SNAP-25, a t-SNARE Which Binds to Both Syntaxin and Synaptobrevin via Domains That May Form Coiled Coils*

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The membrane proteins SNAP-25, syntaxin, and synaptobrevin (vesicle-associated membrane protein) have recently been implicated as central elements of an exocytic membrane fusion complex in neurons. Here we report that SNAP-25 binds directly to both syntaxin and synaptobrevin. The SNAP-25-binding domain of syntaxin lies between residues 199 and 243, within the region previously shown to mediate synaptobrevin binding (Calakos, N., Bennett, M. K., Peterson, K. E., and Scheller, R. H. (1994) Science 263, 1146-1149). The syntaxin-binding domain of SNAP-25 encompasses most of the amino-terminal half of SNAP-25, including its putative palmitoylation sites. Truncation of the carboxyl-terminal 9 residues of SNAP-25, which yields a fragment corresponding to that generated by botulinum neurotoxin A, diminishes the interaction of SNAP-25 with synaptobrevin, but not with syntaxin. Sequence analysis revealed that the regions that mediate the interaction between SNAP-25 and syntaxin contain heptad repeats characteristic of certain classes of a-helices. Similar repeats are also present at the carboxyl terminus of SNAP-25 and in synaptobrevin. These domains have a moderate to high probability of forming coiled coils. We conclude that SNAP-25 can interact with both syntaxin and synaptobrevin and that binding may be mediated by a-helical domains that form intermolecular coiled-coil structures.

Neurons release their neurotransmitter by regulated exocytosis of synaptic vesicles. Recently, major progress has been made in our understanding of the molecular mechanisms that underlie exocytic membrane fusion (reviewed by Rothman and Warren (1994) and Südhof et al. (1993)). A complex consisting of membrane constituents of both the synaptic vesicle as well as the synaptic plasma membrane is thought to act as the core of an exocytic docking and fusion machine. These components include the synaptic vesicle protein synaptobrevin (also referred to as vesicle-associated membrane protein (Trimble et al., 1988)) and the plasmalemmal proteins syntaxin and SNAP-25 (Bennett et al., 1992; Oyler et al., 1989; Söllner et al., 1993a).

Several lines of evidence indicate that these proteins are essential for exocytosis. First, tetanus and botulinum neurotoxins irreversibly block neuronal exocytosis by selectively cleaving synaptobrevin, syntaxin, or SNAP-25 (reviewed by Huttner (1993) and Niemann et al. (1994)). Second, Söllner et al. (1993a) have shown that a set of soluble proteins, essential for intracellular fusion events, selectively interact with the same set of membrane proteins. These soluble proteins are NSF and α, β, γ-SNAPs. Since SNAPs must bind to membrane receptors before NSF, the receptors have been designated as SNAREs, with the vesicular protein (synaptobrevin) designated as a v-SNARE and the plasmalemmal proteins (SNAP-25 and syntaxin) designated as t-SNAREs. ATP cleavage by NSF appears to dissociate this complex, an event proposed to underlie membrane fusion (Söllner et al., 1993b; Rothman and Warren, 1994). Third, homologues of all three membrane proteins, as well as NSF and α-SNAP, have been identified in yeast, where genetic evidence has implicated each of these proteins in the final steps of intracellular membrane fusion. Each trafficking pathway appears to require its own specific set of SNAREs, whereas the yeast homologues of NSF and α-SNAP are thought to operate in all steps. Together, these findings suggest that the principal mechanisms of exocytic membrane fusion have been highly conserved during evolution (reviewed by Bennett and Scheller (1993) and Ferro-Novick and Jahn (1994)).

Both synaptobrevin and syntaxin are small integral membrane proteins with single putative transmembrane domains located at the carboxyl-terminal ends of their sequences. SNAP-25 does not contain a transmembrane domain, but is palmitoylated, probably at cysteine residues present in the middle of the molecule (Oyler et al., 1989; Hess et al., 1992). In nervous tissue, two isoforms of syntaxin (1A and 1B (Bennett et al., 1992)), synaptobrevin (I and II (Elferink et al., 1989)), and SNAP-25 (A and B (Bark et al., 1993; Bark and Wilson, 1994)) have been characterized. In addition, one synaptobrevin homologue (cellubrevin (McMahon et al., 1993)) and several syntaxin homologues (Bennett et al., 1993) have been identified in non-neuronal cells, indicating that the SNAREs comprise multidomain protein families.

Recent experiments have demonstrated that the neuronal SNARE proteins are complexed in the absence of NSF and SNAPs (Söllner et al., 1993b). Furthermore, a direct interaction between syntaxin and synaptobrevin has been characterized (Calakos et al., 1994). This interaction exhibits a moderate to low affinity, but is selective for individual isoforms of syntaxin: synaptobrevins I and II bind to syntaxins 1 and 4, but not to syntaxin 2 or 3. Therefore, these interactions may contribute to the selective targeting and fusion of membrane-bound vesicles with the appropriate acceptor membrane. However, the role of SNAP-25 in exocytic membrane fusion, while essential (Blasi et al., 1993a), has yet to be defined. Is SNAP-25 a subunit of the t-SNARE syntaxin, perhaps modulating the interaction of syn-

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taxin with v-SNAREs, or can SNAP-25 itself function as a t-SNARE by directly interacting with v-SNAREs?

To address these issues, we have investigated potential interactions between SNAP-25 and other components of the SNARE complex. We demonstrate that SNAP-25 binds to both syntxin and synaptotubin and have examined the structural requirements that underlie these interactions.

MATERIALS AND METHODS

Plasmid Construction and Purification of Recombinant Proteins—cDNA encoding rat SNAP-25 (Blasi et al., 1993b; Blasi et al., 1994) and cDNAs encoding syntxin subclone II (Elferink et al., 1989) and syntxin 1A (Bennett et al., 1993) were kindly provided by T. C. Südhof and R. H. Scheller, respectively. All full-length and truncated coding sequences were amplified using the polymerase chain reaction with oligonucleotides containing appropriate restriction sites for subsequent subcloning into the desired plasmids. For in vitro transcription, SNAP-25 and syntxin were subcloned into CDM8 as described previously (Blasi et al., 1993b). X-100 was added to the lysate cation. PCR products were extracted, precipitated, and added directly to the coupled transcription-translation reaction (described below).

For the production of glutathione S-transferase fusion proteins, syntxin was as full-length and various regions of syntxin and SNAP-25 were subcloned into pGEX-1 or pGEX-2T (Pharmacia Biotech Inc.). JM109 cells (500 ml) expressing glutathione S-transferase or glutathione S-transferase fusion proteins were grown to A660 = 0.4, induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside, and harvested after 2−4 h. Cells were pelleted; resuspended in 10 ml of 20 mM Tris, pH 7.2, 150 mM NaCl (TS buffer) containing protease inhibitors (1 mM phenylmethylsulfonfyl fluoride, 2 μg/ml pepstatin, and 20 μg/ml aprotinin); and sonicated on ice 3 times with a SS34 rotor. The supernatant was mixed with 0.5 ml of 15% phenylmethylsulfonyl fluoride, 2 pg/ml pepstatin, and 20 pg/ml aprotinin; and sonicated on ice 3 x 30 s with a probe sonicator. Triton X-100 was added to the lysate (0.5%) and mixed for 20 min at 4 °C, and the suspension was centrifuged twice at 12,000 rpm for 20 min in an SS34 rotor. The supernatant was mixed with 0.5 ml of a 50% slurry of glutathione-Sepharose 4B (Pharmacia Biotech Inc.) for 30 min at 4 °C with mixing, batch-washed three times with 50 ml of TS buffer, and stored as a 50% slurry in TS buffer at 4 °C. Aliquots were subjected to SDS-PAGE and visualized by Comassie Blue staining, and the concentration of the fusion proteins was determined by comparison with glutathione S-transferase standards.

Full-length syntxin subclone II was obtained by treating the glutathione-One-Pharohase-immobilized fusion protein, derived from pGEX-2T, with 2.5 units of thrombin (Sigma)/100 pl of beads for 1 h at room temperature in TS buffer plus 0.5% Triton X-100 (Guan and Dixon, 1991). The supernatant containing the purified syntxin subclone II was collected, and the thrombin was inhibited by the addition of 1 μM phenylmethylsulfonfyl fluoride.

Full-length recombinant syntaxin 1A was prepared by subcloning into pPrCh1 (Invitrogen), resulting in a fusion protein that contains a C-terminal truncation mutants (Fig. 1, shown in Fig. 3). To map the domain of syntaxin 1A, six different cuts were made between 15 residues of the T7 promoter by subcloning into the EcoRI site of a modified version of CDM8 (Seed, 1987) as described previously (Blasi et al., 1993a, 1993b). Radiolabeled proteins were generated by coupled in vitro transcription-translation using the TuT system (Promega), in the presence of [35S]methionine, according to the manufacturer’s instructions. Full-length syntaxin was translated in the presence of microsomes (Promega) as described previously (Blasi et al., 1993b).

Binding Assays—Binding assays were carried out by adding radiolabeled or purified recombinant proteins to immobilized recombinant fusion proteins. Binding was monitored by SDS-PAGE or fluorography for separation of the beads from the unbound material. Syntaxin 1A quantitatively bound to immobilized SNAP-25, but not to the control beads, which contained only glutathione S-transferase (Fig. 1A), as described previously (Blasi et al., 1993b).

RESULTS

To assay for a direct interaction between syntaxin and SNAP-25, we prepared full-length [35S]Met-labeled syntaxin 1A by in vitro transcription-translation in the presence of microsomes. Labeled syntaxin was mixed with glutathione S-transferase or a GST-SNAP-25 fusion protein immobilized on glutathione-Sepharose beads in the presence of detergent. Binding was analyzed by SDS-PAGE and fluorography following separation of the beads from the unbound material. Syntaxin 1A quantitatively bound to immobilized SNAP-25, but not to the control beads, which contained only glutathione S-transferase (Fig. 1A). Similar results were obtained using translated syntaxin 1B (data not shown). To map the domain of syntaxin 1A that mediates this interaction, we generated a series of carboxyl-terminal truncation mutants (Fig. 1, top) and tested them for binding using the same procedure. As shown in Fig. 1B, truncation of syntaxin 1A to residue 243 did not result in a
loss of binding activity. However, further truncation of the carboxy terminus to residue 221 reduced binding by 60%, and truncation to residue 199 abolished binding.

These data show that the region between residues 199 and 243 of syntaxin is important for binding to SNAP-25. However, these findings do not distinguish whether this domain is sufficient for binding, whether it contains only a part of the binding domain, or whether the deletions caused misfolding of the truncated molecule, resulting in loss of binding activity. To address this issue, we generated a glutathione S-transferase fusion protein that contained residues 199–243 of syntaxin 1A (Fig. 2A). As a control, we also fused the entire cytoplasmic domain of syntaxin 1A to glutathione S-transferase (Fig. 2A). As shown in Fig. 2B, translated SNAP-25 bound, as expected, to the cytoplasmic domain of syntaxin 1A. Furthermore, this interaction was preserved in the 45-amino acid segment of syntaxin.

Together, these data demonstrate that SNAP-25 binds to syntaxin 1A in the absence of other synaptic proteins. Binding was confined to a relatively short linear sequence of syntaxin 1A, within residues 199–243. In the following experiments, we used a similar approach to identify the region of SNAP-25 that mediates its interaction with syntaxin. For this purpose, we carried out systematic carboxyl- and amino-terminal deletion analyses of SNAP-25. In these experiments, the deletion mutants were fused to glutathione S-transferase and immobilized using glutathione-Sepharose. Binding was assayed as described for Fig. 2 above using the [35S]Met-labeled cytoplasmic domain of syntaxin 1A generated by in vitro translation as the ligand.

We first examined the effect of carboxyl-terminal deletions of SNAP-25 on syntaxin 1A binding. As shown in Fig. 3B, syntaxin binding was preserved in a fusion protein composed of the amino-terminal 100 residues of SNAP-25, demonstrating that the binding activity resides in the amino-terminal half of SNAP-25. To further define this binding domain, we examined syntaxin 1A binding to shorter segments of SNAP-25. A carboxyl-terminal deletion to residue 81 diminished binding by 38%, and further truncation to residue 61 abolished binding (Fig. 3B). We then prepared amino-terminal deletion mutants of SNAP-25 that were fused to glutathione S-transferase in order to determine the amino-terminal border of the syntaxin-binding domain (Fig. 4A). Syntaxin binding was preserved in a SNAP-25 mutant lacking the first 20 residues (Fig. 4B), a stretch that is poorly conserved between different species (Risunger et al., 1993). Further amino-terminal deletions of SNAP-25 resulted in the loss of detectable binding activity (Fig. 4B).

The data described so far indicate that the amino-terminal half of SNAP-25 is crucial for its interaction with syntaxin 1A. To confirm this assignment, we compared the abilities of SNAP-25 fusion proteins, consisting of either the amino- or carboxyl-terminal half of the protein (Fig. 5A), to bind syntaxin. For these experiments, we purified recombinant syntaxin 1A from Escherichia coli and carried out immunoblot analysis to assay for binding. As expected, syntaxin 1A bound to the full-length and the amino-terminal half of the molecule, but not to the carboxyl-terminal region (Fig. 5C).

Interestingly, the syntaxin-binding domain of SNAP-25 identified in the experiments described above does not contain the region of SNAP-25 that is cleaved by BoNT/A. BoNT/A, a potent inhibitor of exocytosis, has been shown to act by hydrolyzing...
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SNAP-25 between residues 197 and 198 (Schiavo et al., 1993; Binz et al., 1994), resulting in the loss of the carboxyl-terminal 9 residues. Thus, it is unlikely that BoNT/A cleavage affects the interaction between SNAP-25 and syntaxin. To confirm that the fragment that results from BoNT/A cleavage retains syntaxin binding activity, we prepared a SNAP-25 fusion protein that lacked the last 9 amino acids. As shown in Fig. 5C, residues 1–197 of SNAP-25 bound recombinant syntaxin 1A as well as full-length SNAP-25.

What, then, is affected by BoNT/A cleavage of SNAP-25? It has been shown previously that the third membrane component of the SNARE complex, synaptobrevin, interacts directly with syntaxin, although with a relatively low affinity (Calakos et al., 1994; see the Introduction). Since there is some similarity between the synaptobrevin-binding domain of syntaxin and the carboxyl-terminal domain of SNAP-25 (Risinger et al., 1993), we examined whether synaptobrevin is also capable of binding to SNAP-25 and whether this interaction involves the carboxyl-terminal 9 residues of SNAP-25. As shown in Fig. 5C (lower panel), recombinant synaptobrevin II purified from E. coli bound to full-length GST-SNAP-25 fusion protein immobilized on Sepharose beads. This binding was reduced by 73% when the last 9 amino acids of SNAP-25 were removed even though the amount of this mutant used in the binding experiment was higher than that of the full-length protein (Fig. 5, A and B). These findings show that the carboxyl terminus of SNAP-25 is involved in interactions with synaptobrevin and suggest that the perturbation of these interactions by BoNT/A may underlie...
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**DISCUSSION**

In this study, we have demonstrated that SNAP-25 binds directly to both syntaxin and synaptobrevin. The region of syntaxin that binds to SNAP-25 was localized to residues 199-243. This domain is contained within the region previously shown to mediate the interaction of syntaxin with synaptobrevin (residues 194-267 (Calakos et al., 1994)). Therefore, it is possible that SNAP-25 may modulate the interaction of syntaxin with synaptobrevin and vice versa (discussed in more detail below).

In contrast, the domain of SNAP-25 that binds to syntaxin or synaptobrevin (described in more detail below) could not be confined to such a small region. GST-SNAP-25 fusion proteins containing either residues 1-100 or 21-206 bind syntaxin (Figs. 3B and 4B). However, a fusion protein composed of residues 21-100 interacts only weakly with syntaxin (data not shown). These data indicate that while SNAP-25 residues 21-100 contain crucial determinants for syntaxin binding, additional residues adjacent to these borders influence the binding affinity. Interestingly, the binding domain contains a set of four cysteine residues thought to be modified by palmitoylation (Fig. 6) (Hess et al., 1992). While our work with bacterially expressed proteins clearly demonstrates that palmitoylation is not required for SNAP-25 to bind to syntaxin, it is possible that acylation may modulate the interaction. For instance, palmitoylation of GAP-43 regulates its interaction with the α-subunit of Go (Sudo et al., 1992), and palmitoylation of the β2-adrenergic receptor is required for efficient coupling to Go (O'Dowd et al., 1989).

What types of interactions underlie the binding between syntaxin and SNAP-25? It has been previously reported that syntaxin contains heptad repeats characteristic of α-helices that form coiled-coil structures (Inoue et al., 1992; Spring et al., 1994; Calakos et al., 1994). Coiled coils consist of two or more right-handed α-helices wrapped around one another with a left-handed superhelical twist and are found in a variety of proteins. For instance, the leucine zipper motif, found in a number of DNA-binding proteins, forms intermolecular coiled coils that mediate dimerization (Landschulz et al., 1988; Ellenberger et al., 1992), and coiled coils bundle the α-helices of a number of fibrous proteins, such as tropomyosin and keratin (reviewed by Cohen and Parry (1990)). Amino acids residues within the heptad repeats that form coiled coils are assigned positions a through g. While there are preferences for each residue, the most conspicuous elements in these repeats are hydrophobic amino acids at the α and d positions (Parry, 1982; Lupas et al., 1991). Interestingly, the SNAP-25-binding domain of syntaxin contains six heptad repeats in register (Fig. 6). Analysis of the propensity of these repeats to form coiled coils revealed an average score of 1.25 (Fig. 6). For comparison, a mean score of 1.44 has been reported for the GCN4 leucine zipper sequence (Lupas et al., 1991). According to Lupas et al. (1991), scores above 1.1 generally correspond to regions that form extended amphipathic α-helices in globular proteins, and scores above 1.3 have a high probability of forming coiled-coil structures. Therefore, the heptad repeats in the SNAP-25-binding domain of syntaxin likely to form an amphipathic α-helix with a modest probability of forming coiled coils. Syntaxin contains two additional sets of five and six heptad repeats in register at its amino terminus, with average scores of 1.33 and 1.64, respectively (Fig. 6). While this region is not required for the interaction of syntaxin with synaptobrevin or SNAP-25, it may be involved in oligomerization or binding to other proteins such as Munc-18 (also referred to as RabSec1 or RabSec1 (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994)) or synaptotagmin (Bennett et al., 1992).

The presence of heptad repeats in the SNAP-25-binding domain of syntaxin prompted an evaluation of the primary sequence of SNAP-25. Indeed, the amino-terminal half of SNAP-25, i.e. the region that interacts with syntaxin, contains two

**Fig. 6.** Schematic representation of the regions that mediate the interaction of SNAP-25 with syntaxin. The regions that mediate the interaction between SNAP-25 and syntaxin are delineated with brackets. Sets of multiple heptad repeats in register are delineated with coils and are designated A–C. The syntaxin-binding domain of SNAP-25 contains sets A and B, and the SNAP-25-binding domain of syntaxin contains set C. An analysis of each set of heptad repeats is given in the table. The scores reflect the propensity of these regions to form coiled-coil structures. Scores below 1.3 generally correspond to amphipathic α-helices in globular proteins, and scores above 1.3 have a high probability of forming coiled-coil structures (Lupas et al., 1991). The cleavage site of BoNTA is also indicated. TMR, transmembrane domain. The 4 cysteine residues in SNAP-25 that may serve as palmitoylation sites are also indicated.

| SNAP-25 | syntaxin |
|---------|---------|
| set of heptad repeats | A | B | C | A | B | C |
| sequence of set | 1-42 | 45-60 | 127-208 | 30-64 | 66-115 | 189-231 |
| no. of repeats within set | 6 | 6 | 7 | 5 | 6 | 5 |
| ave. score of set | 1.60 | 1.70 | 1.44 | 1.53 | 1.64 | 1.25 |
sets of six heptad repeats interrupted by a break in frame. These heptad repeats have high probabilities of forming coiled coils, yielding average scores of 1.60 and 1.70 (Fig. 6). Therefore, the association of SNAP-25 with syntaxin may involve the interaction of α-helical domains, perhaps by forming intermolecular coiled-coil structures.

In addition, Dascher et al. (1991) have noted that synaptobrevin and the yeast SLY2 and SLY12 gene products contain regions that, when arranged in an α-helical conformation, may form coiled-coil structures. Applying the algorithm of Lupas et al. (1991), we identified five heptad repeats in register between residues 51 and 88 of synaptobrevin II. The average score of this segment was 1.48 (Fig. 6). Interestingly, these repeats (residues 51–88) are immediately preceded by a region (residues 37–55) with properties similar to those of fusion peptide sequences from viral fusion proteins (Jahn and Südhof, 1994). A similar arrangement between an amphipathic fusion peptide and a coiled-coil motif has been observed in the influenza hemagglutinin fusion protein (Cam and Kim, 1993). In the hemagglutinin glycoprotein, low pH induces a conformational change thought to mediate synaptobrevin binding, since no binding was observed under these conditions, binding is abolished not only between synaptobrevin and the yeast SLY2 and SLY12, but also between the t-SNAREs themselves. Since the individual interactions of syntaxin or SNAP-25 with synaptobrevin appear to be relatively weak, it is possible that SNAP-25-syntaxin heterodimers form high affinity v-SNARE-binding sites that are modular by cyclical association and dissociation of the t-SNAREs. In addition, or alternatively, hetero-oligomerization of SNAP-25 and syntaxin may contribute to the specificity of t-SNARE-v-SNARE interactions. For instance, different combinations of SNAP-25s and syntaxins may bind to different sets of v-SNAREs, reducing the absolute number of t-SNAREs required for selective targeting.

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