Protease Resistance of Syntaxin·SNAP-25·VAMP Complexes

IMPLICATIONS FOR ASSEMBLY AND STRUCTURE*

Michelle A. Poirier‡, Joe C. Hao‡, Per N. Malkus‡, Charles Chan‡, Michael F. Moore§, David S. King‡§, and Mark K. Bennett‡¶

From the ‡Department of Molecular and Cell Biology, §Cancer Research Laboratory, and ¶Howard Hughes Medical Institute, University of California, Berkeley, California 94720

A stable ternary complex formed with vesicle-associated membrane protein 2 (VAMP2) and plasma membrane proteins syntaxin 1A and synaptosome-associated protein of 25 kDa (SNAP-25) is proposed to function in synaptic vesicle exocytosis. To analyze the structural characteristics of this synaptic protein complex, recombinant binary (syntaxin 1A·SNAP-25), recombinant ternary, and native ternary complexes were subjected to limited trypsin proteolysis. The protected fragments, defined by amino-terminal sequencing and mass spectrometry, included a carboxyl-terminal region of syntaxin 1A, the cytoplasmic domain of VAMP2, and amino- and carboxyl-terminal regions of SNAP-25. Furthermore, separate amino- and carboxyl-terminal fragments of SNAP-25, when combined with VAMP2 and syntaxin 1A, were sufficient for stable complex assembly. Analysis of ternary complexes formed with full-length proteins revealed that the carboxyl-terminal transmembrane anchors of both syntaxin 1A and VAMP2 were protected from trypsin digestion. Moreover, the stability of ternary complexes was increased by inclusion of these transmembrane domains. These results suggest that the transmembrane domains of VAMP2 and syntaxin 1A contribute to complex assembly and stability and that amino- and carboxyl-terminal regions of SNAP-25 may function as independent domains.

Neurotransmitter release represents a specialized form of regulated secretion wherein neurotransmitter-containing synaptic vesicles selectively dock and fuse with the plasma membrane. This process constitutes an essential step in chemical synaptic transmission and is a likely target for regulatory reactions that modulate the strength of synaptic signaling. The identification and biochemical characterization of numerous synaptic vesicle and presynaptic plasma membrane proteins have provided fundamental insight into the molecular mechanisms underlying neurotransmitter release (for reviews see Refs 1 and 2).

Among the important findings that have emerged from molecular studies of neurotransmitter release is the central role of a protein complex composed of two presynaptic plasma membrane proteins, syntaxin 1 and synaptosome-associated protein of 25 kDa (SNAP-25), along with one synaptic vesicle protein, vesicle-associated membrane protein 2 (VAMP2; also known as synaptobrevin). Several lines of evidence suggest a primary role for syntaxin 1, SNAP-25, and VAMP2 in synaptic vesicle exocytosis. First, each represents a substrate for cleavage by distinct clostridial neurotoxins, toxins that irreversibly inhibit neurotransmitter release in vivo (3, 4). Furthermore, genetic studies in Drosophila have shown that loss of syntaxin 1 or VAMP2 prevents Ca2+-dependent exocytosis (5). Finally, the ternary complex formed by all three proteins acts as a receptor for soluble N-ethylmaleimide-sensitive factor (NSF) attachment proteins (SNAPs), cytosolic proteins required in multiple membrane trafficking events (6). Consequently, the syntaxin 1·SNAP-25·VAMP2 ternary complex is commonly referred to as the synaptic SNAP receptor (SNARE) complex, and its individual components are generally termed SNARE proteins.

Recent biochemical studies have shown that syntaxin 1, SNAP-25, and VAMP2 interact directly to form both binary and ternary complexes (7–12). The ternary complex is noteworthy because of its unusual stability, as judged by resistance to dissociation in the presence of the denaturing detergent SDS (9). Several lines of evidence support the potential biological relevance of the SDS-resistant state. First, temperature-sensitive SDS-resistant complexes are present in brain homogenate directly solubilized with SDS (9). In addition, the SDS-resistant SNARE complex is a bona fide receptor for α-SNAP and NSF (13). Finally, ternary SNARE complex assembly is unaffected by clostridial neurotoxin cleavage of individual SNAREs, yet SDS-resistance is compromised (9). These observations suggest that SDS-resistant ternary complexes may exist in vivo and could be required for neurotransmitter release at the nerve terminal. In vitro binding studies utilizing deletion mutants of syntaxin 1A, SNAP-25, and VAMP2 have demonstrated that the following critical regions are required for binary and ternary SNARE complex formation (7–11, 14): (i) a carboxyl-terminal, membrane-proximal region of syntaxin 1A (sufficient for both binary and ternary interactions with VAMP2 and SNAP-25); (ii) the cytoplasmic domain of VAMP2 (sufficient for both binary and ternary interactions with syntaxin 1A and SNAP-25); (iii) an amino-terminal region of SNAP-25 (sufficient for binary interaction with syntaxin 1A and for ternary complex assembly); and (iv) a carboxyl-terminal region of SNAP-25 (required in conjunction with the amino-terminal region of

*This work was supported by National Institutes of Health Grant GM 51313 and the McKnight Fund for Neuroscience. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: SNAP-25, synaptosome-associated protein of 25 kDa; VAMP2, vesicle-associated membrane protein 2; NSF, N-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; NTA, nitroblue tetrazolium; TPCK, 1-tosylamide-2-phenylethylchloromethyl ketone; AEBSP, 4-(2-aminoethyl)benzene-sulfonyl fluoride; GST, glutathione S-transferase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; RP-HPLC, reversed-phase HPLC.
SNAP-25 for interaction with VAMP2 and stabilization of the ternary complex). Interestingly, within these critical regions are multiple heptad repeat domains that are predicted to form an α-helical coiled coil (15), a protein interaction motif that can generate a variety of stable structures (16). This suggests that a coiled-coil structure may contribute to the assembly and stability of SNARE complexes.

Although the domains of syntaxin 1A, SNAP-25, and VAMP2 necessary and sufficient for pairwise and ternary interactions have been well characterized in recent years, the minimal interacting regions within binary and ternary complexes have not been defined. Even less is known regarding the role of syntaxin 1A and VAMP2 carboxyl-terminal transmembrane domains in SNARE complex assembly. In the present study, we have utilized limited proteolysis of binary and ternary synaptic SNARE complexes to (i) identify the minimal interacting regions within binary and ternary complexes, (ii) demonstrate that these minimal regions are sufficient for assembly of stable, SDS-resistant ternary complexes, and (iii) characterize the role of the carboxyl-terminal transmembrane anchors of syntaxin 1A and VAMP2 in SNARE complex assembly and stability. The results presented provide further insight into synaptic SNARE complex structure.

**EXPERIMENTAL PROCEDURES**

**Materials**—A mouse monoclonal antibody against rat VAMP2 was obtained from Chemicon International (Temecula, CA). A rabbit polyclonal antibody against a carboxy-terminal mouse SNAP-25 peptide (amino acids 195–206) was acquired from StressGen Biotechnologies (Victoria, British Columbia, Canada). Rabbit polyclonal antiserum against rat syntaxin 1A, rat VAMP2 cytoplasmic domain, and mouse SNAP-25 were generated, and HPC-1 (anti-syntaxin 1A) ascites fluid was supplied by Berkeley Antibody (Richmond, CA). Molecular biology reagents were purchased from Stratagene (La Jolla, CA), New England Biolabs (Beverly, MA), and Boehringer Mannheim. Bovine serum albumin, human thiobolin, glutathione-agarose, TPA-treated bovine pancreas trypsin, and reagent grade chemicals were obtained from Sigma. 4-(2-Aminothienyl)benzenesulfonyl fluoride (AEBSF) was purchased from ICN Biochemicals (Costa Mesa, CA). Nickel-nitrilotriacetic acid (NTA)-agarose and M15/pREP4 cells were obtained from Qiagen (Chatsworth, CA)

**Plasmid Construction**—Two vector systems were used for expression of recombinant fusion proteins in *Escherichia coli*. The pGEX-KG vector (17), a derivative of the pGEX-2T vector (Pharmacia Biotech, Uppsala, Sweden), allows for expression of recombinant proteins fused with glutathione S-transferase (GST) at the amino terminus. The pQE-30 vector (Qiagen) allows for expression of recombinant proteins with an N-terminal histidine (His6) tag (His6).

Plasmids encoding GST fusion proteins of full-length rat syntaxin 1A (amino acids 4–288), syntaxin 1A cytoplasmic domain (amino acids 4–266), syntaxin 1A carboxy-terminal membrane proximal fragment (amino acids 191–266; syntaxin 1A-16), full-length mouse SNAP-25 (amino acids 1–206), rat VAMP2 cytoplasmic domain (amino acids 1–94), and a VAMP2 cytoplasmic domain internal deletion mutant (amino acids 1–91, A41–50) were previously described (7, 10, 12, 18). A plasmid encoding a GST-SNAP-25 amino-terminal fusion protein (amino acids 1–95) was prepared by digesting the full-length clone with HindIII (which cuts both in the SNAP-25 insert and at the 3′-end of the pGEX-KG polylinker), followed by religation. Plasmids encoding GST-SNAP-25 (carboxyl terminus; amino acids 125–206), GST-VAMP2 (full-length; amino acids 1–116), His-SNAP-25 (amino acids 1–206), and His-syntaxin 1A-16 were prepared by insertion of the appropriate thiogalactopyranoside for that at 0 °C; (iii) following cell lysis of GST-VAMP2 (amino acids 1–116) and GST-syntaxin 1A bacterial cultures, the lysates were incubated at 4 °C for 30 min prior to centrifugation; (iv) during glutathione-agarose affinity chromatography, 10 mM methionine was included in the PBST (phosphate-buffered saline, 0.05% Tween 20) and 200 μM AEBSF was added to inhibit trypsin cleavage (250 mM NaCl, 2.5 mM CaCl2). DTT was used in the thrombin cleavage buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 25 mM CaCl2). (v) DTT was used for purification of proteins retaining the transmembrane domains (VAMP2 (amino acids 1–116) and syntaxin 1A (amino acids 4–288)), all buffers were supplemented with 0.5% Triton X-100.

**His6 fusion protein expression in E. coli**—strain M15/REP4 (His6-SNAP-25; His6-syntaxin 1A-16) was induced with 0.1 M isopropyl-β-D-thiogalactopyranoside at A600 = 0.5 (His6-SNAP-25) or at A600 = 0.6 (His6-syntaxin 1A-16). The bacterial lysate for His6-SNAP-25 was prepared as described previously (19), while the lysate for His6-syntaxin 1A-16 was prepared as described above. His6 fusion proteins were purified by incubation with nickel-NTA-agarose in nickel binding buffer (20 mM HEPES, pH 7.5, 500 mM KCl, 10% glycerol, 50 mM imidazole, 10 mM methionine, and 1 mM DTT) at 4 °C for 1 h. Fusion proteins were eluted from the nickel-NTA resin with nickel elution buffer (nickel binding buffer supplemented with 250 mM imidazole).

Protein concentrations were estimated using BCA reagent (Pierce) or by Coomassie Blue staining of protein bands after SDS-PAGE with bovine serum albumin as a standard for both assays.

**Trypsin Proteolysis Studies**—Trypsin-digested recombinant proteins (5 μg each) in thrombin cleavage buffer supplemented with 10 mM methionine and 1 mM DTT were incubated overnight at 4 °C and divided into three or four aliquots, depending on the number of trypsin conditions to be analyzed. Trypsin-treated protein in PBST was added to achieve a trypsin:total protein ratio of 1:500, 1:200, 1:50, or 1:25. Digests were carried out for 1 h at room temperature and stopped by the addition of AEBSF. Samples were incubated with nickel beads (in the presence of 1 mM DTT) for 1 h at 4 °C. The beads were washed extensively with PBST-Met, and bound proteins/protein complexes were eluted with SDS-PAGE sample buffer. Protein fragments remaining in the supernatant (lacking the His6 tag) were isolated by trichloroacetic acid precipitation. The samples were either boiled or heated at 37 °C, resolved by SDS-PAGE, and visualized by Coomassie staining or Western blotting.

**Reversed-phase HPLC (RP-HPLC) Purification and Mass Spectrometry Analysis of Trypsin-digested Fragments**—Trypsin-digested SNARE complexes were eluted from nickel-NTA-agarose resin with PBST-Met supplemented with 1 mM DTT and 300 mM imidazole. To resolve the protected fragments, samples were subjected to RP-HPLC. Binary complex (1:200 trypsin condition) and ternary complex (1:25 trypsin condition) samples were resolved on a C4 column (2.1 mm diameter; Vydac, Hesperia, CA) with a flow rate of 175 μl/min. After a 10-min wash at 5% B, the samples were eluted with a gradient from 5 to 95% B over 90 min. Buffer A contained 0.1% trifluoroacetic acid, 0.2% acetonitrile/H2O, and buffer B contained 0.1% trifluoroacetic acid/acetonitrile. Eluted protein fragments were detected by monitoring A200 and analyzed either by SDS-PAGE and Western blotting or by electrospray ionization mass spectrometry (Hewlett Packard 5969A) and amino-terminal sequencing.

**Immunoprecipitation**—Anti-syntaxin 1 monoclonal antibody (HPC-1; 300 μg of Ig) was bound to protein G-Sepharose beads (0.5 ml) and cross-linked with 20 mM dimethylmethylene. The antibody beads (9.2 ml) were then mixed with either Triton X-100 solubilized rat brain membranes (LP1; 12 mg of total protein) or recombinant ternary complex (55–60 μg of total protein) for 2 h at 4 °C. The beads were washed extensively with PBST containing 0.5% Triton X-100, and syntaxin 1-containing complexes were eluted with 0.1 M glycine, pH 2.0, containing 0.5% Triton X-100. The eluate was neutralized with 1 M Tris-HCl, pH 8.0, and the protein concentration was determined. The immunoprecipitated complexes were subjected to trypsin digestion (1:200 trypsin:synaptotagmin:total protein) and analyzed by SDS-PAGE and Western blotting.

**Temperature Sensitivity Analysis of Trypsin-digested SDS-resistant SNARE Complexes**—To test the heat lability of SDS-resistant SNARE complexes, trypsin-digested samples (1:200 trypsin condition) were divided into 11 aliquots and incubated at 50–100 °C in 5 °C increments for 3 min using a programmable thermal cycler (Bio-Rad Laboratories, Watertown, MA). Samples were resolved by SDS-PAGE and analyzed by Western blotting using a rabbit anti-syntaxin 1A antibody.

**SDS-PAGE and Western Blotting**—SDS-PAGE was performed by the method of Laemmli (20). Western blots were probed with one of the following antibodies: mouse anti-syntaxin 1A (1:5000, HPC-1) (Berkley Antibody), rabbit anti-syntaxin 1A (1:1000), rabbit anti-SNAP-25 (SNAP-25(N)) (1:50,000), rabbit anti-SNAP-25 (19S-206) (SNAP-25(C))
H, heptad repeat region. TM, transmembrane domain. CCCC, cysteine residues palmitoylated within SNAP-25. Numbers indicate amino acid boundaries of heptad repeat regions and transmembrane domains.

(1:4000) (StressGen Biotechnologies), mouse anti-VAMP2 (1:4000) (Chemicon International), and rabbit anti-VAMP2 (1:1000). The blots were processed with a chemiluminescence detection system (Renaissance enhanced luminal reagent (NEN Life Science Products)).

RESULTS

Assembly of Binary and Ternary SNARE Complexes Results in Protection from Trypsin Digestion—Domain mapping studies using recombinant full-length SNARE proteins and deletion mutants (7–12) have demonstrated that the minimal interacting regions required for assembly of binary and ternary SNARE complexes include heptad repeat domains present in syntaxin 1A (H3), VAMP2 (H1 and H2), and SNAP-25 (H1, H2, and H3; Fig. 1). In this study, we have used limited proteolysis to further delineate the regions required for complex formation.

Before analyzing the protease sensitivity of SNARE complexes, we determined whether the individual SNARE proteins have intrinsic resistance to trypsin. His6-syntaxin 1A-16 (amino acids 191–266), His6-SNAP-25 (amino acids 1–206), and VAMP2 (amino acids 1–94) were treated with increasing concentrations of trypsin. Digests were processed with a chemiluminescence detection system (Fig. 2A), and those lacking a His6 tag were isolated by precipitation of the nicked bead supernatant with trichloroacetic acid (Fig. 2B). Samples were resolved by SDS-PAGE and analyzed by Western blotting with antibodies recognizing (i) syntaxin 1A; (ii) the carboxy-terminal 12 amino acids of SNAP-25; (iii) the amino-terminal region of SNAP-25; and (iv) VAMP2. Each of the individual proteins was digested at low trypsin concentrations (Fig. 2, A and B), although an amino-terminal fragment of SNAP-25 was partially protected (Fig. 2B). This amino-terminal SNAP-25 fragment is distinct from those protected in either binary or ternary SNARE complexes (see below), and its resistance to proteolysis may be the consequence of recombinant protein aggregation.

The protease sensitivity of complexes containing His6-syntaxin 1A-16 was analyzed by treating preformed complexes with increasing concentrations of trypsin. The complexes were isolated by nickel affinity chromatography and resolved by SDS-PAGE. The total protein pattern was visualized by Coomassie staining, and individual proteins were identified by Western blotting. Treatment of a His6-syntaxin 1A-16/SNAP-25 binary complex with low concentrations of trypsin (1:500 and 1:200) resulted in the protection of a single fragment derived from syntaxin 1A and separate amino- and carboxy-terminal fragments of SNAP-25 (Fig. 3A). In contrast, binary complexes generated with VAMP2 and either syntaxin 1A or SNAP-25 were not resistant to trypsin digestion (data not shown). Treatment of the His6-syntaxin 1A-16/SNAP-25/VAMP2 ternary complex with trypsin resulted in a pattern of syntaxin 1A and SNAP-25 protection similar to that observed for the binary complex (Fig. 3B). However, protection was maintained even at the highest trypsin concentration (1:50), and the syntaxin 1A-16 and SNAP-25 carboxy-terminal fragments each displayed a lower electrophoretic mobility relative to the binary complex protected fragments. Furthermore, VAMP2 was largely protected from proteolysis when assembled in a ternary complex (Fig. 3B). These results demonstrate that the His6-syntaxin 1A-16/SNAP-25 binary complex is partially resistant to proteolysis, and this resistance is further increased following the addition of VAMP2 to form the ternary SNARE complex.

To more precisely define the protected regions of each protein, the protease-resistant fragments generated by trypsin digestion were analyzed by RP-HPLC, amino-terminal sequencing, and mass spectrometry. A ternary complex not exposed to trypsin (PBST control) was also analyzed in order to distinguish between trypsin proteolysis and potential proteolysis during expression and purification of the recombinant proteins (see Table I). Separation of the trypsin-digested ternary complex by RP-HPLC yielded four sets of peaks (Fig. 4A). SDS-PAGE and Western blot analysis demonstrated that each peak consisted of a single prominent species (Fig. 4B) corresponding to one of the protected core fragments (Fig. 4C). Amino-terminal sequence and mass spectrometry analyses were carried out to further define the amino- and carboxy-terminal boundaries of the protected fragments and to deduce the nearest internal protected trypsin sites (Table I). As summarized in Fig. 5, the protected fragments ranged in size from 70 to 94 amino acids and included, but were not limited to, all of the heptad repeat domains within the SNARE proteins analyzed. To determine whether trypsin protection extends beyond the amino-terminal boundary of syntaxin 1A-16, we examined the syntaxin 1A fragment generated following trypsin treatment of a ternary SNARE complex incorporating syntaxin 1A cytoplasmic domain (amino acids 4–266). The protected syntaxin 1A fragment included the 22 amino acids preceding the start of syntaxin 1A-16 but did not include either of the first two heptad repeat domains of syntaxin 1A (Fig. 5).
... The HPLC-purified, trypsin-digested His<sub>6</sub>-syntaxin 1A-16·SNAP-25 binary complex components revealed that a similar set of syntaxin 1A and SNAP-25 fragments were protected (Table I). However, the binary complex protected fragments displayed greater amino- and carboxyl-terminal heterogeneity. These observations further suggest that the structure of the ternary complex is more stable and compact than that of the syntaxin 1A-16·SNAP-25 binary complex.

A Central Region of SNAP-25 Is Required for Multimerization of SDS-resistant Ternary SNARE Complexes—One of the unique properties of both native and recombinant ternary SNARE complexes is their resistance to denaturation in SDS (9, 10, 13). We have analyzed the effect of trypsin treatment on the temperature sensitivity and multimerization of SDS-resistant ternary SNARE complexes. To carry out these analyses, control and trypsin-treated (1:50) samples were incubated at increasing temperatures in SDS-PAGE sample buffer prior to separation by SDS-PAGE. In the absence of trypsin, anti-syntaxin 1A Western blotting revealed three prominent SDS-resistant ternary complexes (with apparent molecular masses of 50, 100, and 200 kDa), all of which were dissociated at >70 °C (Fig. 6A). The 50-kDa complex corresponds to a heterotrimer (composed of syntaxin 1A, SNAP-25, and VAMP2), while the higher molecular weight complexes represent SDS-resistant multimers of this heterotrimer (9, 10). Following trypsin digestion, a single 32-kDa SDS-resistant complex was observed with a temperature sensitivity similar to the non-trypsin-treated complexes. This result demonstrates that trypsin digestion does not compromise the stability of the SDS-resistant SNARE complex but does abolish its ability to form multimers.

The loss of SNARE complex multimerization following trypsin treatment could result from digestion of (i) the amino-terminal region of syntaxin 1A (amino acids 4–157); (ii) the central region of SNAP-25 (amino acids 95–124); or (iii) both of these regions. To distinguish between these possibilities, we analyzed SDS-resistant complex assembly with a set of syntaxin 1A and SNAP-25 deletion mutants. A carboxyl-terminal fragment of syntaxin 1A (syntaxin 1A-16) yielded SDS-resistant multimers when combined with SNAP-25 and VAMP2 (Fig. 6B), demonstrating that the amino terminus of syntaxin 1A is not required for SNARE complex multimerization. In contrast, only the lowest M<sub>r</sub> SDS-resistant complex was observed when separate amino- and carboxyl-terminal SNAP-25 fragments were combined with syntaxin 1A and VAMP2 (Fig. 6C and D). These observations suggest that separate amino- and carboxyl-terminal fragments of SNAP-25 can promote the assembly of
an SDS-resistant SNARE complex but that intact SNAP-25 is essential for forming multimers.

Formation of a Stable SDS-resistant Ternary Complex Is Required for Protection from Trypsin Digestion—Previous studies have demonstrated that ternary SNARE complexes can exist in SDS-resistant or SDS-sensitive states (9, 10). To examine the relationship between SDS resistance and trypsin protection, we have utilized SNAP-25 and VAMP2 deletion mutants incapable of forming SDS-resistant complexes. As shown in Fig. 7, an amino-terminal fragment of SNAP-25 formed a ternary complex with syntaxin 1A-16 and VAMP2 (as judged by the similar recovery of VAMP2 when compared with full-length SNAP-25 (see also Ref. 10)) that was sensitive to both SDS denaturation and trypsin digestion. This trypsin sensitivity was observed with anti-syntaxin 1A and anti-VAMP2 antibodies (Fig. 7) as well as with an anti-SNAP-25 antibody (data not shown). The addition of a SNAP-25 carboxy-terminal fragment restored both SDS resistance and trypsin protection. Similarly, a VAMP2 internal deletion mutant (Δ41–50) that forms SDS-sensitive ternary complexes with syntaxin 1A-16 and SNAP-25 (Fig. 7 and Ref. 10) was not protected from trypsin digestion. Taken together, these results suggest that a correlation exists between the formation of a stable SDS-resistant ternary complex and protection from trypsin proteolysis.

The Transmembrane Domains of Syntaxin 1A and VAMP2 Are Preserved following Trypsin Proteolysis and Promote Ternary SNARE Complex Stability—Since previous studies of SNARE complex assembly have focused primarily on syntaxin 1A and VAMP2 proteins lacking their carboxyl-terminal transmembrane domains, little is known about the contribution of these domains to ternary SNARE complex structure and stability. We have examined the trypsin sensitivity of native complexes immunoprecipitated from a rat brain homogenate and recombinant complexes formed with full-length versions of syntaxin 1A (amino acids 4–266), VAMP2 (amino acids 1–116), and SNAP-25 (amino acids 1–94) (9, 10). To examine the mobility shift that occurs in SNAP-25 of the transmembrane domain in these ternary complexes, we have performed amino-terminal sequencing of the protected fragments derived from native and recombinant full-length syntaxin 1A. As shown in Table I, the mobility shift for syntaxin 1A cytoplasmic domain, amino-terminal sequencing of the syntaxin 1A-derived fragments demonstrated that they share a common amino terminus (starting at amino acid 159; Fig. 5), suggesting that the difference in their mobilities is due to retention of the carboxyl-terminal transmembrane anchor. Furthermore, the absence of a mobility shift for native or recombinant full-length VAMP2 following trypsin digestion of SNARE complexes indicates that the carboxyl-terminal transmembrane domain of VAMP2 is retained. Assembly of ternary SNARE complexes incorporating just one full-length protein (either syntaxin 1A or VAMP2) also resulted in protection of the transmembrane domain (data not shown). These results suggest that carboxyl-
terminal trypsin sites accessible in ternary complexes assembled with the cytoplasmic domains of syntaxin 1A and VAMP2 are inaccessible when full-length versions of either or both proteins are used (Table I and Fig. 5).

To examine whether the transmembrane domains of syntaxin 1A and VAMP2 contribute to ternary SNARE complex stability, we have analyzed the temperature sensitivity of ternary complexes in which syntaxin 1A and VAMP2 components include or lack their respective transmembrane domains. Trypsin-treated complexes were heated at increasing temperatures in SDS-PAGE sample buffer prior to electrophoresis and Western blotting. The stability of native complexes (Fig. 9A) and recombinant complexes containing full-length syntaxin 1A and VAMP2 (Fig. 9B) was comparable, with full dissociation occurring above 90 °C. Reduced stability was observed for recombinant ternary complexes lacking the transmembrane region of either VAMP2 (Fig. 9C) or syntaxin 1A (Fig. 9D). A further decrease in stability was observed with a recombinant complex lacking both syntaxin 1A and VAMP2 transmembrane domains (Fig. 9E). Moreover, SDS-resistant multimers were observed with transmembrane domain-containing ternary complexes even following trypsin treatment (and consequent digestion of the central region of SNAP-25). These data demonstrate that the transmembrane domains of syntaxin 1A and VAMP2 both promote multimerization and enhance the stability of the SDS-resistant ternary complex.

**DISCUSSION**

In the present work, we describe trypsin proteolysis studies aimed at further characterizing the minimal interacting domains within binary and ternary SNARE complexes. We have observed that syntaxin 1A/SNAP-25 binary and ternary SNARE complexes are partially protected from trypsin digestion. The protected fragments include a syntaxin 1A carboxyl-terminal region adjacent to the membrane anchor, SNAP-25 amino- and carboxyl-terminal regions, and the cytoplasmic domain of VAMP2. When present, the transmembrane domains of syntaxin 1A and VAMP2 are also protected from digestion. Moreover, the protected regions include heptad repeat domains within the three SNARE proteins, supporting a coiled-coil mechanism for SNARE complex assembly.

Exposure of the individual SNARE proteins to trypsin revealed that the cytoplasmic domain of VAMP2 and the carboxyl terminus of SNAP-25 display no inherent trypsin resistance, consistent with their lack of secondary structure (21, 22). Syntaxin 1A-16, estimated to have 80% α-helical content (14) and...
observed to be trimeric in solution, was only minimally protected from proteolysis. Unexpectedly, an amino-terminal fragment of SNAP-25, previously shown to have minimal secondary structure (23), was resistant to trypsin degradation. This protease protection may result from aggregation of recombinant SNAP-25 in solution.2

The protease protected fragments derived from a syntaxin 1A-16-SNAP-25 binary complex are not coincident with the minimal interacting regions identified in domain mapping studies. Protection of syntaxin 1A extended well beyond the H3 domain (amino acids 189–231) and beyond the minimal region implicated in SNAP-25 binding (amino acids 189–220 (14)). SNAP-25 protection included both amino- and carboxyl-terminal regions of the protein, the former of which is necessary and sufficient for syntaxin 1A binding (8, 9). Thus, although the carboxyl terminus of SNAP-25 is not required for a pairwise interaction with syntaxin 1A, it appears to be an elemental constituent of the syntaxin 1A-16-SNAP-25 binary complex. Consistent with a role for the carboxyl terminus of SNAP-25 in syntaxin 1A-SNAP-25 binary complex assembly, a carboxyl-terminal fragment of SNAP-25 has recently been shown to interact with a complex composed of syntaxin and the amino terminus of SNAP-25 (23). Analysis of the proteolyzed binary complex fragments also revealed the presence of syntaxin 1A-16 species lacking the His6 tag used to isolate the trypsin-digested complexes. A recently reported complex consisting of two syntaxin 1A molecules per SNAP-25 molecule (22) might explain such a finding. Cleavage of the His6 tag from one syntaxin 1A-16 component in a 2:1 complex would leave one His6 tag for binary complex purification.

Relative to the syntaxin 1A-SNAP-25 binary complex, the syntaxin 1A-16-SNAP-25-VAMP2 ternary complex exhibited more extensive protection of individual fragments and resistance to higher trypsin concentrations. These observations extend previous results demonstrating that the ternary SNARE complex is protected from the proteolytic action of clostridial neurotoxins (9) and suggest that protease resistance is a general property of the complex. The prospect that the ternary SNARE complex is a highly stable structure is further supported by two additional properties: thermal stability (22) and resistance to SDS denaturation (9). We have demonstrated that SDS-resistance is not compromised by trypsin proteolysis, that the electrophoteric mobility of the SDS-resistant complex following trypsin treatment (~32 kDa) is significantly greater than predicted from the sum of its component parts (>40 kDa), and that the four protease protected components of the ternary SNARE complex are sufficient for SDS-resistant complex assembly. These findings indicate that a compact and stable structure, perhaps consisting of a multi-stranded coiled-coil, constitutes the core of the ternary SNARE complex and that the protease protected amino- and carboxyl-terminal fragments of SNAP-25 represent physically distinct modules that function together in promoting SNARE complex stability.

The current studies have also revealed that the carboxyl-terminal transmembrane domains of both syntaxin 1A and VAMP2 are retained following trypsin digestion of native and recombinant ternary SNARE complexes. Importantly, trypsin cleavage sites that are accessible in the absence of the transmembrane anchors are inaccessible in their presence. Although these hydrophobic domains are not essential for complex assembly, they significantly increase the stability of SDS-resistant ternary SNARE complexes, suggesting that the membrane-anchoring domains of syntaxin 1A and VAMP2 are closely associated within the ternary SNARE complex. How might the
transmembrane domains enhance protease protection and complex stability? One possibility is that these hydrophobic regions may influence the conformation of the respective cytoplasmic domains. The protease resistance and temperature stability of ternary complexes generated with a single transmembrane domain (provided by either syntaxin 1A of VAMP2) is consistent with this possibility, as is the influence of the transmembrane domains of syntaxin 1A and VAMP2 on other protein-protein interactions (24). Alternatively (or additionally), the transmembrane domains of syntaxin 1A and VAMP2 might interact directly to enhance SNARE complex stability. Such a scenario would require syntaxin 1A and VAMP2 to be both aligned in a parallel fashion and anchored in the same membrane, as has been recently demonstrated (25–27). Further studies will be required to establish the influence of membrane-anchoring regions on SNARE complex structure and function.

Multimerization has been observed with native SDS-resistant ternary complexes as well as with recombinant ternary complexes generated with bacterially expressed proteins (9, 10, 13). Although a function for SNARE complex multimerization has not been established, it is worth noting that a well-characterized membrane fusion protein, influenza hemagglutinin, functions as a homotrimer and that multiple homotrimers participate in the fusion event (28). Our results demonstrate that the central domain of SNAP-25 plays an important role in ternary complex multimerization. This region of SNAP-25 may support the formation of higher order SDS-resistant SNARE complex multimers by linking ternary complexes directly or by connecting amino- and carboxyl-terminal domains of a single SNAP-25 incorporated into separate ternary complexes. A second mechanism of multimerization can be attributed to the carboxyl-terminal transmembrane domains of syntaxin 1A and VAMP2. It is possible that one or both hydrophobic transmembrane anchors from a single ternary complex interact directly with those from a different ternary complex to promote multimerization.

Previous studies have demonstrated that not all ternary SNARE complexes are SDS-resistant. SDS-sensitive ternary complexes can be generated with SNARE proteins subjected to clostridial neurotoxin proteolysis (9, 13), with internal deletion mutants of VAMP2 (10), or with SNAP-25 mutants lacking the carboxyl-terminal domain. In the present work, we have shown that SDS-sensitive ternary complexes are protease-sensitive. This observation indicates that SDS-sensitive ternary complexes are structurally distinct and suggests that they may represent an intermediate between more stable binary and SDS-resistant ternary complexes. Since SDS-resistant ternary complexes can form when the SNARE proteins are anchored in the same lipid bilayer (26) (a putative pre docking or postfusion complex), it is tempting to speculate that the SDS-sensitive ternary complex, if it were to exist in vivo, might correspond to a complex in which the SNARE proteins are anchored in opposing membranes (a putative docking complex). If this were the case, a transition between SDS-sensitive and SDS-resistant conformations might play a role in promoting the membrane fusion reaction. Such a scenario would provide an explanation for the impaired function of SNAREs whose capacity to form SDS-resistant complexes is compromised (3, 4, 9, 10, 13, 29). Further experiments will be required to characterize the membrane-anchoring topology and functional significance of SDS-sensitive ternary SNARE complexes.

Our results demonstrate that the interacting regions within syntaxin 1A, SNAP-25, and VAMP2 defined by trypsin proteolysis are larger than the heptad repeat domains and, in all cases, are larger than the regions deemed sufficient for binding. In addition, we have established that the transmembrane anchors of syntaxin 1A and VAMP2 influence the structure and stability of the ternary SNARE complex. These findings provide the foundation for future high resolution structural studies of binary and ternary SNARE complexes. Such studies will further our understanding of how these complexes are assembled and may provide insight into conformational changes underlying synaptic vesicle docking and/or fusion in vivo.

Acknowledgments—We thank Lino Gonzalez, Jr. for molecular mass calculations, and all potential tryptic fragments, for critical reading of the manuscript, and for valuable discussions. We thank Xiao-Rong Peng for helpful discussions throughout the course of this work.

REFERENCES

1. Bennett, M. K., and Scheller, R. H. (1994) Annu. Rev. Biochem. 63, 63–100
2. Sudhof, T. C. (1995) Nature 375, 645–653
3. Schiavo, G., Rossetto, O., and Montecucco, C. (1994) Semin. Cell Biol. 5, 221–229
4. Niemann, H., Blasi, J., and Jahn, R. (1994) Trends Cell Biol. 4, 179–185
5. Brodie, K., Prokop, A., Bellen, H. J., O’Kane, C. J., Schulze, K. L., and Sweeney, S. T. (1995) Neuron 15, 663–673
6. Sollner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H., and Rothman, J. E. (1995) Cell 79, 409–418
7. Calakos, N., Bennett, M. K., Peterson, K. E., and Scheller, R. H. (1994) Science 263, 1146–1149
8. Chapman, E. R., An, S., Barton, N., and Jahn, R. (1994) J. Biol. Chem. 269, 27427–27432
9. Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Sudhof, T. C., and Niemann, H. (1994) EMBO J. 13, 5551–5561
10. Hao, J. C., Salem, N., Peng, X-R., Kelly, R. B., and Bennett, M. K. (1997) J. Neurosci. 17, 1596–1603
11. Kee, Y., Lin, R. C., Hsu, S.-C., and Scheller, R. H. (1995) Neuron 14, 991–998
12. Pevenaer, J., Hsu, S.-C., Braun, J. E., Calakos, N., Ting, A. E., Bennett, M. K., and Scheller, R. H. (1994) Neuron 13, 353–361
13. Pellegrini, L. L., O’Conner, V., Lotzepeich, F., and Betz, H. (1995) EMBO J. 14, 4705–4713
14. Zhang, P., Chen, Y. A., Tam, D., Chung, D., Scheller, R. H., and Miljanich, G. P. (1997) Biochemistry 36, 4317–4326
15. Lupas, A., Van Dyke, M., and Stock, J. (1991) Science 252, 1162–1164
16. Lupas, A. (1996) Trends Biochem. Sci. 21, 375–382
17. Guan, K., and Dixon, J. E. (1991) Anal. Biochem. 192, 262–267
18. Bennett, M. K., Calakos, N., and Scheller, R. H. (1992) Science 257, 255–259
19. Bennett, M. K., Miller, K. G., and Scheller, R. H. (1995) J. Neurosci. 15, 1701–1707
20. Laemml, U. (1970) Nature 227, 680–685
21. Corniffe, F., Deloye, P., Fouraux-Zaluzki, M. C., Roques, B.-P., and Poulan, B. (1995) J. Biol. Chem. 270, 16826–16832
22. Fasshauer, D., Otto, H., Eliaison, W. K., Jahn, R., and Brunger, A. T. (1997) J. Biol. Chem. 272, 28036–28041
23. Fasshauer, D., Bruner, D., Shen, B., Jahn, R., and Brunger, A. T. (1997) J. Biol. Chem. 272, 4582–4590
24. Hanson, P. I., Otto, H., Barton, N., and Jahn, R. (1995) J. Biol. Chem. 270, 16955–16961
25. Hanson, P. I., Roth, R., Morisaki, H., Jahn, R., and Heuser, J. E. (1997) Cell 90, 523–535
26. Otto, H., Hanson, P. I., and Jahn, R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6197–6201
27. Lin, R. C., and Scheller, R. H. (1997) Neuron 19, 1087–1094
28. Danielli, T., Pelletier, S. L., Henis, Y. I., and White, J. M. (1996) J. Cell Biol. 133, 559–569
29. Regazzi, R., Sadoul, K., Meda, P., Kelly, R. B., Halban, P. A., and Wollheim, C. B. (1996) EMBO J. 15, 6951–6959

3 P. N. Malkus and M. K. Bennett, unpublished observations.