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A conformational switch in syntaxin during exocytosis: role of munc18

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Syntaxin 1, an essential protein in synaptic membrane fusion, contains a helical autonomously folded N-terminal domain, a C-terminal SNARE motif and a transmembrane region. The SNARE motif binds to synaptobrevin and SNAP-25 to assemble the core complex, whereas almost the entire cytoplasmic sequence participates in a complex with munc18-1, a neuronal Sec1 homolog. We now demonstrate by NMR spectroscopy that, in isolation, syntaxin adopts a 'closed' conformation. This default conformation of syntaxin is incompatible with core complex assembly which requires an 'open' syntaxin conformation. Using site-directed mutagenesis, we find that disruption of the closed conformation abolishes the ability of syntaxin to bind to munc18-1 and to inhibit secretion in PC12 cells. These results indicate that syntaxin binds to munc18-1 in a closed conformation and suggest that this conformation represents an essential intermediate in exocytosis. Our data suggest a model whereby, during exocytosis, syntaxin undergoes a large conformational switch that mediates the transition between the syntaxin–munc18-1 complex and the core complex.
Keywords: exocytosis/munc18/protein NMR/SNARE/syntaxin

Introduction

Membrane fusion is a key event in a wide variety of cellular processes, including vesicular traffic along the secretory pathway, organelle inheritance and neurotransmitter release. Intense research using multiple approaches has led to the general belief that all types of intracellular membrane fusion, from yeast to man, share a common mechanism, with specialized features in different pathways (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994; Südhof, 1995). Central components of the membrane fusion machinery are proteins called SNAREs, which form a complex known as the core or SNARE complex (Söllner *et al*., 1993; reviewed in Hanson *et al*., 1997a). Extensive characterization of the synaptic core complex formed by the vesicular SNARE synaptobrevin/VAMP and the plasma membrane SNAREs syntaxin and SNAP-25 has shown that the complex consists of a highly stable bundle of four parallel α-helices (Hayashi *et al*., 1994; Kee *et al*., 1995; Hanson *et al*., 1997b; Lin and Scheller, 1997; Fasshauer *et al*., 1998; Poirier *et al*., 1998a,b; Sutton *et al*., 1998). However, the exact role of the core complex in fusion remains unclear. The parallel arrangement of the helices in the core complex, which brings together the membrane-proximal sequences of syntaxin and synaptobrevin, suggests that core complex formation may directly execute fusion (Südhof *et al*., 1993; Hanson *et al*., 1997b; Lin and Scheller, 1997). However, recent studies of homotypic vacuolar fusion (Ungermann *et al*., 1998) have shown that core complexes can be disassembled without affecting the fusion step. Thus, while there is overwhelming evidence that the core complex plays an essential role in membrane fusion, it is still unclear whether core complex assembly helps to set up fusion or executes the actual fusion reaction, and the sequence of events that leads to fusion is not yet well understood. This is in part because other protein–protein interactions that may also be critical for fusion are not as well characterized as core complex formation.

Most of the sequences of synaptobrevin and of SNAP-25 participate in the core complex, whereas only the C-terminal third of the cytoplasmic region of syntaxin is involved in core complex formation (see Figure 1A). Analysis of the N-terminal region of the neuronal isoform syntaxin 1 by nuclear magnetic resonance (NMR) spectroscopy (Fernandez *et al*., 1998) showed that residues 28–144 form an independently folded domain (Nsyx) consisting of an up-and-down three-helix bundle, while residues 1–27 and 145–180 are unfolded. The stable fold of Nsyx and its high sequence conservation in plasma membrane syntaxins suggest that this domain is functionally important and participates in binding reactions. Indeed, the N-terminal region of syntaxin is involved in binding to proteins with key roles in neurotransmitter release, such as synaptotagmin (Shao *et al*., 1997), munc13 (Betz *et al*., 1997) and munc18-1 (Hata *et al*., 1993; Kee *et al*., 1995). The interaction of syntaxin 1 with munc18-1 [also known as n-Sec1 (Pevsner *et al*., 1994a) or rb-Sec1 (Garcia *et al*., 1994)] is particularly interesting because all intracellular membrane fusion processes studied to date involve a munc18 homolog. Furthermore, mutations in munc18 homologs cause severe phenotypes associated with complete blocks in neurotransmitter release and/or general secretion (Ossig *et al*., 1991; Hosono *et al*., 1992; Schekman, 1992; Harrison *et al*., 1994; M.Verhage and T.C.Südhof, unpublished). The syntaxin–munc18 complex is very tight, and munc18 can be purified from brain homogenates in a single step on a GST–syntaxin affinity column (Hata *et al*., 1993). This interaction involves both the N- and C-terminal regions of syntaxin and competes with binding to SNAP-25 and synaptobrevin (Pevsner *et al*., 1994b).

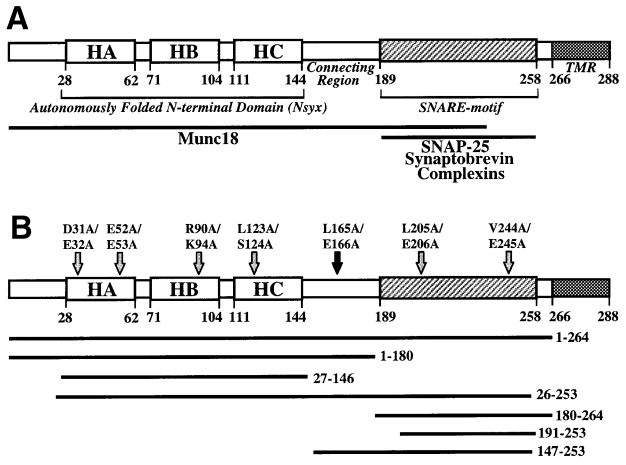

Fig. 1. Domain structure of syntaxin. Schematic diagrams of the syntaxin sequence are shown. Numbers below the diagrams correspond to amino acid residues in syntaxin 1A. The three α-helices from the autonomously folded N-terminal domain (Nsyx) are indicated as HA, HB and HC. The C-terminal cytoplasmic region involved in core complex formation (SNARE motif) is indicated by a striped bar. TMR (shadowed bar) designates the transmembrane region. (**A**) Syntaxin regions involved in binding to the indicated proteins are represented by solid bars below the diagram. (**B**) The location of the syntaxin 1 fragments studied is shown below the diagram with residue numbers indicated on the right. Arrows above the diagram indicate the nature and positions of amino acid substitutions studied. The L165A/E166A mutation, which disrupts the closed conformation of syntaxin and abolishes binding to munc18, is designated by the black arrow.

In addition to binding to other proteins, some evidence suggests that the N-terminal region of syntaxin may play a role in exocytosis through binding to the C-terminal SNARE motif (also called the H3 region). An N-terminal fragment of syntaxin was shown to bind to a C-terminal fragment and to inhibit binding of the latter to synaptobrevin (Calakos *et al*., 1994). An N- to C-terminal interaction in syntaxin was proposed to prevent reassembly of the core complex after disassembly by α-SNAP/NSF (Hanson *et al*. 1995). The N-terminal region of the yeast syntaxin homolog SSO1p has also been shown to interact with its C-terminal region and to cause a kinetic barrier for formation of a binary complex between SSO1p and the yeast SNAP-25 homolog Sec9p (Nicholson *et al*., 1998). Recently, a model was proposed whereby the C-terminus of SSO1p folds back onto the N-terminus to form a four-helix bundle; this conformation is referred to as the 'closed' conformation of SSO1p (Fiebig *et al*., 1999). Based on this model, the possibility was suggested that binding to Sec9p may release the N-terminal domain, resulting in an 'open' conformation of SSO1p. This possibility is consistent with electron microscopy (EM) images of the assembled synaptic core complex, where the N-terminal region of syntaxin appears as a separate domain with a random orientation with respect to the bulk of the complex (Hanson *et al*., 1997b).

The possibility that the N- and C-terminal regions of syntaxin interact with each other and with munc18 raises a number of questions about the mechanism of membrane fusion. Since both interactions are incompatible with core complex formation, are they related? Does munc18 bind to an open or a closed conformation of syntaxin? Since the current data indicate that the N- to C-terminal interaction in syntaxin and SSO1p is weak, is it physiologically relevant? In addition, there is no direct demonstration that an N- to C-terminal interaction occurs intramolecularly, and it is unclear whether the 44 residue region connecting the Nsyx domain with the SNARE motif (Figure 1A) serves only as a linker or has a specific role. To address some of these questions, we have analyzed syntaxin 1 using NMR spectroscopy. We have also prepared a series of mutants of syntaxin 1 and examined the functional and structural consequences of the mutations by a combination of NMR spectroscopy, biochemical experiments and transfection in PC12 cells. Our results reveal that isolated syntaxin 1 adopts a compact closed structure that includes the Nsyx domain, the connecting region and half of the SNARE motif. A mutation in the connecting region disrupts this default conformation of syntaxin 1, prevents binding to munc18-1 and abolishes the functional effects of transfected syntaxin 1 in PC12 cells. These results suggest that during exocytosis, syntaxin switches between two conformations, a closed conformation that binds to munc18 and an open conformation that forms the core complex.

Results

^{1}H–^{15}N HSQC spectra of N- and C-terminal fragments of syntaxin 1

To study whether an interaction between the N- and C-terminal regions of syntaxin occurs intramolecularly, we acquired a series of ^{1}H–^{15}N HSQC spectra of different uniformly ^{15}N-labeled fragments of syntaxin 1A. These spectra contain one cross-peak for each non-proline residue in a protein and for side chains containing NH groups. The spectra can be considered as protein fingerprints, and changes in these fingerprints report binding interactions of the protein under study. In a similar manner, intramolecular interactions between regions of a protein can be studied by comparing the ^{1}H–^{15}N HSQC spectrum of an individual fragment with the spectrum of the same sequence in the context of the full-length protein. Thus, to investigate interactions between the N- and C-terminal regions of syntaxin 1, we first recorded ^{1}H–^{15}N HSQC spectra of N- and C-terminal fragments separately and used these spectra as reference for the spectra of longer fragments containing both regions. The fragments of syntaxin 1 studied are summarized in Figure 1B. All ^{1}H–^{15}N HSQC spectra were acquired under identical conditions (pH 7.4, 25°C, 600 MHz) to facilitate comparisons among them at an optimal solubility of all fragments. The syx27–146 and syx1–180 fragments had been analyzed previously under different conditions (pH 6.5, 32°C, 500 MHz) (Fernandez *et al*., 1998). The ^{1}H–^{15}N HSQC cross-peaks of syx27–146 were reassigned under the conditions of this study to aid in the analysis described below.

In Figure 2A, the ^{1}H–^{15}N HSQC spectrum of the entire N-terminal region (syx1–180) is superimposed with that of the Nsyx domain (syx27–146) to illustrate that the presence of residues 1–26 and 147–180 in the longer fragment causes very little perturbation on the amide groups of the structured domain. Basically all cross-peaks from syx27–146 (black) coincide with a cross-peak from syx1–180 (red) or exhibit very small shifts, and the few exceptions correspond to the N- and C-terminal residues of sx27–146. Cross-peaks from syx1–180 that do not have a corresponding partner in the spectrum of syx27–146,

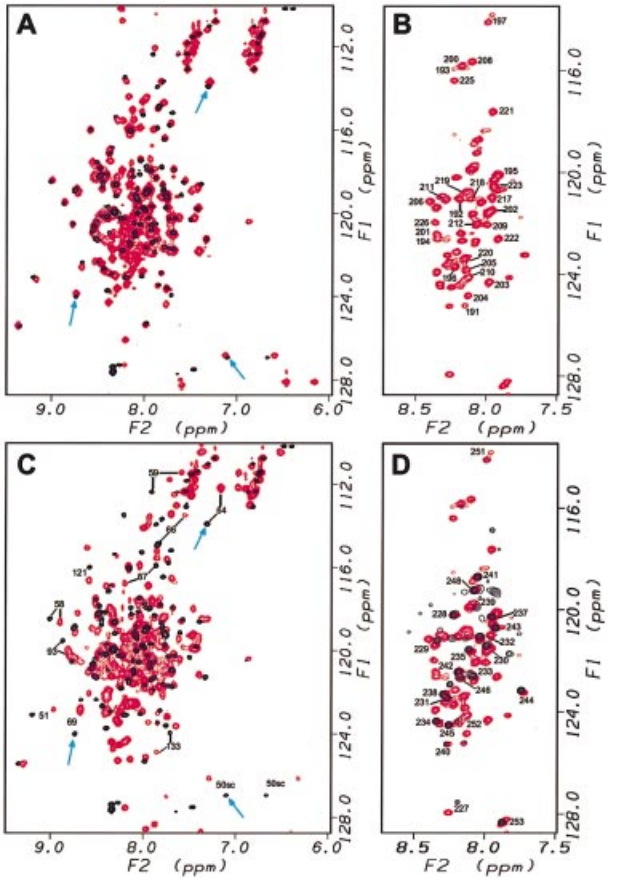

Fig. 2. $^{1}H–^{15}N$ HSQC spectra of syntaxin 1 fragments reveal a closed conformation. (**A**) Superposition of the $^{1}H–^{15}N$ HSQC spectra of syx1–180 (red) and syx27–146 (black). (**B**) $^{1}H–^{15}N$ HSQC spectrum of syx191–253. (**C**) Superposition of the $^{1}H–^{15}N$ HSQC spectra of syx26–253 (red) and syx27–146 (black). (**D**) Superposition of the $^{1}H–^{15}N$ HSQC spectrum of syx26–253 plotted at high contour levels (black) with the $^{1}H–^{15}N$ HSQC spectrum of syx191–253 (red). The cross-peak assignments for the syx191–253 fragment are indicated in (B) and (D) with residue numbers; cross-peaks that coincide with sharp cross-peaks in the syx26–253 fragment are labeled in (D) and other cross-peaks are labeled in (B). Some NH groups could not be observed because of fast exchange with the solvent. In (C), some of the cross-peaks that could be assigned for the N-terminal residues of syx26–253, and the corresponding cross-peaks observed for the same residues in syx27–146, have been labeled with the residue number. Significant shifts with respect to the spectrum of syx27–146 were observed for almost all N-terminal cross-peaks that could be assigned for syx26–253, which include residues 41, 50–52, 54, 56, 58–60, 63, 64, 66, 68, 69, 72, 82, 87, 88, 91, 93, 95, 100, 105, 106, 121, 133 and 138. Blue arrows in (A) and (C) point at cross-peaks discussed in the text. Note that cross-peaks corresponding to Asn and Gln side chains at the lower right corner of the spectra in (A) and (C) are folded in the ^{15}N dimension (F1).

and thus correspond to residues 1–26 or 147–180, have very little chemical shift dispersion in the ^{1}H dimension (F2 in the contour plots). These observations reflect the fact that only residues 28–144 are structured in the syx1–180 fragment (Fernandez *et al.*, 1998).

The $^{1}H–^{15}N$ HSQC spectra obtained for the syx180–264 fragment, which contains the entire SNARE motif, were of low quality due to severe aggregation, and several attempts to increase the solubility of the fragment failed to improve the spectra sufficiently (data not shown). Much higher quality data (Figure 2B) were obtained for a shorter fragment (syx191–253) that comprises most of the SNARE motif and has a more acidic isoelectric point due to removal of the polybasic sequence at the C-terminus of the cytoplasmic region of syntaxin 1. Cross-peak assignments for this fragment (Figure 2B and D) were obtained from ^{15}N-edited three-dimensional NMR experiments. The small dispersion of ^{1}H chemical shifts (F2 dimension) shows that this fragment is basically unfolded. This conclusion is supported by the circular dichroism (CD) spectrum of the fragment, which is characteristic of a mostly random conformation (not shown). A C-terminal fragment extended at the N-terminus (syx147–253) exhibited $^{1}H–^{15}N$ HSQC and CD spectra with very similar characteristics to syx191–253 (not shown), indicating that this fragment is also unfolded. These results demonstrate that the SNARE motif of syntaxin 1 does not constitute an independently folded domain and that the α-helical conformation observed in the core complex is induced by formation of the complex.

Syntaxin 1 adopts a closed conformation

With the $^{1}H–^{15}N$ HSQC spectra of the N- and C-terminal fragments of syntaxin 1 available as a reference, we next studied fragments containing both regions of the protein. The entire cytoplasmic region of syntaxin 1 (syx1–264) also has a tendency to aggregate and yields low quality $^{1}H–^{15}N$ HSQC spectra (not shown). However, the cross-peaks from residues 1–25 can be identified easily in these spectra as sharp signals that coincide with those observed for the same residues in the syx1–180 fragment, indicating that these N-terminal amino acids remain unfolded in the full cytoplasmic region of syntaxin 1. We therefore analyzed a fragment (syx26–253) that comprises most of the cytoplasmic region but lacks the 25 N-terminal residues and the polybasic sequence at the very C-terminus. This fragment yielded much better quality data, and the positions of the $^{1}H–^{15}N$ HSQC cross-peaks in well-resolved regions of the spectrum coincided with those of the broader cross-peaks of syx1–264, indicating that the structured regions of syx1–264 are preserved in syx26–253.

In Figure 2C, the $^{1}H–^{15}N$ HSQC spectrum of syx26–253 (red) is shown superimposed with that of syx27–146, the Nsyx domain (black). The spectrum of syx26–253 is complex and severely overlapped due to the large molecular weight, high helical content (as judged by CD) and scarcity of aromatic residues. However, it is clear from examination of the well-resolved regions of the spectra that many of the cross-peaks from residues 27–146 within the syx26–253 fragment are shifted substantially with respect to the corresponding cross-peaks in the syx27–146 fragment. This observation demonstrates that the Nsyx domain interacts with other parts of the molecule in the syx26–253 fragment, consistent with the formation of a closed conformation. The interaction is intramolecular since the resonance line widths of the structured regions of syx26–253 are characteristic of a monomeric form and inconsistent with a dimer or multimer. In addition, the positions of the $^{1}H–^{15}N$ HSQC cross-peaks are invariant through a protein concentration range from 50 to 700 μM (data not shown).

A total of 27 cross-peaks from residues 27–146 within the syx26–253 fragment could be assigned unambiguously. These cross-peaks correspond to residues throughout the entire Nsyx domain (see legend of Figure 2), and most

of them exhibit significant shifts with respect to the corresponding cross-peaks of the isolated syx27–146 fragment. Thus, the whole Nsyx domain is involved in the interaction with the remainder of the molecule. The assignment of cross-peaks from syx26–253 was aided by the observation that their shifts with respect to the cross-peaks of the syx27–146 fragment occur in the same direction as the slight shifts observed for syx1–180 (e.g. see arrows in Figure 2A and C). This in turn indicates that residues 147–180 of syx1–180 are folding back onto the Nsyx domain in a similar manner as they are observed to do in the syx26–253 fragment but very infrequently, presumably because of the lack of additional C-terminal residues that are required to complete the closed conformation.

To investigate further which part of the syntaxin 1 sequence is involved in formation of the closed conformation, we took advantage of the fact that unfolded regions of a protein have much sharper and more intense resonances than the folded regions, particularly for a large species such as the syx26–253 fragment. Thus, plotting the ^{1}H–^{15}N HSQC spectrum of syx26–253 at high contour levels yields only cross-peaks from the residues that remain highly flexible. A superposition of such a plot with the ^{1}H–^{15}N HSQC spectrum of syx191–253 (Figure 2D) reveals that most of the sharp cross-peaks from the syx26–253 fragment (black) coincide with the C-terminal 26 residues of syx191–253 (red). These results show that residues 227–253, which represent part of the SNARE motif, remain unfolded in syx26–253. The fact that sharp cross-peaks are not observed for residues 145–226, which correspond to the connecting region and the rest of the SNARE motif, suggests that this sequence is structured in syx26–253.

In principle, it could be possible that residues 145–226 do not yield sharp cross-peaks because they interconvert slowly between a number of conformations. However, the fact that the presence of these residues causes specific shifts in the cross-peaks of the Nsyx domain argues against this possibility. In addition, careful comparison of the well-resolved regions of the ^{1}H–^{15}N HSQC spectra from the syx26–253 and syx27–146 fragments reveals the presence for the larger fragment of multiple cross-peaks that are not from the Nsyx domain and do not correspond to chemical shifts characteristic of unfolded structure. Thus, these cross-peaks must arise from the formation of a specific structure in residues 145–226 which packs against the Nsyx domain. The resulting closed conformation is stably folded since no unusual temperature dependence was observed in the ^{1}H–^{15}N HSQC cross-peaks of syx26–253 within a range of 10–37°C (data not shown).

A possible model for the closed conformation, analogous to that proposed for the yeast syntaxin homolog SSO1p (Fiebig *et al*., 1999), would predict that the N-terminal half of the SNARE motif (residues 189–226) forms an α-helix that packs against the Nsyx domain, resulting in a four-helix bundle. However, the 44 residues that form the connecting region between the Nsyx domain and the SNARE motif (residues 145–188) do not yield sharp cross-peaks, indicating that this region does not act merely as a linker. At least part of this region could form another α-helix, leading to a five-helix bundle model (see Figure 3, left). Quantification of the α-helical content of syx26–253 by CD did not allow a distinction between four- and five-helix bundle models, but the severe perturbation of the closed conformation caused by an L165A,E166A mutation (see below) indicates that the connecting region plays a critical role in the formation of the closed conformation of syntaxin 1, favoring the five-helix bundle model. Altogether, our data show that isolated syntaxin adopts a closed conformation that does not simply reflect weak binding between the C-terminal and the N-terminal regions but indeed represents a compact structure spanning residues 28–226. This default conformation is incompatible with core complex formation since an N-terminal fragment of syntaxin inhibits binding of a C-terminal fragment to synaptobrevin (Calakos *et al*., 1994), and the N-terminal region of syntaxin appears to be flexibly linked to the bulk of the core complex on EM images of the complex (Hanson *et al*., 1997b). Thus, syntaxin can exist in at least two basically different conformations that we refer to as open (in the core complex) and closed (the default conformation of isolated syntaxin) (Figure 3).

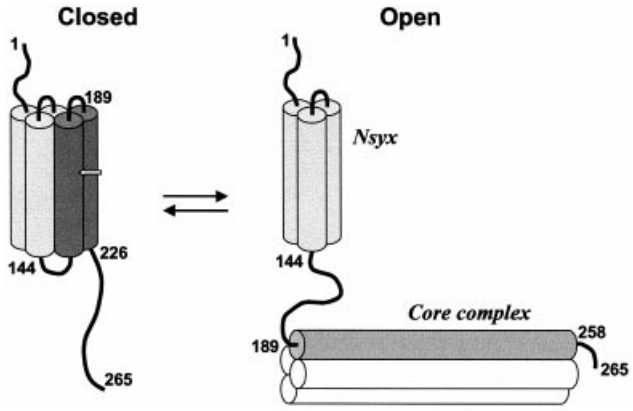

Fig. 3. Models of the open and closed conformations of syntaxin 1. The five-helix bundle model for the closed conformation of syntaxin 1 proposed from our NMR data is shown on the left. The three helices of the Nsyx domain and the two helices predicted for residues 145–225 are shaded in light and dark gray, respectively. The white arrow points to the approximate position predicted for the mutation that disrupts the closed conformation (L165A,E166A). On the right, a model of the open conformation of syntaxin 1 is shown. This model is based on the elucidated three-dimensional structures of the autonomously folded N-terminal domain (Nsyx) (Fernandez *et al*., 1998) and the complex formed by the SNARE motifs of syntaxin, synaptobrevin and SNAP-25 (core complex) (Sutton *et al*., 1998), and on EM images of the whole core complex (Hanson *et al*., 1997b). In the core complex, the SNARE motif of syntaxin is shaded in gray. The numbers in both models indicate approximate limits of secondary structure elements.

Mutational analysis of syntaxin 1

Since almost the entire cytoplasmic region of syntaxin is involved in the interaction with munc18 (Hata *et al*., 1993; Kee *et al*., 1995; and Figure 1A), it is most likely that munc18 binds to either the closed or the open conformation, but not to both. To distinguish between the two possibilities, we introduced a series of mutations in syntaxin 1 and studied whether they disrupt munc18-1 binding and/or perturb the closed conformation of syntaxin 1. Since substantial perturbation of protein structures is generally difficult to accomplish with a single amino acid mutation, and we expected that munc18-1 binding would also be difficult to disrupt due to the high affinity of

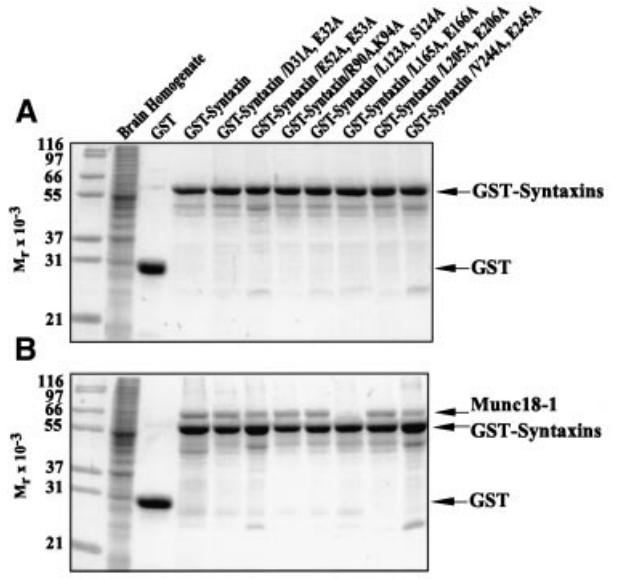

Fig. 4. Binding of munc18-1 from brain homogenates to GST–syntaxin 1 fusion proteins. The panels show Coomassie Blue-stained SDS–polyacrylamide gels. Glutathione–agarose beads containing bacterially expressed GST or various GST–syntaxin fusion proteins before (**A**) or after (**B**) incubation with solubilized total rat brain homogenate were analyzed on SDS–PAGE together with total brain homogenate. The 65 kDa band corresponding to munc18-1 was pulled down by all GST–syntaxin fusion proteins except the L165A,E166A mutant. Molecular weight standards are indicated on the left.

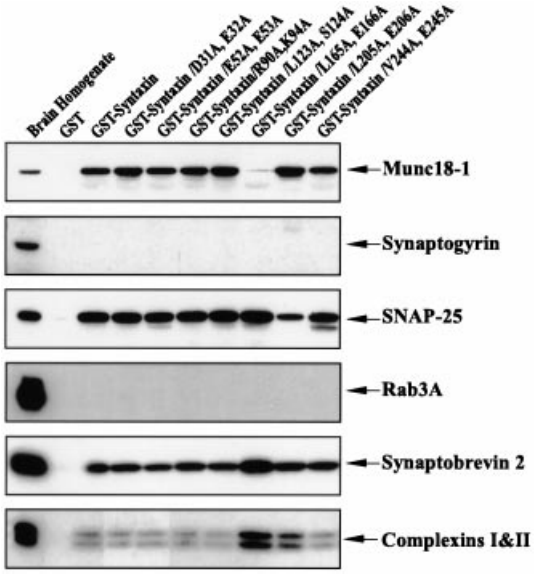

Fig. 5. Binding of brain proteins to immobilized GST–syntaxin 1 fusion proteins. Aliquots of the same beads analyzed in Figure 4B were run on separate gels, transferred onto nitrocellulose and probed with antibodies to the different proteins indicated on the right. Immunoblots were developed using ECL detection.

the interaction, mutations were introduced in pairs (see Figure 1B). The residues targeted for mutation were selected among those that are conserved in plasma membrane syntaxins because conserved residues are more likely to be functionally significant.

The mutations were first introduced in GST fusions of the full cytoplasmic region of syntaxin 1 (residues 1–264). The fusion proteins were attached to glutathione–agarose and tested for binding to munc18-1 in pull-down experiments with rat brain extracts. Under the conditions used, munc18-1 is the only brain protein that binds to syntaxin 1 in sufficient amounts to be readily visible on Coomassie Blue-stained gels (Figure 4). All mutants bound efficiently to munc18-1 except the mutant containing the L165A,E166A substitutions, where binding was abolished. This result was confirmed by immunoblotting with a monoclonal antibody specific for munc18-1 (Figure 5). In GST pull-down experiments, synaptobrevin II, SNAP-25 and complexins I and II also bind to syntaxin 1. However, this binding reaction is substoichiometric and cannot be detected by Coomassie Blue staining, in contrast to munc18-1 binding (Hata *et al*., 1993; McMahon *et al*., 1995). Therefore, protein blots were probed with antibodies to these proteins to analyze whether the mutations affect their binding to syntaxin 1 (Figure 5). Synaptogyrin and Rab3A antibodies were used as negative controls. The only noticeable differences between wild-type and the mutants in these experiments were observed for the L165A,E166A and L205A,E206A substitutions. The L165A,E166A mutant binds higher levels of complexins and synaptobrevin II. Interestingly, this is the same mutant that does not bind munc18-1. The L205A,E206A mutant has slightly increased binding to complexins and substantially decreased binding to SNAP-25, which was not unexpected since the residues mutated are in direct contact with SNAP-25 in the crystal structure of the core complex (Sutton *et al*., 1998).

A mutation that abolishes munc18-1 binding disrupts the closed conformation of syntaxin 1

To analyze the effect of the L165A,E166A mutation on the structure of syntaxin 1, we introduced this mutation into the syx26–253 fragment and acquired its ^{1}H–^{15}N HSQC spectrum. In Figure 6A, the spectrum (red) is superimposed with that of syx27–146 (black) which we used as a reference point for the 'open' conformation. Comparison of Figure 6A with Figure 2C shows that the cross-peak shifts that were observed in syx26–253 (closed conformation) with respect to syx27–146 are largely reduced when the L165A,E166A mutation is introduced. This is illustrated better for three representative cross-peaks (indicated by the arrows in Figures 2C and 6A) in the expansions shown in Figure 6B–D, where spectra from syx27–146 (black), syx1–180 (blue), syx26–253 (green) and the syx26–253/L165A,E166A mutant (red) are superimposed. Note that the cross-peaks of the four different fragments are well aligned. A similar behavior was observed for many of the cross-peaks that could be assigned unambiguously. These results indicate an equilibrium between two basic conformations, open and closed. Here again, syx27–146 represents an open conformation, syx26–253 represents the closed conformation and syx1–180 represents a mostly open conformation. The observation that the positions of the cross-peaks from the syx26–253/L165A,E166A mutant are much closer to the cross-peak positions corresponding to the open conformation suggests that the mutant is in fast equilibrium between the two conformations, with the open structure being predominant.

To ensure that mutations which do not disrupt binding of syntaxin 1 to munc18-1 at the same time do not perturb

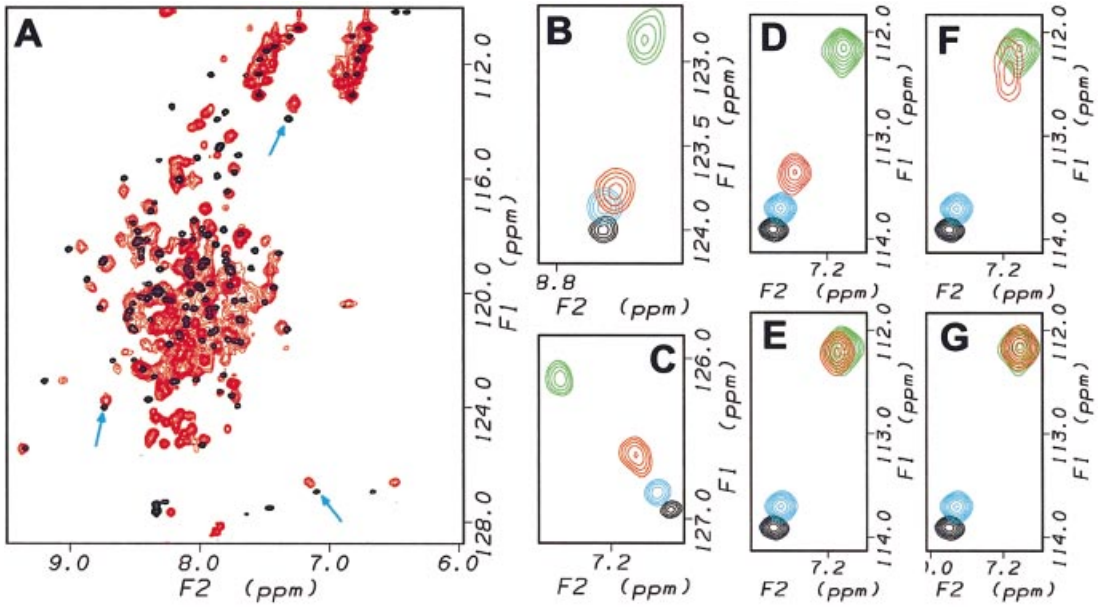

Fig. 6. A mutation that abolishes binding to munc18-1 disrupts the closed structure of syntaxin 1. (**A**) Superposition of the $^{1}H–^{15}N$ HSQC spectra of syx26–253/L165A,E166A (red) and syx27–146 (black). (B–D) Superposition of the $^{1}H–^{15}N$ HSQC spectra of syx27–146 (black), syx1–180 (blue), syx26–253 (green) and syx26–253/L165A,E166A (red) expanded at the region containing the cross-peaks of E69 (**B**), N50 side chain (**C**) and S64 (**D**). These cross-peaks are indicated by the blue arrows in (A) and in Figure 2A and C. (E–G) Superposition of $^{1}H–^{15}N$ HSQC spectra analogous to that in (D), but showing in red the cross-peak of S64 for syx26–253/E52A,E53A (**E**), syx26–253/R90A,K94A (**F**) or syx26–253/L123A,S124A (**G**) instead of syx26–253/L165A,E166A.

the closed conformation of syntaxin 1, we prepared three syx26–253 fragments containing either the E52A,E53A, the R90A,K94A or the L123A,S124A substitutions. With the exception of cross-peaks corresponding to residues near the mutations, the $^{1}H–^{15}N$ HSQC spectra of these three mutants were very similar to that of the wild-type syx26–253 fragment. This is illustrated in Figure 6E–G for the diagnostic cross-peak from S64, which is in the same or similar position as in the wild-type for all three mutants. The slight shift and the broadening observed for the S64 cross-peak of the R90A,K94A mutant (Figure 6F) were also observed for other identified cross-peaks from the N-terminal region of this mutant. This suggests that the R90A,K94A mutation destabilizes the closed conformation without opening it or abolishing binding to munc18-1.

The disruption of the closed conformation of syx26–253 by the L165A,E166A mutation, but not by the E52A,E53A, the R90A,K94A or the L123A,S124A substitutions, is also illustrated by comparison of their $^{1}H–^{15}N$ HSQC spectra plotted at high contour levels with the spectrum of the wild-type syx26–253 fragment (Figure 7). The $^{1}H–^{15}N$ HSQC spectrum of the syx26–253/L165A,E166A mutant contains a larger number of sharp cross-peaks (Figure 7B). These cross-peaks most likely correspond to the region between residues 145 and 226, since the cross-peaks assigned to the N-terminal domain suggest that its overall structure is not perturbed. The picture that emerges from these observations is that the L165A,E166A mutation disrupts at least partially the structure formed by residues 145–226 within syx26–253. This yields high flexibility for some residues in this region and reduces the ability of residues 145–226 to fold back onto the N-terminal domain. As a result, there is an equilibrium between 'open-like' and closed (but disrupted) conformations. Overall, the structural consequences of the L165A,E166A substitutions abolish binding to munc18-1 (Figures 4 and 5). In addition, the perturbation of the closed structure caused by the mutation increases the ability of the C-terminal region to bind to complexin and synaptobrevin. The apparent lack of an effect of the mutation on SNAP-25 binding could be due to a saturation effect of the ECL detection method used.

Is the closed conformation of syntaxin 1 important for exocytosis?

The fact that syntaxin 1 assumes two distinct conformations, a closed state involved in binding to munc18-1 and an open state in the core complex, raises the question of whether the transition between these states is an essential event in exocytosis, i.e. if syntaxin 1 undergoes a conformational switch during exocytosis. To examine the role of the two conformational states of syntaxin in exocytosis, we used PC12 cells transfected with human growth hormone (hGH). PC12 cells are neuroendocrine cells which secrete neurotransmitters and neuropeptides as a function of stimulation (Greene and Tischler, 1976). Secretion from PC12 cells occurs by exocytosis and requires core complex assembly from SNAREs since secretion is inhibited with botulinum and tetanus toxins (Banerjee *et al*., 1996). Transfected PC12 cells secrete hGH in a Ca^{2+}-dependent manner when stimulated with α-latrotoxin or by KCl depolarization. Co-transfection of hGH with a protein of interest leads to their co-expression in the same cells. This makes it possible to study the effect of the co-expressed protein on hGH secretion (Holz *et al*., 1993; Sugita *et al*., 1999a,b).

The transfection experiments in PC12 cells were performed with constructs that express the entire cytoplasmic region (residues 1–264) of wild-type syntaxin 1 or of syntaxin 1 with the mutations described above. To ensure that all proteins are expressed similarly in mammalian cells, we transfected these constructs into COS7 cells and

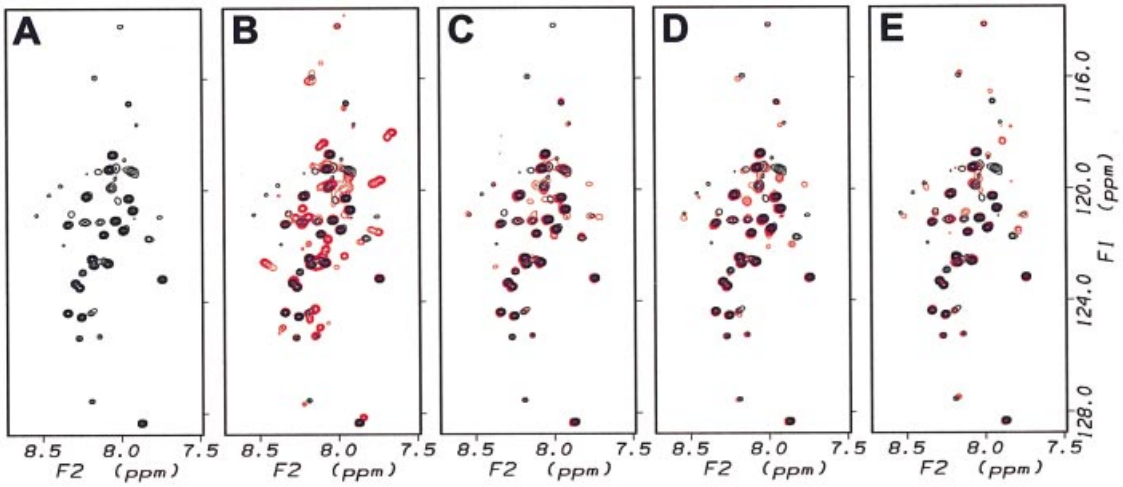

Fig. 7. A mutation that abolishes binding to munc18-1 yields a larger number of flexible residues in syntaxin 1. (**A**) ^{1}H–^{15}N HSQC spectrum of syx26–253 plotted at high contour levels. (B–E) ^{1}H–^{15}N HSQC spectra of syx26–253/L165A,E166A (**B**), syx26–253/E52A,E53A (**C**), syx26–253/R90A,K94A (**D**) or syx26–253/L123A,S124A (**E**) plotted at high contour levels (red) and superimposed with the spectrum of wild-type syx26–253 shown in (A) (black).

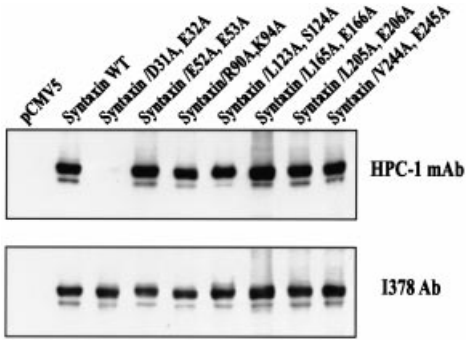

Fig. 8. Expression of wild-type and mutant syntaxins in mammalian cells. COS7 cells were transfected either with the pCMV5 expression vector alone or with pCMV5 constructs encoding the wild-type or mutated cytoplasmic region of syntaxin 1A (residues 1–264). Syntaxin expression was analyzed by immunoblotting with monoclonal antibody HPC-1 (upper panel) or with polyclonal antibody I378 at 3 days after transfection, which corresponds to the time point when the PC12 secretion experiments were performed (see Figure 9). Note that a D31A/E31A mutant was not recognized by the HPC-1 antibody.

probed the total cell extracts with two different anti-syntaxin antibodies. This experiment was carried out in COS7 cells instead of PC12 cells because the transfection efficiency in PC12 cells is so low that the transfected cells cannot be analyzed easily by immunoblotting (Sugita *et al*., 1999a). The immunoblotting results demonstrated similar levels of the wild-type and mutant syntaxins in the transfected cells (Figure 8). Interestingly, the monoclonal antibody HPC-1 (upper panel) failed to detect the D31A/E32A mutant, suggesting that the two residues mutated in syntaxin form part of the epitope recognized by the HPC-1 antibody. The transfection experiment confirmed that the mutations introduced into syntaxin do not cause major changes in its expression level and stability in mammalian cells.

Transfection of the wild-type cytoplasmic region of syntaxin 1 into PC12 cells inhibited exocytosis by 50–70% over baseline, independently of whether exocytosis was induced by KCl depolarization or application of α-latrotoxin (Figure 9 and data not shown). It is probable that the truncated syntaxin 1 has a dominant-negative effect because it substitutes for endogenous syntaxin in protein–protein interactions but is not membrane bound, thereby uncoupling the protein–protein interactions from the membrane. When we examined the effects on exocytosis of the various syntaxin mutants, we found that most mutants still inhibited hGH release. Strikingly, only a single mutation, the L165A,E166A substitution that disrupted the closed conformation of syntaxin and abolished munc18-1 binding, had no inhibitory effect on secretion (Figure 9). It is interesting that even the mutant with decreased SNAP-25 binding (L205A,E206A) still inhibited secretion, suggesting that the inhibitory effect of truncated syntaxin on secretion is not caused by its ability to bind to SNAP-25. This conclusion was confirmed in independent experiments which showed that truncation of syntaxin 1 to residue 243, which preserves the closed conformation and munc18-1 binding, does not abolish the inhibitory effect of transfected syntaxin 1 on exocytosis despite the fact that the SNARE-binding sequence is partly removed. In contrast, truncation to residue 180, which abolishes the closed conformation and munc18-1 binding, did not have an inhibitory effect (data not shown). Thus, inhibition of secretion by transfected syntaxin 1 correlates precisely with its ability to adopt a closed conformation and its binding activity towards munc18-1. Together, these results suggest that the closed conformation of syntaxin 1 plays an essential role in the fusion reaction, most likely via munc18-1 binding. These data agree well with studies in knockout mice indicating that munc18-1 levels in neurons are limiting for exocytosis (M.Verhage and T.C.Südhof, unpublished) and suggest that a munc18-1–syntaxin complex is an essential intermediate in fusion.

Discussion

Syntaxin in membrane fusion

A convergence of results from many systems from yeast to man has identified the core complex as a central intermediate in membrane fusion (reviewed in Hanson *et al*., 1997a). However, the exact function of the core complex remains unclear. Assembly of the core complex

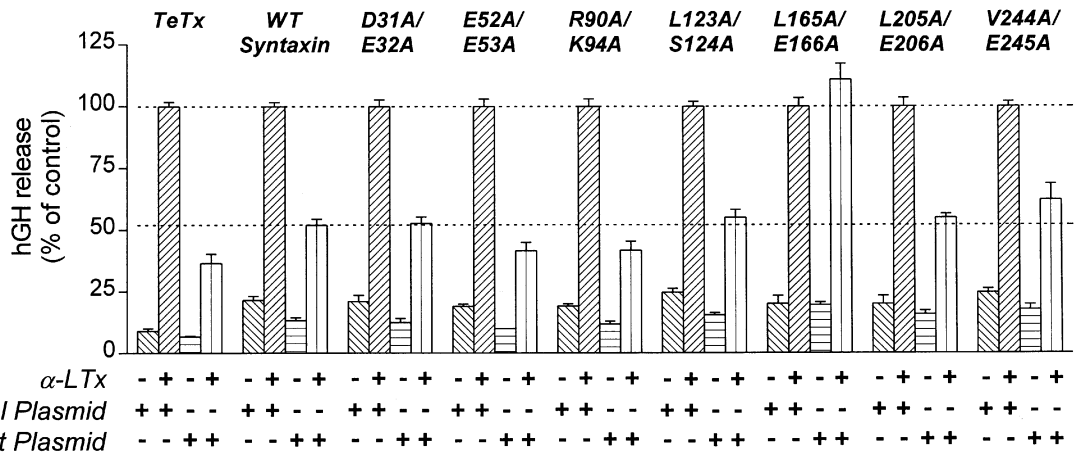

Fig. 9. The cytoplasmic region of syntaxin 1 inhibits hGH secretion from transfected PC12 cells: effects of mutations. PC12 cells were co-transfected in parallel with an hGH expression plasmid and a control plasmid (hatched bars) or with an hGH expression palsmid and a test plasmid encoding either tetanus toxin light chain (TeTx) or wild-type and mutant syntaxins. All transfected syntaxins contained residues 1–264 of syntaxin 1A with the mutations identified above the bars. Transfected cells were divided into two aliquots that were treated for 10 min with physiological saline buffer containing no additions or containing 0.3 nM α-latrotoxin. In each experiment, every test of a syntaxin construct was accompanied by a control transfection, and the amount of hGH secreted after α-latrotoxin treatment from the control transfected cells was set as 100%. The secreted amounts of hGH under all other conditions were calculated as a percentage of the stimulated control. Results from multiple experiments were pooled and are expressed as means ± SEMs. The 100% level and the level of release for transfection of wild-type syntaxin 1 are marked by dotted lines.

could drive fusion (Südhof *et al*., 1993; Hanson *et al*., 1997b; Lin *et al*., 1997), but it is uncertain whether core complex formation occurs prior to fusion or executes the actual fusion reaction. In the former case, the energy released by complex formation may be used for 'loading' the system like a spring, and other proteins would contribute to the fusion reaction. In the latter case, a minimalistic model postulates that SNARE interactions alone mediate membrane fusion. This possibility was supported by reconstitution experiments using recombinant SNAREs and synthetic phospholipid vesicles (Weber *et al*., 1998). Three lines of evidence argue against this minimalistic model. First, in yeast vacuole fusion, SNARE complexes are assembled and can be fully disassembled before fusion occurs, suggesting that complexes functionally precede fusion but are not sufficient for fusion (Ungermann *et al*., 1998). Secondly, in isolated nerve terminals, the abundance of SNARE core complexes can be regulated independently of fusion, indicating that core complex formation sets up the fusion reaction but does not actually execute it (G.Lonart and T.C.Südhof, unpublished). Finally, at least one other class of proteins in addition to SNAREs performs an essential role in all fusion reactions studied, namely the Sec1/munc18-1 proteins (SM proteins) (Ossig *et al*., 1991; Hosono *et al*., 1992; Schekman 1992; Harrison *et al*., 1994; M.Verhage and T.C.Südhof, unpublished). SM proteins bind to syntaxin-like SNAREs, which therefore participate in at least two critical protein complexes: core complexes and syntaxin–SM protein complexes. This places syntaxin in an ideal position to couple the functions of the SM proteins and the core complex. Such coupling could be associated with an N- to C-terminal interaction in syntaxin. Hence, establishing how the intramolecular and intermolecular reactions of syntaxin are interrelated is crucial for an understanding of the mechanism of membrane fusion. Following up on this idea, we have analyzed the conformational behavior of isolated syntaxin 1, the structural requirements for the binding of syntaxin 1 to munc18-1 and the functional consequences of perturbing the structural characteristics and binding properties of syntaxin 1. In conjunction with previous studies, our data show that syntaxin can exist in two fundamentally different conformations (open and closed). Furthermore, our results suggest that during exocytosis a large conformational switch in syntaxin mediates a transition between the syntaxin–munc18-1 complex and the core complex.

Closed conformation of syntaxin 1

Previous structural analyses involving syntaxin 1 fragments had revealed two different structural motifs: at the N-terminus, an autonomously folded three-helix bundle (residues 28–144; Fernandez *et al*., 1998), and at the C-terminus, a long α-helix in the core complex (residues 189–258; Sutton *et al*., 1998). EM images of the core complex indicated that the N-terminal domain of syntaxin is flexibly linked to the bulk of the complex (Hanson *et al*., 1997b). However, when syntaxin was studied outside of the core complex, an interaction was observed between the N- and C-terminal fragments from syntaxin 1 (Calakos *et al*., 1994). Similar data were obtained for the yeast syntaxin SSO1p (Nicholson *et al*., 1998; Fiebig *et al*., 1999). Based on these results, a natural expectation was that the C-terminus of syntaxin 1 (and SSO1p) could fold back onto its N-terminal domain into a 'closed' conformation, with the 44 residue region between the two domains serving as a flexible linker. However, the relevance of the interaction between the N- and C-terminal fragments of syntaxin was questionable because its low affinity contrasts with the high affinity of other interactions involving syntaxin, e.g. formation of the core complex and the munc18–syntaxin complex. The affinity of the N-terminus for the C-terminus could increase in an intramolecular interaction, but little intramolecular stabilization

would be expected with a flexible linker of >40 residues. In addition, there was no direct demonstration that the N- to C-terminal interaction is intramolecular. The N-/C-terminal interaction could in fact be an artifact due to the 'stickiness' of the C-terminus of syntaxin which has been observed to bind to a large variety of proteins (e.g. Sheng *et al*., 1994; Kee *et al*., 1995; Kee and Scheller, 1996; Naren *et al*., 1998).

Our results show that the N- and C-terminal regions of syntaxin indeed interact intramolecularly but the interaction is not simply a result of weak affinity between the two regions. The interaction occurs in the context of a compact, stable conformational state where residues 145–188 do not serve as a linker. Rather, these residues form an integral part of the structure, which is highlighted by the severe structural perturbation caused by the L165A,E166A substitution. This represents a fundamental difference from previous models: interactions that require an open conformation of syntaxin do not compete with a weak N- to C-terminal interaction; instead, they involve opening of the compact conformation that syntaxin 1 adopts by default. This default conformation of syntaxin 1 is likely to be conserved in plasma membrane syntaxins since evolutionary conservation is observed throughout their sequences (Fernandez *et al*., 1998; see also Advani *et al*., 1998). Recent NMR and CD studies of SSO1p provide strong support for this assumption (Fiebig *et al*., 1999). Although the NMR signals from the structured part of SSO1p were not monitored, assignment of the sharp resonances of the cytoplasmic region of SSO1p indicated that only residues 1–30 and 227–265 are unstructured, strikingly resembling our results. It is interesting that the C-terminal end of the closed conformation of syntaxin 1 (residue 226) coincides with the point where Gln226 forms a polar layer in the middle of the core complex (Sutton *et al*., 1998) and where the sequence of the syntaxin 1C isoform diverges from that of syntaxin 1A (Jagadish *et al*., 1997).

Does munc18-1 binding involve opening of the closed conformation of syntaxin, or is the closed conformation responsible for binding to munc18-1? Our results reveal a strict correlation between the effect of mutations on munc18 binding and on the conformation of syntaxin: mutations that preserved the closed structure did not prevent munc18 binding, while the L165A/E166A substitution disrupts the closed conformation and at the same time abolishes munc18 binding. It is possible that this mutant does not bind munc18 because of a direct involvement of L165 and/or E166 in the interaction. However, the fact that the mutation of these residues perturbs the closed conformation of syntaxin implies that they are involved in the stabilization of this conformation rather than in direct binding to munc18-1.

Implications for fusion

Our transfection experiments in PC12 cells support the notion that the closed conformation of syntaxin 1 and the formation of a syntaxin–munc18 complex are physiologically important. The inhibitory effect of the transfected cytoplasmic region of syntaxin 1 on secretion probably occurs through binding to an endogenous factor(s) that is critical for secretion, effectively sequestering it from the exocytotic machinery. *A priori*, primary candidates for this factor are proteins that bind strongly to syntaxin 1 *in vitro*, such as synaptobrevin/SNAP-25 and munc18-1. Our results indicate that the inhibition of secretion occurs through binding to munc18-1 since the only mutation that disrupts munc18-1 binding (L165A/E166A) is also the only mutation that eliminates the inhibition. The inhibitory effect does not appear to be mediated by binding to SNAP-25 since the L205A,E206A mutation decreases binding to SNAP-25 but does not preclude the inhibition. In addition, the L165A,E166A mutation increases binding to synaptobrevin; if the inhibitory effect occurred through binding of the transfected syntaxin 1 to synaptobrevin, the mutation would be expected to preserve or increase inhibition instead of abolishing it. At present, we cannot completely rule out the possibility that the transfected syntaxin 1 inhibits secretion through binding to a protein other than munc18-1 or the SNAREs, e.g. munc13 (Betz *et al*., 1997).

The finding that the inhibition of secretion by the transfected cytoplasmic region of syntaxin 1 probably occurs by sequestering munc18-1 from the exocytotic machinery points to a critical role for munc18-1 in membrane fusion. Such a role is in agreement with the strong phenotypes observed in mutants of munc18 and munc18 homologs. At the same time, the requirement for a closed conformation of syntaxin 1 for munc18-1 binding implies that the closed conformation constitutes an obligatory intermediate in membrane fusion. All these results suggest that syntaxin switches between two conformations during exocytosis, a closed conformation that binds munc18-1 and an open conformation that forms the core complex.

An N- to C-terminal interaction in syntaxin has been proposed to regulate core complex formation, perhaps by preventing reassembly after the action of α-SNAP/NSF disassembles the complex (Hanson *et al*., 1995; Nicholson *et al*., 1998). Since munc18-1 binding requires a closed conformation of syntaxin 1 and prevents formation of the core complex, the question can be raised: is the primary role of the closed conformation of syntaxin 1 and its interaction with munc18-1 to ensure the proper timing of core complex formation? This possibility is difficult to reconcile with the results of our transfection experiments in PC12 cells and with the complete block of secretion caused by mutations in munc18. It is most likely that the multiprotein complex that regulates membrane fusion acts as a 'well oiled machine' which requires a minimal number of pieces to function properly. This would imply that the munc18–syntaxin complex performs a function in fusion that is as central as that of the core complex itself. As a result, at least one additional factor, perhaps with an enzymatic role, would be required to catalyze the conformational change in syntaxin 1 that mediates the transition between the munc18–syntaxin complex and the core complex. Unraveling the nature of the factor, and which of the two complexes involving syntaxin occurs first during exocytosis, are among the main challenges toward understanding the mechanism of intracellular membrane fusion.

Materials and methods

Construction of expression vectors

pCMV-SyntIA (residues 1–264, wild-type) was constructed by subcloning a 0.8 kb *Eco*RI–*Hin*dIII fragment from pGEX-SyntIA (McMahon

and Südhof, 1995) into the same site of pCMV5. Syntaxin mutants (D31A,E32A; E52A,E53A; R90A,K94A; L123A,S124A; L165A,E166A; L205A,E206A; and V244A,E245A) were made initially as full length in pCMV5, using pCMV-HPC-1 (McMahon and Südhof, 1995) as a template (after a *Bss*HII site was introduced as a silent mutation) and Chameleon™ double-stranded, site-directed mutagenesis kit (Stratagene). The 0.8 kb PCR fragments on these mutant constructs were then digested by *Eco*RI and *Bgl*II and subcloned into the same site of pCMV5, resulting in pCMV-SyntIA mutants (residues 1–264). pCMV-SyntIA (residues 1–180) was constructed by subcloning a 0.55 kb *Eco*RI–*Hin*dIII fragment from pGEX-SyntIA (residues 1–180) (Hata *et al*., 1993; McMahon and Südhof, 1995). pCMV-SyntIA (residues 1–253 and 1–226) were constructed by digesting PCR fragments on pCMV-SyntIA (residues 1–264) with *Eco*RI and subcloning them into the *Eco*RI site of pCMV5.

Expression vectors for recombinant GST–syntaxin 1A (residues 1–264) fusion proteins containing mutations were constructed by subcloning 0.8 kb *Eco*RI–*Hin*dIII fragments from pCMV constructs into pGEX-KG (Guan and Dixon, 1991). Expression vectors for GST–syntaxin 1A fragments 1–180 and 27–146 were described earlier (Fernandez *et al.*, 1998). GST–syntaxin 1A fragments 180–264, 26–253, 147–253 and 191–253 were made using customary designed primers (Operon Technologies, Inc.) and standard PCR cloning techniques, and subcloned into the *Nco*I–*Sal*I cloning sites of pGEX-KG (for the 180–264 fragment) or into the *Bam*HI–*Eco*RI sites of the pGEX-KT expression vector (Hakes and Dixon, 1992).

Protein expression and purification

Recombinant proteins for NMR studies were expressed in minimal media using $^{15}NH_4Cl$ as the sole nitrogen source. Briefly, BL21(DE3) cells containing the corresponding expression plasmid were grown till OD_{600} ~0.6 in the presence of 100 μg/ml ampicillin and induced overnight at room temperature with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) in the presence of an additional 100 μg/ml ampicillin. Cells were harvested by centrifugation, resuspended in ice-cold phosphate-buffered saline (PBS) containing 2 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol (DTT) and 0.2 mM AEBSF, and snap-frozen in liquid N_2. Cells were thawed on ice and broken by passing twice through an Avestin (Ottawa, Canada) cell disrupter at >12 000 p.s.i. After centrifugation of the lysate for 30 min at 18 000 *g* at 4°C, the resulting supernatant was incubated overnight at 4°C with glutathione–agarose beads (Sigma). The beads were washed sequentially in PBS containing 1% (w/v) Triton X-100 or 1 M NaCl followed by five washes in PBS and thrombin cleavage buffer (50 mM Tris pH 8.0, 200 mM NaCl, 2.5 mM $CaCl_2$), and cleaved with thrombin (Sigma). Cleaved proteins were eluted and purified by anion-exchange chromatography FPLC (Pharmacia, MonoQ) in 20 mM Na phosphate (pH 7.5) buffer and a 0–1 M NaCl gradient. Buffer was exchanged a few times for 20 mM Na phosphate (pH 7.4) containing Sigma protease inhibitor cocktail and 2 mM DTT to ensure that the final NaCl concetration in the NMR sample was <10 mM.

NMR experiments

All NMR spectra were acquired on a Varian INOVA600 spectrometer using a PFG-triple resonance probe. Most ^1H–^{15}N HSQC spectra were acquired at 25°C with 100–150 μM protein samples dissolved in 20 mM phosphate (pH 7.4). For the wild-type syx26–253 fragment, additional ^1H–^{15}N HSQC spectra were acquired at 25°C with protein concentrations between 50 and 700 μM, and with 400 μM protein concentration at temperatures ranging from 10 to 37°C. The spectral widths used in the ^1H (F2) and ^{15}N (F1) dimensions were 9000 and 1140 Hz, respectively. Cross-peak assignments for the ^1H–^{15}N HSQC spectrum of the syx27–146 fragment under the conditions of this study were obtained from a three-dimensional HNCO spectrum and by comparison with our previous assignments obtained at pH 6.5 and 32°C (Fernandez *et al.*, 1998; BMRB accession No. 4198). Assignments for the ^1H–^{15}N HSQC cross-peaks of the syx191–253 fragment were first obtained at 5°C using three-dimensional ^{15}N-edited TOCSY-HSQC and NOESY-HSQC spectra since at 25°C the NOEs are very weak and several ^1H–^{15}N HSQC cross-peaks broaden beyond detection due to fast exchange with the solvent. Assignments at 25°C were then obtained from a temperature titration using ^1H–^{15}N HSQC spectra, and confirmed with a three-dimensional ^{15}N-edited TOCSY-HSQC spectrum. Pulse-field gradient and sensitivity-enhanced pulse sequences were used for all experiments (Kay *et al*., 1994; Zhang *et al*., 1994).

Protein binding experiments

GST pull-down assays from total solubilized rat brain homogenate were performed essentially as described (Hata *et al*., 1993). Aliquots of 40 μl of glutathione–agarose beads containing ~0.4–0.5 nmol of different syntaxin mutants or GST alone were incubated overnight at 4°C with 2 ml of pre-cleared total rat brain homogenate (~40 mg protein). Beads were washed five times with 1.5 ml of ice-cold binding buffer (40 mM HEPES pH 7.4, 100 mM NaCl, 1% CHAPS and 2 mM EDTA) and analyzed by 12% SDS–PAGE followed by Coomassie Blue staining. For immunoblotting, ~1/10 part of each sample was separated by SDS–PAGE, transferred to nitrocellulose and probed with the corresponding antibody. Blots were washed, incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham) and developed with an ECL Western blotting detection system (Amersham). Monoclonal antibodies to synaptobrevin (Cl 69.1), Rab3A (Cl 42.2) and synaptogyrin (Cl 80.1) were a gift of Dr R.Jahn. The monoclonal antibody to SNAP-25(SMI81) was from Sternberger Monoclonals Inc. The monoclonal antibody to munc18 was from Transduction Laboratories. Polyclonal antibodies for complexins I and II were described previously (McMahon *et al*., 1995).

Protein expression in COS7 cells

COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) under 5% CO_2 and transfected using DEAE–dextran with chloroquine and a 2 min glycerol shock as described (Gorman, 1985; Sugita *et al*., 1999b) with 6.6 μg of DNA for 900 000 cells in a 10 cm dish. Cells were washed with PBS 3 days after transfections and harvested in 1.0 ml of SDS sample buffer. Proteins were extracted after passage through a 25 gauge needle (10 times), and aliquots (8 μl) were analyzed by standard SDS–PAGE and immunoblotting using ECL detection with antibodies previously described (McMahon *et al*., 1995).

PC12 cell secretion experiments

PC12 cells were cultured at 37°C in 5% CO_2 in 75 cm^2 flasks (uncoated) in RPMI 1640 with heat-inactivated 10% horse serum and 5% FBS, penicillin (50 U/ml) and streptomycin (50 U/ml). Confluent cells were harvested, triturated by passage through a 5 ml serological pipet, and plated onto collagen-coated, 6-well 35 mm dishes (Costar) at 40–50% confluency. About 40–48 h later (70–80% confluency), 2.4 μg of plasmid DNA was transfected by lipofectamine (Gibco-BRL) according to the manufacturer's specifications in DMEM without serum and antibiotics to increase transfection efficiency. After 6 h, 4 ml of complete RPMI 1640 medium was added. PC12 cells from dishes transfected with the same plasmids were harvested 2 days later, pooled and re-plated on 12-well 22 mm dishes (Corning) at a ratio of two 22 mm wells for one transfected 35 mm well. One day after re-plating, secretion experiments were performed with all test and control conditions carried out on the same pool of transfected cells. Controls were treated with physiological saline solution (PSS; 145 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 15 mM HEPES pH 7.4). KCl-triggered secretion was stimulated by a 15 min incubation with high K^+ saline solution (PSS containing 95 mM NaCl and 56 mM KCl). α-latrotoxin was added for 10 min at 0.3 nM in PSS. The percentage of hGH secreted into the medium and retained in the cells was measured by radioimmunoassay (Nichols Institute, CA).

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