Accelerated Publications

Binding of the Munc13-1 MUN Domain to Membrane-Anchored SNARE Complexes†

Rong Guan, Han Dai, and Josep Rizo*

Departments of Biochemistry and Pharmacology, University of Texas Southwestern Medical Center, 6000 Harry Hines Boulevard, Dallas, Texas 75390

Received November 28, 2007; Revised Manuscript Received December 26, 2007

ABSTRACT: The core of the membrane fusion machinery that governs neurotransmitter release includes the SNARE proteins syntaxin-1, SNAP-25 and synaptobrevin, which form a tight "SNARE complex", and Munc18-1, which binds to the SNARE complex and to syntaxin-1 folded into a closed conformation. Release is also controlled by specialized proteins such as complexins, which also bind to the SNARE complex, and unc13/Munc13s, which are crucial for synaptic vesicle priming and were proposed to open syntaxin-1, promoting SNARE complex assembly. However, the biochemical basis for unc13/Munc13 function and its relationship to other SNARE interactions are unclear. To address this question, we have analyzed interactions of the MUN domain of Munc13-1, which is key for this priming function, using solution binding assays and cofloatation experiments with SNARE-containing proteoliposomes. Our results indicate that the Munc13-1 MUN domain binds to membrane-anchored SNARE complexes, even though binding is barely detectable in solution. The MUN domain appears to compete with Munc18-1 but not with complexin-1 for SNARE complex binding, although more quantitative assays will be required to verify these conclusions. Moreover, our data also uncover interactions of membrane-anchored syntaxin-1/SNAP-25 heterodimers with the MUN domain, Munc18-1 and complexin-1. The interaction with complexin-1 is surprising, as it was not observed in previous solution studies. Our results emphasize the importance of studying interactions within the neurotransmitter release machinery in a native membrane environment, and suggest that unc13/Munc13s may provide a template to assemble syntaxin-1/SNAP-25 heterodimers, leading to an acceptor complex for synaptobrevin.

The release of neurotransmitters by Ca^{2+}-triggered synaptic vesicle exocytosis is a critical event in interneuronal communication that involves docking of synaptic vesicles to active zones, vesicle priming to a release-ready state, and Ca^{2+}-evoked fusion (*1*). Release is governed by a sophisticated protein machinery that includes components with homologues in most types of intracellular membrane traffic, such as the Sec1/Munc18 (SM)[1] protein Munc18-1 and the SNARE proteins synaptobrevin/VAMP, syntaxin-1 and SNAP-25 (*2*−*6*). These proteins are believed to underlie a conserved fusion mechanism. Release also depends critically on proteins with specialized roles such as the active zone proteins unc13/Munc13s (*7*−*9*), the Ca^{2+} sensor synaptotagmin-1 (*10*) and complexins (*11*), which help to provide the exquisite temporal and spatial regulation of synaptic exocytosis.

The strong nature of some of the interactions within the release machinery facilitated their analysis at or near atomic resolution in solution, which yielded key mechanistic insights. Thus, synaptobrevin, syntaxin-1 and SNAP-25 form a tight "SNARE complex" consisting of a parallel four-helix bundle through sequences called SNARE motifs, which led to the notion that assembly of this complex is key for membrane fusion (*12*−*14*). Syntaxin-1 also forms a tight complex with Munc18-1 when folded into a "closed conformation" that hinders SNARE complex formation and involves an interaction of its N-terminal H_{abc} domain (*15*) with its SNARE motif (*16*, *17*), suggesting that syntaxin-1 undergoes a large conformational switch during exocytosis. Munc18-1 was also found to bind to the SNARE complex (*18*, *19*), which may underlie how SM proteins and SNAREs generally cooperate in membrane fusion (*6*). Biophysical studies have also revealed the structural basis for a Munc13 homodimer to Munc13-RIM heterodimer switch that may connect vesicle priming with presynaptic plasticity (*20*), and for the tight interaction between complexins and the SNARE

† This work was supported by Grant I-1304 from the Welch Foundation and NIH Grant NS37200 to J.R.

* To whom correspondence should be addressed. Phone: 214-645-6360. Fax: 214-645-6291. E-mail: jose@arnie.swmed.edu.

[1] Abbreviations: DLS, dynamic light scattering; DOPS, 1, 2-dioleoyl phosphatidylserine; NMR, nuclear magnetic resonance; β-OG, octyl-β-D-glucopyranoside; POPC, 1-palmitoyl, 2-oleoyl phosphatidylcholine; SM, Sec1/Munc18; SNARE, soluble *N*-ethylmaleimide sensitive factor attachment protein receptor; SNAP-25, synaptosomal associated protein of 25 kDa; TCEP, tris(2-carboxyethyl)phosphine; VAMP, vesicle associated membrane protein.

10.1021/bi702345m CCC: $40.75
Published on Web 01/18/2008

complex, which may stabilize the SNARE complex in preparation for fast Ca^{2+}-triggered release (*21*). However, it is noteworthy that many studies have reported SNARE−synaptotagmin-1 interactions [reviewed in (*1*, *6*, *22*)], but no such complex has been crystallized. Recent data suggested that difficulties in analyzing synaptotagmin-1/SNARE complex interactions arise because multiple binding modes exist in solution, and membrane anchoring of the SNARE complex is critical to increase specificity and stabilize the correct binding mode (*23*) [see also (*24*)]. Note also that complexin displaces synaptotagmin-1 from soluble SNARE complexes, but the opposite result is obtained on membranes (*25*). It seems likely that other crucial interactions within the neurotransmitter release machinery may also be strongly influenced by membranes or may even require membranes to be observable, given the very nature of the biological process they govern.

These arguments suggest that inclusion of membranes in biochemical experiments may be crucial to properly answer many questions that remain to be addressed to understand the mechanism of release. Perhaps the most crucial among these questions is to determine which interactions underlie the function of unc13/Munc13s. The total abrogation of release caused by genetic ablation of unc13 in *Caenorhabditis elegans* or the two major isoforms (Munc13-1 and Munc13-2) in mice (*7*−*9*) demonstrated the critical functional importance of these proteins, and a role in opening syntaxin-1 to promote SNARE complex formation was proposed based on the finding that overexpression of a constitutively open syntaxin-1 mutant (*16*) can partially rescue this phenotype in *C. elegans* (*26*), and on a report of a Munc13-1/syntaxin-1 interaction (*27*). However, it was later found that the protein fragments used in the latter study were not properly folded, and that an autonomously folded module containing these fragments, called the MUN domain, does not form binary complexes with syntaxin-1 in solution and yet rescues release in Munc13-1/2 double KO mice (*28*). These findings do not rule out that unc13/Munc13s may function in opening syntaxin 1, but show that the biochemical basis for this function remains to be determined.

In the study described here, we tested the hypothesis that the Munc13-1 MUN domain interacts with assembled SNARE complexes, but binding was barely detectable in solution. This observation, together with the arguments described above, led us to investigate whether the postulated interaction might require a membrane environment for the SNARE complex. Liposome cofloatation assays indicate that, indeed, the MUN domain binds to membrane-anchored SNARE complexes. Our data also suggest that the MUN domain partially competes with Munc18-1 but not with complexin-1 for SNARE complex binding, although more quantitative studies will be required to verify these conclusions. Moreover, cofloatation assays also reveal binding of the MUN domain, Munc18-1 and complexin-1 to membrane-anchored syntaxin-1/SNAP-25 heterodimers. These results support the proposed role of unc13/Munc13s in promoting SNARE complex assembly, and emphasize the importance of including membranes to study interactions within the release machinery.

EXPERIMENTAL PROCEDURES

Recombinant Proteins. Plasmids to express full-length rat Munc18-1, full-length rat complexin-1, full-length rat syntaxin-1A, the MUN domain of rat Munc13-1 (residues 859−1531), the cytoplasmic region of syntaxin-1A (residues 2−253), the SNARE motifs of human SNAP-25B (11−82 and 141−203), and the SNARE motif or the cytoplasmic domain of rat synaptobrevin-2 (29−93 or 1−96, respectively) as GST fusion proteins were described previously (*16*, *18*, *21*, *28*, *29*). Most of these proteins were expressed in bacteria as GST-fusion proteins, isolated by affinity chromatography, cleaved with thrombin and purified by ion exchange or gel filtration chromatography basically as described in these references. For full-length rat syntaxin-1A, cleavage with thrombin was performed in cleavage buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$) containing 1% (w/v) β-OG and the protein was purified by ion exchange chromatography on a MonoQ column (GE healthcare life sciences) in 20 mM Tris, pH 8.0, 0.5 mM TCEP, also containing 1% (w/v) β-OG, with a NaCl gradient. A plasmid encoding tag-free full-length rat syntaxin-1A as well as His_6-tagged full-length human SNAP-25B in a polycistronic vector with separate T7 promoters was made using custom-designed primers and standard PCR cloning techniques, and subcloned into the pETDuet (Novagen) coexpression vector. The plasmid was transformed into *Escherichia coli* Rosetta (DE3) competent cells, and the proteins were expressed at 25 °C with induction by 0.4 mM IPTG. The harvested cell pellets were resuspended into 30 mL of suspension buffer (50 mM Tris, pH 8.0, 300 mM KCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 20 mM imidazole, 0.5 mM ABESF), and disrupted by passing twice through a high-pressure cell extruder at ∼7,500 psi (model EmulsiFlex-C5, Avestin Inc.). The cell lysate was clarified by centrifugation at 18,000 rpm, mixed with 1.33 mL of 75% slurry of prewashed Ni-NTA agarose resin (QIAGEN) per liter of culture, and rotated in the cold room for 2 h at 4 °C. Nonspecifically bound proteins were removed by multiple washes with suspension buffer. The resin was washed twice with 5 bed volumes of 50 mM Tris, pH 8.0, 300 mM KCl, 1% (w/v) β-OG, 0.5 mM ABESF, and the recombinant proteins were released with 14 mL of Ni-NTA elution buffer (50 mM Tris, pH 8.0, 300 mM KCl, 1% (w/v) β-OG, 250 mM imidazole). 10 μL/mL sigma inhibitor cocktail, 0.5 mM ABESF and 1 mM DTT were added, and the eluted syntaxin/SNAP-25 complex was further purified by ion exchange chromatography on a monoQ column (GE healthcare) in 20 mM Tris, pH 8.0, 1% (w/v) β-OG, 0.5 mM TCEP, with a NaCl gradient.

Assembly of SNARE Complexes. Soluble SNARE complexes formed with the cytoplasmic region of syntaxin-1 and the SNARE motifs of synaptobrevin and SNAP-25 were assembled and purified as described (*18*). SNARE complexes for reconstitutions were assembled by incubating the syntaxin-1/His-SNAP-25 complex with a 1.5 excess of synaptobrevin-2 cytoplasmic fragment at room temperature for 2 h or overnight. Complex formation was checked by SDS−PAGE and Proto Blue or Coomassie Blue staining with boiled and nonboiled samples.

Proteoliposome Preparation. Starting liposomes contained 15% (w/w) DOPS and 85% (w/w) POPC, or 15% DOPS, 83.5% POPC and 1.5% *N*-(lissamine rhodamine B sulfonyl)-

1,2-dipalmitoyl phosphatidylethanolamine (Avanti Polar Lipids). Lipids in chloroform were mixed and the solvent was evaporated under a nitrogen gas stream, and dried overnight under vacuum. The lipid film was hydrated with reconstitution buffer (25 mM HEPES, pH 7.4, 100 mM KCl, 0.1 mM EGTA, 1 mM freshly added DTT) to give a final lipid concentration of 15 mM. The hydrated lipids were vigorously vortexed for >5 min. The resulting large multilamellar vesicles were disrupted by 5 freeze/thaw cycles in liquid N_2, and were forced through an 80 nm polycarbonate filter at least 21 times using a Mini-extruder (Avanti Polar Lipids, Alabaster, AL) to yield large unilamellar vesicles (LUV) of 100 nm mean diameter, as measured by DLS. The liposomes were stored at 4 °C and used within 3 days. For reconstitutions, the liposomes (100 μL; 15 mM lipids) were mixed at room temperature with 200 μL of 15 μM syntaxin-1/SNAP-25 heterodimer, preassembled SNARE complex or full-length syntaxin-1 dissolved in reconstitution buffer containing 1% (w/v) β-OG. The mixture was kept at room temperature for 30 min under gentle stirring, and the detergent β-OG was then removed by a three-step dialysis against reconstitution buffer (25 mM HEPES, pH 7.4, 100 mM KCl, 0.1 mM EGTA, and 1 mM freshly added DTT) containing Biobeads SM2 polystyrene beads (Bio-Rad Laboratories) (2 times with 1 L of buffer, 1 g of Biobeads for 2 h, plus one time with 2 L of buffer, 2 g of Biobeads overnight).

Gel Filtration Binding Assays. The assays were performed with a Superdex S200 10/300 GL column (GE healthcare) injecting 500 μL samples that contained a 5 μM concentration of each protein or complex (2.5 mM lipids for proteoliposomes), dissolved in 20 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM DTT. Binding reactions were incubated for 2 h at 4 °C before injection into the column.

1D ^{13}C-Edited ^{1}H NMR Experiments. The spectra were obtained by acquiring the first trace of a ^{1}H−^{13}C HSQC spectrum (1,500 scans; 30 min total acquisition time) on a Varian INOVA600 spectrometer at 25 °C in 20 mM Tris, pH 8.0, 150 mM NaCl, using H_2O/D_2O 95:5 (v/v) as the solvent.

Cofloatation Assays. Mixtures of the desired recombinant proteins and 75 μL of reconstituted proteoliposomes, all in reconstitution buffer, were diluted to 180 μL with reconstitution buffer and incubated at 4 °C for 1 h. The samples were mixed with an equal volume of 80% (w/v) HistoDenz (Sigma) and placed on the bottom of 5 × 41 mm ultracentrifuge tubes. The proteoliposomes were then overlaid with 150 μL of 35% (w/v) HistoDenz and 150 μL of 30% (w/v) HistoDenz. Additional reconstitution buffer (15 μL) was placed on the top of the density gradient. The samples were centrifuged at 48,000 rpm using an SW55 rotor (Beckman) for 4 h at 4 °C. The top of the gradient (150 μL) was then analyzed by SDS−PAGE and Proto Blue staining. For the experiment of Figure 2B, the gradient was divided into five equal samples of 135 μL each and analyzed by SDS−PAGE and Proto Blue staining.

RESULTS

Analysis of MUN Domain/SNARE Complex Interactions in Solution. The finding that the Munc13-1 MUN does not form binary complexes with diverse cytoplasmic fragments of syntaxin-1 (*28*), together with multiple failed attempts to detect interactions of the Munc13-1 MUN domain with other individual components of the release apparatus in solution [Nan Shen and Josep Rizo, unpublished results] and the notion that unc13/Munc13s may promote SNARE complex formation, led us to hypothesize that the MUN domain may provide a template to assemble SNARE complexes and hence that it may bind to such complexes. We first tested this hypothesis in solution using gel filtration assays. The method is illustrated in Figure 1A for the Munc18-1/SNARE complex interaction, which provides a positive control and is manifested by a shift in the elution volume of Munc18-1 due to SNARE complex binding (Figure 1A) [note that only a slight shift is observed for the SNARE complex because its radius of gyration is not substantially altered upon Munc18-1 binding; see ref (*18*)]. However, no shift in the

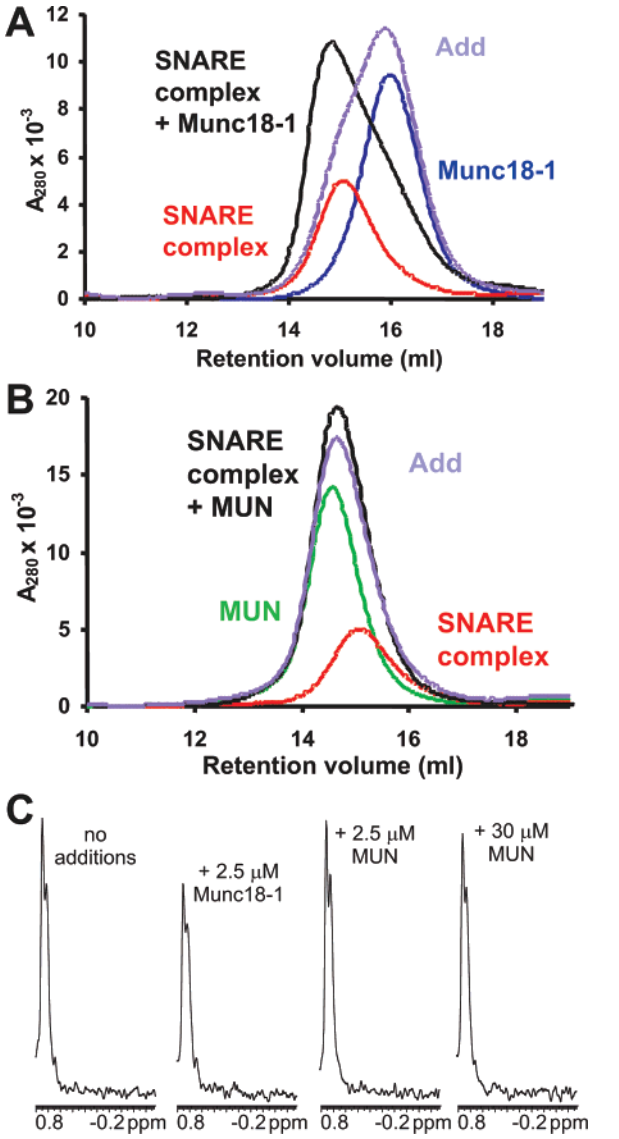

FIGURE 1: Analysis of MUN domain/SNARE complex interactions in solution. (A, B) Gel filtration elution profiles of purified Munc18-1 (blue), MUN domain (green), soluble SNARE complex (red), and mixtures of SNARE complex with Munc18-1 (A, black trace) or MUN domain (B, black trace). The purple lines show the addition of the red and blue traces in (A) and of the green and red traces in (B). (C) Methyl regions of 1D ^{13}C-edited ^{1}H NMR spectra of 2 μM SNARE complex containing uniformly ^{13}C-labeled syntaxin-1 in the absence or presence of unlabeled Munc18-1 or MUN domain.

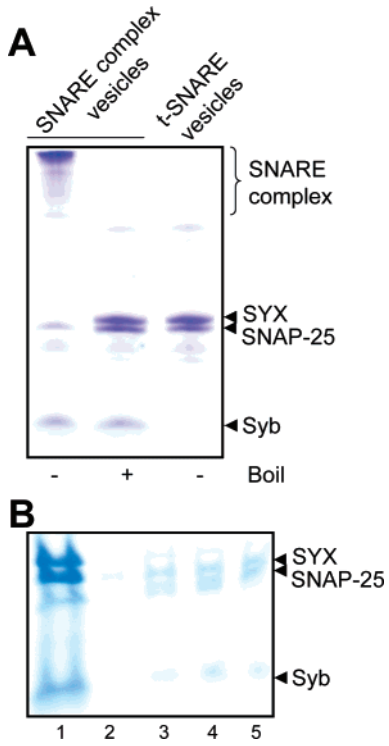

FIGURE 2: SDS−PAGE analysis of SNARE proteoliposomes. (A) Syntaxin-1/SNAP-25 heterodimers (t-SNARE), or SNARE complexes formed by incubation of these heterodimers with a 1.5-fold excess of the synaptobrevin cytoplasmic region in detergent solution, were reconstituted into preformed liposomes, and the resulting proteoliposomes were analyzed by SDS−PAGE and Proto Blue staining with or without boiling the samples as indicated. (B) SNARE complex proteoliposomes were subjected to a HistoDenz gradient, and the top fraction was analyzed with a second HistoDenz gradient. The figure shows the analysis by SDS−PAGE and Proto Blue staining of the five fractions collected in the second gradient (1−5 correspond to fractions taken from the top to the bottom of the gradient). All samples were boiled.

elution profile of the MUN domain or the SNARE complex was observed when they were incubated together and coinjected in the gel filtration column, and the chromatogram of the mixture (black curve in Figure 1B) was superimposable within experimental error with the addition of the individual chromatograms of the MUN domain and the SNARE complex (purple curve in Figure 1B).

Since interactions of moderate affinity may not be observable by gel filtration, we used a one-dimensional (1D) ^{13}C-edited NMR method (*30*) that does not suffer from this drawback. The method is based on detecting a decrease in the intensity of the strongest methyl resonance (SMR) of 1D ^{13}C-edited NMR spectra of a ^{13}C-labeled protein upon binding to an unlabeled protein due to the increase in correlation time and the resulting resonance broadening. We previously used this method to demonstrate binding of Munc18-1 to the SNARE complex (*18*), and here we also used this interaction as a positive control. Thus, the SMR intensity of the 1D ^{13}C-edited NMR spectrum of 2 μM SNARE complex containing ^{13}C-labeled syntaxin-1 exhibited a clear decrease upon addition of 2.5 μM Munc18-1 (Figure 1C). However, no such decrease was observed in the presence of 2.5 μM MUN domain, and increasing the MUN domain concentration to 30 μM led to only a slight decrease in SMR intensity that is practically within experimental error. Overall, the gel filtration and NMR data show that, if there is any interaction between the MUN domain and the SNARE complex in solution, it is of very weak affinity.

Analysis of Interactions between the MUN Domain and Membrane-Anchored SNARE Complexes. Our failure to conclusively detect an interaction of the MUN domain with the SNARE complex in solution, together with the finding that membrane anchoring of the SNARE complex strongly enhances its specific binding to synaptotagmin-1 (*23*), led us to hypothesize that a membrane environment may be required for binding of the MUN domain to the SNARE complex. To test this hypothesis, we coexpressed full-length syntaxin-1 and SNAP-25, and purified the resulting heterodimers. SNARE complexes were formed by incubation with the cytoplasmic region of synaptobrevin in detergent, and they were then reconstituted into preformed liposomes with a 1:500 protein to lipid ratio [higher protein densities led to aggregation on the membrane (*23*, *31*)]. Interestingly, analysis of the proteoliposomes by SDS−PAGE and Proto Blue staining revealed quantitative formation of SDS-resistant SNARE complexes, with no detectable isolated syntaxin-1 and only a small amount of remaining SNAP-25 (Figure 2A). This finding suggests that the coexpressed syntaxin-1/SNAP-25 heterodimers have a 1:1 stoichiometry that contrasts with the 2:1 stoichiometry observed with separately expressed syntaxin-1 and SNAP-25 (*32*), as half of the syntaxin-1 molecules would remain free after SNARE complex formation if the heterodimers had a 2:1 stoichiometry. The 1:1 stoichiometry was also suggested by analysis of the purified syntaxin/SNAP-25 heterodimers by SDS−PAGE before (data not shown) or after incorporation into liposomes (Figure 2A), since the bands of syntaxin-1 and SNAP25 had similar intensities and standard samples showed that both proteins have a similar staining capacity.

To test for binding of the MUN domain to proteoliposomes containing SNARE complexes, we first employed gel filtration experiments using also Munc18-1 as a positive control. However, no coelution of the MUN domain or Munc18-1 with the SNARE complex liposomes was observed (Figure 3). While the lack of binding of Munc18-1 to these proteoliposomes contrasts with the almost quantitative interaction observed with soluble SNARE complexes (Figure 1A) and might suggest that the presence of the membrane weakens the Munc18-1/SNARE complex interaction, it is likely that dissociation of membrane-anchored Munc18-1/SNARE complex assemblies during chromatography may be favored by the large difference in elution volumes between Munc18-1 and the proteoliposomes (in contrast to the similar elution volumes of Munc18-1 and soluble SNARE complexes).

To use a less stringent method to test for binding of the MUN domain to membrane-anchored SNARE complexes, we performed cofloatation assays in a HistoDenz gradient [ref (*19*)] (Figure 4). In these experiments, we also included Munc18-1 and complexin-1 in different combinations to investigate whether they might cooperate or compete with the potential MUN domain/SNARE complex interaction. The SNARE complex proteoliposomes used for these assays contained 1.5% rhodamine-labeled lipids for easy detection, and they were first separated from the excess of synaptobrevin used to ensure full SNARE complex assembly (see Figure 2A) using a HistoDenz gradient. The isolated proteoliposomes were then incubated with the MUN domain,

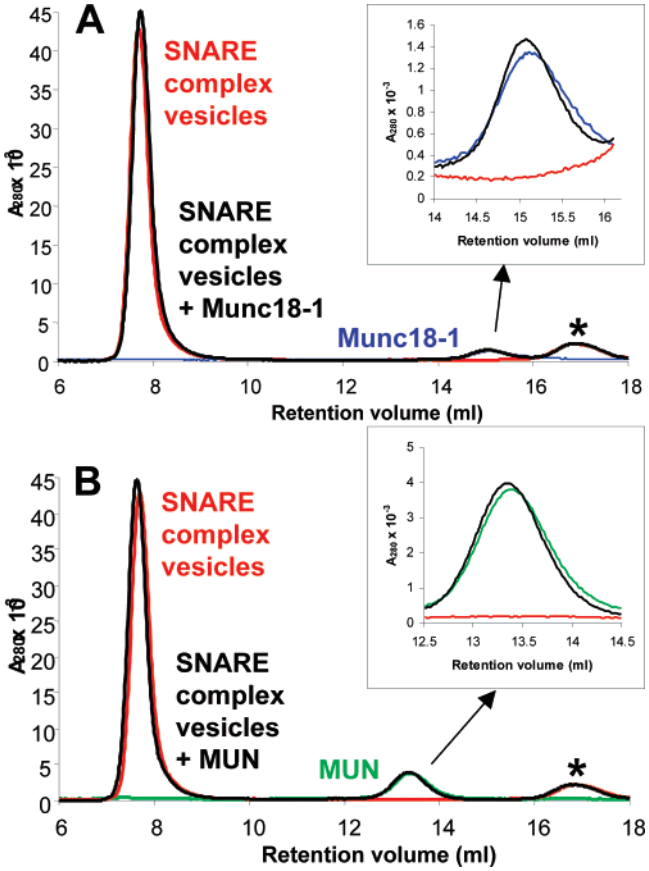

FIGURE 3: Analysis of MUN domain/SNARE complex proteoliposome interactions by gel filtration. (A, B) Elution profiles of Munc18-1 (blue), MUN domain (green), SNARE complex proteoliposomes (red), and mixtures of the proteoliposomes with Munc18-1 (A, black trace) or MUN domain (B, black trace). The insets show expansions of the regions where Munc18-1 (A) or the MUN domain (B) elute. The ∗ indicates a peak corresponding to excess synaptobrevin cystoplasmic region.

Munc18-1 and/or complexin-1 (in equimolar amounts with respect to the SNARE complex unless otherwise indicated), and were subjected to an additional HistoDenz gradient followed by SDS−PAGE analysis of the gradient fractions monitored by Proto Blue staining. Figure 2B illustrates an experiment performed with the SNARE complex proteoliposomes without additions, and where the samples were boiled before running the gel to visualize the separate SNARE proteins. Note that the presence of HistoDenz in the gradient fractions distorts the migration of the different proteins in the gels, but it is clear that the majority of the SNARE proteins floated to the top fraction of the gradient, where most of the lipids were also found based on detection of their rhodamine fluorescence. This pattern of distribution of the SNAREs and the phospholipids was also observed in all subsequent experiments with different protein additions, as assessed by analysis of boiled samples where syntaxin-1, synaptobrevin and SNAP-25 can be detected separately and with nonboiled samples where the three proteins run together in diffuse bands corresponding to SDS-resistant SNARE complexes (see Figure 4). In Figure 4 we present the data obtained with nonboiled samples because boiling partially degrades Munc18-1, and we focus on the top fraction of each experiment, which contains the proteins that bound to the SNARE complex proteoliposomes.

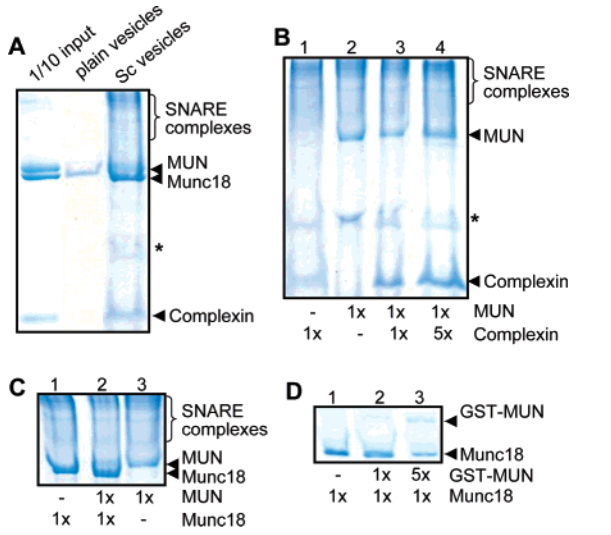

FIGURE 4: The MUN domain binds to membrane-anchored SNARE complexes. (A) Cofloatation assays of equimolar mixtures of MUN domain, Munc18-1 and complexin-1 with protein free liposomes (plain vesicles) or SNARE complex proteoliposomes (Sc vesicles). The samples were subjected to a HistoDenz gradient, and the top fraction of each gradient was analyzed by SDS−PAGE and Proto Blue staining without boiling. The left lane shows one tenth of the input used for the experiment performed with SNARE complex proteoliposomes before the gradient. Note that the SNARE complexes run in a diffuse pattern at the top of the gel. The ∗ indicates the position of residual free syntaxin-1 and SNAP-25. (B−D) Cofloatation assays with SNARE complex proteoliposomes and different combinations of proteins as indicated were performed and analyzed as in (A).

Importantly, inclusion of MUN domain, Munc18-1 and complexin-1 revealed clear binding of the three proteins to the SNARE complex-containing proteoliposomes, whereas only a trace amount of Munc18-1 bound to protein-free liposomes used as control (Figure 4A) (the identity of this band as Munc18-1, as opposed to MUN domain, was confirmed in separate control experiments where either Munc18-1 or the MUN domain was included; data not shown). In addition, the MUN domain also bound to the SNARE complex proteoliposomes in the absence of complexin-1 and Munc18-1 (Figure 4B, lane 2). Hence, these results indicate that the MUN domain binds directly to the membrane-anchored SNARE complexes and that the membrane indeed plays an important role in enhancing the MUN domain/SNARE complex interaction. It is unclear from the data in Figure 4A whether the MUN domain, Munc18-1 and complexin-1 can bind simultaneously to the membrane-anchored SNARE complexes, since only a fraction of each protein cofloated with the proteoliposomes (compare with the lane containing one tenth of the input in Figure 4A). Experiments performed with a constant amount of MUN domain and variable amounts of complexin-1, up to a 5-fold excess, suggested that these two proteins do not compete for binding to membrane-anchored SNARE complexes (Figure 4B). However, the degree of MUN domain binding observed in the presence of an equimolar amount of Munc18-1 appeared to be somewhat lower than in its absence (Figure 4C), suggesting that the two proteins compete at least partially for binding to membrane-anchored SNARE complexes. This conclusion was supported by competition experiments between Munc18-1 and GST-MUN domain (note that in these experiments we could not use an excess

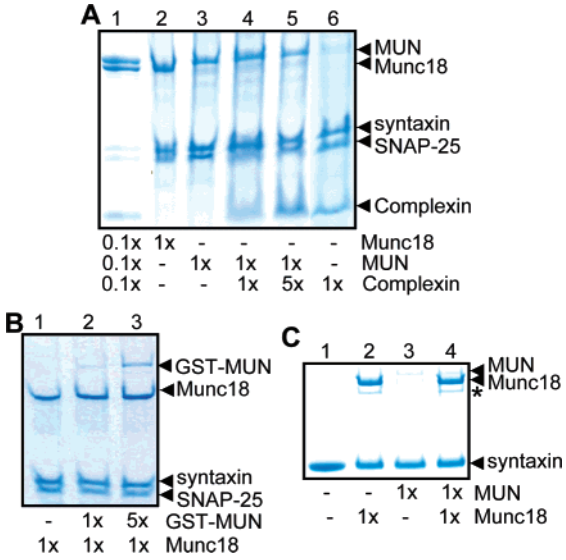

FIGURE 5: The MUN domain, Munc18-1 and complexin-1 bind to membrane-anchored syntaxin-1/SNAP-25 heterodimers. Cofloatation assays with proteoliposomes containing reconstituted syntaxin-1/SNAP-25 heterodimers (A, B) or syntaxin-1 (C) and different protein combinations were performed and analyzed as in Figure 4. The left lane in panel A shows one tenth of a sample containing the proteoliposomes, MUN domain, Munc18-1 and complexin-1 in the same amounts used for the cofloatation assays. In (C), the * indicates a degradation band of Munc18-1.

of Munc18-1 because of its limited solubility, and that an excess of MUN domain yielded inconclusive results due to the close migration of Munc18-1 and the MUN domain in SDS−PAGE). Thus, although the GST-MUN domain appeared to bind more weakly to the membrane-anchored SNARE complex than the MUN domain, addition of an excess of the fusion protein decreased Munc18-1 binding (Figure 4D). Note however that these competition results need to be interpreted with caution, since there was some variability between experiments and the distortions caused by the HistoDenz in the gels hindered quantitation of the data.

Analysis of Interactions of Membrane-Anchored Syntaxin-1/SNAP-25 Heterodimers. Since it is unclear to what extent interactions of the SNARE complex are shared by syntaxin-1/SNAP-25 heterodimers, we also used cofloatation assays to investigate whether the MUN domain, Munc18-1 and complexin-1 bind to syntaxin-1/SNAP-25 heterodimers reconstituted into proteoliposomes. Indeed, clear binding was observed for the three proteins (Figure 5A). As observed for SNARE complex interactions, competition assays between the MUN domain and complexin-1 suggested that both proteins can bind simultaneously to the membrane-anchored syntaxin-1/SNAP-25 heterodimers (Figure 5A). Moreover, experiments performed with Munc18-1 and variable amounts of GST-MUN domain suggested that they also can bind simultaneously to the heterodimers (Figure 5B), in contrast to the results obtained for the SNARE complex. The binding of the MUN domain to the syntaxin-1/SNAP-25 heterodimers uncovered by these results further supports its proposed role in promoting SNARE complex assembly. Note that the interaction of Munc18-1 with these heterodimers observed here had been suggested by studies using lawns of plasma membrane (*33*) but had not been described with purified components, whereas the binding of complexin-1 to membrane anchored syntaxin-1/SNAP-25 heterodimers observed in our assays contrasts with previous data suggesting that this interaction does not occur in solution (*34*), indicating that the membrane may play an important role in this interaction.

Although the MUN domain does not bind to the cytoplasmic region of syntaxin-1 in solution (*28*), the importance of the membrane in the diverse interactions uncovered here led us to test in a final set of experiments whether the MUN domain might bind to membrane-anchored full-length syntaxin-1 using also cofloatation assays. Munc18-1 used as a positive control exhibited robust binding to syntaxin-1 proteoliposomes, but practically no binding was observed for the MUN in the absence or presence of Munc18-1 (Figure 5C). These results indicate that the MUN domain does not interact with membrane-anchored syntaxin-1 in the absence of SNAP-25, or with syntaxin-1/Munc18-1 complexes.

DISCUSSION

Great advances have been made in characterizing high-affinity complexes involved in synaptic exocytosis, but many questions about the interactions that mediate this process remain unanswered. Particularly important is to understand the basis for the crucial function of the Munc13-1 MUN domain in release (*28*). Recent findings suggesting that interactions within the release machinery can depend critically on membranes (*23*, *25*), together with the proposed function of unc13/Munc13 in promoting SNARE complex assembly (*26*, *27*) and extensive failed attempts to detect binding of the Munc13-1 MUN domain to putative targets in solution, led us to hypothesize that the MUN domain binds to SNARE complexes anchored on membranes. Our cofloatation assays now support this hypothesis and suggest that the MUN can also bind to membrane-anchored syntaxin-1/SNAP-25 heterodimers. Moreover, our results also indicate that these heterodimers bind to Munc18-1, as suggested by previous studies (*33*), and to complexin-1, in contrast to solution data (*34*). Overall, our data support the notion that the MUN domain is involved in SNARE complex assembly, and emphasize the importance of performing biochemical studies of the release machinery in a membrane environment, which is not surprising given the fact that this machinery is assembled between two membranes.

Our cofloatation data need to be interpreted with caution, as they could be influenced by the presence of HistoDenz. However, the control with protein-free liposomes, which revealed only a small degree of Munc18-1 binding, indicates that the cofloatation of the MUN domain, Munc18-1 and complexin-1 with proteoliposomes containing SNARE complexes or syntaxin-1/SNAP-25 heterodimers is not simply due to nonspecific protein aggregation on the liposomes. This conclusion is further supported by the partial competition observed between Munc18-1 and the MUN domain for binding to membrane-anchored SNARE complexes, which also indicates specificity. Moreover, all the binding interactions observed in these cofloatation assays were consistently observed in multiple experiments performed with separate preparations. However, we did observe some variability in the amount of binding in different experiments, and quantitation of the results was hindered by the gel distortions caused by HistoDenz. Thus, while our cofloatation data were

highly reproducible from a qualitative point of view, more quantitative methods will be required to verify the results of our competition assays.

Our data suggest that the membrane can influence multiple SNARE interactions. Membrane anchoring of the SNARE complex appears to weaken Munc18-1 binding, given the contrast between the gel filtration results obtained with soluble (Figure 1A) or membrane-anchored complex (Figure 3A). This conclusion needs to be further tested, since this contrast may arise from the large difference in elution volumes between Munc18-1 and proteoliposomes, but it is plausible that Munc18-1 binding to fully assembled SNARE complexes is hindered by steric interactions with the membrane that may not occur in vivo if binding involves partially assembled SNARE complexes. Conversely, the membrane appears to be key for binding of the MUN domain to SNARE complexes and of complexin-1 to syntaxin-1/SNAP-25 heterodimers, which likely arises from cooperativity of interactions with lipids and the SNAREs. Note that the membrane is not required for binding of complexin-1 to the SNARE complex (*24*, *29*), but this interaction involves the SNARE motifs of syntaxin-1 and synaptobrevin (*21*) and hence is impossible for the syntaxin-1/SNAP-25 heterodimer. These observations suggest that the modes of binding of complexin-1 to the SNARE complex and the heterodimer are drastically different, and that only the latter is stabilized by the membrane. In this context, functional studies recently led to the proposal that the N-terminus of complexin-1, which precedes the SNARE complex binding region, may bind to phospholipids as well as to the C-termini of the syntaxin-1 and SNAP-25 SNARE motifs (*35*), but further research will be required to verify this proposal.

The significance of the interactions of the MUN domain with membrane-anchored SNARE complexes and syntaxin-1/SNAP-25 heterodimers remains to be demonstrated, but these interactions bode well for the proposed role of unc13/Munc13 in promoting SNARE complex formation. This proposal arose from the finding that a constitutively open syntaxin-1 mutant partially rescues release in unc13 nulls in *C. elegans* (*26*), and a report of an interaction of Munc13-1 with the syntaxin-1 N-terminus (*27*). However, it was later found that the Munc13-1 fragments used in this study are not properly folded, and that the Munc13-1 MUN domain, which is autonomously folded and does not bind to syntaxin-1 in solution, rescues release in Munc13-1/2 double KO mice (*28*). Our findings that the MUN domain does not bind to reconstituted full-length syntaxin-1 but binds to membrane-anchored SNARE complexes and syntaxin-1/SNAP-25 heterodimers suggest that the MUN domain may indeed interact with syntaxin-1, but as part of a concerted mechanism involving multiple interactions with syntaxin-1, SNAP-25 and the plasma membrane. It is tempting to speculate that the MUN domain may thus provide a template to assist in formation of the syntaxin-1/SNAP-25 heterodimer, leading to an "acceptor complex" for synaptobrevin. A similar role can be proposed for Munc18-1, as it also binds to membrane-anchored syntaxin-1/SNAP-25 heterodimers and SNARE complexes, but it is unclear whether the MUN domain and Munc18-1 can perform this role simultaneously or sequentially. It is also worth noting that the contrast between the 1:1 stoichiometry of coexpressed syntaxin-1/SNAP-25 heterodimers suggested by our data and the 2:1 stoichiometry observed for binding between soluble, separately expressed syntaxin-1 and SNAP-25 (*32*) may be related to the finding that liposome fusion induced by full-length neuronal SNAREs was observed only when syntaxin-1 and SNAP-25 were coexpressed (*36*). These observations suggest that some key states of the complexes formed by the release machinery may be kinetically hindered.

Clearly, further research will be required to test all these ideas and to investigate the nature of the various complexes discussed above. Our data suggest that definitive tests of these ideas will require quantitative studies of protein complexes in a native membrane environment, preferably between two apposed membranes. Particularly valuable for this purpose will be fluorescence spectroscopy approaches, which have already been used to study interactions of membrane-anchored SNARE complexes with complexin and synaptotagmin-1 (*23*, *24*). It will also be critical in future research to consider the possibility that some conformational states may be reachable only under certain experimental conditions.

NOTE ADDED IN PROOF

Using single molecule fluorescence resonance energy transfer experiments, Weninger et al. have also found that the Munc13-1 MUN domain binds to membrane-anchored t-SNARE complxes (*37*).

ACKNOWLEDGMENT

We thank Yi Xu for providing the sample of ^{13}C-labeled SNARE complex, and Irina Dulubova and Thomas C. Südhof for helpful comments on the manuscript.

REFERENCES

1. Sudhof, T. C. (2004) The synaptic vesicle cycle, *Annu. Rev. Neurosci. 27*, 509−547.
2. Rizo, J., and Sudhof, T. C. (2002) Snares and munc18 in synaptic vesicle fusion, *Nat. Rev. Neurosci. 3*, 641−653.
3. Brunger, A. T. (2005) Structure and function of SNARE and SNARE-interacting proteins, *Q. Rev. Biophys.* 1−47.
4. Jahn, R., and Scheller, R. H. (2006) SNAREs—engines for membrane fusion, *Nat. Rev. Mol. Cell Biol. 7*, 631−643.
5. Toonen, R. F., and Verhage, M. (2003) Vesicle trafficking: pleasure and pain from SM genes, *Trends Cell Biol. 13*, 177−186.
6. Rizo, J., Chen, X., and Arac, D. (2006) Unraveling the mechanisms of synaptotagmin and SNARE function in neurotransmitter release, *Trends Cell Biol. 16*, 339−350.
7. Augustin, I., Rosenmund, C., Sudhof, T. C., and Brose, N. (1999) Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles, *Nature 400*, 457−461.
8. Richmond, J. E., Davis, W. S., and Jorgensen, E. M. (1999) UNC-13 is required for synaptic vesicle fusion in C. elegans, *Nat. Neurosci. 2*, 959−964.
9. Varoqueaux, F., Sigler, A., Rhee, J. S., Brose, N., Enk, C., Reim, K., and Rosenmund, C. (2002) Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming, *Proc. Natl. Acad. Sci. U.S.A. 99*, 9037−9042.
10. Fernandez-Chacon, R., Konigstorfer, A., Gerber, S. H., Garcia, J., Matos, M. F., Stevens, C. F., Brose, N., Rizo, J., Rosenmund, C., and Sudhof, T. C. (2001) Synaptotagmin I functions as a calcium regulator of release probability, *Nature 410*, 41−49.
11. Reim, K., Mansour, M., Varoqueaux, F., McMahon, H. T., Sudhof, T. C., Brose, N., and Rosenmund, C. (2001) Complexins regulate a late step in Ca2+-dependent neurotransmitter release, *Cell 104*, 71−81.
12. Hanson, P. I., Roth, R., Morisaki, H., Jahn, R., and Heuser, J. E. (1997) Structure and conformational changes in NSF and its

membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy, *Cell 90*, 523−535.
13. Poirier, M. A., Xiao, W., Macosko, J. C., Chan, C., Shin, Y. K., and Bennett, M. K. (1998) The synaptic SNARE complex is a parallel four-stranded helical bundle, *Nat. Struct. Biol. 5*, 765−769.
14. Sutton, R. B., Fasshauer, D., Jahn, R., and Brunger, A. T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution, *Nature 395*, 347−353.
15. Fernandez, I., Ubach, J., Dulubova, I., Zhang, X., Sudhof, T. C., and Rizo, J. (1998) Three-dimensional structure of an evolutionarily conserved N-terminal domain of syntaxin 1A, *Cell 94*, 841−849.
16. Dulubova, I., Sugita, S., Hill, S., Hosaka, M., Fernandez, I., Sudhof, T. C., and Rizo, J. (1999) A conformational switch in syntaxin during exocytosis: role of munc18, *EMBO J. 18*, 4372−4382.
17. Misura, K. M., Scheller, R. H., and Weis, W. I. (2000) Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex, *Nature 404*, 355−362.
18. Dulubova, I., Khvotchev, M., Liu, S., Huryeva, I., Sudhof, T. C., and Rizo, J. (2007) Munc18-1 binds directly to the neuronal SNARE complex, *Proc. Natl. Acad. Sci. U.S.A. 104*, 2697−2702.
19. Shen, J., Tareste, D. C., Paumet, F., Rothman, J. E., and Melia, T. J. (2007) Selective activation of cognate SNAREpins by Sec1/Munc18 proteins, *Cell 128*, 183−195.
20. Lu, J., Machius, M., Dulubova, I., Dai, H., Sudhof, T. C., Tomchick, D. R., and Rizo, J. (2006) Structural Basis for a Munc13-1 Homodimer to Munc13-1/RIM Heterodimer Switch, *PLoS Biol. 4*, e192.
21. Chen, X., Tomchick, D. R., Kovrigin, E., Arac, D., Machius, M., Sudhof, T. C., and Rizo, J. (2002) Three-dimensional structure of the complexin/SNARE complex, *Neuron 33*, 397−409.
22. Bai, J., and Chapman, E. R. (2004) The C2 domains of synaptotagmin—partners in exocytosis, *Trends Biochem. Sci. 29*, 143−151.
23. Dai, H., Shen, N., Arac, D., and Rizo, J. (2007) A Quaternary SNARE-Synaptotagmin-Ca(2+)-Phospholipid Complex in Neurotransmitter Release, *J. Mol. Biol. 367*, 848−863.
24. Bowen, M. E., Weninger, K., Ernst, J., Chu, S., and Brunger, A. T. (2005) Single-molecule studies of synaptotagmin and complexin binding to the SNARE complex, *Biophys. J. 89*, 690−702.
25. Tang, J., Maximov, A., Shin, O. H., Dai, H., Rizo, J., and Sudhof, T. C. (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis, *Cell 126*, 1175−1187.
26. Richmond, J. E., Weimer, R. M., and Jorgensen, E. M. (2001) An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming, *Nature 412*, 338−341.
27. Betz, A., Okamoto, M., Benseler, F., and Brose, N. (1997) Direct interaction of the rat unc-13 homologue Munc13-1 with the N terminus of syntaxin, *J. Biol. Chem. 272*, 2520−2526.
28. Basu, J., Shen, N., Dulubova, I., Lu, J., Guan, R., Guryev, O., Grishin, N. V., Rosenmund, C., and Rizo, J. (2005) A minimal domain responsible for Munc13 activity, *Nat. Struct. Mol. Biol. 12*, 1017−1018.
29. Pabst, S., Hazzard, J. W., Antonin, W., Sudhof, T. C., Jahn, R., Rizo, J., and Fasshauer, D. (2000) Selective interaction of complexin with the neuronal SNARE complex. Determination of the binding regions, *J. Biol. Chem. 275*, 19808−19818.
30. Arac, D., Murphy, T., and Rizo, J. (2003) Facile detection of protein-protein interactions by one-dimensional NMR spectroscopy, *Biochemistry 42*, 2774−2780.
31. Liu, T., Tucker, W. C., Bhalla, A., Chapman, E. R., and Weisshaar, J. C. (2005) SNARE-driven, 25-millisecond vesicle fusion in vitro, *Biophys. J. 89*, 2458−2472.
32. Fasshauer, D., Otto, H., Eliason, W. K., Jahn, R., and Brunger, A. T. (1997) Structural changes are associated with soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor complex formation, *J. Biol. Chem. 272*, 28036−28041.
33. Zilly, F. E., Sorensen, J. B., Jahn, R., and Lang, T. (2006) Munc18-Bound Syntaxin Readily Forms SNARE Complexes with Synaptobrevin in Native Plasma Membranes, *PLoS Biol. 4*.
34. Pabst, S., Margittai, M., Vainius, D., Langen, R., Jahn, R., and Fasshauer, D. (2002) Rapid and selective binding to the synaptic SNARE complex suggests a modulatory role of complexins in neuroexocytosis, *J. Biol. Chem. 277*, 7838−7848.
35. Xue, M., Reim, K., Chen, X., Chao, H. T., Deng, H., Rizo, J., Brose, N., and Rosenmund, C. (2007) Distinct domains of complexin I differentially regulate neurotransmitter release, *Nat. Struct. Mol. Biol. 14*, 949−958.
36. Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H., and Rothman, J. E. (1998) SNAREpins: minimal machinery for membrane fusion, *Cell 92*, 759−772.
37. Weninger, K., Bowen, M. E., Choi, U. B., Chu, S., and Brunger, A. T. (2008) Accessory proteins stabilize the acceptor complex for synaptobrevin, the 1:1 syntaxin/SNAP-25 complex, *Structure*, in press.

BI702345M