An α-Helical Minimal Binding Domain within the H3 Domain of Syntaxin Is Required for SNAP-25 Binding†

Pingyu Zhong,‡ Yu A. Chen,§ Deborah Tam,‡ David Chung,‡ Richard H. Scheller,§ and George P. Miljanich*,‡

Neurex Corporation, 3760 Haven Avenue, Menlo Park, California 94025, and Department of Molecular and Cellular Physiology, Howard Hughes Medical Institute, Stanford University, Stanford, California 94305

Received October 9, 1996; Revised Manuscript Received January 30, 1997⊗

ABSTRACT: The interaction between the proteins syntaxin 1A and SNAP-25 is a key step in synaptic vesicle docking and fusion. To define the SNAP-25 binding domain on syntaxin, we have prepared peptides that span the syntaxin H3 domain (residues 191−266), the region previously shown to be important for binding to SNAP-25, and then determined the affinities of these peptides for binding to SNAP-25. A minimal binding domain was identified within a region of 32 amino acids (residues 189−220). Its affinity for SNAP-25 is substantially enhanced by C-terminal extension (residues 221−266). Circular dichroism revealed the presence of substantial α-helicity in the H3 domain and in the 32-mer minimal binding domain, but not in H3 peptides that do not bind to SNAP-25. At temperatures that denature the α-helix of the minimal binding domain peptide, SNAP-25 binding is lost. Selected mutations in evolutionarily conserved residues of the amphiphilic α-helix within the minimal binding domain (e.g., residues 205 and 209) greatly reduce the affinity for SNAP-25 but have no major effect on secondary structure, suggesting that these residues may interact directly with SNAP-25. The H3 domain peptide and the minimal binding domain peptide inhibit norepinephrine release from PC12 cells. These results suggest that specific amino acid residues in the H3 domain, positioned by the underlying α-helical structure, are important for its binding to SNAP-25 and support the notion that this interaction is important for presynaptic vesicular exocytosis.

Neurons communicate with each other by secreting neurotransmitters from membranous vesicles in presynaptic nerve terminals. The molecular mechanisms by which synaptic vesicles are directed to release sites and undergo exocytosis upon calcium entry have been extensively studied over the past few years, leading to the identification of many proteins that play critical roles in this process. The emerging theme from these studies is that vesicular docking and fusion are mediated through a series of dynamic protein−protein interactions (Söllner et al., 1993a,b; Scheller, 1995) that provide specificity for vesicle targeting and the machinery for docking and membrane fusion (Bennett & Scheller, 1994; Jahn & Südhof, 1994). As proposed by a current model for vesicle docking and fusion, a 7S complex composed of two synaptic vesicle proteins (vSNAREs),[1] VAMP (also known as synaptobrevin) and synaptotagmin, and two plasma membrane proteins (tSNAREs), SNAP-25 and syntaxin, is assembled. The binding between vSNAREs and tSNAREs brings the vesicle within close proximity of the release sites at the plasma membrane and thus serves as the molecular docking mechanism. Subsequent binding of the cytosolic proteins, αSNAP and NSF, concurrent with a loss of synaptotagmin, results in the formation of a 20S complex composed of syntaxin, SNAP-25, VAMP, αSNAP, and NSF (Söllner et al., 1993a,b). Hydrolysis of ATP by NSF activates the complex, leading to membrane fusion through unknown intermediates (Morgan et al., 1994). Thus, the interaction between SNAP-25 and syntaxin appears to be of critical importance in the vesicle docking and fusion process. This conclusion is supported by *in vitro* binding studies. SNAP-25 binds to syntaxin with moderate affinity *in vitro* (Pevsner et al., 1994). Similarly, binding of VAMPII to syntaxin is substantially stabilized by the presence of SNAP-25, leading to a nearly 10-fold increase in affinity (Pevsner et al., 1994). Interestingly, this potentiation is syntaxin isoform-specific; VAMPII binding to syntaxin 1A, the major neuronal isoform of syntaxin, can be enhanced in dramatic fashion compared to that of other syntaxin isoforms (Pevsner et al., 1994), consistent with the expression of SNAP-25 predominantly in neurons (Bark & Wilson, 1993; Bark et al., 1995). It is, therefore, reasonable to postulate that the syntaxin−SNAP-25 interaction is crucial for promoting the docking of VAMP-containing vesicles at plasmalemmal release sites with high affinity and high specificity.

The SNAP-25 binding region on syntaxin is located within the H3 domain (amino acids 191−266), which is next to the C-terminal membrane anchor (Figure 1). The H3 domain mediates not only SNAP-25 binding but also the binding of syntaxin to VAMP, αSNAP, synaptotagmin, and, in part, n-sec1. Within the H3 domain, the N-terminal portion (amino acids ∼191−221) is likely to contain the SNAP-25 binding site (Chapman et al., 1994; Kee et al., 1995).

† Y.A.C. was supported by a predoctoral fellowship from the Howard Hughes Medical Institute.

* Correspondence to Dr. George P. Miljanich, Neurex Corp., 3760 Haven Ave., Menlo Park, CA 94025. Telephone: 415-833-1218. Fax: 415-853-1538.

‡ Neurex Corp.

§ Stanford University.

⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1997.

[1] Abbreviations: CD, circular dichroism; GST, glutathione *S*-transferase; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; NE, norepinephrine; NSF, *N*-ethylmaleimide-sensitive fusion factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; vSNARE, vesicle SNARE; tSNARE, target SNARE; SNAP-25, synaptosomal-associated protein of 25 kDa; VAMP, vesicle-associated membrane protein.

S0006-2960(96)02540-8 CCC: $14.00

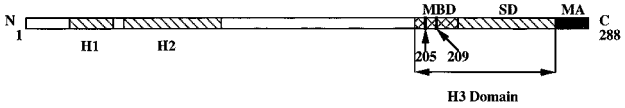

FIGURE 1: Functional domains of syntaxin 1A. A schematic representation of various functional domains of syntaxin 1A is illustrated. The three regions, H1−H3, are predicted to be amphiphilic α-helices. Within the H3 domain, a minimal binding domain (MBD) for SNAP-25 and a supporting domain (SD) are located N-terminally and C-terminally, respectively. Leucine 205 and isoleucine 209 are likely to be key residues for the binding of SNAP-25. The last 20 amino acid residues of syntaxin serve as a membrane anchor (MA).

Sequence analysis of the H3 domain predicts a strong propensity for α-helical formation. Further analysis reveals a repeating pattern of hydrophobic amino acids within the helical heptad repeats characteristic of amphiphilic α-helices (Inoue et al., 1992; Spring et al., 1993). That is, within each set of seven amino acids, $(abcdefg)_n$, positions *a* and *d* are regularly occupied by hydrophobic residues. These amino acids commonly form an interface for protein−protein interactions through coiled coils. Consistent with their functional importance, positions *a* and *d* constitute the most evolutionarily conserved sites in the H3 domain. Although direct evidence for the existence of amphiphilic α-helicity in the H3 domain and its relationship to SNAP-25 binding has not been previously reported, one expects that altering the specific sequence in the H3 domain responsible for SNAP-25 binding or disrupting the α-helical structure presumed to be required for this binding would interfere with syntaxin−SNAP-25 interaction. Kee et al. (1995) demonstrated that a syntaxin 1A mutant containing point mutations at amino acid residues 198, 202, 205, and 209, which are at *a* and *d* positions in the predicted heptad repeats, has significantly reduced SNAP-25 binding activity. However, protein−protein interactions in this and other studies were assayed by SDS−PAGE and Western blot, which provide only a qualitative measure of affinity. Furthermore, it is not known whether the loss of SNAP-25 binding activity of these syntaxin mutants is in fact due to the alteration of the primary sequence alone or to disruption of the putative underlying helical structure.

In the present study, we focus on further defining the molecular features of syntaxin 1A binding to SNAP-25. A family of peptides spanning different sequences of the H3 domain of syntaxin 1A was prepared through chemical synthesis or recombinant expression and partial protease digestion. The affinities of these peptides for SNAP-25 were determined in a quantitative *in vitro* binding assay. On the basis of this analysis, a putative minimal SNAP-25 binding domain spanning amino acids 189−220 was identified. It was also found that a supporting domain located in the region C-terminal to the minimal binding domain significantly enhances the affinity of syntaxin for SNAP-25 but, by itself, does not bind to SNAP-25. To investigate the secondary structural requirements for the binding, we studied the solution structure of the SNAP-25 binding domain of syntaxin with circular dichroism (CD) spectroscopy. These measurements revealed that substantial amphiphilic α-helical structure is present in the H3 domain and in the minimal binding domain and strongly suggested that the helical structure is necessary for syntaxin−SNAP-25 binding. In addition, selected point mutations in evolutionarily conserved residues of the putative amphiphilic α-helix within the minimal binding domain greatly reduce the affinity for SNAP-25 without affecting the secondary structure. Finally, the affinities of the H3 domain peptides for SNAP-25 are correlated with their potencies for inhibiting norepinephrine secretion from PC12 cells. These results support the notion that the interaction between syntaxin and SNAP-25 is required for presynaptic vesicle exocytosis.

EXPERIMENTAL PROCEDURE

Recombinant Protein Preparation. GST (glutathione S-transferase) fusion protein constructs of syntaxin 1A11 (residues 4−266), SP191−266 (residues 191−266), SP220−266 (residues 220−266), and syntaxin 1A11 point mutants (m1−m6, m12, m13, m34, and m35) were subcloned in the expression vector pGEX-KG. Syntaxin 1A11 is derived from syntaxin 1A and lacks the first three N-terminal amino acids and the C-terminal membrane anchor. Previous studies have shown that syntaxin 1A11 contains sufficient sequence for binding to a number of exocytotic proteins, including SNAP-25 (Pevsner et al., 1994; Chapman et al., 1994; Calakos et al., 1994; Hayashi et al., 1994; Kee et al., 1995; Kee & Scheller, 1996). Positions of the point mutations are as follows: m1 (R198A and I202A, i.e., Arg198 and Ile202 were mutated to Ala), m2 (L205A and I209A), m3 (L212A and F216A), m4 (V223A and Q226A), m5 (I230A and I233A), m6 (A240V and V244A), m12 (R198A, I202A, L205A, and I209A), m13 (R198A, I202A, L212A, and F216A), m34 (L212A, F216A, V223A, and Q226A), and m35 (V223A, Q226A, I230A, and I233A). Purification was performed by binding the fusion proteins to a glutathione−agarose column followed by thrombin cleavage as previously described (Pevsner et al., 1994; Kee et al., 1995). For competition binding assays, protein concentrations were determined by SDS−PAGE and scanning densitometry. For circular dichroism (CD) spectroscopy, and PC12 cell secretion assays, proteins from the glutathione−agarose column and thrombin cleavage were further purified by reverse phase HPLC. Protein concentrations were determined by amino acid analysis.

Preparation of Peptide Fragments of the H3 Domain. Peptide sequences and preparative sources are listed in Table 1. Synthetic peptides SP189−220, SP204−220, SP186−211, SP209−240, and SP238−266 were synthesized on the basis of the sequence of the H3 domain of syntaxin 1A. P1, whose sequence is unrelated to the H3 domain, was used as a control peptide. Solid phase synthesis of the peptides was performed on an Applied Biosystems 430A peptide synthesizer, starting from *p*-methylbenzhydrylamine resin. Peptides were cleaved from the resin with hydrogen fluoride and purified by reverse phase HPLC. Peptide concentrations were determined by amino acid analysis. For peptide fragments from partial protease digestion, a panel of peptides of different sizes was prepared by partial digestion (chymotrypsin, 100:1 protein:enzyme by weight, 37 °C for 20 min) of recombinant SP191−266, or of synthetic peptides described above, followed by fractionation by reverse phase HPLC. The purity of each peptide preparation was confirmed by analytical HPLC. The concentration of each purified peptide was determined by amino acid analysis.

In Vitro Transcription and in Vitro Translation. A 0.6 kb *Bam*HI fragment of rat SNAP-25 cDNA was ligated into pBluescript KSII$^-$ to generate RNA using T7 polymerase.

Table 1: Sequences of Syntaxin 1A H3 Domain-Related Peptides[a]

Code	amino acid sequence	Source
	191 201 * * 211 221 231 241 251 261	
SP191-267:	ALSEIETRHS EIIKLENSIR ELHDMFMDMA MLVESQGEMI DRIEYNVEHA VDYVERAVSD TKKAVKYQSK ARRKKII	F
	.....	
SP186-211:	SISKQ ALSEIETRHS EIIKLENSIR E	S
SP209-240:	IR ELHDMFMDMA MLVESQGEMI DRIEYNVEHA	S
SP238-267:	EHA VDYVERAVSD TKKAVKYQSK ARRKKII	S
SP206-216:	ENSIR ELHDMF	S/C
SP189-205:	KQ ALSEIETRHS EIIKL	S/C
SP189-216:	KQ ALSEIETRHS EIIKLENSIR ELHDMF	S/C
SP189-220:	KQ ALSEIETRHS EIIKLENSIR ELHDMFMDMA	S/C
SP204-220:	KLENSIR ELHDMFMDMA	S/C
SP204-216:	KLENSIR ELHDMF	S/C
SP217-220:	MDMA	F/C
SP191-216:	ALSEIETRHS EIIKLENSIR ELHDMF	F/C
SP220-267:	A MLVESQGEMI DRIEYNVEHA VDYVERAVSD TKKAVKYQSK ARRKKII	F
SP223-235:	VESQGEMI DRIEY	F/C
SP236-243:	NVEHA VDY	F/C
SP244-257:	VERAVSD TKKAVKY	F/C
SP236-257:	NVEHA VDYVERAVSD TKKAVKY	F/C
SP191-222:	ALSEIETRHS EIIKLENSIR ELHDMFMDMA ML	F/C
SP191-235:	ALSEIETRHS EIIKLENSIR ELHDMFMDMA MLVESQGEMI DRIEY	F/C
SP191-253:	ALSEIETRHS EIIKLENSIR ELHDMFMDMA MLVESQGEMI DRIEYNVEHA VDYVERAVSD TKK	F/C

[a] The peptides prepared from fusion protein (F), chemical synthesis (S), or partial chymotrypsin digestion (C) of either a fusion protein or a synthetic peptide are illustrated. The nomenclature for the peptides is based on their positions in the sequence of syntaxin 1A; i.e., the H3 domain is named SP191−266 for the syntaxin peptide spanning residues 191−266. The extent of the SNAP-25 minimal binding domain (189−220) is indicated by dots. Asterisks indicate the most important amino acid residues (205 and 209) for SNAP-25 binding.

The RNA template was used to perform *in vitro* translation in the presence of [^{35}S]methionine. The specific activity of the ^{35}S-labeled SNAP-25 was about 14 000 Ci/mmol.

Scintillation Proximity Binding Assay. A simple microtiter plate binding assay employing scintillation proximity assay methodology was developed to study synaptic vesicle protein−protein interactions. Syntaxin GST fusion protein diluted in 50 mM Tris at pH 8.0 was first coated onto wells of a Scintistrip, a microtiter plate in which scintillant is covalently incorporated, by incubation overnight at 4 °C. The remaining nonspecific binding sites were blocked by incubation in 0.01% BSA for 1 h at 4 °C. [^{35}S]Methionine-labeled SNAP-25 generated through *in vitro* translation was diluted in binding buffer [20 mM Hepes (pH 7.4), 120 mM potassium acetate, 2 mM EDTA, and 0.05% Tween 20], and aliquots of the diluted labeled SNAP-25 were added into the precoated and preblocked wells to reach a final concentration of approximately 0.03 nM. The binding incubation was performed in the presence of a soluble test competitor at room temperature for 1.5 h, except where indicated otherwise. Finally, the microtiter plate was washed in cold buffer (0.05% Tween 20 replaced by 5% glycerol in the binding buffer) to remove the soluble ligand and free [^{35}S]methionine. After air-drying, bound [^{35}S]SNAP-25 was determined using a 96-well liquid scintillation counter (Wallac Microbeta). For all experiments, the total bound counts per minute in the absence of competing ligand was approximately 5% of the total specific counts per minute loaded. Data were expressed as a percentage of maximal binding determined in the absence of competitor.

CD Spectroscopy. CD spectra were obtained at 0 °C using an Aviv 60 DS spectropolarimeter. Samples were dissolved to a concentration of approximately 0.03−0.06 mM in 1 mM potassium phosphate at pH 7.0 and 100 mM potassium fluoride for spectroscopic studies or 1 M NaCl and 1 mM sodium borate, sodium phosphate, and sodium citrate at pH 7.0 for thermal denaturation studies. Mean residue ellipticity is defined as the ellipticity normalized by the molar concentration of amino acid residues of a tested peptide. Total ellipticity is defined as the ellipticity normalized by the molar concentration of a tested peptide. Thermal denaturation was studied at 222 nm with step increases (2−5 °C) in temperature. Melting point (T_m) is defined as the temperature at which 50% of the α-helical structure is denatured; i.e., mean residue ellipticity at 222 nm decreases to 50% of the maximal value obtained at 0 °C.

[^{3}H]Norepinephrine (NE) Secretion Assay in PC12 Cells. Procedures to prepare permeabilized PC12 cells by "cell

cracking" are described in detail elsewhere (Hay & Martin, 1992). In brief, PC12 cells labeled with [^{3}H]NE were permeabilized by a single passage through a stainless steel ball homogenizer. One to two million cracked cell ghosts were then added to KGlu buffer [20 mM Hepes (pH 7.2), 120 mM potassium glutamate, 20 mM potassium acetate, 2 mM EGTA, and 0.1% BSA] containing 2 mM ATP, rat brain cytosol (~1 mg/mL final protein concentration), 1 μM free calcium, and a test compound as indicated, for 1 h of preincubation on ice. NE release was triggered by raising the temperature to 22 or 35 °C. Secretion was terminated after 15 min by chilling on ice followed by centrifugation at 2000 *g* for 40 min. The released [^{3}H]NE in the supernatant was quantified by scintillation counting. Pellets were solubilized in 1% Triton X-100 and similarly counted. [^{3}H]NE release is expressed as a percentage of the total cellular [^{3}H]NE: [100([^{3}H] in supernatant)]/([^{3}H] in supernatant + [^{3}H] in pellet).

Materials. [^{35}S]Methionine and [^{3}H]NE were purchased from Amersham. Kits for *in vitro* translation and *in vitro* transcription were purchased from Promega (San Diego, CA). Glutathione−agarose beads, glutathione (reduced form), human plasma thrombin, and chymotrypsin were purchased from Sigma Chemicals (St. Louis, Mo). Scintistrip microtiter plates were purchased from Wallac Inc. (Turko, Finland).

RESULTS

Minimal SNAP-25 Binding Domain of Syntaxin 1A. The syntaxin−SNAP-25 binding assay relies on the ability of radioactive label bound to the well wall of a microtiter plate impregnated with scintillant to produce photons; unbound radiolabeled ligand will not. In the present study, ^{35}S-radiolabeled SNAP-25 is brought into proximity of the scintillant by specific binding to the GST−syntaxin 1A11 fusion protein that is preimmobilized on the well wall.

The specificity of binding of SNAP-25 to syntaxin in this assay was evaluated in two ways. In the first, the binding assay was performed in the presence of increasing concentrations of soluble SNAP-25. The IC_{50} for competition by the soluble SNAP-25 was found to be 0.45 ± 0.03 μM (mean ± SE). This value is in good agreement with the previously reported K_d value for SNAP-25 binding to syntaxin 1A (0.4 μM; Pevsner et al., 1994). In the second, the binding assay was performed in the presence of increasing concentrations of either soluble syntaxin 1A11 or SP191−266 (the intact H3 domain, Figure 1 and Table 1). These proteins bind to [^{35}S]SNAP-25 to form soluble complexes which are removed during the wash following incubation. Thus, the presence of soluble syntaxin peptide reduces the amount of [^{35}S]SNAP-25 binding to the syntaxin 1A11 fusion protein immobilized on the well wall in a concentration-dependent manner. The IC_{50} for the competition by syntaxin or syntaxin peptide can be used as a relative measure of its affinity for SNAP-25 (Table 2). The level of nonspecific binding, determined by the residual counts per minute in the presence of saturating concentrations of SNAP-25, syntaxin 1A11, or SP191−266, accounted for approximately 15% of the total binding observed in the absence of competitor.

Semiquantitative studies previously showed that a C-terminally truncated syntaxin 1A that contains amino acids 4−221 binds to SNAP-25, whereas another truncated syntaxin 1A that is only 31 residues shorter on the C-terminal side does not (Kee et al., 1995). Moreover, the H3 domain of syntaxin 1A (amino acids 191−266) was shown to have SNAP-25 binding activity (Kee et al., 1995). On the basis of these results, it was postulated that a putative binding domain for SNAP-25 resides approximately within the region from amino acids 191 to 221 of syntaxin. However, more quantitative studies of the syntaxin−SNAP-25 interaction are required to define this binding domain at a higher resolution. Furthermore, it is not clear whether this region by itself is sufficient for SNAP-25 binding, whether a smaller fragment within this region is sufficient, or what contribution the additional C-terminal region of the H3 domain makes to syntaxin−SNAP-25 interactions. Thus, a number of synthetic peptides and chymotryptic fragments of the H3 domain were generated and tested for their affinities for [^{35}S]SNAP-25 in the competition binding assay (Figure 2 and Table 2). Peptides SP189−220 and SP191−222 are both 32-mers and overlap the putative binding domain [amino acids 191−221, from Pevsner et al. (1994) and Kee et al. (1995)]. SP186−211 and SP209−240 partially overlap the putative binding domain and contain sequences either N-terminal or C-terminal, respectively, to the region containing the putative SNAP-25 binding domain. SP191−216, SP204−220, and its chymotryptic fragments were produced in order to more

Table 2: SNAP-25 Binding Affinities of Peptides of the H3 Domain and Syntaxin 1A Mutants[a]

H3 peptides	IC_{50} (μM)	syntaxin 1A mutants	IC_{50} (μ)
SP191−266	0.043 ± 0.004	1A11	0.062 ± 0.008
SP186−211	>100	m1	0.35 ± 0.05
SP209−240	>100	m2	1.7 ± 0.28
SP238−266	>100	m3	0.43 ± 0.04
SP189−205	>100	m4	0.09 ± 0.002
SP189−216	>100	m5	0.22 ± 0.04
SP189−220	2.7 ± 0.5	m6	0.04 ± 0.005
SP204−220	>100	m12	2.2 ± 0.33
SP191−216	>100	m13	3.5 ± 0.47
SP191−222	4.6 ± 0.9	m34	1.2 ± 0.47
SP191−235	0.18 ± 0.025	m35	0.24 ± 0.03
SP191−253	0.05 ± 0.006		
SP220−266	>100		

[a] Binding affinities were determined as described in Experimental Procedures. Maximal binding in the absence of competitor (control) was approximately 3000−4000 cpm, whereas the background binding was about 15% of the control. IC_{50}'s were calculated using computerized curve fitting. The data are expressed as the mean ± sd ($n \geq 3$).

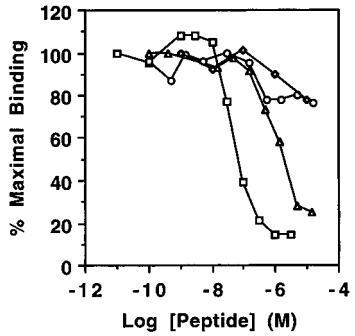

FIGURE 2: Minimal SNAP-25 binding domain of syntaxin 1A. [^{35}S]SNAP-25 binding to the immobilized GST−syntaxin 1A11 fusion protein was performed in the presence of increasing concentrations of competing peptides: (square) SP191−266, (triangle) SP189−220, (circle) SP204−220, and (diamond) SP191−216. The data are presented for one representative experiment ($n \geq 3$, maximal standard deviation of <10% of the mean).

precisely pinpoint critical epitopes within the putative binding domain. Furthermore, SP238−266 and its related peptides and SP220−266 were generated to encompass various epitopes in the C-terminal region and the entire C-terminal region, respectively, outside the putative binding domain. Finally, an orderly increase in the size of the putative binding domain was generated by increasing the length of the peptide in the C-terminal direction (SP189−220, SP191−235, SP191−253, and SP191−266, the entire H3 domain).

As shown in Figure 2 and Table 2, a minimal binding domain for SNAP-25, a sequence that substantially overlaps the region of amino acids 191−221, is sufficient for SNAP-25 binding. SP189−220, the 32-mer peptide that spans amino acid 189−220, inhibits [^{35}S]SNAP-25 binding to immobilized syntaxin 1A11 with an IC_{50} of 2.7 ± 0.5 μM. Another peptide within this region (SP191−222; see Table 2) appears to have a somewhat decreased affinity. SP204−220, a 17-mer peptide that has a sequence identical to the C-terminal portion of the minimal binding domain, has a very low level of activity, suggesting a requirement for the N terminus of this domain. On the other hand, peptides containing the N-terminal sequence alone, e.g., SP191−216, did not displace [^{35}S]SNAP-25 binding. IC_{50}'s for all peptides assayed are given in Table 2. Note that SP189−216 lacks only the last four amino acids (MDMA) of SP189−220 but is inactive. Taken together, these results show that only peptides that contain virtually the entire core of the 32-mer region are active. This 32-mer from amino acids 189−220 is therefore the minimal binding domain for the interaction of syntaxin and SNAP-25.

While the minimal binding domain (residues 189−220) is an effective competitor of [^{35}S]SNAP-25 binding, it is about 50-fold less potent than SP191−266 (IC_{50} = 0.043 ± 0.004 μM), which represents the entire H3 domain (Figure 2 and Table 2). This observation is consistent with previous studies demonstrating that truncation of the C-terminal region results in a substantial reduction of SNAP-25 binding (Chapman et al., 1994). In peptide SP191−235 (Figure 2), 15 additional residues extend C-terminally from the 32-mer minimal binding domain. This extension enhances the affinity for SNAP-25 by more than 10-fold (Table 1). Extension C-terminally by 33 residues (SP191−253) increases the affinity to that of SP191−266 and syntaxin 1A11 (Table 2). In order to define whether the C-terminal region contains additional binding sites for SNAP-25, peptides that represent the sequence of this region were tested for [^{35}S]SNAP-25 competitive binding. Interestingly, all peptides spanning various C-terminal epitopes of the H3 domain (SP209−240, etc.) and the peptide spanning the entire C-terminal region (SP220−266) but lacking amino acids 189−220 fail to bind to SNAP-25. Therefore, it appears that the C-terminal portion of the H3 domain is not sufficient for SNAP-25 binding.

Previous studies have also demonstrated that the H3 domain is required for binding of syntaxin 1A to other SNARE proteins such as VAMPII (Kee et al., 1995). Interestingly, a heterotrimeric complex can be formed among syntaxin 1A, SNAP-25, and VAMPII (Pevsner et al., 1994). Using the binding assay described above, we have observed that SP191−266, and to a lesser extent SP189−220, enhanced [^{35}S]SNAP-25 binding to the immobilized VAMPII−GST fusion protein in a concentration-dependent manner (data not shown). This suggests that the SNAP-25 minimal binding domain is also involved in the binding of syntaxin to VAMPII.

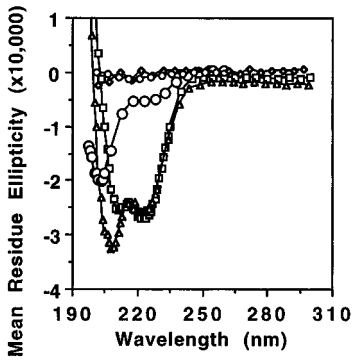

FIGURE 3: CD spectroscopic analysis of the H3 domain and related peptides. CD spectra were measured as described in Experimental Procedures. Data are expressed as mean residue ellipticities (millidegrees per centimeter per residue per micromolar × 10 000): (square) SP191−266, (triangle) SP189−220, (circle) SP204−220, (diamond) SP191−216, and (small circle) SP220−266.

SNAP-25 Binding and the α-Helical Content of the H3 Domain. Sequence analysis predicts that the H3 domain of syntaxin 1A contains substantial α-helical content (Inoue et al., 1992; Spring et al., 1993). In the present study, experiments were directed toward answering the following three questions regarding the relationship of the α-helical structure of the H3 domain to its SNAP-25 binding activity. (1) Does the predicted α-helical structure in the H3 domain in fact exist? (2) If there are α-helices in the H3 domain, how does the 32-mer domain contribute to this α-helicity? (3) Is α-helical structure required for SNAP-25 binding? To answer these questions, circular dichroism (CD) spectroscopy was performed to analyze the secondary structures of SP191−266 and related peptides.

(1) Is the H3 domain α-helical as predicted? As predicted, the H3 domain (SP191−266) yields a CD spectrum typical of an α-helical conformation. That is, a characteristic double peak with maxima at 208 and 222 nm is observed (Figure 3). In parallel, SP189−220, the SNAP-25 minimal binding domain, and SP191−235, a peptide containing the minimal binding domain and a C-terminal extension, also show the presence of α-helicity. Similar CD spectra were observed for SP191−222 and SP191−253 (data not shown). The CD intensity at 222 nm suggests that SP189−220 is ∼70% helical and SP191−235 and SP191−266 are ∼80% helical. In sharp contrast, little α-helix was found in SP204−220 and SP191−216, inactive peptides lacking only short N-terminal or C-terminal sequences of the minimal binding domain, respectively.

(2) How does the 32-mer minimal binding domain contribute to the formation of the α-helix of the H3 domain? Surprisingly, compared to the high helical content of SP191−266, minimal α-helix was found in the peptide that spans the entire C terminus of H3 but lacks the 32-mer minimal binding domain (SP220−266, Figure 3). Other smaller C-terminal peptides (e.g. SP238−266) also show insignificant α-helical content (data not shown). Therefore, the C-terminal region of the H3 domain cannot adopt an α-helical conformation independent of the minimal binding domain, even though its amino acid sequence is enriched in helix-forming amino acids and contains the seven heptad repeats typical of amphiphilic α-helices.

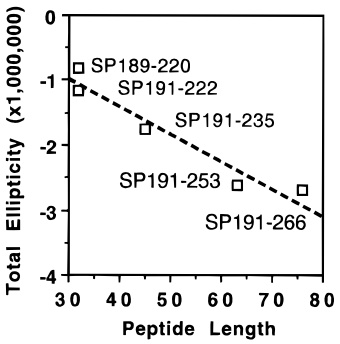

FIGURE 4: Length-dependent helicity of H3 domain peptides. The relationship between total ellipticities at 222 nm (millidegrees per centimeter per micromolar × 1 000 000) and peptide length (number of amino acid residues) is plotted. The regression line was drawn by computerized linear fitting. Square symbols from the top are SP189−220, SP191−222, SP191−235, SP191−253, and SP191−266.

If the C-terminal region fails to form α-helices, one might predict that addition of such a nonhelical sequence to a helical sequence, i.e., the 32-mer minimal binding domain, would dilute the mean residue ellipticity of the peptide containing the combined sequences. However, the mean residue ellipticity at 222 nm is maintained (Figure 3) when the sequence of the 32-mer minimal binding domain was extended C-terminally from SP189−220 to SP191−235, to SP191−253, and then to the entire H3 domain (SP191−266). Thus, it appears that addition of C-terminal amino acids to the 32-mer minimal binding domain results in recruitment of additional helix from the C-terminal sequence, even though such a sequence, by itself, is unable to form α-helix. In support of this notion, a plot of total ellipticity versus peptide length is approximately linear (Figure 4). These results suggest that the C-terminal sequence requires the 32-mer minimal binding domain to be helical and, further, that the helicity of the entire H3 domain is dependent upon the integrity of the 32-mer region.

(3) Is the α-helical structure required for SNAP-25 binding? If α-helical structure is required for SNAP-25 binding, the temperature dependence of the α-helicity of H3 domain peptides should correlate with the temperature dependence of their SNAP-25 binding. To test this hypothesis, we first measured the thermal stability of the α-helicity of each peptide of interest. In these thermal denaturation experiments (Figure 5), the ellipticity of each peptide tested was recorded at 222 nm while the temperature was increased in steps from 0 to 80 °C and the melting temperature (T_m) was then calculated. Of those tested, the least stable α-helix was found to be SP191−222, with a T_m of 27 °C. T_m's for SP189−220, SP191−235, and SP191−253 are approximately the same (T_m = 35 °C). Interestingly, the α-helix of the intact H3 domain (SP191−266) is further stabilized (T_m = 54 °C) compared to SP191−253 by addition of 13 amino acids at the C terminus. Although all denaturation curves are well fit by sigmoidal functions, the intact H3 domain exhibits higher apparent cooperativity compared to the minimal binding domain peptide (SP189−220). The α-helical content in all peptides tested can be fully recovered by cooling (data not shown).

Next, several peptides of interest were subjected to the [^{35}S]SNAP-25 competition binding assay at 0, 22, and 35 °C (Figure 6). Within such a temperature range, the α-helicity of the syntaxin 1A is maintained (data not shown).

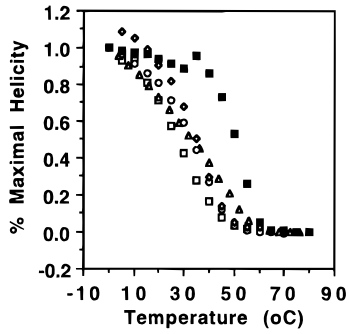

FIGURE 5: Differential stability of α-helicity of the H3 domain and related peptides. Thermal denaturation of α-helicity was determined as described in Experimental Procedures. Ellipticity was recorded at 222 nm at temperatures from 0 to 80 °C with 2−5 °C step increases. The data are expressed as a percentage of maximal mean residue ellipticity at 222 nm measured at 0 °C: (square) SP191−222, (triangle) SP189−220, (circle) SP191−235, (diamond) SP191−253, and (filled square) SP191−266.

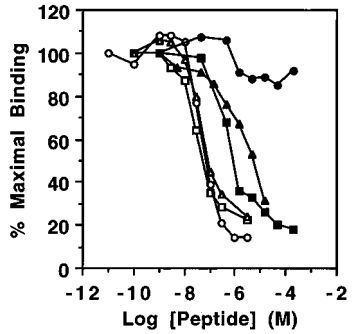

FIGURE 6: Effect of temperature on the binding of H3 domain peptides to SNAP-25. [^{35}S]SNAP-25 binding to the immobilized fusion protein of GST−syntaxin 1A11 was performed in the presence of various concentrations of soluble SP191−266 (open symbols) or SP189−220 (closed symbols) at 0 °C (square), 22 °C (triangle), and 35 °C (circle), respectively. The data are from one representative experiment ($n \geq 3$, maximal standard deviation of <10% of the mean).

Furthermore, its binding to [^{35}S]SNAP-25 is not degraded as the temperature is increased to 35 °C. As predicted, the IC_{50} for the intact H3 domain, which contains thermally stable α-helix, is essentially the same at all three temperatures. In contrast, SP189−220, which loses 50% of its helical content at 35 °C, significantly loses its ability to bind to SNAP-25 with increasing temperatures. The control peptide, Peptide P1 (ELAALEAKLAALEAKAGY), which shows an α-helical content similar to that of SP189−220, but whose sequence has no resemblance to that of the H3 domain, does not bind to SNAP-25 at any temperature (data not shown). Together, these results strongly suggest that α-helical structure is required for SNAP-25 binding and, further, suggest that information for specific binding resides in the primary sequence as well.

Contribution to SNAP-25 Binding of the Hydrophobic Amino Acid Residues along the Putative Amphiphilic α-Helices of the H3 Domain. What information for specific binding resides in the primary sequence of the H3 domain? According to the simple heptad repeat model of α-helical structure, hydrophobic amino acid residues at the repeating positions *a* and *d* in the α-helices of the H3 domain will form a ribbon of hydrophobic surface. Many of these residues are evolutionarily conserved in syntaxin homologues from yeast to mammals (Inoue et al., 1992; Spring et al., 1993), suggesting their functional importance. If these amino

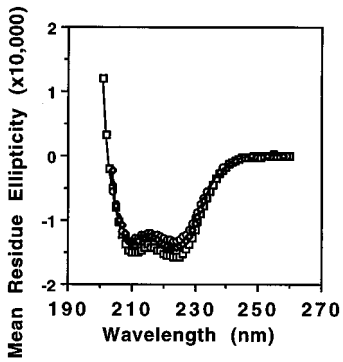

FIGURE 7: Comparison of CD spectroscopic analysis of syntaxin 1A and its selected mutants. CD spectra were measured as described in Experimental Procedures. Data are expressed as mean residue ellipticities (millidegrees per centimeter per residue per micromolar × 10 000: (square) wild type syntaxin 1A, (triangle) m5, (circle) m6, and (diamond) m12.

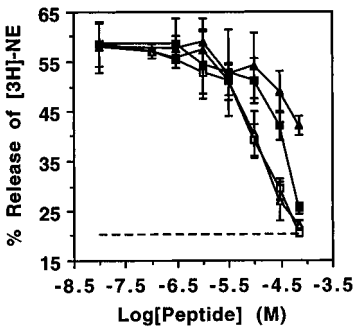

FIGURE 8: The soluble H3 domain and the minimal binding domain differentially inhibit [^{3}H]NE secretion from permeabilized PC12 cells. SP191−266 (open symbols) or SP189−220 (closed symbols) was introduced into permeabilized PC12 cells, and [^{3}H]NE secretion was evoked at 22 °C (square) and 35 °C (triangle) in the presence of MgATP, rat brain cytosol, and calcium. Data are expressed as the percentage of [^{3}H]NE released from the total cellular [^{3}H]NE pool (mean ± sd, $n = 3-4$). Basal release was 20% of the total cellular [^{3}H]NE (dashed line).

acids are critically involved in the syntaxin−SNAP-25 interaction, substitution of the larger hydrophobic residues at positions *a* and *d* with a smaller amino acid (e.g., alanine) should reduce the binding affinity of syntaxin for SNAP-25. In order to pinpoint the contributions of the various *a* and *d* hydrophobic residues to SNAP-25 binding, a series of syntaxin 1A11 mutants was generated, and these mutants were tested for their ability to displace [^{35}S]SNAP-25 binding to immobilized syntaxin 1A11 (Table 2). The mutant proteins, m1−m3, m12, and m13, possess one or two pairs of substitutions at the *a* and *d* sites within the 32-mer minimal binding domain, whereas m4−m6 and m35 carry substitutions in the region C-terminal to the minimal binding domain. The substitutions in the mutant m34 straddle the minimal binding domain and this C-terminal region.

As predicted, all mutations within the minimal binding domain produced significant loss of affinity for SNAP-25 binding (mutations at amino acids 198, 202, 205, 209, 212, and 216), whereas mutations in the C-terminal portion of H3 resulted in much weaker effects (Table 2). Among them, the most substantial effects are found with m2, m12, m13, and m34, which decrease the apparent affinity for SNAP-25 by about 20−50-fold as compared to that of wild type syntaxin 1A11. Moderate effects were achieved by m1, m3, m5, and m35, which decreased the binding affinity by 3−7-fold. Minimal changes in binding affinity were observed for m4 and m6. Although the hydrophobic *a* and *d* residues 198, 202, 205, 209, 212, and 216 are important sites for syntaxin−SNAP-25 binding, their individual roles are obviously not equivalent. Among the mutants carrying one single pair of point mutations, alanine substitution at positions 205 and 209 resulted in the most significant loss of affinity of SNAP-25 binding to syntaxin. Thus, residues 205 and 209 appear to be particularly critical for this protein−protein interaction.

The substantial loss in SNAP-25 binding affinity resulting from point mutations at positions *a* and *d* in the H3 domain could be due to either disruption of the α-helical structure or loss of specific binding sites, both of which are necessary for SNAP-25 binding. To resolve this issue, the α-helical contents of three mutants were determined. Among them, SNAP-25 binding affinity is reduced insignificantly in m6, moderately in m5, and substantially in m12. As shown in Figure 7, there is no major reduction in the α-helical content of any of the three mutants compared to that of wild type syntaxin. Similar data were obtained from m2 (data not shown). Both m2 and m12 carry point mutations at residues 205 and 209. Therefore, loss of SNAP-25 binding affinity in these mutants is not likely to be due to loss of α-helical structure but, instead, may be due to loss of specific protein−protein contacts.

Secretion of [^{3}H]Norepinephrine from PC12 Cells. [^{3}H]Norepinephrine ([^{3}H]NE) secretion from membrane-permeabilized PC12 cells at two different temperatures, 22 and 35 °C, was used to demonstrate the functional significance of the interaction between SNAP-25 and the H3 domain. Three peptides, the H3 domain (SP191−266), the minimal binding domain (SP189−220), and the truncated minimal binding domain (SP204−220), were each introduced into permeabilized PC12 cells. It was expected that exogenous SP191−266 (which lacks syntaxin's plasma membrane anchoring domain) will compete with endogenous membrane-anchored syntaxin for binding with exocytotic proteins, including SNAP-25, disrupt complex formation, and thus inhibit secretion. Consistent with this expectation, at 22 °C, intact H3 domain inhibited [^{3}H]NE secretion with an IC_{50} of 9 ± 1 μM ($n = 4$); the inhibition was complete, as the highest concentration of peptide reduced secretion to basal levels (Figure 8). SP189−220, which has a lower affinity for [^{35}S]SNAP-25 than the H3 domain, inhibited [^{3}H]NE secretion with a higher IC_{50} of 40 ± 1 μM ($n = 4$). In contrast, SP204−220, a peptide that cannot bind to [^{35}S]SNAP-25, produced only minimal inhibition at 100 μM (data not shown). This minor effect is likely nonspecific, because the same mass of GST control protein gave a similar concentration-dependent inhibition (data not shown). The temperature dependence of inhibition is consistent with the higher α-helical stability of SP191−266 than that of SP189−220. That is, inhibition of [^{3}H]NE secretion by SP191−266 at both temperatures is similar, whereas a significantly weaker inhibitory effect of SP189−220 was seen at 35 °C, a temperature at which the α-helical content of SP189−220 is substantially reduced.

DISCUSSION

A large body of information accumulated over the past few years has provided a first glimpse into the molecular mechanisms that mediate the exocytotic release of neu-

rotransmitter. As more proteins involved in the vesicle docking and fusion process are identified, one important task is to define how these proteins interact with each other to promote exocytosis. The present study focuses on a key step in the complex series of protein−protein interactions leading to membrane fusion, i.e., the interaction between syntaxin 1A and SNAP-25. In the present study, we have demonstrated the following. (1) A minimal binding domain for interaction with SNAP-25 is located in a 32-amino acid sequence (residues 189−220) in the H3 domain of syntaxin. (2) The H3 domain and the SNAP-25 minimal binding domain within it contain substantial α-helical secondary structure, as predicted. (3) This α-helical structure is required for the interaction between syntaxin and SNAP-25. (4) Amino acid residues 205 and 209 at the *d* and *a* positions, respectively, in the putative α-helix of the H3 domain are particularly important for SNAP-25 binding, suggesting that they could constitute binding sites that directly interact with SNAP-25. (5) The interaction between syntaxin and SNAP-25 is likely required for secretion of norepinephrine from PC12 cells.

We have also found the following. (1) Different portions of the H3 domain have different capacities for adopting α-helical structure, and the 32-mer minimal binding domain is critical for α-helical formation over the entire H3 domain. (2) Optimal α-helical stability of the H3 domain requires the last 13 amino acids of the C-terminal region of the domain (amino acid residues 254−266). (3) The C-terminal region of the H3 domain appears to function as a "supporting domain", conferring optimal SNAP-25 binding activity to the minimal binding domain.

The minimal binding domain we have identified agrees well with a previous finding suggesting that the SNAP-25 binding domain on syntaxin resides within the region between amino acid residues 191 and 221 (Chapman et al., 1994; Kee et al., 1995). The present study extends this initial observation by showing that this region alone is sufficient for binding and by further showing that peptides containing only partial sequences of the minimal binding domain do not show significant binding. Thus, these partial sequences lack some of the key binding sites and/or lose the ability to form the stable α-helical structure of the minimal binding domain.

The importance of interacting amphiphilic α-helices to the biological functions of proteins and peptides has been demonstrated in a variety of systems (Kasier & Kezdy, 1984). For example, tropomyosin, keratin, apolipoprotein E, and the leucine zipper motif of several DNA-binding proteins involve the interaction of α-helical structures (Cohen & Parry, 1990; O'Shea et al., 1991). Moreover, α-helical coiled coil motifs are important in proper protein folding, and structures in which two or more α-helices wrap around each other to form a left-handed supercoil have been found in many proteins. Formation of the hydrophobic face of an amphiphilic α-helix requires that hydrophobic amino acid side chains repeat in a characteristic pattern in the primary sequence of a helical region, reflecting the inherent heptad structure, $(abcdefg)_n$, of α-helices. Positions *a* and *d* are usually hydrophobic residues, while positions *e* and *g* are frequently charged residues. Previous identification of such heptad repeats in the sequences of syntaxin, SNAP-25, and VAMP prompted the hypothesis that these proteins may interact with each other through α-helical coiled coil motifs, as well. However, there has been no direct evidence to support this hypothesis. Our CD measurements showing that the H3 domain and the SNAP-25 minimal binding domain adopt an α-helical structure provide the first experimental evidence supporting this hypothesis for the interaction of these two proteins.

In our temperature dependence studies, SP189−220, with a T_m of 35 °C for helix denaturation, loses significant affinity for [^{35}S]SNAP-25 as the temperature was increased over the range from 0 to 22 to 35 °C, whereas SP191−266, with a T_m of 54 °C, maintains approximately the same affinity over this same range (Figure 5). These results provide additional evidence for the hypothesis that the α-helical structure in syntaxin is required for its interaction with SNAP-25.

Secondary structure is, of course, only one key factor in the interaction between syntaxin and SNAP-25. The present study has also begun to resolve features of primary structure that are required for this protein−protein interaction. A control peptide (P1) that contains a similar level of α-helical content but bears no relation to the primary sequence of the minimal binding domain does not bind to SNAP-25, suggesting that specific binding sites positioned on the α-helical minimal binding domain are necessary. To search for these binding sites, we have examined the effects of point mutations of the hydrophobic amino acid residues on the amphiphilic α-helix of the H3 domain. Since these hydrophobic residues are highly conserved evolutionarily (Kee et al., 1995), one suspects that they play important roles in the interaction between syntaxin and other exocytotic proteins. Recent studies from a variety of proteins have shown that point mutations of key amino acid residues can either remove a specific functional site without disrupting the secondary structure or subtantially disturb the helical structure (Li et al., 1993; Steif et al., 1995; Utani et al., 1995). In both cases, these mutations could lead to loss of function. Tandem alanine substitutions at residues 205 and 209 (leucine and isoleucine, respectively) cause a major loss of SNAP-25 affinity, but this loss is not accompanied by significant disruption of the α-helix as monitored by CD spectroscopy. Therefore, leucine 205 and isoleucine 209 are likely to participate in the binding sites for SNAP-25.

In the present study, we have linked the biochemical and structural data to functional activity. We show that the intact H3 domain (SP191−266) and the 32-mer minimal binding domain (SP189−220) are able to nearly completely inhibit calcium-dependent norepinephrine (NE) secretion from PC12 cells. The H3 domain (SP191−266) more potently inhibited secretion than the minimal binding domain (SP189−220), while a fragment of the minimal binding domain (SP204−220) was inactive, consistent with the binding affinity of SP191−266 being higher than that of SP189−220 and with the lack of binding activity of SP204−220 (Figure 8 and Table 1). Moreover, temperature-dependent inhibition of secretion by these peptides (Figure 8) is also correlated with their α-helical thermal stability and their temperature-dependent affinities for SNAP-25 (Figures 5 and 6). These results provide functional evidence that the interaction of the α-helical minimal binding domain of syntaxin with SNAP-25 is required for calcium-dependent exocytosis. We note here that, for these peptides, the IC_{50} values obtained from the binding assay are lower than the IC_{50} values obtained from the [^{3}H]NE release assay, namely, about 2 orders of magnitude for SP191−266 and 1 order for SP189−220. One possible explanation is the likely low efficiency

of delivering relatively large peptides to the intracellular space of permeabilized cells. This notion is supported by the greater difference in the binding and functional activities for the 9 kDa peptide, SP191−266, compared to that for the 3.8 kDa peptide, SP189−220. That is, while the extracellular concentration of the peptide may be micromolar in the release assay, the concentration at the intracellular site of exocytosis will probably be lower for SP189−220 and lower still for the larger peptide, SP191−266. In addition, conditions at the site of exocytosis, such as concentrations of endogenous syntaxin and SNAP-25, will likely be very different from conditions in the binding assay. Furthermore, both peptides may interact with multiple exocytotic proteins, and if this is the case, it would be unlikely that their interactions with SNAP-25 alone would determine the IC_{50} of these peptides in the functional assay.

In contrast to previous studies, our data further suggest that, in addition to the minimal binding domain, a second functionally and structurally distinct domain within H3 is also involved in the interaction of SNAP-25 with syntaxin, i.e., a supporting domain (Figure 1). This supporting domain is involved in at least two events: enhancement of SNAP-25 binding affinity and stabilization of the helicity of H3. How does this C-terminal supporting domain enhance SNAP-25 binding affinity? SP191−235 and SP191−253 exhibit 15- and 50-fold increases, respectively, in SNAP-25 binding affinity compared to SP189−220 (Table 2) but fail to show corresponding increases in thermal stability of the α-helices (though the C-terminal region does appear to introduce more cooperativity, Figure 3). Thus, helical stability alone cannot entirely account for the enhancement of SNAP-25 binding affinity. In addition, our data from CD spectroscopy do not reveal a striking change in α-helicity per residue of the peptides extended C-terminally from the 32-mer minimal binding domain. Therefore, an increase in the mean α-helical content also cannot account for the increase in SNAP-25 binding affinity. Third, H3 domain peptides that lack the minimal binding domain (SP220−266 and others) fail to bind to SNAP-25 significantly (Table 2), whereas SP191−266 (sequence of the minimal binding domain plus SP220−266) possesses higher affinity for SNAP-25 than the minimal binding domain. Taken together, these results suggest at least two alternative mechanisms, neither of which can be ruled out by the current data. (1) No SNAP-25 binding sites reside within the C-terminal region outside of the minimal binding domain, but conformational changes in the minimal binding domain induced by this region and which are not detectable by CD spectroscopy confer optimal binding affinity. (2) Additional SNAP-25 binding sites exist within the C-terminal region but only when it is helical. As shown in Figure 3, this helical structure can be achieved only when the C-terminal region is contiguous with the minimal binding domain. Therefore, we further postulate that the minimal binding domain is the critical region for maintaining the helicity of the entire H3 domain. This hypothesis predicts that intramolecular cooperativity between the 32-mer minimal binding domain and the C-terminal supporting domain is necessary for syntaxin to function optimally.

Thus, the 32-mer domain may be indirectly important for syntaxin binding to several other proteins in the exocytotic process. The binding domains on syntaxin for n-sec1 (within residues 4−240), αSNAP (within residues 191−240), VAMP (within residues 191−266), and synaptotagmin (within residues 220−266) are all located in or contain part of the H3 domain (Pevsner et al., 1994; Chapman et al., 1994; Hayashi et al., 1994; Calakos et al., 1994; Kee et al., 1995; Kee & Scheller, 1996). Part of the binding site for αSNAP and VAMP (Kee et al., 1995) could be contributed by the 32-mer minimal binding domain. Importantly, the 32-mer domain may be required for syntaxin to present an optimal C-terminal α-helical conformation of the H3 domain which is likely to be needed for interaction with these proteins.

Finally, it is generally appreciated that dysfunction in synaptic transmission underlies many neuropathologic conditions. Virtually all neuroactive drugs have been aimed at modulating synaptic transmission, whether directed at metabolic pathways, reuptake systems, receptors, or signal transduction pathways. Further understanding of the protein−protein interactions that mediate exocytosis may open new opportunities for modulating synaptic transmission therapeutically. Recent results suggest that isoforms of at least some of these exocytotic proteins may be differentially expressed in cells and tissues, including brain regions (Bark et al., 1995; Bennett et al., 1993; Ullrich & Sudhof, 1995). This suggests that (1) at least some of the numerous possible combinations of isoforms will provide specific protein−protein interactions susceptible to novel selective agents with the potential for treating neurological disorders and (2) these agents can reasonably be expected to have pharmacological profiles distinct from those of current therapeutics. Elucidation of the structural features of the interactions between exocytotic proteins may aid in the development of such agents.

ACKNOWLEDGMENT

The authors thank Dr. Laszlo Nadasdi, Dr. Laszlo Urge, Mei Zhou, Dennis Hom, Diego Silva, Paulina Tran-Tau, and Fang Chang of Neurex Corp. and Drs. Yun Kee, Jason Bock, Shu-Chan Hsu, Renee Hollomon, Peizhi Lou, and Karen Peterson of Stanford University for their generous technical support and constructive comments. The authors also thank Jane Del Carlo of Neurex Corp. for her expertise in preparation of the manuscript.

NOTE ADDED IN PROOF

See also Fasshauer et al. (1997).

REFERENCES

Bark, I. C., & Wilson, M. C. (1993) *Gene 139*, 291−292.

Bark, I. C., Hahn, K. M., Ryabinin, A. E., & Wilson, M. C. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1510−1514.

Bennett, M. K., & Scheller, R. H. (1994) *Annu. Rev. Biochem. 63*, 63−100.

Bennett, M. K., Garcia-Arraras, J. E., Elferink, L. A., Peterson, K., Fleming, A. M., Hazuka, C. D., & Scheller, R. H. (1993) *Cell 74*, 863−873.

Calakos, N., Bennett, M. K., & Scheller, R. H. (1994) *Science 263*, 831−844.

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

Cohen, C., & Parry, D. A. D. (1990) *Proteins: Struct., Funct., Genet. 7*, 1−15.

Fasshauer, D., Bruns, D., Shen, B., Jahn, R., & Brünger, A. T. (1997) *J. Biol. Chem. 272*, 4582−4590.

Hay, J. C., & Martin, T. F. (1992) *J. Cell Biol. 119*, 139−151.

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

Inoue, A., Obata, K., & Akagawa, K. (1992) *J. Biol. Chem. 267*, 10613−10619.

Jahn, R., & Südhof, T. C. (1994) *Annu. Rev. Neurosci. 17*, 219−246.
Kasier, E. T., & Kezdy, F. J. (1984) *Science 259*, 249−255.
Kee, Y., & Scheller, R. H. (1996) *J. Neurosci. 16*, 1975−1981.
Kee, Y., Lin, R. C., Hsu, S., & Scheller, R. H. (1995) *Neuron 14*, 991−998.
Li, X., Rock, F., Chong, P., Cockle, S., Keating, A., Ziltener, H., & Klein, M. (1993) *J. Biol. Chem. 268* (30), 22377−22384.
Morgan, A., Dimaline, R., & Burgoyne, R. D. (1994) *J. Biol. Chem. 269*, 29347−29350.
O'Shea, E. K., Klemm, J. D., Kim, P. S., & Alber, T. (1991) *Science 254*, 539.
Pevsner, J., Hsu, S.-C., Braun, J. E. A., Calakos, N., Ting, A. E., Bennett, M. K., & Scheller, R. H. (1994) *Neuron 13*, 353−361.
Scheller, R. H. (1995) *Neuron 14*, 1−5.
Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., & Rothman, J. E. (1993a) *Nature 362*, 318−324.
Söllner, T., Bennet, M. K., Whiteheart, S. W., Scheller, R. H., & Rothman, J. E. (1993b) *Cell 75*, 409−418.
Spring, J. K., Kato, M., & Bernfield, M. (1993) *Trends Biochem. Sci. 18*, 124−125.
Steif, C., Hinz, H.-J., & Cesarieni, G. (1995) *Proteins 23*, 83−96.
Ullrich, B., & Südhof, T. C. (1995) *Neuropharmacology 34*, 1371−1377.
Utani, A., Nomizu, M., Sugiyama, S., Miyamoto, S., Roller, P., & Yamada, Y. (1995) *J. Biol. Chem. 270* (7), 3292−3298.

BI9625408