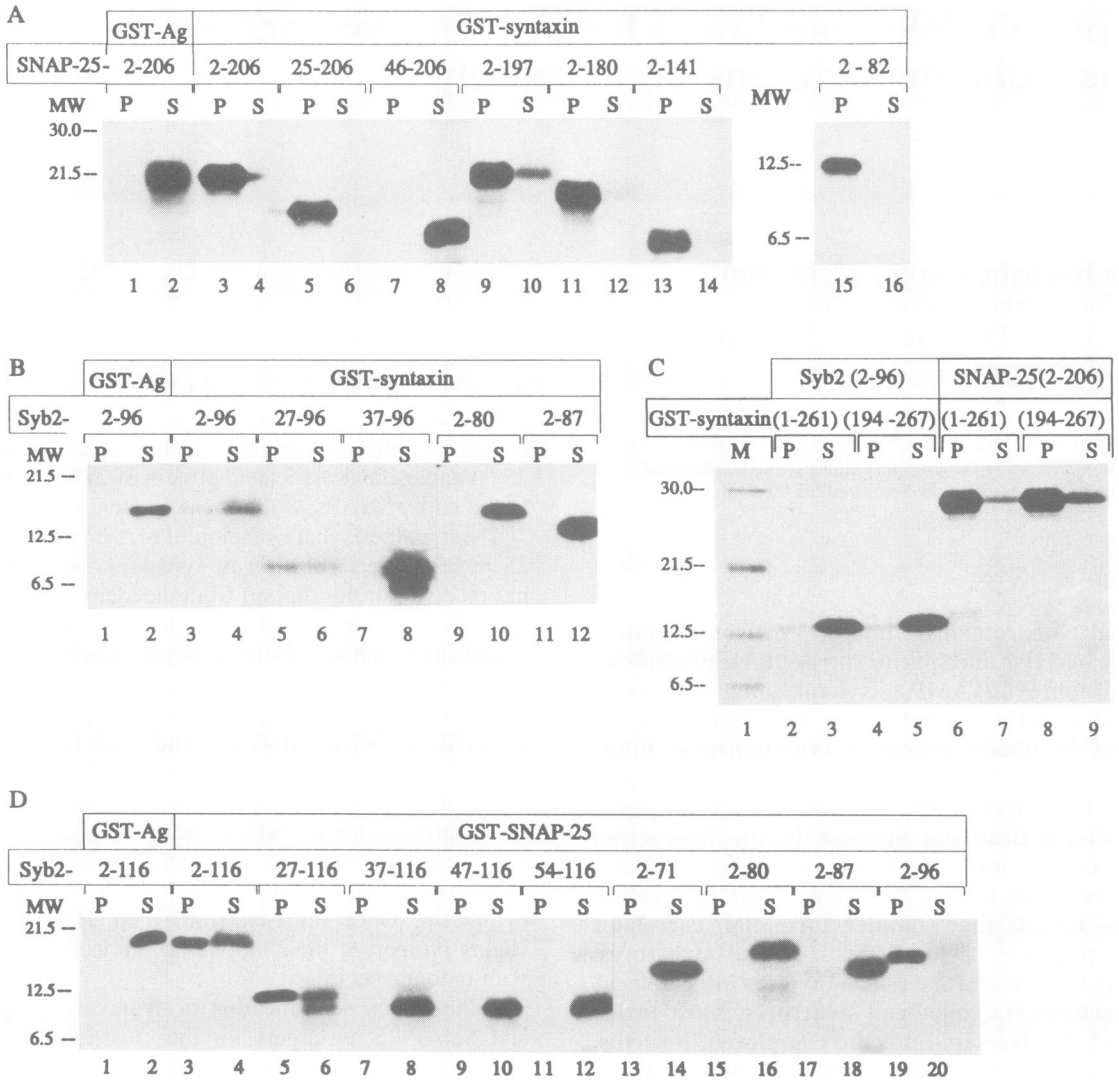

Fig. 1. Binding of synaptobrevin, syntaxin and SNAP-25 to each other in binary interactions: identification of sequences involved in binding. (**A**) Binding of SNAP-25 and fragments of SNAP-25 to GST−syntaxin (residues 1–261) immobilized on glutathione agarose beads. Full-length SNAP-25(2–206) and deletion mutants (numbers specify amino acid residues) were radiolabeled by *in vitro* transcription and translation and incubated with GST−syntaxin agarose matrix. Unbound (S) and bound material (P) were analyzed by SDS−PAGE and autoradiography. No binding is observed with a matrix displaying only GST−Ag. (**B**) Binding of radiolabeled synaptobrevin deletion mutants to GST−syntaxin. The conserved domain of synaptobrevin (residues 27–96) is required for binding to GST−syntaxin. (**C**) Synaptobrevin [Syb2(2–96)] and SNAP-25(2–206) bind to both GST−syntaxin(1–261) and GST−syntaxin(194–267). (**D**) Binding of radiolabeled synaptobrevin deletion mutants to GST−SNAP-25(1–206). The entire conserved domain of synaptobrevin is required for binding to GST−SNAP-25. Numbers to the left of each gel indicate the positions of molecular weight markers.

neurotoxins interfere with this complex formation, suggesting a mechanism of action for these proteases.

Results

SNAP-25 binds to syntaxin Ia via its N-terminal domain

SNAP-25, syntaxin and synaptobrevin are isolated from brain in a complex that is likely to represent an active intermediate in the membrane fusion process (Söllner *et al.*, 1993a,b). However, the genesis of this complex and the nature of its underlying interactions are unclear except for the binding of syntaxin to synaptobrevin (Söllner *et al.*, 1993b; Calakos *et al.*, 1994). Therefore, we systematically studied the potential interactions of different components in the complex with each other.

We first asked if the two plasma membrane proteins, syntaxin and SNAP-25, directly bind to each other and what sequences are responsible for this binding. To examine these questions, a series of radiolabeled N- and C-terminal fragments of SNAP-25 were produced by *in vitro* translation and tested for binding to the cytoplasmic domain of syntaxin 1a fused to glutathione-S-transferase (GST; Hata *et al.*, 1993). With this assay, SNAP-25 was found to bind tightly to syntaxin yielding a complex that was salt-resistant to up to 2 M NaCl (Figure 1A and data not shown). This binding is specific because (i) no binding was observed with GST and (ii) competition with unlabeled SNAP-25 carrying a C-terminal His6 tag (SNAP-25-His6) abolished binding (Figure 2). No competition of binding was observed with unrelated proteins such as bovine serum albumin or a synthetic synaptobrevin-specific peptide (residues 1–93; data not shown).

The binding of SNAP-25 to syntaxin was not affected

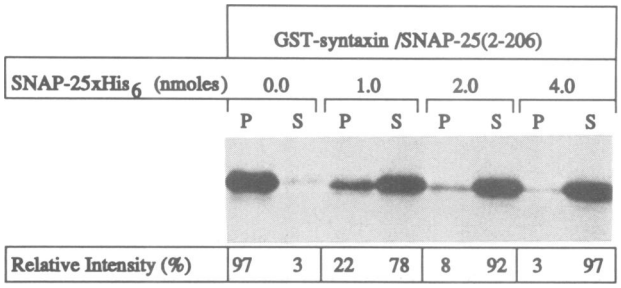

Fig. 2. Binding competition assay of *in vitro*-generated SNAP-25 to syntaxin. GST−syntaxin (0.2 nmol) was immobilized on glutathione agarose beads and incubated overnight in binding buffer containing constant amounts of radiolabeled SNAP-25(2–206) and various amounts of unlabeled SNAP-25-His6, as indicated. Beads were collected by centrifugation. The supernatant and washed pellet fractions were analyzed by SDS−PAGE and autoradiography. Intensities of bands were measured by laser densitometry. Values represent the mean values of two independent experiments.

significantly by a deletion of 24 residues from the N-terminus of SNAP-25, but was abolished by N-terminal deletions of 35 and 45 amino acids (Figure 1A and data not shown). Proteolysis of SNAP-25 by BoNT/A or BoNT/E (cleaving between Gln197 and Arg198, or Arg180 and Ile181, respectively; Schiavo *et al.*, 1993c; Binz *et al.*, 1994) had no effect on binding (Figure 1A, lanes 9–12). Studies with additional C-terminal deletion mutants indicated that SNAP-25 interacts specifically with syntaxin via a sequence comprising residues 25–82 (Figure 1A, lanes 13–16). Similar experiments designed to localize the binding site of SNAP-25 on syntaxin revealed that only the C-terminal third of syntaxin (residues 194–267) is required for this interaction (Figure 1C, lanes 6–9).

Synaptobrevin binds to both syntaxin and SNAP-25

We next examined the interactions between (i) synaptobrevin and syntaxin, and (ii) synaptobrevin and SNAP-25. For this purpose we again used a series of radiolabeled synaptobrevin fragments produced by *in vitro* translation and GST−syntaxin or GST−SNAP-25 fusion proteins. A weak but specific binding of synaptobrevin to syntaxin was observed (Figure 1B). In agreement with a previous report (Calakos *et al.*, 1994), this binding involved the C-terminal third of syntaxin (Figure 1C, lanes 2–5), i.e. the same domain of syntaxin that is required for the binding of SNAP-25 (Figure 1C, lanes 6–9). Surprisingly, synaptobrevin was also found to interact directly with SNAP-25 (Figure 1D). Again, both of these interactions appeared to be specific because (i) they occurred only with the GST fusion proteins and not with GST alone (Figure 1B and D) and (ii) radiolabeled synaptobrevin could be specifically displaced from GST−syntaxin or GST−SNAP-25 by the addition of 2.0 or 0.4 nmol of unlabeled synaptobrevin peptide (residues 1–93), respectively (data not shown).

Interestingly, N- and C-terminal deletions of synaptobrevin affected its binding to GST−syntaxin (Figure 1B) or GST−SNAP-25 (Figure 1D) in a similar manner: (i) a deletion of the N-terminal residues 2–26, a region that diverges in synaptobrevins from various species and isoforms, slightly enhanced binding to SNAP-25 or syntaxin (Figure 1B and D, lanes 3–6); (ii) a synaptobrevin mutant lacking the C-terminal transmembrane anchor region

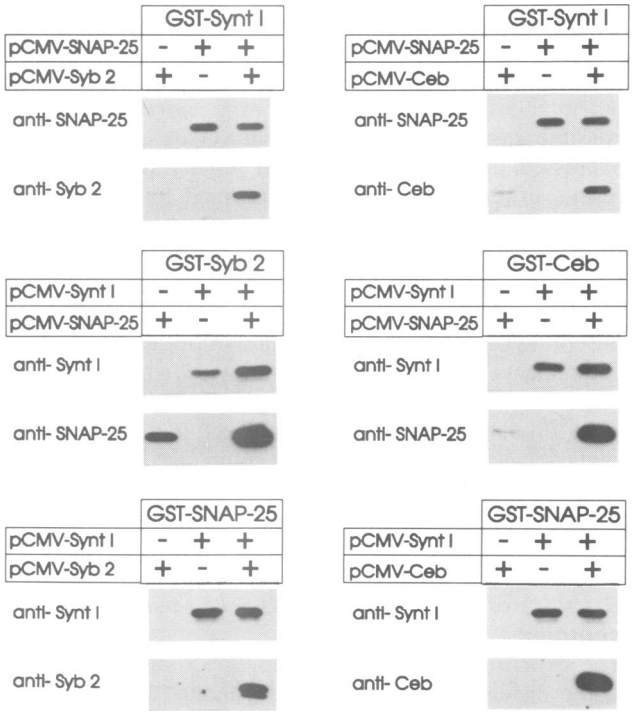

Fig. 3. Relative binding of synaptobrevin/cellubrevin, syntaxin and SNAP-25 to each other in binary and ternary reactions. Cell lysates containing full-length SNAP-25, synaptobrevin, cellubrevin and syntaxin expressed in COS cells were mixed as indicated and tested for binding to GST−syntaxin 1a (GST−Synt 1), GST−synaptobrevin 2 (GST−Syb 2), GST−cellubrevin (GST−Ceb) and GST−SNAP-25, as indicated. Bound material was analyzed by SDS−PAGE and immunoblotting. Note the potentiation of binding of SNAP-25 and syntaxin to synaptobrevin in the presence of all three proteins, as opposed to binary interactions in which binding is hardly visible at this exposure.

showed enhanced binding to syntaxin (data not shown) and to SNAP-25 (Figure 1D, compare lanes 3 and 4 with 19 and 20); and (iii) deletion mutants of synaptobrevin starting at Ala37 or ending at Lys87 bound only poorly compared with the full-length molecule. Together, these data suggest that the binding of synaptobrevin to both syntaxin and SNAP-25 is relatively weak and requires the presence of the entire conserved domain encompassing residues Thr27−Met96 (Archer *et al.*, 1990).

In vitro complex formation of syntaxin, SNAP-25 and synaptobrevin

The experiments described above suggest that in binary systems syntaxin, synaptobrevin and SNAP-25 interact directly with each other, whereby the binding strength between the two presynaptic membrane proteins clearly exceeds the binding strengths observed in reactions between synaptobrevin and either syntaxin or SNAP-25. These binding assays were performed with *in vitro*-translated material. To see whether *in vivo*-folded polypeptides showed the same binding characteristics, we expressed the different proteins by transfection in COS cells (Figure 3). Synaptobrevin, cellubrevin (a ubiquitous synaptobrevin homolog; McMahon *et al.*, 1993), SNAP-25A and syntaxin Ia were transfected into COS cells. Bacterial recombinant GST fusion proteins containing the cytoplasmic domains of these proteins were attached

to glutathione beads and used as a matrix for affinity purifications of proteins expressed in COS cells; bound proteins were detected by immunoblotting. To exclude potential artifacts, experiments were carried out in all possible combinations of bacterial versus eukaryotically expressed proteins in binary and ternary reactions. The results of these experiments confirmed the conclusions obtained with *in vitro*-translated material, demonstrating weak but significant binding of synaptobrevin or cellubrevin to both syntaxin and SNAP-25, as well as strong binding of syntaxin to SNAP-25. Unexpectedly, the binding of synaptobrevin or cellubrevin to the other two components was dramatically increased when both syntaxin and SNAP-25 were present together (Figure 3, right lanes in all panels). These findings support the idea that syntaxin and SNAP-25 form tight complexes in the absence of synaptobrevin (or cellubrevin), whereas binary interactions between synaptobrevin and syntaxin or SNAP-25 are comparatively weak.

Stability of the syntaxin–SNAP-25–synaptobrevin complex

The experiments described in Figure 3 suggest that the stable heterodimer containing syntaxin and SNAP-25 provides high-affinity binding sites for synaptobrevin (or cellubrevin). To study the formation of this ternary complex in a reconstituted system, we examined the binding reaction of purified proteins comprising the cytoplasmic domains of the three polypeptides: a synthetic synaptobrevin 2 peptide (residues 1–93), GST–syntaxin(1–261) and SNAP-25(1–206)-His6. As described above, only poor binding of the synaptobrevin peptide to GST–syntaxin was observed (Figure 4, lane 4). GST–syntaxin and SNAP-25 bound to each other much more efficiently, yielding a saturable heterodimer with an ~1:1 stoichiometry (Figure 4, lane 5, see the legend for details). The GST–syntaxin–SNAP-25 heterodimer showed a dramatically increased affinity for synaptobrevin, yielding ternary complexes which, according to densitometer scanning, contained GST–syntaxin, SNAP-25 and synaptobrevin in the approximate ratio of 1:1:0.7 (lane 6).

Encouraged by the high affinity of the syntaxin–SNAP-25 heterodimer for synaptobrevin, we tested the stability of the ternary complex against denaturation by sodium dodecyl sulfate (SDS). SDS–PAGE analysis of the ternary complex revealed that samples that were incubated in SDS–PAGE sample buffer at 37 instead of 100°C contained two new high molecular weight bands with apparent M_r of 113 000 and 230 0000, respectively (Figure 4, lane 7). These were selectively absent from boiled samples (lane 6). The complexes were not dissociated by freezing and thawing or upon prolonged storage in sample buffer (lane 8), and were stable even to exposure at temperatures of up to 60°C for 5 min (data not shown).

To analyze the composition of the high molecular weight complexes, we isolated them from SDS–polyacrylamide gels by electroelution and boiled and re-analyzed them by SDS–PAGE. Laser densitometer scannings of the Coomassie-stained gels were performed and the molar ratios calculated assuming that the staining intensities were proportional to the total mass of each polypeptide. These analyses revealed that the 113 and 230 kDa complexes contained syntaxin, SNAP-25 and synaptobrevin in nearly equimolar amounts (see the legend to Figure 4 for details).

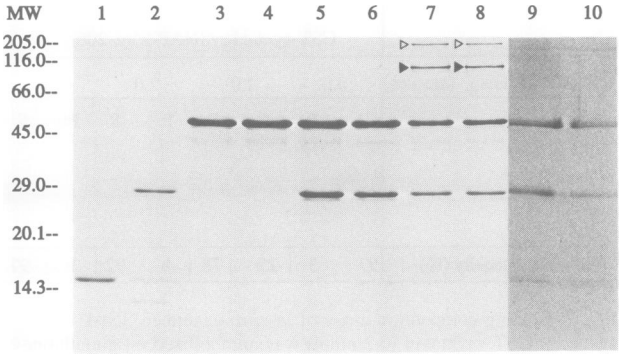

Fig. 4. Assembly of synaptobrevin, SNAP-25 and syntaxin complexes *in vitro* from purified components and the generation of SDS-resistant complexes. The figure shows a Coomassie-stained SDS–PAGE gel of a synthetic synaptobrevin peptide (lane 1, residues 1–93), recombinant SNAP-25-His6 (lane 2) and GST–syntaxin (lane 3). These three proteins were incubated overnight in binding buffer and glutathione agarose beads in the following combinations: GST–syntaxin and synaptobrevin (lane 4); GST–syntaxin and SNAP-25-His6 (lane 5); GST–syntaxin, SNAP-25-His6 and synaptobrevin (lanes 6–10). Beads were washed. Bound material was eluted by incubation in sample buffer at 37°C and analyzed by SDS–PAGE either after boiling (lanes 4–6) or without boiling (lanes 7 and 8). The two high molecular weight species in lanes 7 and 8 that only appear in unboiled samples (open and closed arrowheads) were cut out, electroeluted and re-analyzed by SDS–PAGE after boiling to determine the composition [lane 9, 113 kDa complex (closed arrowhead); lane 10, 230 kDa species (open arrowhead)], revealing that they consist exclusively of GST–syntaxin, SNAP-25-His6 and synaptobrevin. For the determination of the stoichiometry of the GST–syntaxin–SNAP-25 heterodimer we performed laser densitometer scannings of lane 5 and four additional gels. After correction for the molecular masses, the ratio of syntaxin to SNAP-25 was determined to be 1:0.99 ± 0.09 (five independent experiments). Similar measurements for the ternary complexes in lanes 6, 9 and 10 revealed the following molar ratios of syntaxin to SNAP-25 to synaptobrevin: 1:1.03 ± 0.10:0.72 ± 0.11 (three distinct experiments, lane 6); 1:1.05:0.81 (mean value of two independent experiments, lane 9); and 1:0.99:0.91 (mean value of two independent experiments, lane 10).

At present, the relationship between the two SDS-resistant complexes is unclear. It should be noted that even larger molecular weight complexes were observed when higher protein concentrations of the three constituents were applied. As both the 113 and 230 kDa molecular species failed to dissociate in the presence of SDS at 37°C, we may conclude that their electrophoretic migration in SDS–PAGE does not reflect the true molecular mass of the complexes. Therefore, although it is clear that the two complexes contain equimolar amounts of the three constituents, the absolute composition of the complexes remains to be established.

We then used SDS resistance as an assay to study the role of SNAP-25 in the formation of the complex. Constant amounts of GST–syntaxin attached to glutathione agarose were preloaded with increasing amounts of SNAP-25 and incubated with constant amounts of radiolabeled synaptobrevin. After the removal of unbound material, the agarose beads were washed; free and bound proteins were analyzed by SDS–PAGE after incubation at either 37 or 100°C (Figure 5). As expected, only ~15% of the total radiolabeled synaptobrevin bound to GST–syntaxin in the absence of SNAP-25 (Figure 5A and B, lanes 1) and

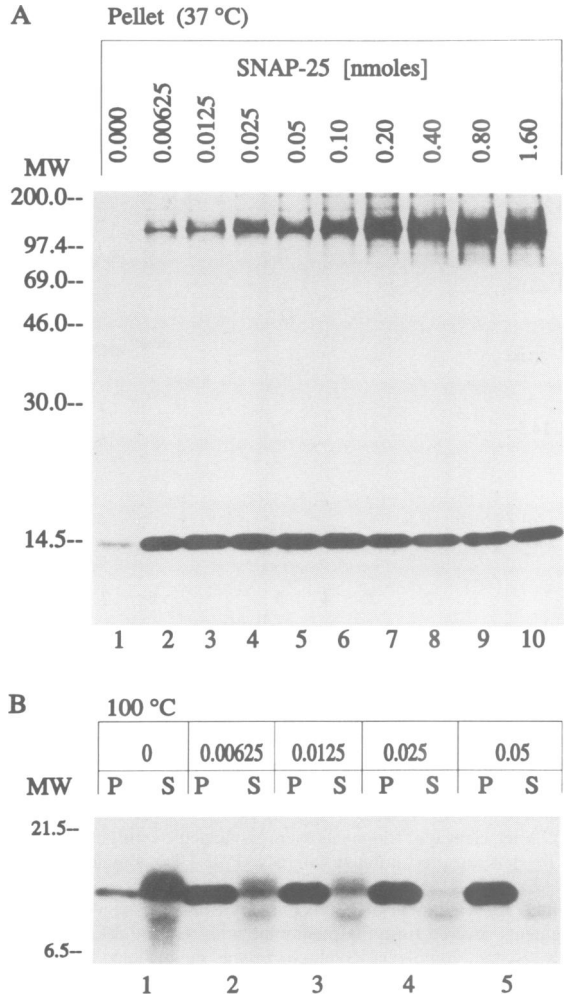

Fig. 5. SNAP-25 induces high-affinity binding sites for synaptobrevin on syntaxin. GST−syntaxin (0.2 nmol) was incubated with constant amounts of radiolabeled synaptobrevin (<1 pmol) with the indicated amounts of SNAP-25. Binding to GST−syntaxin was analyzed by SDS−PAGE and autoradiography of the radiolabeled synaptobrevin. In (**A**), only bound material was analyzed with samples that were not boiled prior to electrophoresis. In (**B**), bound (P) and unbound synaptobrevin (S) are compared at low SNAP-25 concentrations (range 0.00–0.05 nmol) following boiling. The concentration of SNAP-25 used in the assay is indicated on top of each gel; numbers on the left indicate positions of molecular weight markers.

dissociated quantitatively in sample buffer at 37°C. The addition of only 6.25 pmol of SNAP-25 to the GST−syntaxin caused a significant increase in the binding of synaptobrevin and the formation of an SDS-resistant complex (Figure 5). With increasing concentrations of SNAP-25, there was a dose-dependent increase in the binding of synaptobrevin to GST−syntaxin and in the formation of the SDS-resistant complexes. Both of these activities were linearly dependent on the SNAP-25 concentration but had a different concentration dependence. Synaptobrevin binding to GST−syntaxin as a function of SNAP-25 was maximal at much lower SNAP-25 concentrations than the formation of the SDS-resistant complex (compare Figure 5A with B). Thus, the formation of high-affinity ternary complexes and the development of stable SDS-resistant complexes are distinct processes that can be experimentally differentiated.

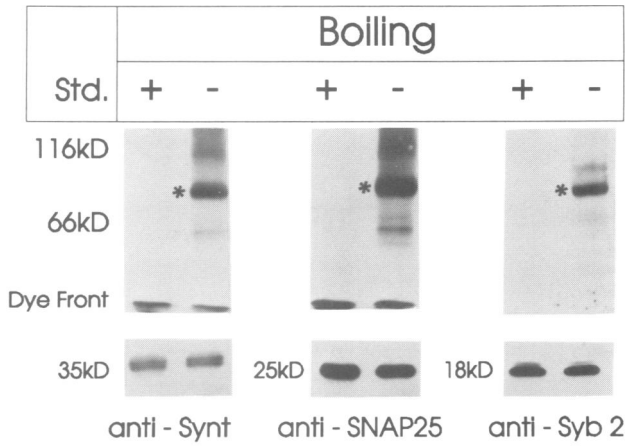

Fig. 6. Identification of an SDS-resistant complex of synaptobrevin, syntaxin and SNAP-25 in rat brain homogenates. Solubilized rat brain homogenate (10 mg/ml protein) was mixed with sample buffer and analyzed by SDS−PAGE and immunoblotting on 7 (top panels, to resolve high molecular weight complexes) and 15% gels (bottom panels, to visualize monomeric proteins). Immunoblots were probed with antibodies to syntaxin 1a, SNAP-25 and synaptobrevin 2, as indicated. The 80 kDa high molecular weight complex that is only present in unboiled samples is identified by an asterisk.

Identification of the native SDS-resistant complex in rat brain

The question arises as to whether the stoichiometric SDS-resistant complex between syntaxin, SNAP-25 and synaptobrevin, as reconstituted *in vitro* in the experiments described above, is physiologically relevant or an *in vitro* artifact. To address this question, we investigated whether a complex with similar properties could be detected in brain. Rat brain homogenates were analyzed by SDS−PAGE and immunoblotting for the synaptobrevin, SNAP-25 and syntaxin after either boiling or incubation at 37°C (Figure 6). A high molecular weight complex with an M_r ~80 000 could be detected with antibodies to syntaxin, SNAP-25 and synaptobrevin in the samples incubated at 37°C, but not in the boiled samples (asterisks in Figure 6). The difference in the electrophoretic mobility of this complex in comparison with that containing the pure recombinant and synthetic proteins in Figure 4 may be ascribed to an absence of the GST and His6 tags and the presence of the membrane anchor domains. Further analyses showed that the complex does not contain α-SNAP, NSF, synaptotagmin or Munc-18 (data not shown). In the experiments shown in Figure 6, brain was first homogenized in Triton X-100 before analysis. Mixing experiments with purified proteins suggested that the SDS-resistant complex cannot form in SDS sample buffer. When brain was homogenized in SDS sample buffer instead of Triton X-100, the amount of SDS-resistant trimeric complex decreased (data not shown). This suggests that (i) some of the complex forms after homogenization and (ii) in native brain, only small percentages of synaptobrevin, syntaxin and SNAP-25 are present in the SDS-resistant complex. It is possible that the second high molecular weight complex observed in the reconstituted reactions (Figure 4) is also present in brain because it would not be apparent in these gels; however, it is clear that in this more physiological material the supposed heterotrimer is the major species of the complex.

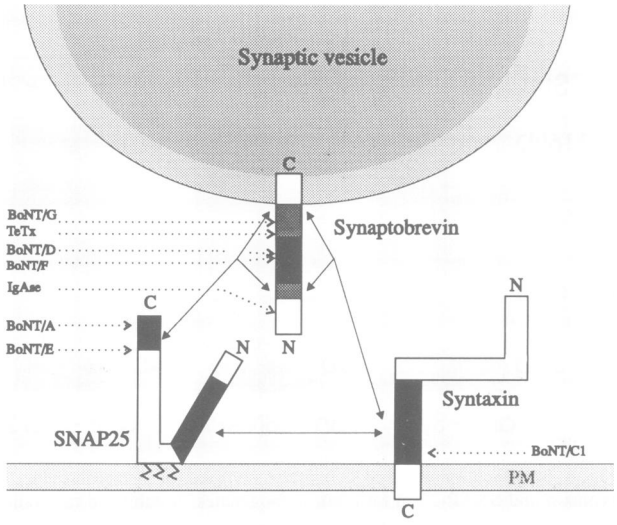

Fig. 7. Schematic drawing of the critical sequences of synaptobrevin, syntaxin and SNAP-25 involved in binding reactions and of the positions of cleavage by different clostridial neurotoxins.

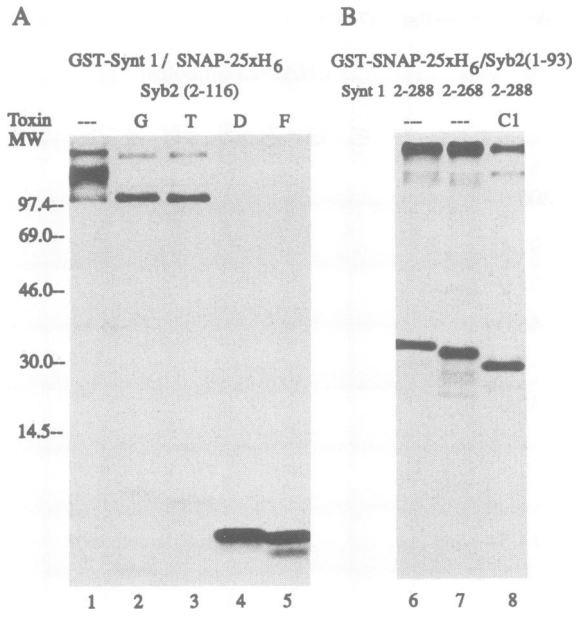

Fig. 8. Effects of cleavage of synaptobrevin and syntaxin by botulinum and tetanus toxins on complex formation. (**A**) Effect of neurotoxin cleavage on synaptobrevin assembly into SDS-resistant complexes. Only some toxin cleavage reactions inhibit complex formation (BoNT/F, BoNT/D), whereas others sever synaptobrevin from the membrane but still assemble into complexes (BoNT/G, TeTx). Synaptobrevin 2 (residues 2–116) was radiolabeled *in vitro* and incubated with BoNT/F, BoNT/D, TeTx and BoNT/G or control buffer (−). After cleavage, fragments were incubated overnight with GST−syntaxin pre-saturated with SNAP-25-His6. Bound material was eluted and analyzed by SDS−PAGE and autoradiography without boiling. (**B**) Treatment of syntaxin 1a by BoNT/C1 generates soluble fragments severed from the membrane with reduced affinity for ternary SDS-resistant complexes. Syntaxin 1a was radiolabeled by *in vitro* translation, reconstituted into membranes and digested with BoNT/C1. The soluble cleavage product was isolated in the supernatant after sedimentation of the membranes through a sucrose cushion and incubated overnight with GST−SNAP-25 in the presence of synaptobrevin [Syb2(1–93)]. For quantitative comparisons, two truncated soluble syntaxins were incubated in the same reactions in parallel. Bound material was eluted from the matrix at 37°C and analyzed by SDS−PAGE without boiling and autoradiography. Numbers on the left indicate positions of molecular weight markers.

Effects of clostridial neurotoxins on syntaxin−synaptobrevin−SNAP-25 complexes

The eight clostridial neurotoxins inhibit neurotransmission by proteolyzing synaptobrevin, syntaxin or SNAP-25 selectively and at seven distinct sequences (summarized in Figure 7). The questions arise as to why the proteolytic cleavage by the different neurotoxins inhibits synaptic vesicle exocytosis and whether inhibition relates to complex formation.

To address these questions, we first studied the effect of cleavage of synaptobrevin by four different toxins (TeTx, BoNT/D, BoNT/F and BoNT/G) on the ability of synaptobrevin to complex with syntaxin or SNAP-25. After cleavage, the synaptobrevin products generated by all toxins were unable to bind to syntaxin or SNAP-25 in binary interactions (data not shown). This result agrees well with the synaptobrevin sequence requirement for binding to syntaxin and SNAP-25 (Figure 1). Surprisingly, in ternary interactions in the presence of both syntaxin and SNAP-25, synaptobrevin cleavage products of all toxins regained binding (Figure 8A). With BoNT/D and BoNT/F that cleave in the middle of synaptobrevin, both the N- and the C-terminal fragments showed this property (Figure 8A, lanes 4 and 5). When the binding complexes were tested for SDS resistance, only the TeTx and BoNT/G fragments that contain almost the complete conserved domain of synaptobrevin were able to assemble into SDS-resistant complexes (Figure 8A). In agreement with this finding, an analysis of N- and C-terminal deletion mutants of synaptobrevin indicated that a core region between residues 37 and 70 is essential to confer SDS resistance (data not shown). Therefore, cleavage of synaptobrevin by the different toxins preserves the ability of the proteolyzed synaptobrevin to form ternary complexes in spite of diminishing binary interactions; it either abolishes the ability of synaptobrevin to form SDS-resistant complexes (BoNT/D and /F) or severs the complex-forming domain from the vesicle membrane (TeTx and BoNT/B and BoNT/G; Table I).

We next studied the effect of BoNT/C1 on syntaxin. BoNT/C1 cleaves syntaxin just outside of the transmembrane region, producing a soluble cytoplasmic fragment of syntaxin (Blasi *et al.*, 1993b). This fragment continues to bind to the synaptobrevin−SNAP-25 complex but cannot efficiently form SDS-resistant complexes (Figure 8B).

In a final set of experiments we studied the effects of SNAP-25 cleavage by BoNT/A and BoNT/E on complex formation. In many ways, BoNT/A and BoNT/E are the most interesting clostridial neurotoxins because they cleave SNAP-25 at its very C-terminus and do not interfere with either a conserved domain (as do BoNT/D and BoNT/F for synaptobrevin) or membrane attachment of SNAP-25 (as do BoNT/G and TeTx for synaptobrevin and BoNT/C1 for syntaxin). Therefore, the mechanism of action of BoNT/A and BoNT/E cannot consist of a simple severing of the complex from the membrane or interference with fundamental interactions. Interestingly, the effect particularly of BoNT/A on neurotransmitter release is also comparatively mild, suggesting that the toxin may interfere with synaptic vesicle exocytosis only partly (McMahon *et al.*, 1992).

Table I. Effects of clostridial neurotoxins on assembly of the SNARE complex

Toxin	Target (rat brain)	Cleavage site	Functional effects of neurotoxin cleavage			
			Severance of complex from membranes[a]	Inhibition of binary interactions	Inhibition of ternary interactions	Inhibition of assembly into SDS-resistant complex[b]
TeTx (BoNT/B)	synaptobrevin 2	Gln76–Phe77	+	syntaxin, inhibited SNAP-25, inhibited	no effect	no effect
BoNT/G	synaptobrevin 2	Ala81–Ala82	+	syntaxin, inhibited SNAP-25, inhibited	no effect	no effect
BoNT/F	synaptobrevin 2	Gln58–Lys59	–	syntaxin, inhibited SNAP-25, inhibited	no effect	inhibited
BoNT/D	synaptobrevin 2	Lys59–Leu60	–	syntaxin, inhibited SNAP-25, inhibited	no effect	inhibited
BoNT/C[a]	syntaxin 1a		+	SNAP-25, no effect synaptobrevin, no effect	no effect	reduced
BoNT/A	SNAP-25A	Gln197–Arg198	–	syntaxin, no effect synaptobrevin, reduced	no effect	50%
BoNT/E	SNAP-25A	Arg180–Ile181	–	syntaxin, no effect synaptobrevin, inhibited	no effect	inhibited

[a]Note that the assembled ternary complex is resistant to neurotoxin cleavage.
[b]Determined by incubation for 30 min at 37°C.

In binary reactions, BoNT/A and BoNT/E cleavage of GST–SNAP-25 dramatically inhibited binding of synaptobrevin (Figure 9A), suggesting that the C-terminal domain of SNAP-25 is required for its interaction with synaptobrevin. Since C-terminally deleted SNAP-25 mutants continue to bind to GST–syntaxin (Figure 1A), we used GST–syntaxin matrices saturated with the BoNT/A and BoNT/E products of SNAP-25 to study the binding of radiolabeled synaptobrevin. Similar to what had been observed with synaptobrevin cleavage products, toxin treatment of SNAP-25 had no effect on the assembly of ternary complexes (Figure 9B, lanes 5–8). However, complexes containing BoNT/A-digested SNAP-25 were only partially resistant to SDS (Figure 9B, lane 11); BoNT/E-digested SNAP-25 was not (lane 12). Thus, the C-terminus of SNAP-25 is not required for the formation of ternary synaptobrevin–syntaxin–SNAP-25 complexes, but plays an essential role in the generation of SDS resistance. The effects of different neurotoxins on the biochemical properties of their targets are summarized in Table I.

Resistance of fusion complexes against neuroselective proteases

Our results suggest that syntaxin, synaptobrevin and SNAP-25 form a very tight, SDS-resistant complex that occurs *in vivo* and cannot be formed after cleavage with some of the clostridial neurotoxins. We next examined if the proteins in the complex are accessible to toxin cleavage. None of the toxins cleaving synaptobrevin were active on the complex (Figure 10A, lanes 3–5), and toxins affecting SNAP-25 showed only weak activity (lanes 6 and 7). As a positive control, the complex was also digested with IgA protease. This enzyme selectively cleaves synaptobrevin 2 outside of the conserved domain between Pro20 and Ala21. Cleavage occurred even in the SDS-resistant ternary complex, suggesting that the N-terminal region of synaptobrevin is exposed on the surface (Figure 10A, lane 8). Control experiments showed that GST fusion proteins can be cleaved even after immobilization on the matrix (Figure 10B). Together these data suggest that the toxins do not attack the assembled fusion complex but proteolyze their targets either when the complex is disassembled by means of the NSF–SNAP complex (Söllner *et al.*, 1993b) or when syntaxin, synaptobrevin and SNAP-25 are retrieved for function after fusion.

Discussion

Botulinum toxins and tetanus toxin are potent inhibitors of synaptic vesicle exocytosis in nerve terminals. These toxins act as specific proteases that in nerve terminals cleave only a single substrate at a single site. The targets for the toxins were identified as the synaptic vesicle protein synaptobrevin (BoNT/B, /D, /F and /G, and TeTx; Link *et al.*, 1992; Schiavo *et al.*, 1992, 1993a,b; Yamasaki *et al.*, 1994a,b) and the presynaptic plasma membrane proteins SNAP-25 (BoNT/A and /E; Blasi *et al.*, 1993a; Schiavo *et al.*, 1993b,c; Binz *et al.*, 1994) and syntaxin (BoNT/C1; Blasi *et al.*, 1993b). The identification of these three synaptic proteins as toxin targets suggests that they function directly in synaptic vesicle exocytosis. In support of this notion, the same three proteins were shown to be present in brain as a heterotrimeric complex that acts as a receptor for α/β/γ-SNAPs and NSF (abbreviated as SNARE, 'SNAP/NSF receptor'), proteins with a known function in vesicular membrane traffic (Söllner *et al.*, 1993a,b). Thus, two independent experimental approaches discovered an exocytotic function for the same three proteins without, however, identifying potential mechanisms for this function and the relationship between the two approaches. In our study we have now determined the essential domains within synaptobrevin, syntaxin and SNAP-25 that control assembly into various ternary complexes and used clostridial neurotoxins as tools to determine the potential function of such complexes in membrane fusion.

By employing a variety of expression strategies and seven different neurotoxins, four major observations were made. (i) We demonstrate that each of the three synaptic

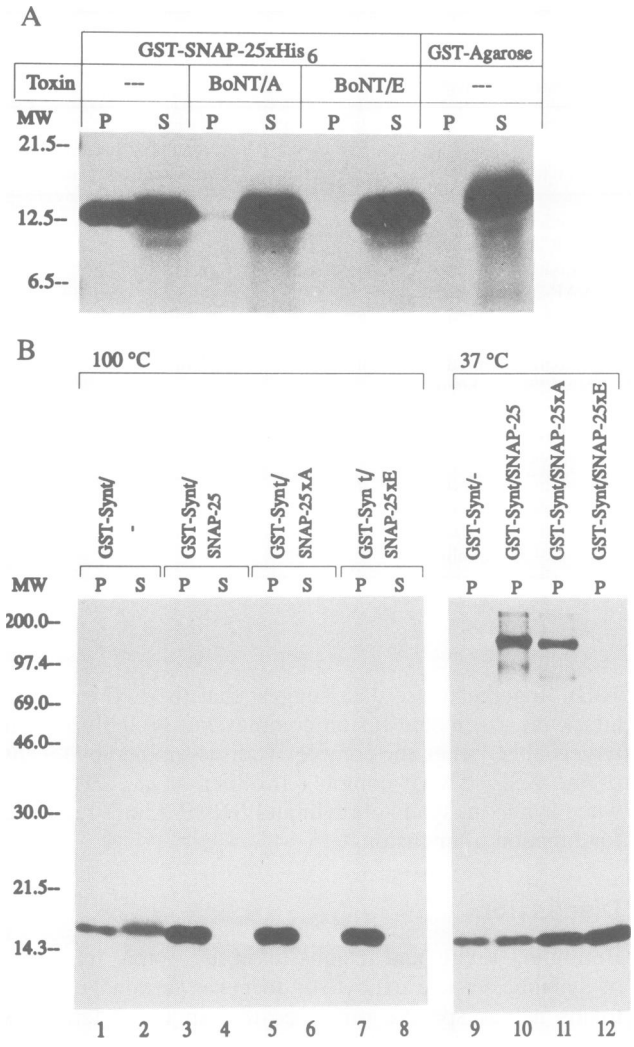

Fig. 9. Effects of SNAP-25 cleavage by BoNT/A and BoNT/E on the formation of SDS-resistant ternary complexes. (**A**) BoNT/A and BoNT/E inhibit binding of synaptobrevin to GST−SNAP-25 in binary reactions. Aliquots of GST−SNAP-25-His6 were incubated in binding buffer alone or with BoNT/A or BoNT/E. After the removal of uncleaved material by passage over Ni-NTA agarose, the flow-through was coupled to glutathione agarose and used for binding of radiolabeled synaptobrevin. BoNT/A treatment drastically reduces binding of synaptobrevin; BoNT/E abolishes binding of synaptobrevin. (**B**) Effects of BoNT/A and BoNT/E on the binding of synaptobrevin to syntaxin−SNAP-25 heterodimers and the formation of ternary SDS-resistant complexes. Agarose beads carrying affinity-bound heterodimers of GST−syntaxin−SNAP-25 or GST−syntaxin and BoNT/A- or BoNT/E-cleaved SNAP-25 were incubated overnight with constant amounts of radiolabeled synaptobrevin(2–96). Bound (P) and unbound material (S) were separated and analyzed by SDS−PAGE and autoradiography after boiling (left panel) or after incubation at 37°C in SDS sample buffer (right panel).

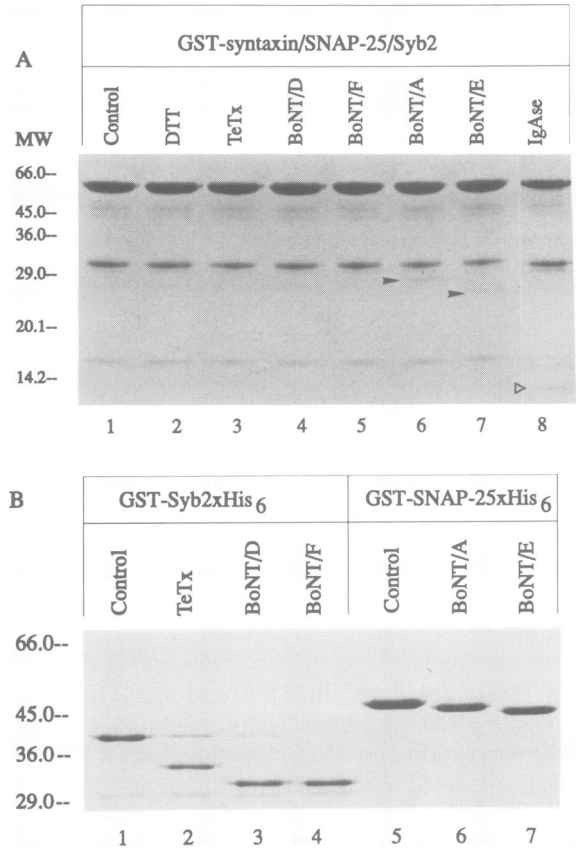

Fig. 10. Neurotoxins fail to proteolyze synaptobrevin and SNAP-25 in the assembled complex. (**A**) Beads containing equimolar concentrations of GST−syntaxin, synaptobrevin and SNAP-25-His6 assembled into high-affinity complexes were incubated for 90 min with binding buffer (control), binding buffer containing various toxins as indicated, or IgA protease (IgAse). DTT represents a control incubation in dithiothreitol, as some of the toxin preparations contained this reducing agent to prevent oxidation of the toxin. None of the toxins proteolyze the ternary complex, whereas IgAse can cleave indicating exposure of the N-terminus of synaptobrevin. (**B**) The same toxins cleave GST fusion proteins attached to the agarose matrix in a non-complexed form in parallel incubations, indicating that the toxins are active.

proteins, synaptobrevin, SNAP-25 and syntaxin, binds to the other in the absence of the third. We mapped the minimal domains for these interactions to sequences with a high probability of forming coiled-coils (see below). (ii) We show that the interaction between SNAP-25 and syntaxin generates a high-affinity binding site for synaptobrevin that results in the formation of a stable stoichiometric complex that is resistant to SDS and also present in brain. (iii) We delineate two distinct mechanisms of action for the inhibitory activity of clostridial neurotoxins: (a) severing the attachment of a complex to the synaptic vesicle or plasma membrane or (b) inhibition of the formation of ternary SDS-resistant complexes. (iv) We show that clostridial toxins largely fail to proteolyze ternary complexes and can only cleave free target proteins.

Together these findings have several important implications for neurotransmitter release. The unusual properties of the complex of synaptobrevin, syntaxin and SNAP-25 suggest that it represents a low-energy state that may be a critical intermediate in the fusion reaction. After homogenization of rat brain in SDS sample buffer, the relative amounts of SDS-resistant complex are low. Homogenization in less denaturing detergents leads to increased generation of the complex, suggesting that part of the complex observed in brain forms after homogenization (Figure 6). An essential role for the stable SDS-resistant ternary complex in exocytosis is suggested by the experiments with BoNT/A and /E that cleave SNAP-25, and with BoNT/D and BoNT/F that cleave synaptobrevin. The respective cleavage products obtained with these toxins still assemble into ternary complexes, and the ternary

Table II. α-Helical regions with coiled-coil-forming potentials in synaptobrevin, syntaxin and SNAP-25

Protein	Predicted α-helix	Average score[a]	Number of heptad repeats in register
Synaptobrevin	Arg30–Arg56	1.17	4 (3)
	Asp57–Tyr88	1.50	5
SNAP-25	Met1–Ala42	1.62	6
	Arg45–Cys85	1.76	5
	Asn159–Leu203	1.54	7
Syntaxin	Met30–Ser64	1.33	5
	Asp68–Asp112	1.64	6
	Lys189–Asp231	1.25	6

[a]Scores calculated according to Lupas *et al.* (1991).

complexes obtained with these cleaved complexes have normal membrane associations (that are abolished by the action of some of the other toxins). Nevertheless, cleavage inhibits exocytosis, and the only biochemical parameter this correlates with is the conversion of the ternary complex into an SDS-resistant complex. Therefore this conversion may play a pivotal role in making synaptic vesicles competent for exocytosis.

The ternary complex of synaptobrevin, syntaxin and SNAP-25 is largely insensitive to cleavage by clostridial neurotoxins. *In vivo* TeTx inhibits neurotransmitter release but does not inhibit or reverse docking (Neale *et al.*, 1989; Hunt *et al.*, 1994). It remains to be determined if the toxins only attack synaptobrevin, syntaxin and SNAP-25 before assembly of the complex, or if the complex dissociates before membrane fusion and allows the toxins to act between this dissociation and the fusion reaction. Measurements on neurotransmitter release in TeTx-poisoned nerve terminals suggest that after TeTx poisoning, the fast phase of neurotransmitter release, presumably due to docked vesicles, is unimpaired, whereas the slow phase is inhibited (McMahon *et al.*, 1992). This result is best interpreted by a model in which vesicles that were docked before TeTx action are still competent to fuse and are not sensitive to TeTx, suggesting that the toxins act primarily before assembly of the SNARE complex. The accumulation of docked vesicles in resting nerve terminals poisoned by TeTx (Hunt *et al.*, 1994) does suggest, however, that the initial phase of docking of synaptic vesicles may not involve complex formation between synaptobrevin, SNAP-25 and syntaxin, and that the three proteins display their primary function after the initial docking of the vesicles.

What structural features mediate the tight binding of synaptobrevin, SNAP-25 and syntaxin into an SDS-resistant complex? Our deletion analyses pointed to four interactive domains: the conserved core region of synaptobrevin (residues 27–96), the N- and C-terminal domains of SNAP-25 (residues 26–82 and 180–206, respectively) and the C-terminal domain of syntaxin (residues 194–261) adjacent to the transmembrane anchor domain. As pointed out previously (Inoue *et al.*, 1992; Spring *et al.*, 1993; Jahn and Südhof, 1994), each of these regions has a high propensity for the formation of α-helical coiled-coils (Table II). Coiled-coils are formed by two or more right-handed α-helices that are wound around each other into a tight compact structure with a left-handed super-helical twist. This type of intermolecular interaction is found in transcription factors containing leucine zippers (O'Shea *et al.*, 1991; Ellenberger *et al.*, 1992), fibrous proteins of the keratin family (Cohen and Parry, 1990) and the fusogenic hemagglutinin spike of influenza virus (Carr and Kim, 1993). According to Lupas *et al.* (1991), scores >1.1 are significant for extended α-helices and scores >1.3 characterize regions with a high propensity for helical coiled-coils, with a top score of 1.91 for the GCN4 leucine zipper. The propensity scores in Table II range from 1.17 for an amphiphilic helix in synaptobrevin to 1.7 for a region within the N-terminal domain of SNAP-25. Whereas the presence and function of such helices in the three proteins remain to be verified by crystallography, perhaps employing the SDS-resistant complex, additional data in the literature link coiled-coil formation to membrane fusion. The influenza virus hemagglutinin (HA) forms homotrimers that mediate binding to the cell surface, internalization of the virus particle and fusion with cellular membranes. The driving force for membrane fusion was suggested recently to reside in a 36 residue peptide forming a loop in the X-ray structure of uncleaved native hemagglutinin (Carr and Kim, 1993). This peptide sequence has a high propensity to extend two flanking α-helices, thereby generating (together with the other two HA molecules) a three-stranded intermolecular coiled-coil. Through this conformational change, the fusogenic peptide at the N-terminus of the HA2 subunit is thought to be propelled into the cellular membrane to induce fusion. It is intriguing to speculate that a similar spring-loaded mechanism may exist in the synaptobrevin–syntaxin–SNAP-25 complex: the three proteins could first associate by forming an extended heterotrimeric coiled-coil in which the core domain of synaptobrevin, the N-terminal domain of SNAP-25 and the C-terminal domain of syntaxin are wrapped around each other in a rod-like coiled-coil. Since each of the individual helices contains a predicted loop in the middle, the rod could be triggered to collapse into a stump of half the original length. Such a mechanism could expose a fusogenic peptide and would cut the distance between the presynaptic and the vesicle membrane in half and perhaps drive the ternary complex from a 'docked' SDS-sensitive phenotype into a fusogenic SDS-resistant phenotype of lower energy.

Materials and methods

Buffers

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. HEPES buffer: 20 mM HEPES–NaOH pH 7.4, containing 100 mM NaCl and 0.1% (v/v) Nonidet P-40. Binding buffer: 4 mM HEPES–NaOH pH 7.4, 100 mM NaCl, 3.5 mM $CaCl_2$, 3.5 mM $MgCl_2$, 1 mM EDTA, 0.1% (v/v) Nonidet P-40. Washing buffer: 50 mM Tris–HCl pH 8.0, 0.1 M NaCl, 2.5 mM $MgCl_2$, 0.1% (v/v) Nonidet P-40. Homogenization buffer: 10 mM HEPES–NaOH, pH 7.4, containing 150 mM NaCl and 1 mM EGTA.

Plasmid constructions and expression

GST–syntaxin 1a (residues 1–261), GST–syntaxin(194–267), GST–SNAP-25A(1–206), GST–synaptobrevin 2(1–96) and GST–cellubrevin(1–103) were subcloned into pGexKG (Guan and Dixon, 1991) using fragments generated by PCR with the appropriate restriction sites introduced in the PCR primers. Fusion proteins were affinity-purified on GST–agarose according to the protocols of the manufacturer. For expression in COS cells, the corresponding full-length cDNAs were subcloned into pCMV vectors (Anderson *et al.*, 1989). Vectors were

transfected into COS cells using the DEAE–dextran method. Expression and purification of SNAP-25-His6 were described previously (Binz *et al.*, 1994). For *in vitro* transcription and translation, pSP72 or pSP73 (Promega) or Bluescript vectors (Stratagene) were used (Mayer *et al.*, 1988). N-terminal deletion mutants Syb2(27–116), Syb2(37–116), Syb2(47–116) and Syb2(54–116) were generated by PCR (Yamasaki *et al.*, 1994a). C-terminal deletions were generated by linker insertion using the following oligonucleotides (restriction sites): Syb2(2–71), AGATGTAAGCTTCC, AGCTTACATCTGGA (*Bgl*I); Syb2(2–80), CTAGCATGTGATATCACATG (*Spe*I); and Syb2(2–87), AATGATGTAAGCTTAA, AAGCTTACATCATTTT (*Bsg*I). pSyb2(2–96) was generated by PCR using the following two primers: CACACTCGAGATGTCGGCTACCGCTGCCACC and ACAATCGATTTACATCATCTTGAGGTTTTTCC. Clones encoding the N-terminal deletion mutants Syb2(27–96) or Syb2(37–96) were generated by replacement of the *Xho*I–*Acc*I fragment of pSyb2(2–96) by those of pSyb2(27–116) or pSyb2(37–116), respectively. Deletion mutants of SNAP-25 were generated by PCR using the following 5′ primers: SNAP-25(14–206), CTCTGGATCCATGCAGAGGAGGGCTGACCAG; SNAP-25(25–206), CTCTGGATCCATGTCCCTGGAAAGCACCCGTCGC; SNAP-25(46–206), CTCTGGATCCATGACTTTGGTTATGTTGGATGAG; SNAP-25(36–206), CTCTGGATCCATGGTTGAAGAGAGTAAAGACGCTG and the downstream primer CTCTGAATTCTTAACCACTTCCCAGCATCTTTGTTGC; and SNAP-25(2–82), CTCTGGATCCATGGCCGAAGACGCG and CTCTGAATTCTTAGCCTAAATCCTTTAAATTTTTCTC.

In vitro transcription and translation

Plasmids were linearized downstream from the genes and transcribed *in vitro* and translated in the presence of [^{35}S]methionine (Mayer *et al.*, 1988).

In vitro protein binding studies

For *in vitro* binding studies, 0.2 nmol of GST–syntaxin or GST–SNAP-25 were incubated for 2 h at 4°C (head over head rotation) with 20 µl slurry of glutathione agarose in binding buffer. After six washes with binding buffer, the matrix was resuspended in 400 µl of binding buffer; 3 µl of the *in vitro* translation mixture were added and rotation was continued for 16 h. The matrix was centrifuged and the unbound material (S fraction) in the supernatant was recovered by precipitation with trichloroacetic acid. The matrix was washed six times with 400 µl of washing buffer. Bound material in the pellet was eluted by incubation (30 min at 37°C) with 60 µl of sample buffer (P fraction). For competition assays, incubations were carried out in the presence of various concentrations of the appropriate unlabeled purified ligand. Samples were processed as above and subjected to SDS–PAGE and autoradiography. Intensities of bands were quantified with an LKB model 2202 ultrascan densitometer.

Brain homogenates and cell lysates

Rat brain was homogenized in homogenization buffer. The total homogenate was solubilized by the addition of 1% Triton X-100. Alternatively, rat brain was homogenized directly in sample buffer. Only the soluble fractions were loaded onto the gel. COS cell lysates were prepared in a similar manner except that cracking of cells was achieved by 20 passages through a 21-gauge needle.

Toxin treatment

Standard incubations of *in vitro*-translated synaptobrevin, SNAP-25 or membrane-associated syntaxin have been described previously (Blasi *et al.*, 1993b; Binz *et al.*, 1994; Yamasaki *et al.*, 1994a,b). Cleavage of SNAP-25-His6 or GST–SNAP-25-His6 with DTT-reduced BoNT/A or BoNT/E (200 nM final concentration) was performed in HEPES buffer for 1 h at 37°C. After addition of the same amount of the toxin, incubation was continued for another 1 h and uncleaved material was removed by passage through Ni-nitrilotriacetic acid (NTA)–agarose. For toxin treatment of the ternary complex, GST–syntaxin (0.2 nmol), SNAP-25-His6 (0.6 nmol) and the synthetic 93mer synaptobrevin peptide (0.6 nmol) were pre-assembled on the column (saturating conditions). After six washing steps with washing buffer, the matrix was suspended in 30 µl of binding buffer and one of the following toxin L chains or DTT-reduced holotoxins was added: TeTx (400 nM final concentration), BoNT/D (100 nM) or BoNT/F, BoNT/A or BoNT/E (each 200 nM). Alternatively, IgA protease (1 µM final concentration) was added. After incubation for 1.5 h at 37°C under gentle rotation, the suspension was centrifuged to recover bound material in the pellet and released material in the supernatant fractions. Elution was performed for 30 min at 37°C with 60 µl of sample buffer. To control cleavability of matrix-associated monomers, GST–SNAP-25-His6 or GST–synaptobrevin 2-His6 (0.2 nmol each) were coupled to 20 µl glutathione agarose, washed and incubated with the individual toxins as above for the ternary complex.

SDS–PAGE

SDS–PAGE was carried out as described by Laemmli (1970) using 15% gels.

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