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Binding of Munc18-1 to Synaptobrevin and to the SNARE Four-Helix Bundle[†]

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ABSTRACT: Sec1/Munc18 (SM) proteins and soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) form part of the core intracellular membrane fusion machinery, but it is unclear how they cooperate in membrane fusion. The synaptic vesicle SNARE synaptobrevin and the plasma membrane SNAREs syntaxin-1 and SNAP-25 assemble into a tight SNARE complex that includes a four-helix bundle formed by their SNARE motifs and is key for fusion. The neuronal SM protein Munc18-1 binds to syntaxin-1 and to the SNARE complex through interactions with the syntaxin-1 N-terminal region that are critical for neurotransmitter release. It has been proposed that Munc18-1 also binds to synaptobrevin and to the SNARE four-helix bundle and that such interactions might be crucial for membrane fusion, but definitive, direct evidence of these interactions has not been described. Using diverse biophysical approaches, we now demonstrate that Munc18-1 indeed binds to synaptobrevin and to the SNARE four-helix bundle. Both interactions have similar affinities (in the low micromolar range) and appear to involve the same cavity of Munc18-1 that binds to syntaxin-1. Correspondingly, the N-terminal region of syntaxin-1 competes with the SNARE four-helix bundle and synaptobrevin for Munc18-1 binding. Importantly, the Munc18-1 binding site on synaptobrevin is located at the C-terminus of its SNARE motif, suggesting that this interaction places Munc18-1 right at the site where fusion occurs. These results suggest a model in which neurotransmitter release involves a sequence of three different types of Munc18-1−SNARE interactions and in which Munc18-1 plays a direct, active role in membrane fusion in cooperation with the SNAREs.

Intracellular membrane traffic is governed by a conserved machinery formed by members of several protein families (*1*, *2*). Particularly important among these components are Sec1/Munc18 (SM) proteins and soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs),[1] which are believed to form the core of the fusion machinery (*3*−*5*). The primary function of the SNAREs became clear from studies of the synaptic vesicle protein synaptobrevin and the plasma membrane proteins syntaxin-1 and SNAP-25, the SNAREs that control neurotransmitter release. These studies showed that SNAREs form tight "SNARE complexes" through sequences called SNARE motifs (*6*). These motifs form a four-helix bundle (*7*, *8*) that brings the two membranes into close proximity (*9*), which is key for membrane fusion (*1*−*5*). The role of SM proteins is still highly unclear despite intense research.

Early studies showed that the neuronal SM protein Munc18-1 is essential for neurotransmitter release (*10*) and binds tightly to syntaxin-1 (*11*). In addition to a SNARE motif that precedes a C-terminal transmembrane (TM) sequence, syntaxin-1 contains an N-terminal region that includes a three-helix bundle domain called the Habc domain (*12*). Isolated syntaxin-1 forms a closed conformation that involves packing of the SNARE motif against the Habc domain and is incompatible with the SNARE complex but is critical for Munc18-1 binding (*13*). The Munc18-1−syntaxin-1 interaction involves a characteristic cavity formed in Munc18-1 due to its arch-shaped architecture (*14*) and gates entry of syntaxin-1 into SNARE complexes (*15*). However, studies of other SM proteins suggested that this interaction is not general, as the SM protein from the yeast plasma membrane, Sec1p, binds to assembled SNARE complexes rather than to the syntaxin homologue Sso1p (*16*), and the yeast vacuolar syntaxin Vam3p lacks a closed conformation (*17*). Moreover, SM proteins from other membrane compartments were found to bind to an N-terminal sequence of their cognate syntaxins (*18*−*21*).

This apparently confusing picture was partially clarified by a growing body of evidence showing that SM proteins generally bind to SNARE complexes and that the syntaxin N-terminal sequences often contribute to binding (refs *16* and *22*−*27*, but see ref *28*). These findings suggest that formation of SM protein−SNARE complex assemblies might underlie the key functional importance of SM proteins, as predicted in a model in which the core fusion machinery is formed by SM protein−SNARE complex assemblies rather than SNARE complexes alone (*23*, *29*) (Figure S1 of the Supporting Information). Indeed, Munc18-1 binding to the SNARE complex enhances SNARE-dependent lipid mixing in reconstitution assays (*24*, *30*) and is critical for neurotransmitter release (*31*), activities that depend on the interaction of Munc18-1 with the syntaxin-1 N-terminal

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[1]Abbreviations: CPMG, Carr−Purcell−Meiboom−Gill; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; BS3, bis(sulfosuccinimidyl)-suberate; *E*, FRET efficiency; FRET, fluorescence resonance energy transfer; HSQC, heteronuclear single-quantum correlation; MS, mass spectrometry; NMR, nuclear magnetic resonance; SNARE, soluble *N*-ethylmaleimide sensitive factor attachment protein receptor; WT, wild type.

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sequence (*24*, *31*). Moreover, interactions of Munc18-1 with the syntaxin-1 Habc domain within the SNARE complex are also crucial for priming synaptic vesicles to a readily releasable state (*32*). However, these interactions do not appear to be important for downstream events that lead to Ca^{2+}-triggered release (*32*) and, mechanistically, it is unclear how binding of Munc18-1 to the syntaxin-1 N-terminal region can help in the induction of membrane fusion. One possibility is that Munc18-1 could assist in SNARE complex assembly after syntaxin-1 is opened (*14*, *33*), and evidence of this proposal was described recently (*24*). In this scenario, Munc18-1 does not play a direct role in fusion but acts as an accessory factor in SNARE complex assembly. However, it seems difficult to reconcile this model with the strict requirement of Munc18-1 and SM proteins in general for membrane fusion (*34*), and some evidence supported a role for Sec1p in yeast exocytosis after formation of the SNARE complex (*35*).

The model assigning a direct role for Munc18-1 in membrane fusion that we proposed (Figure S1 of the Supporting Information) emerged from the realization that Munc18-1−SNARE complex assemblies might be much more efficient than SNARE complexes alone in exerting mechanical force on the membranes to induce fusion (*29*). Munc18-1 binding to the four-helix bundle formed by the SNARE motifs of syntaxin-1, SNAP-25, and synaptobrevin (below termed the SNARE four-helix bundle) is a central aspect of this model. Some data suggested that Munc18-1 indeed binds to the SNARE four-helix bundle (*23*, *24*), and the stimulation of SNARE-dependent lipid mixing caused by Munc18-1 in reconstitution assays appeared to depend on Munc18-1−synaptobrevin interactions (*24*). However, no direct binding of Munc18-1 to synaptobrevin could be detected in this study (*24*), and isothermal titration calorimetry (ITC) data indicated that Munc18-1 has a similar affinity for the SNARE complex and the syntaxin-1 N-terminal region, suggesting that this region is solely responsible for the SNARE complex binding to Munc18-1 (*36*). Hence, it is still unclear whether Munc18-1 indeed binds to synaptobrevin and to the SNARE four-helix bundle, despite the central importance that these interactions could have to couple the functions of Munc18-1 and the SNAREs in membrane fusion.

In this study, we have addressed these questions using a combination of biophysical approaches. We demonstrate that Munc18-1 binds directly to the SNARE motif of synaptobrevin and to the SNARE four-helix bundle with an affinity in the low micromolar range. Our data suggest that these interactions involve the same cavity of Munc18-1 involved in binding to the syntaxin-1 closed conformation and map the Munc18-1 binding site on synaptobrevin to the very C-terminus of its SNARE motif. These results suggest that synaptobrevin binding places Munc18-1 right at the site of membrane fusion and that Munc18-1 may indeed have a direct role in fusion in cooperation with the SNAREs.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. Vectors for expressing rat Munc18-1 and fragments corresponding to the cytoplasmic regions of rat syntaxin-1A (residues 2−253) or rat synaptobrevin-2 (residues 1−96), to the syntaxin-1A N-terminal region (residues 1−180), or to the SNARE motifs of syntaxin-1A (residues 191−253), synaptobrevin-2 (residues 29−93), and human SNAP-25B (residues 11−82 and 141−203) as GST fusion proteins were described previously (*12*, *13*, *23*, *37*). A vector for expressing squid Munc18-1 as a His-tagged protein was a kind gift from W. Weissenhorn (*38*). Cysteine mutations of Munc18-1 (K125C and K308C), syntaxin-1A(2−253) (D27C), and synaptobrevin(1−96) (S61C) were introduced with the QuickChange site-directed mutagenesis kit (Stratagene). All proteins were expressed and purified as described previously (*12*, *13*, *23*, *37*). SNARE complexes were assembled and purified as described previously (*23*, *32*, *37*).

Fluorescence Spectroscopy. Labeling with fluorescence probes was performed with samples of 50−100 μM single-cysteine mutated proteins in 20 mM HEPES (pH 7.2) and 100 mM KCl via incubation with a 10−20-fold excess of *N*-(2-aminoethyl)maleimide BODIPY-FL, tetramethylrhodamine-5-iodoacetamide dihydroiodide, or Texas Red C5-bromoacetamide (Molecular Probes) at room temperature for 6 h, and 10 mM DTT was added to stop the reaction (*39*). Fluorescence emission spectra (480−680 nm; excitation at 465 nm) were acquired on a PTI fluorimeter using 50−100 nM samples of BODIPY-FL-labeled Munc18-1 dissolved in 20 mM HEPES (pH 7.2), 100 mM KCl, and variable concentrations of SNARE complexes or fragments.

Chemical Cross-Linking. Rat Munc18-1 (17 μM) and 1×−5× synaptobrevin(29−96) were incubated with 5 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) or 1 mM bis-(sulfosuccinimidyl)suberate (BS3) for 1 h at room temperature and the reactions quenched with 25 mM Tris (pH 8.0). Samples of 18 μg of protein were loaded onto SDS gels for PAGE and LC−MS/MS analysis.

Miscellaneous. $^{1}H-^{15}N$ HSQC spectra were recorded on Varian INOVA600 spectrometers at 25 °C using samples dissolved in 20 mM HEPES (pH 7.2) and 120 mM KCl. ITC experiments were performed using a VP-ITC system (MicroCal) at 20 °C in 20 mM HEPES (pH 7.4) and 100 mM KCl with 20 μM squid Munc18-1 and successive injections of 0.4 mM synaptobrevin(49−96) or 0.5 mM synaptobrevin(77−96). The data were fitted using a single-site binding model with Origin for ITC version 5.0.

RESULTS

Munc18-1 Binds to Synaptobrevin and to the SNARE Four-Helix Bundle. To directly test for diverse types of Munc18-1−SNARE interactions using FRET, we prepared two mutants of rat Munc18-1 in which residue 125 or 308 was mutated to cysteine and labeled them with BODIPY-FL as a donor fluorescence probe. Specific labeling of residue 125 or 308 was verified by trypsin digestion and mass spectrometry (MS), and was favored by the exposed nature of these side chains (the native cysteines of Munc18-1 are buried). We will refer to the labeled Munc18-1 proteins as Munc18-125-BP and Munc18-308-BP. We also introduced single-cysteine mutations at residue 27 of the cytoplasmic region of syntaxin-1 (residues 2−253), where the only native cysteine (residue 145) was mutated to serine, and at residue 61 of the cytoplasmic region of synaptobrevin (residues 1−96). The mutants were labeled with Rhodamine or Texas Red as a fluorescence acceptor probe [termed syntaxin-27-Rho or -TR and synaptobrevin-61-Rho or -TR, respectively]. Representative experiments with one probe or the other are shown below, but the nature of the probe did not alter the results observed.

To test the labeled proteins in a well-characterized interaction, we first tested Munc18-1−syntaxin-1 binding. We observed a

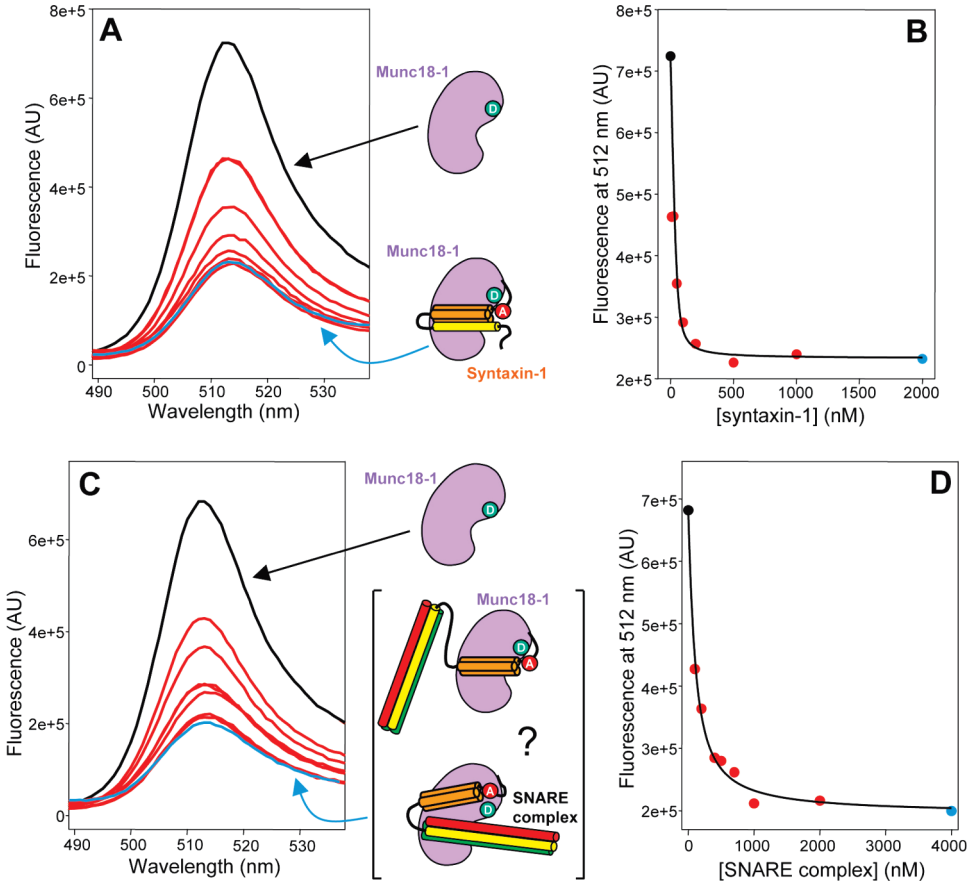

FIGURE 1: Binding of Munc18-1 to closed syntaxin-1 and neuronal SNARE complex monitored by FRET. (A and C) Emission fluorescence spectra of 50 nM Munc18-125-BP and variable concentrations of syntaxin-27-Rho (A) or the SNARE complex containing syntaxin-27-TR (C). The SNARE complex was formed with syntaxin-27-TR and the SNARE motifs of syntaptobrevin and SNAP-25. The diagrams next to the spectra represent Munc18-1 (purple) with a donor fluorescence probe (green), syntaxin-1(2−253) (SNARE motif, yellow; Habc domain, orange) labeled with an acceptor fluorescence probe (red), and SNARE complexes containing syntaxin-1(2−253), synaptobrevin (red), and SNAP-25 (green). In the bracket, two different types of Munc18-1−SNARE complex assemblies in which the SNARE four-helix bundle is or is not interacting with Munc18-1 are represented. (B and D) Plots of the fluorescence emission intensity at 512 nm as function of syntaxin-27-Rho concentration (B) and SNARE complex concentration (D), derived from the spectra shown in panels A and C, respectively. The data were fit to a standard single-binding site model.

high FRET efficiency ($E > 70\%$) between syntaxin-27-Rho and Munc18-125-BP (Figure 1A), as expected from the proximity of residue 125 of Munc18-1 to residue 27 of syntaxin-1 in their complex [~23 Å (*14*)] and assuming a Forster radius of 50 Å. Titrations with variable syntaxin-27-Rho concentrations yielded saturable dose−response curves (e.g., Figure 1A,B) and an approximate dissociation constant (K_D) of 5 nM, consistent with published data (*31*, *32*, *36*). Similar results were obtained with Munc18-308-BP (Figure S2 of the Supporting Information). We also analyzed binding of a SNARE complex containing syntaxin-27-TR or -Rho to Munc18-125-BP or Munc18-308-BP. We observed FRET efficiencies similar to those observed with isolated syntaxin-27-Rho or -TR, as expected if the Habc domain occupies a similar position upon binding of syntaxin-1 or the SNARE complex to Munc18-1 (*31*, *32*, *36*). Titrations with different SNARE complex concentrations yielded saturable binding curves with a K_D of ~100 nM (Figures 1C,D and data not shown), comparable to those determined previously by NMR spectroscopy (*23*, *32*). Hence, all these data show that the fluorescently labeled mutants exhibit interactions analogous to those between the WT proteins.

These data do not clarify whether the SNARE four-helix bundle binds to Munc18-1. To directly address this question, we studied binding of Munc18-1 to SNARE complexes containing synaptobrevin-61-Rho or -TR and lacking the syntaxin-1 N-terminal region (i.e., using a syntaxin-1 fragment spanning residues 191−253). Importantly, these complexes exhibited substantial FRET with Munc18-308-BP and less efficient FRET with Munc18-125-BP (Figure 2A and data not shown). In titration experiments, we were unable to reach complete saturation because of the limited availability of the fluorescently labeled SNARE four-helix bundles, but the data could be fit well with a standard protein−ligand binding equation (Figure 2B), yielding estimated K_D values on the order of 6 μM. Hence, these results demonstrate that Munc18-1 binds to the SNARE four-helix bundle with low micromolar affinity.

We also tested for binding of isolated synaptobrevin-61-Rho or -TR to Munc18-308-BP and observed even more efficient FRET (Figure 2C). Titrations with different synaptobrevin-61-Rho concentrations (Figure 2D) also yielded K_D values on the order of 6 μM. This similarity in affinities suggests that analogous residues mediate the interactions of Munc18-1 with synaptobrevin and with the SNARE four-helix bundle, and hence that synaptobrevin is largely responsible for binding of the SNARE four-helix bundle to Munc18-1. The observation that the FRET efficiency is higher for the synaptobrevin interaction (compare panels A and C of Figure 2) is not inconsistent with this conclusion, since isolated synaptobrevin is known to be highly

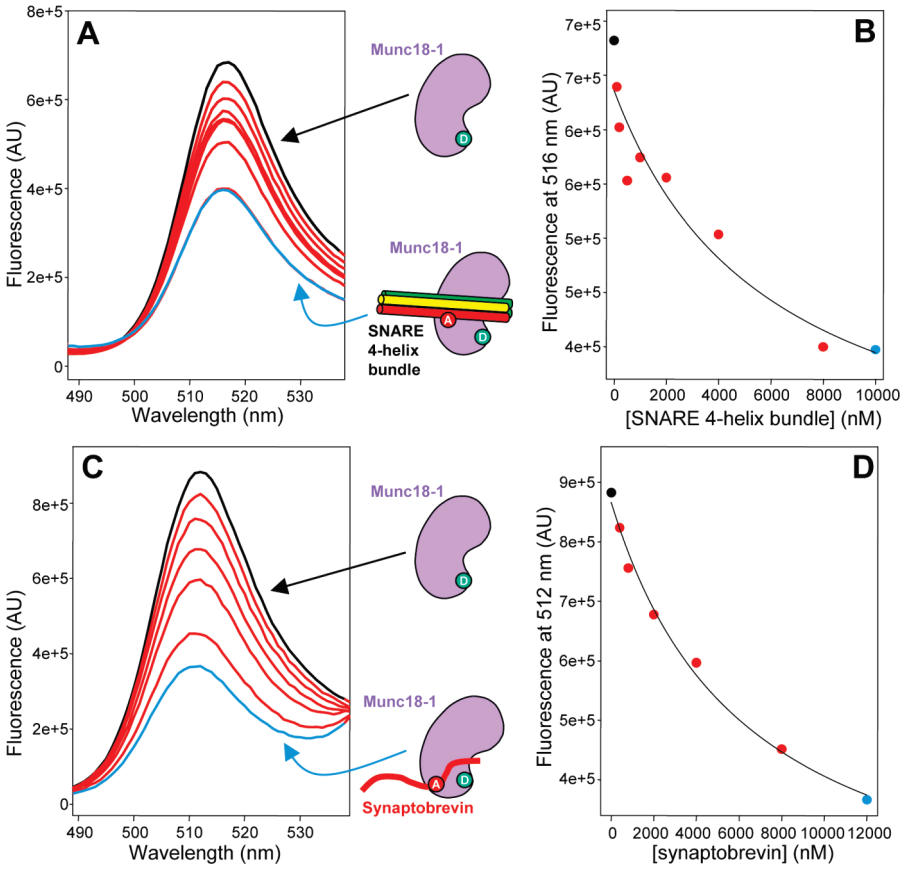

FIGURE 2: Munc18-1 binds to synaptobrevin and the SNARE four-helix bundle. (A and C) Emission fluorescence spectra of 50 nM Munc18-308-BP and variable concentrations of synaptobrevin-61-Rho (C) or the SNARE four-helix bundle containing synaptobrevin-61-TR and the SNARE motifs of syntaxin-1 and SNAP-25 (A). The diagrams next to the spectra represent Munc18-1 (purple) with a donor fluorescence probe (green), synaptobrevin(1−96) (red) with an acceptor fluorescence probe (red), and SNARE four-helix bundles containing labeled synaptobrevin(1−96) (red) and the SNARE motifs of syntaxin-1 (yellow) and SNAP-25 (green). (B and D) Plots of the fluorescence emission intensity at 512 nm as function of synaptobrevin-61-Rho concentration (D) and SNARE four-helix bundle concentration (B), derived from the spectra shown in panels A and C, respectively. The data were fit to a standard single-binding site model.

flexible (*40*). Therefore, if the region of synaptobrevin containing residue 61 remains flexible upon Munc18-1 binding, such flexibility can yield a range of distances between the donor and acceptor probes, and the r^{-6} dependence of the FRET efficiency is expected to yield ensemble-averaged values that are strongly biased in favor of the shorter distances. Note also that addition of 50 μM unlabeled synaptobrevin(1−96) led to a considerable decrease in the efficiency of FRET between Munc18-1 and the SNARE four-helix bundle containing synatobrevin-61-TR (Figure S3A of the Supporting Information), showing that synaptobrevin and the SNARE four-helix bundle compete for Munc18-1 binding and strongly supporting the conclusion that both interactions involve similar residues.

The Syntaxin-1 N-Terminal Region Competes with the SNARE Four-Helix Bundle and Synaptobrevin for Munc18-1 Binding. The syntaxin-1 closed conformation consists of a four-helix bundle formed by the Habc domain and the SNARE motif (*14*). Hence, it is natural to speculate that the SNARE four-helix bundle may bind to the same cavity of Munc18-1 as the syntaxin-1 closed conformation. Since the Habc domain likely remains bound at the same site when the SNARE complex interacts with Munc18-1 (*32*), this domain might hinder binding of the SNARE four-helix bundle to Munc18-1. To test this hypothesis, we first performed FRET experiments with Munc18-308-BP and the SNARE four-helix bundle containing synaptobrevin-61-Rho or -TR. Efficient FRET was observed when excess (10 μM) SNARE four-helix bundle was added to 50 nM Munc18-308-BP, but most of the FRET was lost when 10 μM syntaxin-1 N-terminal region (residues 1−180) was present (Figure 3A). Moreover, we observed no FRET between Munc18-308-BP and the SNARE complex containing the syntaxin-1 N-terminal region and synaptobrevin-61-Rho or -TR (Figure 3B). Although we cannot rule out the possibility that these results arise from conformational changes, the data strongly support the hypothesis that the syntaxin-1 N-terminal region and the SNARE four-helix bundle compete for binding to the same Munc18-1 cavity. The syntaxin-1 N-terminal region also decreased the efficiency of FRET between synaptobrevin-61-Rho and Munc18-308-BP (Figure S3B of the Supporting Information), again suggesting that the same Munc18-1 cavity binds to synaptobrevin.

Munc18-1 Binds to the C-Terminus of the Synaptobrevin SNARE Motif. To gain further insights into the residues involved in Munc18-1−synaptobrevin binding, we first performed chemical cross-linking experiments between WT Munc18-1 and a synaptobrevin fragment spanning its SNARE motif (residues 29−96). No cross-linking was observed when we used EDC, an agent that links carboxyl groups to primary amines (Figure 4A), but efficient cross-linking was obtained upon addition of BS3, an agent that cross-links primary amines with primary amines (see the red arrow in Figure 4A). Trypsin digestion of the cross-linked product and MS analysis revealed one peptide containing a

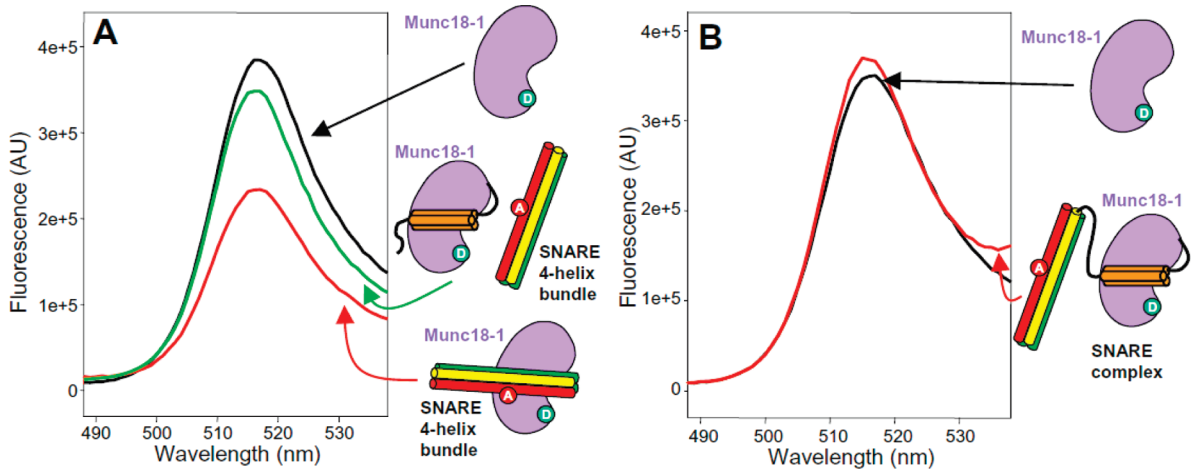

FIGURE 3: The N-Terminal region of syntaxin-1 competes with the SNARE four-helix bundle for Munc18-1 binding. (A) Emission fluorescence spectra of 50 nM Munc18-308-BP alone (black) or in the presence of 10 μM SNARE four-helix bundle containing synaptobrevin-61-TR and the SNARE motifs of syntaxin-1 and SNAP-25, before (red) or after (green) addition of 10 μM syntaxin-1 N-terminal region (residues 1−180). The diagrams next to the spectra represent Munc18-1 (purple) with a donor fluorescence probe (green), the syntaxin-1 N-terminal region (Habc domain colored orange), and SNARE four-helix bundles containing labeled synaptobrevin(1−96) (red) with an acceptor fluorescence probe (red) and the SNARE motifs of syntaxin-1 (yellow) and SNAP-25 (green). (B) Emission fluorescence spectra of 50 nM Munc18-308-BP alone (black) or in the presence of 20 μM SNARE complex formed with synaptobrevin-61-Rho, syntaxin(2−253), and the SNARE motifs of SNAP-25.

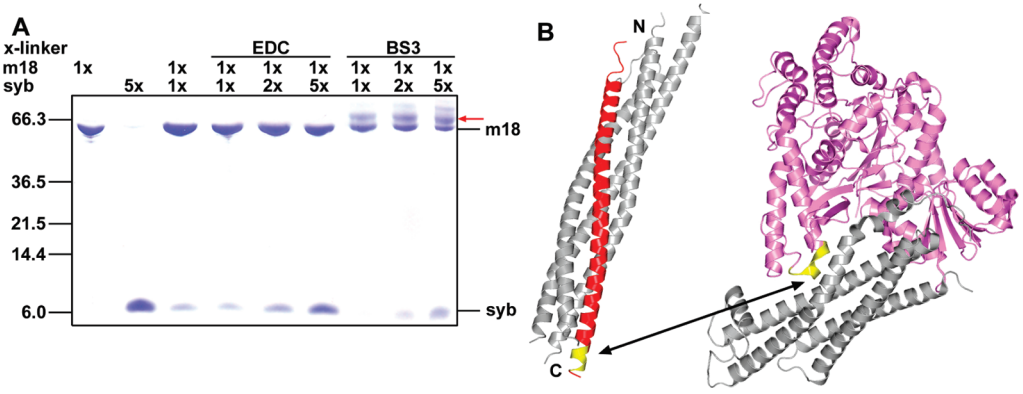

FIGURE 4: Cross-linking of synaptobrevin and Munc18-1. (A) SDS−PAGE of samples containing synaptobrevin(29−96), Munc18-1, or both after cross-linking with EDC or BS3. The relative concentrations of both proteins are indicated above the lanes. The positions of molecular mass markers are indicated at the left. The red arrow at the right indicates the position of the cross-linked product. (B) Ribbon diagrams of the crystal structure of the SNARE complex (*8*) and the Munc18-1−syntaxin-1 complex (*14*) with synaptobrevin colored red and Munc18-1 colored purple (other proteins colored gray). The sequences of synaptobrevin and Munc18-1 that were cross-linked are colored yellow.

sequence from Munc18-1 (KMPQYQK, residues 333−339) cross-linked to a sequence from synaptobrevin (KYWWK, residues 87−91). The sequence of Munc18-1 is indeed located in the cavity that binds to closed syntaxin-1 (Figure 4B). Intriguingly, residues 87−91 of synaptobrevin are at the very C-terminus of the SNARE motif (Figure 4B). This finding could be fundamentally important for the mechanism of membrane fusion, since an interaction with the C-terminus of the synaptobrevin SNARE motif would place Munc18-1 right at the site where membrane fusion occurs.

To investigate the Munc18-1 binding site on synaptobrevin by a different method, we used NMR spectroscopy. We first attempted to acquire $^{1}H-^{15}N$ heteronuclear single-quantum coherence (HSQC) spectra of ^{15}N-labeled synaptobrevin(29−96) in the presence and absence of rat Munc18-1. While the spectra suggested that rat Munc18-1 indeed binds to the C-terminus of synaptobrevin(29−96), obtaining high-quality data was hindered by the insolubility of rat Munc18-1 at the concentrations required for these NMR experiments. To solve this problem, we turned to squid Munc18-1 (sMunc18-1), which shares a high degree of sequence identity with rat Munc18-1 (66.4%) and is more soluble [this better behavior allowed crystallization of isolated sMunc18-1 (*38*)]. Gel filtration experiments showed that sMunc18-1 co-elutes with mammalian syntaxin-1 and the SNARE complex (Figure S4 of the Supporting Information), showing that sMunc18-1 binds tightly to the mammalian SNAREs like rat Munc18-1. Moreover, we were able to obtain $^{1}H-^{15}N$ HSQC spectra of 40 μM synaptobrevin(29−96) in the absence and presence of sMunc18-1. Since synaptobrevin is unstructured (*40*), binding to a large protein such as sMunc18-1 is expected to lead to selective broadening of the cross-peaks from the sequences involved in binding, while regions that do not participate in the interaction are expected to remain flexible and still yield observable cross-peaks. Indeed, we observed that addition of sMunc18-1 caused broadening of some synaptobrevin(29−96) cross-peaks, while many others were unaffected (Figure 5A). Importantly, the majority of broadened cross-peaks correspond to the region encompassing residues 75−95 of synaptobrevin (Figure 5A) at the very C-terminus of its SNARE motif (Figure 5B), in correlation with the cross-linking results obtained

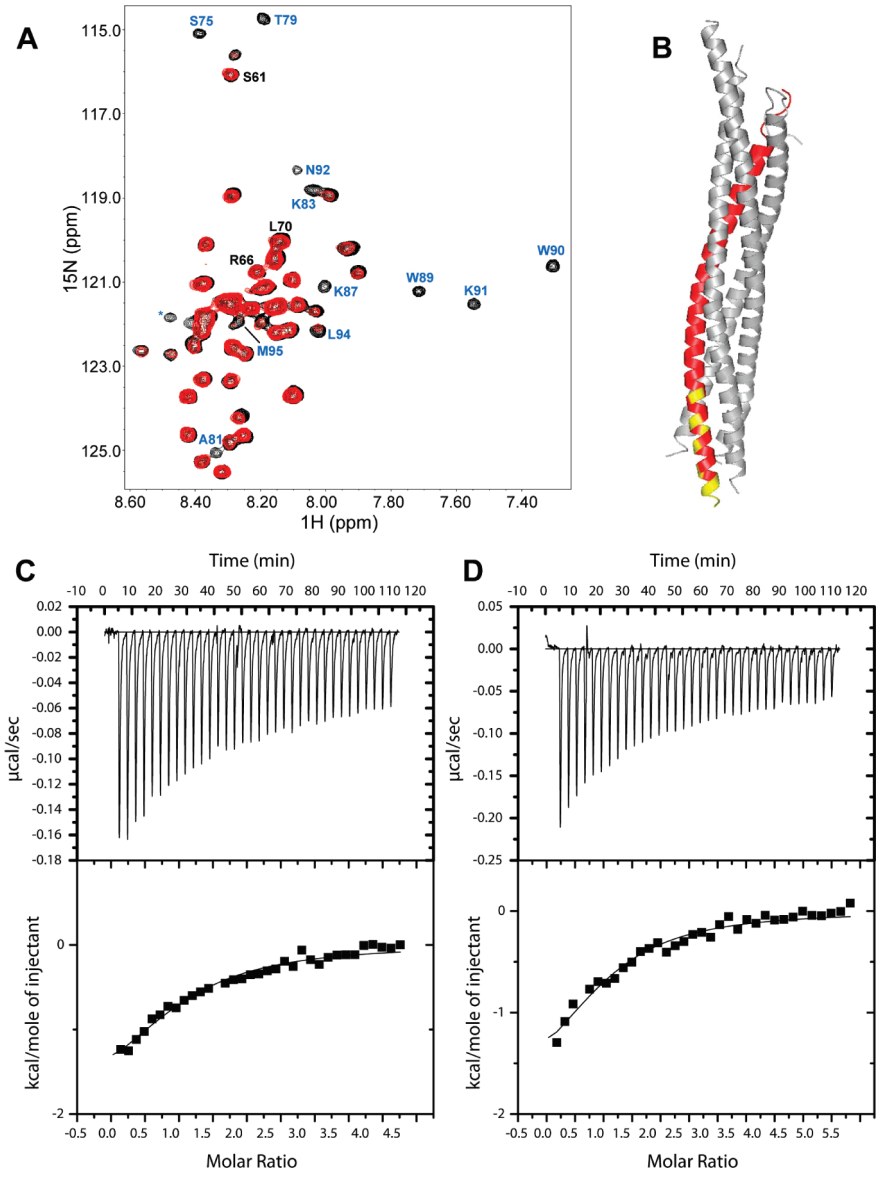

FIGURE 5: sMunc18-1 binds to the C-terminus of the synaptobrevin SNARE motif. (A) $^{1}H-^{15}N$ HSQC spectra of 40 μM synaptobrevin(29−96) in the absence (black) and presence (red) of 40 μM sMunc18-1. Well-resolved cross-peaks that were strongly broadened and correspond to residues at the C-terminus of the synaptobrevin SNARE motif are labeled in blue. Three well-resolved cross-peaks that do not exhibit such strong broadening and correspond to the region spanning residues 60−70 are labeled in black. (B) Ribbon diagram of the SNARE complex with synaptobrevin colored red; residues corresponding to the cross-peaks labeled in blue in panel A are colored yellow. (C and D) ITC analysis of binding of synaptobrevin(49−96) (C) or synaptobrevin(77−96) (D) to sMunc18-1.

with rat Munc18-1. Note that the binding region does not include residues 60−70, which were previously suggested to mediate Munc18-1−synaptobrevin interactions (*24*), although we cannot rule out the possibility that this region might contribute to Munc18-1−SNARE four-helix bundle interactions.

To further confirm our conclusions, we obtained a synthetic peptide corresponding to residues 77−96 of synaptobrevin and acquired ^{1}H NMR spectra of the peptide alone, sMunc18-1 alone, and a mixture of the peptide and sMunc18-1, comparing the intensities in the methyl region of the spectra before and after applying a Carr−Purcell−Meiboom−Gill (CPMG) pulse sequence for 100 ms. The stronger T_2 relaxation of the peptide in the presence of sMunc18-1 showed that the peptide binds to sMunc18-1 (Figure S5 of the Supporting Information). Finally, we also compared the affinity of sMunc18-1 for a fragment containing the C-terminal half of the synaptobrevin SNARE motif (residues 49−96) and the synaptobrevin(77−96) peptide using ITC, obtaining K_D values of 13.3 and 13.5 μM, respectively (Figure 5C,D). These values are comparable to the K_D values obtained by FRET for binding of rat Munc18-1 to synaptobrevin(1−96) and the SNARE four-helix bundle (Figure 2), and their similarity confirms that the C-terminus of the SNARE motif is responsible for synaptobrevin binding to Munc18-1.

DISCUSSION

SM proteins are central components of the intracellular membrane fusion machinery, but their main role is still highly unclear. This uncertainty arises in part because of the diversity of SM protein−SNARE interactions that have been identified and in part because no definitive evidence has been presented for the various models of SM protein function that have been proposed. To explain the very strong blocks in membrane fusion observed in the absence of SM proteins (*4*, *34*), one of these models

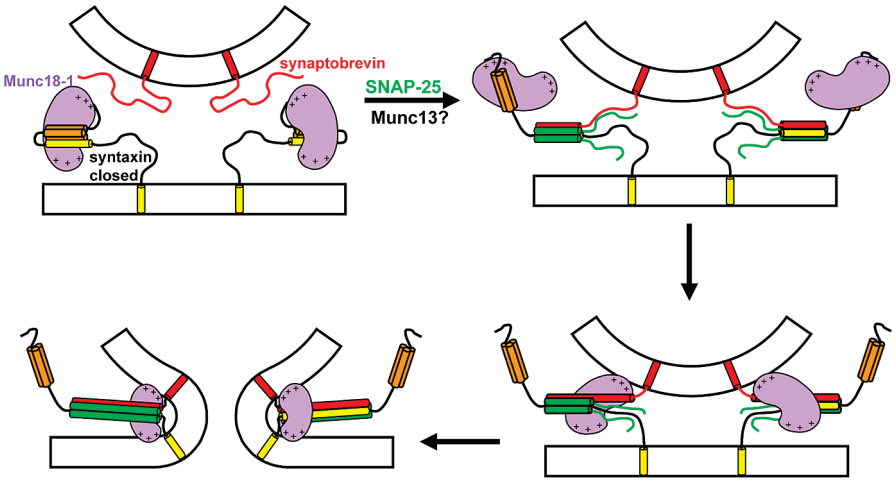

FIGURE 6: Proposed model of neurotransmitter release involving three types of Munc18-1−SNARE interactions. The model assumes that Munc18-1 (purple) is initially bound to closed syntaxin-1 (Habc domain, orange; SNARE motif, yellow) (top left panel). Partial assembly of SNARE complexes of syntaxin-1 with synaptobrevin (red) and SNAP-25 (green) occurs by an unknown mechanism that likely involves Munc13 (not shown); here we propose that Munc18-1 is bound to only the N-terminal region of syntaxin-1 at this stage (top right panel). The next step is proposed to involve the transition of Munc18-1 from the syntaxin-1 N-terminal region to the C-terminus of the synaptobrevin SNARE motif, which could be favored by cooperativity with other interactions such as Munc18-1 binding to the vesicle membrane (bottom right panel). The central aspect of this model is that membrane fusion results from the cooperative action of Munc18-1 and the SNAREs, which would be favored by the binding of Munc18-1 to synaptobrevin and could involve interactions of basic residues of Munc18-1 (indicated by the + signs) with both membranes (bottom left panel; see the text for further details). Other proteins involved in triggering release and conferring its Ca^{2+} sensitivity are not represented for the sake of simplicity, but they are expected to cooperate with Munc18-1 and the SNAREs to trigger release.

predicted that SM proteins bind to the SNARE four-helix bundle, enabling application of force to the membranes by the SNAREs (*29*) (Figure S1 of the Supporting Information). Reconstitution experiments showed that Munc18-1 can strongly enhance the ability of the SNAREs to induce lipid mixing and suggested that this ability depends on Munc18-1−synaptobrevin interactions, but no biochemical evidence of such interactions was described (*24*). We now show that Munc18-1 indeed binds directly to both synaptobrevin and the SNARE four-helix bundle with an affinity in the low micromolar range. Our data suggest that these interactions involve the same cavity of Munc18-1 in which the closed syntaxin-1 binds. Furthermore, we find that the C-terminus of synaptobrevin is responsible for Munc18-1 binding, placing Munc18-1 right at the site where membrane fusion occurs. These results reinforce the notion that Munc18-1 and SM proteins in general play a direct role in membrane fusion and suggest a speculative model in which a sequence of distinct Munc18-1−SNARE interactions occurs during synaptic vesicle exocytosis (Figure 6).

It is likely that several of the models of SM protein function that have been proposed over the years (*4*, *34*) are at least partially correct, since SM proteins may play several roles, but the key question is why SM proteins are so critical for fusion. The binary Munc18-1−syntaxin-1 complex imposes a roadblock that gates entry of syntaxin-1 into SNARE complexes (*13*, *15*, *33*, *41*), but this interaction might still play positive roles by stabilizing both proteins in vivo (*10*, *15*), and by assisting in vesicle docking, since syntaxin-1 and Munc18-1 function in docking in chromaffin cells while synaptobrevin does not (*15*). Importantly, overexpression of SNAP-25 rescues the docking defect in chromaffin cells from Munc18-1 knockout mice, likely by promoting formation of syntaxin-1−SNAP-25 heterodimers, but does not rescue the secretion defect (*42*). Hence, Munc18-1 must play an additional role downstream of docking and syntaxin-1−SNAP heterodimer assembly. There is in fact evidence of the participation of Munc18-1 in more than one of the steps that lead to exocytosis (*4*, *15*, *32*).

Although Munc18-1 binding to closed syntaxin-1 hinders formation of the SNARE complex from an energetic point of view (*33*), Munc18-1 could still play a role in assisting SNARE complex assembly in downstream events (*5*, *13*, *14*). Indeed, Munc18-1 enhances the assembly of SNARE complexes between coexpressed syntaxin-1−SNAP-25 heterodimers and synaptobrevin, which might underlie the stimulation of SNARE-dependent lipid mixing in reconstitution assays (*24*). Since the coexpressed heterodimers appear to be in an inhibited state where the Habc domain hinders full interactions between the SNARE motifs (*43*), it seems likely that Munc18-1 disinhibits this state by binding to the syntaxin-1 N-terminal region. It is unknown whether these events occur in vivo, but in any case, it seems clear that interactions of Munc18-1 with the syntaxin-1 N-terminal region are important for release (*31*, *32*). However, these interactions do not appear to play a role after vesicle priming (*32*), and it seems unlikely that a mere role for Munc18-1 in assisting in SNARE complex assembly underlies its essential nature for release (*10*), as SNARE complexes can be readily assembled in vitro. Moreover, stimulation of SNARE-dependent lipid mixing by Munc18-1 appears to depend on interactions with synaptobrevin (*24*), and some evidence indicates that the SM protein Sec1p functions downstream of SNARE complex formation (*35*).

These observations suggest that the key function of Munc18-1 and SM proteins in membrane fusion involves interactions with the SNARE four-helix bundle, as initially inferred for Sec1p (*16*) and as predicted by our previous model of how SM proteins and SNAREs form the core fusion machinery (*29*) (Figure S1 of the Supporting Information). Some results suggested the existence of such interactions (*23*, *24*, *28*, *44*), but ITC data indicated that Munc18-1 binding to the SNARE complex involves interactions with only the syntaxin-1 N-terminal region (*36*). Our results now show unambiguously that Munc18-1 indeed binds to the SNARE four-helix bundle and to the very C-terminus of the synaptobrevin SNARE motif (Figures 2, 4, and 5). It is very likely that both interactions involve the same residues, since they have

similar affinities and synaptobrevin competes with the SNARE four-helix bundle for Munc18-1 binding (Figure S3 of the Supporting Information). Our data also show that these interactions involve the cavity of Munc18-1 in which closed syntaxin-1 binds (Figures 3 and 4), which explains the ITC data mentioned above (*36*). These results need to be interpreted with caution, since the interactions we report have moderate affinity and their physiological relevance remains to be established. However, the finding that Munc18-1 binds to the very C-terminus of the synaptobrevin SNARE motif is very intriguing, since it places Munc18-1 right at the site of fusion and hence suggests a fundamental new view by which Munc18-1 is intimately and directly involved in fusion. Such a role could explain the critical nature of SM proteins for membrane fusion in vivo.

Our original idea of how Munc18-1 could help in the induction of fusion (*29*) (Figure S1 of the Supporting Information) was based on a simple mechanical principle: flexibility in the linker between the SNARE motifs and TM regions could hinder transduction of the energy of SNARE complex assembly onto the membranes and allow assembled SNARE complexes to diffuse to the middle of the intermembrane space. However, binding of a bulky protein such as Munc18-1 to assembling SNARE complexes would prevent such diffusion and would help in the application of torque on the two membranes to induce fusion, since Munc18-1 would push the membranes away while SNARE complexes bring them together. Application of force by the SNAREs on the membranes would be particularly efficient with Munc18-1 bound to the synaptobrevin C-terminus (Figure 6), as it would be impossible to fully assemble the SNARE complex without strongly bending the membranes (even if there is some flexibility in the linkers). Moreover, Munc18-1 contains two highly positive surfaces surrounding the SNARE-binding cavity (Figure S6 of the Supporting Information) that could bind to the two membranes and help in bending them to induce fusion (Figure 6), as proposed for synaptotagmin-1 (*39*). In this context, only weak binding of Munc18-1 to liposomes has been reported (*43*), but such interactions could be greatly strengthened by the intrinsic cooperativity of the system (see below).

Our model involves three different types of interactions of Munc18-1 with SNAREs (Figure 6): the binary interaction with closed syntaxin-1, the interaction with the syntaxin-1 N-terminal region as syntaxin-1 opens and SNARE complexes start to form via an as yet unknown mechanism that likely involves Munc13 (*43*), and the binding to the C-terminus of synaptobrevin. Although the affinity of Munc18-1 for synaptobrevin is moderate and weaker than its affinity for the syntaxin-1 N-terminal region in binding assays with isolated proteins, the transition from syntaxin-1 to synaptobrevin binding could be favored by strong cooperativity with the proposed interactions of Munc18-1 with both membranes, which would bring the membranes together and could therefore cooperate, in addition, with the pulling forces of the assembling SNARE complexes. Support for these ideas is provided by the finding that the HOPS complex, which includes the SM protein involved in yeast vacuolar fusion Vps33p, stabilizes trans SNARE complexes as opposed to cis SNARE complexes (*44*), since such stabilization must involve binding of HOPS to the two apposed membranes. Moreover, membranes appear to enhance binding of Munc18-1 to the SNARE four-helix bundle (*24*), and the functional importance of the synaptobrevin region that binds to Munc18-1 has been shown by the impairment in neurotransmitter release caused by mutation of two tryptophan residues in this region (*45*), although the molecular target of this region in vivo remains to be determined. Candidates for such targets are also Munc13s, complexins, and syntaptotagmin-1, factors that play key roles in neurotransmitter release and also bind to the SNARE four-helix bundle (*37*, *43*, *46*). These factors could provide additional cooperativity to the system, but they might also compete with Munc18-1 for binding to the four-helix bundle. Clearly, our model remains highly speculative and, to understand the mechanisms of membrane fusion and neurotransmitter release, it will be particularly important to study the interplay between the interactions among all these proteins and the lipids in the context of trans SNARE complexes formed between two apposed membranes. The data presented here have now yielded a hypothesis that can help in guiding these very challenging studies and propose a fundamentally new view of how Munc18-1 might actively cooperate with the SNAREs to induce membrane fusion.

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SUPPORTING INFORMATION AVAILABLE

Additional models and results. This material is available free of charge via the Internet at http://pubs.acs.org.

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