Munc18a Scaffolds SNARE Assembly to Promote Membrane Fusion

Travis L. Rodkey, Song Liu, Meagan Barry, and James A. McNew

Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77251-1892

Submitted May 30, 2008; Revised August 29, 2008; Accepted September 23, 2008

Monitoring Editor: Benjamin S. Glick

Munc18a is an SM protein required for SNARE-mediated fusion. The molecular details of how Munc18a acts to enhance neurosecretion have remained elusive. Here, we use in vitro fusion assays to characterize how specific interactions between Munc18a and the neuronal SNAREs enhance the rate and extent of fusion. We show that Munc18a interacts directly and functionally with the preassembled t-SNARE complex. Analysis of Munc18a point mutations indicates that Munc18a interacts with helix C of the Syntaxin1a NRD in the t-SNARE complex. Replacement of the t-SNARE SNAP25b with yeast Sec9c had little effect, suggesting that Munc18a has minimal contact with SNAP25b within the t-SNARE complex. A chimeric Syntaxin built of the Syntaxin1a NRD and the H3 domain of yeast Sso1p and paired with Sec9c eliminated stimulation of fusion, suggesting that Munc18a/Syntaxin1a H3 domain contacts are important. Additionally, a Syntaxin1A mutant lacking a flexible linker region that allows NRD movement abolished stimulation of fusion. These experiments suggest that Munc18a binds to the Syntaxin1a NRD and H3 domain within the assembled t-SNARE complex, positioning them for productive VAMP2 binding. In this capacity, Munc18a serves as a platform for *trans*-SNARE complex assembly that facilitates efficient SNARE-mediated membrane fusion.

INTRODUCTION

SM (Sec1p/Munc18) proteins are a class of large (~68–78 kDa) evolutionarily conserved cytosolic proteins known to regulate vesicle fusion in the secretory pathway. The original SM protein *unc-18* was isolated in a *Caenorhabditis elegans* screen for uncoordinated (*unc*) mutants showing defects in locomotion resulting from disruption of the nervous system (Brenner, 1974). The *unc-18* orthologue in *Saccharomyces cerevisiae*, Sec1p, was identified later in a screen for temperature-sensitive secretion-deficient strains (Novick and Schekman, 1979; Aalto *et al.*, 1991). Orthologues have been found in plants (KEULE; Assaad *et al.*, 2001), invertebrates (ROP; Salzberg *et al.*, 1993), s-Sec1 (Dresbach *et al.*, 1998), and mammals (Munc18, also known as neuronal Sec1; Hata *et al.*, 1993; Garcia *et al.*, 1994; Pevsner *et al.*, 1994b). Sec1p isoforms were subsequently discovered in each step of the secretory pathway in yeast (Novick *et al.*, 1980; Banta *et al.*, 1990; Ossig *et al.*, 1991; Cowles *et al.*, 1994), and higher organisms (Weimer and Richmond, 2005). In mammals, the SM protein Munc18 has been implicated in exocytosis (Verhage *et al.*, 2000; Fisher *et al.*, 2001; Graham *et al.*, 2004; Ciufo *et al.*, 2005; Schutz *et al.*, 2005; Burgoyne and Morgan, 2007). The three Munc18 isoforms: Munc18a (Munc18-1), Munc18b (Munc18-2), and Munc18c (Munc18-3) display tissue-specific expression, with Munc18a being localized almost exclusively to brain (Pevsner *et al.*, 1994b; Weimer and Richmond, 2005).

SM proteins have been shown to function biochemically by interacting with SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins (Sollner *et al.*, 1993). Most SNAREs are tail-anchored proteins that spe-

cifically distribute along the secretory pathway. They assemble in a combinatorial manner at each vesicular trafficking step in the secretory pathway to mediate membrane fusion (Jahn and Scheller, 2006). SNARE proteins are operationally divided into those primarily present on the transport vesicle (v-SNAREs) and target plasma membrane (t-SNAREs; Sollner *et al.*, 1993). SNARE proteins have two defined functions that are distinct but interrelated. Their first role is to provide the final layer of proofreading to assure that the ensuing fusion event is compartmentally correct (McNew *et al.*, 2000). Specific pairing of v- and t-SNAREs fulfills this proofreading function. Their second role is to provide mechanical energy to drive the fusion reaction, which is achieved by the SNARE complex assembly process (Weber *et al.*, 1998). Each SNARE protein contains an ~60-residue SNARE motif that consists of a characteristic heptad repeat sequence with a strong propensity to form coiled-coils (Weimbs *et al.*, 1997). Membrane fusion is driven by the “zippering” of SNARE motifs in the appropriate combination to provide the mechanical force necessary for fusion of phospholipid bilayers (Weber *et al.*, 1998; Melia *et al.*, 2002).

The association of SM proteins with Syntaxin family proteins has been well documented. Protein expression levels of Syntaxin1a and Munc18a have been closely linked (Voets *et al.*, 2001; Arunachalam *et al.*, 2008), and in vivo data suggest Munc18a stabilizes and traffics Syntaxin1a to the plasma membrane in some cell types (Medine *et al.*, 2007). Biochemical work also has shown that Munc18a interacts strongly with the free t-SNARE Syntaxin1a (Pevsner *et al.*, 1994a), which stabilizes the “closed” conformation of Syntaxin1a within the cavity of the arch-shaped Munc18a molecule (Misura *et al.*, 2000; Yang *et al.*, 2000). This binary Munc18a/Syntaxin1a interaction is thought to prevent the cognate t-SNARE SNAP25 from binding to Syntaxin1a, thereby blocking formation of the assembled t-SNARE complex necessary for vesicle fusion (Yang *et al.*, 2000). However, recent work with SNAREs in native plasma membranes provides

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-05-0538>) on October 1, 2008.

Address correspondence to: James A. McNew (mcnew@rice.edu).

evidence that SNAP25 can enter into a t-SNARE complex, whereas Syntaxin1a is bound to Munc18a as a heterodimer (Zilly *et al.*, 2006), possibly aided by arachidonic acid (Connell *et al.*, 2007) or Munc13 (Hammarlund *et al.*, 2007). The strong affinity between Syntaxin1a and Munc18a derives from the extensive interactions within Syntaxin1a, including an extreme N-terminal peptide, the N-terminal regulatory domain (NRD, also called the Habc domain) and H3 “core” domains and Munc18a (Misura *et al.*, 2000; Burkhardt *et al.*, 2008). Some of these interactions also have been observed between other SM proteins such as Munc18c, Sly1p, and Vps45p and their cognate Syntaxins (Dulubova *et al.*, 2002; Yamaguchi *et al.*, 2002; D’Andrea-Merrins *et al.*, 2007).

Other modes of SM protein/SNARE interactions have also been documented. The yeast SM protein Sec1p binds weakly if at all to Sso1p, the yeast Syntaxin1a homolog. Instead, it binds strongly to the t-SNARE complex (Scott *et al.*, 2004) as well as the fully assembled ternary SNARE complex (Carr *et al.*, 1999; Scott *et al.*, 2004). Sly1p was shown to bind non-Syntaxin t-SNAREs (Peng and Gallwitz, 2004). Vps45p, a yeast SM protein involved in transport from the Golgi to late endosomes (Cowles *et al.*, 1994), has been shown to bind the yeast v-SNARE Snc1p as well as the cognate Syntaxin Tlg2p (Carpp *et al.*, 2006), suggesting that SM proteins could associate with v-SNAREs as well as t-SNAREs.

Although SM proteins have been studied extensively in many systems, their suggested functions have been contradictory (reviewed in Gallwitz and Jahn, 2003; Toonen and Verhage, 2003; Weimer and Richmond, 2005; Burgoyne and Morgan, 2007). SM proteins have been proposed to play both inhibitory and stimulatory roles in the fusion process (Wu *et al.*, 1998, 2001; Verhage *et al.*, 2000; Yang *et al.*, 2000; Scott *et al.*, 2004). Biochemical evidence that Munc18a could block t-SNARE complex formation led to the hypothesis that Munc18a inhibits vesicle fusion. This idea was bolstered by work suggesting that overexpression of the *Drosophila melanogaster* SM protein ROP (ras opposite; Salzberg *et al.*, 1993), which exhibits 65% sequence similarity with Munc18a, inhibited neurotransmission (Wu *et al.*, 1998). However, the results of several other *in vivo* experiments indicated that Munc18a plays a positive role in neurotransmission. Mouse Munc18a knockouts resulted in a lethal phenotype due to severe neurodegeneration immediately after birth (Verhage *et al.*, 2000), although brain development was unaffected, pointing to an absolute requirement for Munc18a in neurosecretion. Furthermore, elimination of Munc18a in mouse chromaffin cells reduced Ca^{2+} -dependent exocytosis of large dense core vesicles by a factor of 10 (Voets *et al.*, 2001). Studies of SM proteins in other organisms have also pointed toward a positive rather than negative role for SM protein function. Loss of function alleles of Sec1p in *S. cerevisiae* or ROP in *D. melanogaster* result in lethal phenotypes (Novick *et al.*, 1980; Harrison *et al.*, 1994; Schulze *et al.*, 1994). Although loss of the Munc18a homolog in *C. elegans* (*unc18*) is not lethal, it leads to severe impairment of synaptic transmission (Weimer *et al.*, 2003).

To elucidate the function of SM proteins, we previously focused on Sec1p, the yeast homolog of Munc18a. Sec1p was shown to bind strongly to the preassembled yeast t-SNARE and ternary SNARE complexes, and that binding directly stimulated fusion of yeast SNAREs in an *in vitro* fusion assay (Scott *et al.*, 2004). This original observation was supported further by a recent study showing that Munc18a also directly promotes membrane fusion *in vitro* and that its stimulatory effect is specific to the neuronal SNAREs Syntaxin 1A, SNAP25, and VAMP2 (Shen *et al.*, 2007).

Here, we used mutational and domain swap analyses to dissect further the interactions of Munc18a with the neuronal SNARE complex. We found that Munc18a interacts directly with the assembled Syntaxin1a/SNAP25 t-SNARE complex and minimally with the v-SNARE VAMP2. Munc18a stimulates neuronal SNARE-mediated fusion by about twofold in an *in vitro* fusion assay, and an ionic interaction between Munc18a and the Syntaxin1a NRD contributes to the accelerated fusion. The stimulatory effect of Munc18a can essentially be abolished by replacing the SNARE domain of Syntaxin with the homologous yeast Sso1 SNARE domain. Interestingly, deletion of the flexible linker region between the NRD and H3 domain of Syntaxin1a (residues 159–182) substantially reduced the positive effect of Munc18a. These results suggest that Munc18a recognizes the Syntaxin1a H3 domain within the assembled t-SNARE complex in addition to known contacts with the NRD. Binding of Munc18a to the assembled t-SNARE complex may place the NRD in a conformation that favors VAMP2 binding and subsequently accelerates SNARE-mediated membrane fusion.

MATERIALS AND METHODS

Cloning

The following SM and SNARE Plasmid constructs were created.

Untagged Syntaxin1a (pJM537). An untagged version of Rat Syntaxin1a was cloned by PCR of pTW34 (Parlati *et al.*, 1999) using oligos 426 (5′-CGC ATA TGA AGG ACC GAA CCC AGG AGC-3′) and 427 (5′-GCC TCG AGC TAT CCA AAG ATG CCC CCG ATG G-3′). The PCR product was cloned into pCR-Blunt II-TOPO using a Zero Blunt PCR cloning kit (Invitrogen, Carlsbad, CA), producing pJM525. The clone was verified by sequencing. The gene was cut out of pJM525-2 by restriction enzyme digest with NdeI and XhoI, and the resulting fragment was ligated into pETDuet-1 (EMD Biosciences, La Jolla, CA) cut with the same enzymes.

Syntaxin1aNRD (1-178)/Sso1ΔNRD (pJM579). A His₆-Syntaxin1a NRD (Rat)/Sso1 ΔNRD (Yeast) chimera was cloned by PCR from the yeast expression vector pJM413 with oligos 301 (5′-GCC AAT TCA TGA AGG ACC GAA CCC AGG-3′) and 103 (5′-GAG ATA TCC TCG AGT TAA CGC GTT TTG ACA ACA GCT GGG-3′). The PCR product was digested with EcoRI and XhoI and then ligated into pET24a(+) (EMD Biosciences) cut with the same enzymes. The clone was verified by sequencing.

Syntaxin1a Δ159-182 (pJM590). An untagged Syntaxin1a Δ159-182 was cloned by PCR of pTW34 with oligos 426 (5′-CGC ATA TGA AGG ACC GAA CCC AGG AGC-3′) and 485 (5′-CCG AAT TCC CGG CCA GTG ATC TCC AGC-3′), producing the sequence coding for amino acids 1-158 (Syntaxin1a NRD), and PCR of pTW34 with oligos 427 (5′-GCC TCG AGC TAT CCA AAG ATG CCC CCG ATG G-3′) and 486 (5′-GCA CAT ATG GAA TTC ATG GAC TCC AGC ATC TCG-3′), producing the sequence coding for amino acids 183–289 (Syntaxin H3 domain). The H3 domain fragment was cut with EcoRI and XhoI and ligated into pET24a(+) cut with the same enzymes, producing pJM583. The NRD fragment (1-158) was cut with NdeI and EcoRI and ligated into pJM583 cut with the same enzymes, producing pJM590. The resulting clone was verified by sequencing.

Syntaxin1a Δ159-182-H₆ (pJM595). The sequence coding for His₆-Syntaxin1a Δ159-182 was cut out of pJM590 by restriction enzyme digest with MscI and XhoI. The resulting fragment was ligated into pTW12 (Weber *et al.*, 1998) cut with the same enzymes.

Munc18a-H₆ R39E (pJM550). A site-directed mutagenesis kit (Stratagene, La Jolla, CA; QuikChange II) was used to create Munc18a-His₆ R39E. PCR of pJM537 as template was performed with oligos 453 (5′-GTG GAC CAG TTA AGC ATG GAG ATG CTG TCT TCC TGC TG-3′) and 454 (5′-CAG CAG GAA GAC AGC ATC TCC ATG CTT AAC TGG TCC AC-3′) in order to create the mutation. The resulting PCR product was sequenced to confirm the mutation.

Munc18a-H₆ E59R (pJM551). A site-directed mutagenesis kit (Stratagene; QuikChange II) was used to create Munc18a-His₆ E59R. PCR of pJM537 as template was performed with oligos 447 (5′-GAG GGG ATC ACA ATT GTG AGG GAT ATC AAC AAG CGC CG-3′) and 448 (5′-CGG CGC TTG TTG ATA TCC CTC ACA ATT GTG ATC CCC TC-3′) in order to create the mutation. The resulting PCR product was sequenced to confirm the mutation.

Munc18a-H₆ T574D (pJM563). A site-directed mutagenesis kit (Stratagene; QuikChange II) was used to create Munc18a-H₆ T574D. PCR of pJM537 as template was performed with oligos 471 (5'-GAT CCA CGC ACA TTC TCG ACC CAC AGA AAC TGC TGG-3') and 472 (5'-CCA GCA GTT TCT GTG GGT CGA GAA TGT GCG TGG ATC-3') in order to replace T574 with an aspartic acid. The resulting PCR product was sequenced to confirm the mutation.

Recombinant Protein Production and Reconstitution

The following SM proteins were expressed and purified.

Munc18a. Munc18a-His₆ (*Rattus norvegicus*) was produced by expressing pJM546 (plasmid received from Dr. Jingshi Shen, University of Colorado at Boulder) in BL21 (DE3) *Escherichia coli* (Stratagene). Cells were grown at 37°C in 4 l of SuperBroth (Teknova, Half Moon Bay, CA) to an OD₆₀₀ of ~0.75 while shaking at 200 rpm in a baffled 6-l flask. Protein expression was induced with 1 mM IPTG while shaking 3 h at 37°C at 200 rpm. Cells were pelleted by centrifugation and then immediately resuspended in 50 ml of buffer A150 (25 mM HEPES, pH 7.4, 150 mM KCl, 10% glycerol, and 1 EDTA-free protease inhibitor tablet; Roche, Indianapolis, IN). Cells were passed through an EmulsiFlex-C5 High Pressure Homogenizer (Avestin, Ottawa, ON, Canada) and lysed by pressure. Cellular debris was removed by centrifugation at 186,000 × *g*_{max} for 1 h at 4°C. Cell extract was filtered at 4°C first through a sterile 1.2 μM filter (Millipore, Bedford, MA) and then through a sterile 0.45-μM filter (Millipore). Extract was then passed over a HiTrap HP Ni²⁺-chelating column (GE Healthcare, Waukesha, WI) in an ÄKTA Prime chromatography system (Amersham Biosciences, Piscataway, NJ) to bind Munc18a-His₆. The column was washed with 10 column volumes of buffer A150 (25 mM HEPES, pH 7.4, 150 mM KCl, 10% glycerol, and 2 mM β-mercaptoethanol [BME]). Protein was eluted in 20 column volumes with a linear gradient of 20 to 500 mM imidazole in buffer A150. Peak fractions were pooled and then immediately dialyzed against 4 l of buffer A500 (25 mM HEPES-KOH, 500 mM KCl, 10% glycerol, and 1 mM DTT, pH 7.4) overnight at 4°C in a microdialysis chamber (Gibson, Worthington, OH) to exchange imidazole with KCl. Protein was then dialyzed 4 h at 4°C against 1 l of buffer A350 (25 mM HEPES, 350 mM KCl, 10% glycerol, and 1 mM DTT), followed by dialysis against 1 l buffer A200 (25 mM HEPES, 200 mM KCl, 10% glycerol, and 1 mM DTT) for 4 h at 4°C to reduce the KCl concentration. Protein was aliquoted, flash frozen in liquid nitrogen, and stored at -80°C. Preps were quantitated with Amido Black assays (Schaffner and Weissmann, 1973), and yields ranged from 9.44 to 12.71 mg/ml (4 ml).

SM Protein Munc18a Mutants. Munc18a-His₆ T574D, Munc18a-His₆ E59R, and Munc18a-His₆ R39E were all expressed and purified as described above for Munc18a-His₆. Yields were (15.46 mg/ml, T574D; 8.98 mg/ml, E59R; and 8.64 mg/ml, R39E) as determined by Amido Black assays.

The following t-SNARE proteins were expressed and purified.

Syntaxin1a/SNAP25b. Syntaxin1a (rat) and His₆-SNAP25b (mouse) were co-expressed from the dicistronic plasmid pTW34. Eight liters of *E. coli* [BL21(DE3)] were grown at 37°C in SuperBroth to an OD₆₀₀ of ~0.70. Protein expression was induced with 0.25 mM IPTG for 4 h at 37°C. Cells were pelleted, resuspended in buffer A400, pH 7.4 (25 mM HEPES, 400 mM KCl, 10% glycerol, and 2 mM BME), and then lysed as described for Munc18a-His₆ in the presence of 1% Triton X-100. Extract was clarified, filtered, and passed over an Ni²⁺-chelating column as described for Munc18a-His₆. The column was washed with 10 column volumes of buffer A400 containing 1% Triton X-100. Triton X-100 was exchanged for *n*-octyl-β-D-glucopyranoside (OG) by washing with 25 column volumes of buffer A100 (25 mM HEPES-KOH, 100 mM KCl, 10% glycerol, and 2 mM BME, pH 7.4) containing 1% OG. Protein was eluted in 20 column volumes with a linear gradient of 20 to 500 mM imidazole in buffer A100 (25 mM HEPES, 100 mM KCl, 10% glycerol, 2 mM BME, and 1% OG). Protein was aliquoted as described above and quantitated with an Amido Black assay. Yields ranged from 4.05 to 5.55 mg/ml. The resulting t-SNARE complex was reconstituted by detergent dilution and dialysis as described (Scott *et al.*, 2003).

Syntaxin1a/Sec9c. Untagged Syntaxin1a (rat) and His₆-Sec9c were coexpressed from pJM537 and pJM418 and purified as described above for Syntaxin1a/His₆-SNAP25b. However, protein was lysed in buffer A400, washed with A400 + 1% TX-100 and A400 + 1% OG, and then eluted with buffer A400 + 1% OG in order to help prevent precipitation as observed from trials eluting with lower [KCl]. Yield was 3.02 mg/ml as determined by an Amido Black assay. The resulting mixed t-SNARE complex was reconstituted as described for Syntaxin1a/His₆-SNAP25b.

Syntaxin1a NRD-Sso1ΔNRD/Sec9c. The His₆-Syntaxin1a NRD-Sso1ΔNRD chimera/His₆-Sec9c t-SNARE complex was expressed separately from pJM579 and pJM418. pJM418 was purified as described previously (Liu *et al.*, 2007a) with a yield of 4.5 mg/ml as determined by an Amido Black assay. His₆-Syntaxin1a NRD-Sso1ΔNRD was reconstituted by itself as described for Syntaxin1a/His₆-

SNAP25b. The resulting proteoliposomes were mixed with a threefold molar excess of His₆-Sec9c overnight at 4°C and then refloated 4 h at 4°C at 218,500 × *g*_{max} in 0%/30%/40% density step gradients to remove unbound Sec9c.

Syntaxin1a Δ159-182/SNAP25b. The Syntaxin1aΔ159-182/His₆-SNAP25b t-SNARE complex was coexpressed from pJM595 and pFP247 (Parlati *et al.*, 1999) and purified as described before for Syntaxin1a/His₆-SNAP25b. Yield was 1.19 mg/ml as determined by an Amido Black assay. Syntaxin1aΔ159-182/His₆-SNAP25b t-SNARE complex was reconstituted as described for Syntaxin1a/His₆-SNAP25b.

Sso1p/Sec9c. His₆-Sso1p (yeast) and His₆-Sec9c (yeast) were expressed and purified separately from pJM88-1 and pJM418, respectively, as previously described (McNew *et al.*, 2000; Liu *et al.*, 2007a). Yields were 2.3 mg/ml for His₆-Sso1p and 4.5 mg/ml for His₆-Sec9c. His₆-Sso1p was reconstituted as described for Syntaxin1a/His₆-SNAP25b, then mixed with a threefold molar excess of His₆-Sec9c, and refloated as described for His₆-Syntaxin1a NRD-Sso1ΔNRD/His₆-Sec9c.

Syntaxin1a Δ159-182. Syntaxin1aΔ159-182-His₆ was expressed from pJM595 as described for Syntaxin1a/His₆-SNAP25b, except that 0.5% glucose was added to the growth medium. Yield was 2.26 mg/ml as determined by an Amido Black assay. Syntaxin1aΔ159-182-His₆ was reconstituted as described for His₆-Sso1p (McNew *et al.*, 2000).

The following v-SNARE proteins were expressed and purified.

VAMP2. VAMP2-His₆ (mouse) was expressed and purified from pTW38 as described previously (Parlati *et al.*, 1999). Yield was 6.41 mg/ml as determined by an Amido Black assay. VAMP2-His₆ was reconstituted into labeled donor proteoliposomes as described (Scott *et al.*, 2003).

Snc1p. Snc1p-His₆ (pJM90-1) was expressed and purified as described previously (McNew *et al.*, 2000). Yield was 4.5 mg/ml. Snc1p-His₆ was reconstituted as described for VAMP2-His₆.

Reconstitution by Detergent Dilution and Dialysis

SNARE proteins were reconstituted into proteoliposomes by detergent dilution and dialysis as previously described (Scott *et al.*, 2003). Protein in 1% OG (final volume 500 μl for t-SNARE acceptor proteoliposomes, 300 μl for v-SNARE donor proteoliposomes) was mixed with a dried film of 85 mol% 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC)/15 mol% 1,2-di-oleoylphosphatidylserine (DOPS) at RT for 15 min and then diluted with the appropriate buffer to reduce OG below its critical micellar concentration (CMC; Scott *et al.*, 2003). OG was then removed from the resulting proteoliposomes by dialysis for ~16 h at 4°C in a microdialysis flow chamber (Gibson). Proteoliposomes were then separated from unincorporated lipid and protein by floatation for 4 h at 4°C at 48,000 rpm (218,500 × *g*_{ave}) in a Beckman SW-55Ti rotor (Fullerton, CA) through a 40%/30%/0% discontinuous density step gradient made of Accudenz (Accurate Chemical and Scientific, Westbury, NY) dissolved in buffer + 1 mM DTT. Proteoliposomes were harvested (400 μl t-SNARE, 75 μl v-SNARE) at the 0%/30% gradient interface. The amount of protein incorporated was determined by Amido Black assays.

In Vitro Fusion Assay

Proteoliposomes to be fused were mixed in 96-well Fluoronunc Polysorp plate (Nunc, Naperville, IL) strips, covered in aluminum foil, and incubated on ice for 3 h. A 3-h preincubation period was empirically determined to produce the maximum stimulation of fusion (data not shown). Ninety-six-well plate strips then were placed in a fluorescent plate reader (Fluoroskan II, MTX Lab Systems, Vienna, VA) preheated to 37°C. Acceptor (t-SNARE) and donor (v-SNARE) proteoliposomes were fused 2 h at 37°C as described previously (Scott *et al.*, 2003) with readings taken every 2 min. Ten microliters of 2.5% (wt/vol) *n*-dodecylmaltoside detergent was added at 2 h to produce maximum NBD fluorescence. NBD fluorescence was normalized to percent of maximum fluorescence.

RESULTS

Munc18a Stimulates In Vitro Fusion of Syntaxin1a/SNAP25b and VAMP2

Yeast Sec1p was shown to strongly and positively regulate membrane fusion in vitro (Scott *et al.*, 2004), and Munc18a appears to promote membrane fusion in a similar manner (Shen *et al.*, 2007). We produced recombinant Munc18a-H₆ in *E. coli* to further characterize in detail its effect on in vitro fusion with the neuronal t-SNAREs Syntaxin1a/His₆-SNAP25b and v-SNARE VAMP2-H₆. Unlike yeast Sec1p,

Munc18a-H₆ was very well expressed (~12.5 mg/l of culture) and largely stable at high concentrations (~140 μ M) under neutral pH conditions at moderately high salt concentrations (> 200 mM KCl, see *Materials and Methods* for details; however, it should be noted that the ability of Munc18a to stimulate SNARE-mediated fusion is highly salt sensitive; see Supplemental Figure S5). The ability to produce Munc18a-H₆ at high concentrations allowed for the addition of soluble Munc18a-H₆ directly to a standard fusion reaction.

For the in vitro fusion assays, full-length Syntaxin1a and H₆-SNAP25b were coexpressed in *E. coli* and reconstituted as a functional t-SNARE complex into acceptor liposomes containing 85 mol% 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC)/15 mol % 1,2-dioleoylphosphatidylserine (DOPS). Fluorescently labeled donor v-SNARE liposomes containing POPC, DOPS, and the headgroup labeled lipids [N-(7-nitro-2,1,3-benzoxadiazole-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine (NBD-DPPE) and N-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl phosphatidylethanolamine (Rh-DPPE)] were produced with recombinant VAMP2-H₆. Fusion is measured as an increase in NBD fluorescence when unlabeled acceptor membrane merges with the fluorescently labeled donor membrane, relieving quenched NBD fluorescence due to fluorescence resonance energy transfer (FRET) between the two fluorescent lipids (Weber *et al.*, 1998; Scott *et al.*, 2003). Syntaxin1a/SNAP25b and VAMP2 proteoliposomes were mixed in the presence or absence of 20 μ M Munc18a-H₆ for 3 h at 4°C, and the effects on SNARE-mediated fusion were examined (Figure 1A).

The addition of Munc18a-H₆ resulted in a marked (about twofold) increase in the extent of fusion at 120 min (Figure 1, A and C), very similar to the effects of Sec1p on exocytic yeast SNAREs (Scott *et al.*, 2004). The degree of Munc18a stimulation documented in Figure 1A is likely to be significantly underestimated due to improved fusion efficiency by SNAREs at low temperature in the presence of Munc18a. Close inspection of the primary NBD fluorescence data (Supplemental Figures S1, top panel, S2A, top panel, and S2B) shows a significant increase in initial fluorescence at the beginning of the fusion reaction after Munc18a-H₆ treatment. We attribute this difference to fusion enhancement during the preincubation phase on ice caused by the presence of Munc18a. Our interpretation of this result is that the normally slowed SNARE-dependent fusion reaction during the 3-h preincubation period without Munc18a is much more active at this temperature in the presence of Munc18a. Given that we must normalize each fusion reaction to the minimum and maximum fluorescence value in each well for reliable comparisons between experiments, the total stimulatory effect of Munc18a is underestimated by the normalization procedure.

The increased fusion mediated by Munc18a is saturable and reaches a maximum of 1.8-fold at 20 μ M Munc18a under these conditions (Figure 1, B and C). When the extent of fusion at 120 min is adjusted to account for fusion during the preincubation period, a more robust stimulation is observed (mean = ~2.2-fold, n = 14, data not shown). The stimulation of fusion measured at 120 min is mirrored by a similarly significant improvement in the initial rate of fusion when Munc18a-H₆ is present. The initial rate (measured as a percent of maximum fusion per minute) increased by 2.8-fold in the presence of Munc18a (Figure 1C).

The observed stimulation of fusion could be the result of Munc18a interaction with any of the participating SNAREs. We next determined if Munc18a was interacting preferentially with one of the SNARE subcomplexes by mixing Munc18a for 3 h with either t-SNARE or v-SNARE proteo-

liposomes independently and then adding the other proteoliposome population immediately before initiation of fusion at 37°C. Preincubation of Munc18a with t-SNARE (Figure 2, $\diamond$) or v-SNARE ($\blacklozenge$) proteoliposomes alone with 20 μ M Munc18a-H₆ resulted in no stimulation of fusion, with initial rate and final extent of fusion essentially equal to SNARE-only control reactions (Figure 2, $\circ$). As expected, inclusion of all components during preincubation resulted in robust stimulation of fusion ($\bullet$) that was completely dependent on SNARE complex formation as shown by inhibiting a fusion reaction with the cytoplasmic domain of VAMP2 (20 μ M CDV, solid line), which agrees with the findings of others (Shen *et al.*, 2007). These results suggest that both the t-SNARE complex and the v-SNARE communicate with Munc18a during the fusion reaction.

Munc18a Binds the Assembled t-SNARE Complex

Given that Sec1p interacts directly with the Sso1p/Sec9c t-SNARE complex to stimulate fusion, we next examined potential direct interactions between Munc18a and the t-SNARE complex. Recent NMR data suggest that Munc18a may also bind directly to a soluble t-SNARE complex lacking the Syntaxin1A TMD (Dulubova *et al.*, 2007). Additionally, recent data suggest that Munc18a binds to the full-length t-SNARE complex by gel filtration and flotation as well as single-molecule fluorescence spectroscopy (Guan *et al.*, 2008; Weninger *et al.*, 2008). We used three methods to investigate Munc18a binding to the preassembled t-SNARE complex. First, we asked if Munc18a could directly bind to the assembled t-SNARE complex reconstituted into proteoliposomes. Syntaxin1a/H₆-SNAP25 acceptor proteoliposomes or protein-free (PF) liposomes were mixed with a threefold molar excess of Munc18a-H₆ for 4 h at 4°C and then the Munc18a/t-SNARE complex proteoliposomes were reisolated from free Munc18a by flotation through a density step gradient. Proteoliposomes were harvested and the extent of Munc18a binding analyzed by SDS-PAGE followed by Coomassie staining. Significant although substoichiometric binding of Munc18a to Syntaxin1a/SNAP25 was detected with minimal nonspecific binding to PF liposomes (Supplemental Figure S3).

Second, we formed potential t-SNARE complex/Munc18a complexes in detergent solution that subsequently were reconstituted as units. This method was used in our previous work with Sec1p and the yeast t-SNAREs Sso1p/Sec9c (Scott *et al.*, 2004). Binding in the absence of lipid was accomplished by mixing Syntaxin1a/H₆-SNAP25 protein in a 0.75% OG detergent solution with a threefold molar excess of Munc18a-H₆ at 4°C for 4 h. The resultant protein mixture was reconstituted into liposomes and floated through a density step gradient to remove unbound Munc18a. Significant but substoichiometric binding of Munc18a to the t-SNARE complex was observed (Figure 3A).

Third, Munc18a/t-SNARE complex interactions were investigated by copurification. Untagged Munc18a was coexpressed with full-length untagged Syntaxin1a and His₆-SNAP25b, and the complex was purified through nickel affinity chromatography. Significant amounts of Munc18a were eluted in fractions of both Munc18a/full-length t-SNARE complex with protein molar ratios similar to those shown in Figure 3A and Supplemental Figure S3 (data not shown).

Evidence that Vps45p binds the v-SNARE Snc1p (Carpp *et al.*, 2006) led us to investigate Munc18a/VAMP2 interactions. Detectable but substoichiometric binding of Munc18a to VAMP2 was observed by both binding and reconstituting in detergent solution (data not shown) and binding to preformed VAMP2 proteoliposomes (Supplemental Figure S4).

Figure 1. Soluble Munc18a Strongly Stimulates *in vitro* fusion. (A) Kinetic fusion graph of unlabeled acceptor neuronal t-SNARE (Syntaxin1a/SNAP25b) proteoliposomes fused with fluorescently labeled donor neuronal v-SNARE (VAMP2) proteoliposomes in the presence (●) or absence (○) of 20 μ M Munc18a. Background fusion (solid line) was measured by the addition of the cytoplasmic domain of VAMP2 (CDV) in a 1.6-fold molar excess of t-SNARE protein to inhibit fusion. The inclusion of 10 μ M Munc18a in the inhibited fusion reaction serves as an additional control for background fusion. Both proteoliposome populations and Munc18a were preincubated 3 h at 4°C and then fused 2 h at 37°C in a standard fusion reaction. Data are represented as percent (%) of maximum fluorescence versus time. Fusion reactions contained 45 μ l of Syntaxin1a/SNAP25 proteoliposomes (55.4 μ g, 0.962 nmol protein, 44 nmol lipid) and 5 μ l of VAMP2 proteoliposomes (2.6 μ g, 0.189 nmol protein, 5.3 nmol lipid). All fusion reactions were brought up to a final volume of 60 μ l with Munc18a, buffer A100, CDV, or a combination of the three. (B) Titration of soluble Munc18a into *in vitro* fusion reactions. Increasing amounts (μ M) of Munc18a were added to a series of independent fusion reactions. Background fusion measured by the addition of CDV to a fusion reaction in the presence of 10 μ M Munc18a was subtracted from the endpoint fusion of each reaction, yielding net endpoint fusion. Net fusion (% of maximum fluorescence) of each reaction at 120 min was plotted against [Munc18a] (○). The titration curve was fit with a sigmoidal equation in the Kaleidagraph package (Abelbeck Software, Reading, PA). Saturation of increase in fusion occurs at 20 μ M Munc18a. (C) Net fold stimulation in initial rate and final extent of *in vitro* fusion resulting from addition of 20 μ M Munc18a as described in A. Endpoint fusion (% maximum fluorescence) for 14 independent fusion reactions of Syntaxin1a/SNAP25 and VAMP2 in the presence and absence of 20 μ M Munc18a was averaged. Endpoint fusion (% maximum fluorescence) of seven independent fusion reactions of Syntaxin1a/SNAP25, CDV, VAMP2, and 10 μ M Munc18a was averaged to determine the average background signal. The average background signal was subtracted from the average endpoint fusion of reactions in the presence and absence of 20 μ M Munc18a to yield net stimulation. Error bars, SEM. The rates of fusion between the 6-min (after temperature change from 4 to 37°C) and 10-min mark were calculated in fusion reactions of Syntaxin1a/SNAP25/VAMP2 in the presence and absence of 20 μ M Munc18a by linear curve fits. The initial rate increased from 0.39%/min in reactions without Munc18a (■, left) to 1.09%/min in reactions with 20 μ M Munc18a (■, right), an improvement of 2.8-fold.

His₆-VAMP2 could also be copurified with untagged Munc18a as described above for the t-SNARE complex, although to a lesser extent (data not shown).

Munc18a Prebound to the Assembled t-SNARE Complex Stimulates Fusion

We next investigated the functional consequences of Munc18a binding to the assembled t-SNARE complex. When Munc18a and Syntaxin1a/SNAP25 were reconstituted in detergent solution as a unit (Figure 3A) and used as acceptor liposomes in a fusion assay, improved fusion was

observed relative to t-SNARE complexes without bound Munc18a (Figure 3B, ●, vs. ○). The stimulation observed by bound Munc18a still required prior preincubation with v-SNARE proteoliposomes. However, the addition of 20 μ M soluble Munc18a to the fusion reaction containing Munc18a bound Syntaxin1a/SNAP25 acceptor proteoliposomes resulted in further stimulation (Figure 3B, ◆). This increased stimulation is likely due to the substoichiometric binding of Munc18a to the t-SNARE complex. These data confirm that the Munc18a bound to the t-SNARE complex is a functional intermediate.

Figure 2. Preincubation of Munc18a with both Syntaxin1a/SNAP25 and VAMP2 is required for stimulation of fusion. Strong stimulation was observed when t- and v-SNAREs were preincubated for 3 h with 20 μ M Munc18 (●) compared with SNARE-only reactions with preincubation (○). However, no stimulation was observed when Syntaxin1a/SNAP25 proteoliposomes were preincubated with 20 μ M Munc18a with VAMP2 proteoliposomes added immediately before fusion (◇). Conversely, when VAMP2 proteoliposomes were preincubated with 20 μ M Munc18a with Syntaxin1a/SNAP25 proteoliposomes added immediately before fusion (◆), no significant stimulation of fusion above that of the SNARE-only control (○) was observed. All reactions contained 45 μ l Syntaxin1a/SNAP25 acceptor proteoliposomes (55.4 μ g, 0.962 nmol protein, 44 nmol lipid), 5 μ l VAMP2 donor proteoliposomes (2.6 μ g, 0.189 nmol protein, 5.3 nmol lipid), and were brought up to a final volume of 60 μ l with Munc18a and buffer A100. Proteoliposomes were preincubated 3 h at 4°C then fused 2 h at 37°C as described in Figure 1.

Ionic Interactions are Required for Munc18a-mediated Stimulation of Fusion

Fully zipped SNARE complexes form a four-helix bundle with a highly charged surface that may interact with regulatory proteins (Sutton *et al.*, 1998). Inspection of the Munc18a/Syntaxin1a crystal structure also suggests that ionic interactions may contribute to this interaction (Misura *et al.*, 2000). To test whether Munc18a-mediated fusion stimulation depends on ionic interactions with SNAREs, potassium chloride was titrated into independent fusion reactions with 20 μ M Munc18a to determine the effect of increased ionic strength. Interestingly, Munc18a-mediated fusion stimulation exhibited a dramatic salt sensitivity, as an increase from 100 to 200 mM KCl essentially abolished stimulation of fusion (Supplemental Figure S5).

Molecular modeling of the Munc18a/Syntaxin1a heterodimer indicated that ionic interactions may exist between arginine 114 in Syntaxin1a and glutamate 59 in Munc18a as well as glutamate 234 in Syntaxin1a and arginine 39 in Munc18a. To test whether those two ionic interactions are required for Munc18-mediated stimulation, site-directed mutagenesis was used to mutate Munc18a R39 and Munc18a E59 to residues carrying the opposite charge (E→R and R→E) in order to destroy the postulated salt bridges. Munc18a R39E and Munc18a E59R mutants were cloned, expressed, and purified as described for wild-type Munc18a. The effects of these mutations were assayed by adding 20 μ M of either Munc18a R39E or Munc18a E59R to fusion reactions of Syntaxin1a/SNAP25b and VAMP2 and by comparing initial rates and endpoint fusion to reactions with

same neuronal SNAREs and 20 μ M wild-type Munc18a. Munc18a R39E showed no significant deficit in initial rate of fusion, although endpoint fusion was slightly impaired, but not statistically significant (Figure 4A, □). In contrast, the initial rate of fusion of Munc18a E59R was ~40% slower than wild type (2.83- vs. 4.46-fold) and stimulation of endpoint fusion was impaired by ~25% (1.56- vs. 2.02-fold; Figure 4B).

Phosphorylation of Munc18a by CDK5 May Enhance Stimulation of Fusion

Several reports suggest that Munc18a may be phosphorylated by CDK5 at T574 (Shuang *et al.*, 1998; Fletcher *et al.*, 1999). This phosphorylation is thought to dissociate Syntaxin1a/Munc18a heterodimers (Shuang *et al.*, 1998). However, phosphorylation of Munc18b T572 by CDK5 enhances Munc18b binding to the preassembled Syntaxin3b/SNAP25b t-SNARE complex (Liu *et al.*, 2007b). Site-directed mutagenesis was used to introduce a phosphomimetic mutant (T574D) in Munc18a to test the potential effects of Munc18a phosphorylation on in vitro fusion. Munc18a T574D protein was expressed, purified, and added solubly (20 μ M) to fusion reactions of Syntaxin1a/SNAP25b and VAMP2 (Figure 4A, △). An ~20% increase in initial rate of fusion was observed over that of wild-type Munc18a, whereas only a 5% increase in endpoint fusion was detected (Figure 4B).

Stimulation of Fusion by Munc18a Is Specific to Syntaxin1a/SNAP25b and VAMP2

The specificity of Munc18a for Syntaxin1a/SNAP25b and VAMP2 has been demonstrated by others with combinations of neuronal t-SNARE complexes and v-SNAREs from plasma membrane as well as other cellular compartments (Shen *et al.*, 2007). Here, we tested the effects of Munc18a on the exocytic yeast t-SNAREs Sso1p/Sec9c and the cognate v-SNARE Snc1p. Addition of 20 μ M Munc18a to fusion reactions of Sso1p/Sec9c and Snc1p failed to stimulate fusion to any degree (Figure 5B, bottom). Although Snc1p eliminates the stimulatory effect of Munc18a on Syntaxin1a/SNAP25b (Shen *et al.*, 2007), it was unclear if Munc18a would affect fusion of Sso1p/Sec9c and VAMP2. This combination of yeast t-SNAREs and neuronal v-SNARE was fully fusogenic, and addition of 20 μ M Munc18a produced a small but reproducible 1.2-fold stimulation of fusion (Figure 5B, top).

The t-SNARE Complex Light Chain Is Not Required for Stimulation of Fusion

Although it is clear that Munc18a binds the Syntaxin1a NRD to stimulate in vitro fusion fully, it was unclear whether Munc18a binds more than just the Syntaxin1a NRD within the assembled t-SNARE complex, including the t-SNARE complex light-chain SNAP25b. To test Munc18a/SNAP25b interactions, a mixed t-SNARE complex composed of Syntaxin1a and the yeast t-SNARE light-chain Sec9c was coexpressed, reconstituted, and fused with VAMP2 in the presence or absence of 20 μ M Munc18a. Fusion of the mixed t-SNARE complex in the absence of Munc18a was equivalent to the wild-type t-SNARE complex in initial rate and final extent (Figure 5A, ○). The addition of 20 μ M soluble VAMP2 (CDV) effectively inhibited fusion of the mixed t-SNARE complex (Figure 5A, bottom, solid line). Although Munc18a stimulated the mixed t-SNARE complex to essentially the same extent as equivalent wild-type t-SNAREs (Figure 5A, ●), a substantial decrease was observed in the initial rate of Munc18a-mediated stimulated fusion with the mixed t-SNARE complex compared with wild-type t-

Figure 3. Munc18a functionally interacts with the assembled t-SNARE complex. (A) Munc18a binds the assembled t-SNARE complex. Syntaxin1a/H₆-SNAP25 protein in 1% OG was mixed with a threefold molar excess of Munc18a for 4 h at 4°C at a final [KCl] of 133 mM and final OG of 0.8%, and the mixture was reconstituted into acceptor liposomes by the standard detergent dilution and dialysis method. Proteoliposomes were run on a Novex NuPage 10% Bis-Tris gel (Encinitas, CA), and binding of Munc18a was resolved by staining the gel with Coomassie blue. Ten microliters of sample was loaded per lane. Lane 1, unlabeled Syntaxin1a/SNAP25 proteoliposomes; lane 2, unlabeled Syntaxin1a/SNAP25/Munc18a proteoliposomes; lane 3, SNARE-free liposomes prepared in the presence of Munc18a. Munc18a binds the assembled t-SNARE complex, although apparently in substoichiometric amounts. (B) Functional consequences of Munc18a binding to Syntaxin1a/SNAP25. Munc18a bound to the preassembled t-SNARE complex stimulates fusion to the same extent as soluble 20 μ M Munc18a added to a fusion reaction of Syntaxin1a/SNAP25 acceptor and VAMP2 donor proteoliposomes and was further stimulatory by addition of soluble 20 μ M Munc18a to the fusion reaction. Forty-five microliters of Munc18a prebound to the t-SNARE complex (●) was preincubated 3 h at 4°C with VAMP2 donor liposomes in the presence (◆) or absence of 20 μ M soluble Munc18a and then was fused for 2 h at 37°C. Fusion of Munc18a prebound to Syntaxin1a/SNAP25 was stimulated to essentially the same extent as fusion of Syntaxin1a/SNAP25 and VAMP2 in the presence of 20 μ M soluble Munc18a (not shown) compared with a control reaction without Munc18a (○). A roughly 1.8-fold stimulation of fusion above the SNARE-only control reaction was observed for Munc18a prebound to the t-SNARE complex and for a reaction with 20 μ M Munc18a added in solution. Addition of soluble 20 μ M Munc18a to a reaction of Munc18a prebound to Syntaxin1a/SNAP25 and VAMP2 resulted in a further 1.56-fold stimulation (~2.8-fold above SNARE-only control). Background fusion was measured by inhibiting a fusion reaction (solid line) with 20 μ M CDV.

SNARE complex (see Figure 7, bottom). It is likely that these differences in rate are attributable directly to the overall differences in rate between yeast (McNew *et al.*, 2000) and neuronal (Weber *et al.*, 1998) SNAREs and not the ability of Munc18a to stimulate fusion.

Replacement of the Syntaxin1a H3 Domain Abolishes Stimulation of Fusion

The results with the mixed t-SNARE complex suggest that interactions between Munc18a and the t-SNARE light chains of SNAP25 are minimal in Munc18a bound to the t-SNARE complex. However, the binary Munc18a/Syntaxin1a structure reveals extensive contacts between Munc18a and the H3 SNARE domain of Syntaxin1a. This interaction was further supported by recent calorimetric data from binding of soluble truncated Syntaxin1a mutants and Munc18a (Burkhardt *et al.*, 2008). We next tested the functional consequences of replacing the H3 domain of Syntaxin1a with that of the yeast homolog Sso1p. A chimera comprised of the NRD of Syntaxin1a and the H3 domain of yeast Sso1p was paired with Sec9c as the t-SNARE light chain. This t-SNARE complex contains the neuronal Syntaxin1a NRD and three yeast SNARE domains in order to negate Munc18a interactions with the Syntaxin1a H3 domain and effectively isolate the Syntaxin1a NRD. This chimeric SNARE complex was fully functional, fused comparably to wild-type SNAREs or the mixed t-SNAREs (Figure 5C, ○), and was inhibited fully by 20 μ M CDV (solid line). However, no stimulation of the initial rate or final extent of fusion above control reactions was observed in the presence of 20 μ M Munc18a (Figure 5C, ●, see also Figure 7).

Deletion of the Syntaxin1a Linker Region Abrogates Stimulation of Fusion

Syntaxin1a is known to assume either an "open" or a putatively autoinhibitory "closed" conformation (Dulubova *et al.*, 1999). The Syntaxin1a NRD is thought to be able to change conformation via a flexible α -helical linker region (residues 159-182; Margittai *et al.*, 2003). Munc18a has been implicated in the regulation of the Syntaxin1a NRD conformational state (Dulubova *et al.*, 1999; Yang *et al.*, 2000). To determine if the Syntaxin1a linker region affects stimulation of fusion by Munc18a, a Syntaxin1a linker region deletion mutant (Syntaxin1a Δ 159-182) was generated, coexpressed with SNAP25b, reconstituted, and fused with VAMP2 in the presence or absence of 20 μ M Munc18a. Liposomes containing t-SNARE complexes produced with Syntaxin1a Δ 159-182 fused normally with VAMP2 (Figure 6, ○). However, stimulation of fusion in the presence of Munc18a was impaired markedly compared with wild-type Syntaxin1a/SNAP25 for this mutant (Figure 6, ●). Both the rate (1.65- vs. 4.6-fold for wild type) and extent (1.3- vs. 2-fold for wild type) of fusion stimulation by Munc18a were largely abolished (Figure 7), whereas binding of Munc18a to free Syntaxin1a Δ 159-182-H₆ was apparently unaffected (data not shown).

DISCUSSION

Despite the pivotal roles Sec1/Munc18 family proteins play in every step of SNARE-mediated membrane fusion events along the secretory pathway, their functions have been obscured by their multiple, nonconserved binding modes to SNAREs as well as seemingly contradictory conclusions

Figure 4. Ionic interactions with SNAREs are important for Munc18a-mediated stimulation of fusion. (A) Representative kinetic fusion graph of Syntaxin1a/SNAP25b proteoliposomes fused with VAMP2 proteoliposomes in the absence (○) or presence of 20 μ M wild-type or mutant (R39E, E59R, and T574D) Munc18a. The fusion assay was performed as described in Figure 1A. Background fusion (solid line) was measured by inhibiting a standard fusion reaction with 20 μ M CDV. Wild-type Munc18a (●) produced about a two-fold stimulation of endpoint fusion at 120 min compared with a SNARE-only control reaction (○). Little deviation from wild-type Munc18a was observed with the Munc18a phosphomimetic T574D (△), whereas a small deficit in endpoint fusion was observed with Munc18a R39E (□), and a more noticeable deficit in endpoint fusion and initial rate was detected with Munc18a E59R (◇). (B) Quantification of differences in net fusion and initial rate of fusion resulting from Munc18a mutations. Top panel, histogram of differences in net fusion at 120 min. Endpoint net fusion from multiple independent fusion reactions was calculated by subtracting endpoint background signal values from endpoint values of SNARE-only reactions and reactions in the presence of Munc18a. Net fold stimulation was then calculated by dividing net fold stimulation of reactions with wild-type or mutant Munc18a by net fold fusion of equivalent

drawn from numerous investigations. However, recent discoveries have started to reveal the multifaceted functions of SM proteins and reconcile these with their distinct binding states.

Among all the SM proteins, Munc18a is of special interest as it specifically regulates neurotransmitter release. The atomic structure of a binary Munc18a/Syntaxin1a complex showed extensive interactions between the Syntaxin1a NRD (Habc domain) and H3 core domains with Munc18a (Misura *et al.*, 2000). Recent refinement of this crystal structure revealed an additional interaction between the extreme N-terminus of Syntaxin1a with Munc18a (Burkhardt *et al.*, 2008), which has also been recently detected by NMR (Khvotchev *et al.*, 2007). SM protein interaction with extreme N-terminal sequences has also been demonstrated for Munc18c (Latham *et al.*, 2006), and the ER SM protein Sly1p (Bracher and Weissenhorn, 2002; Yamaguchi *et al.*, 2002). This mode of interaction has important implications for previous binding studies that utilized N-terminally tagged Syntaxin1a (Rodkey and McNew, 2008). These interactions apparently stabilize Syntaxin1a in its closed conformation and prevent the formation of the t-SNARE complex, which is consistent with the negative role of Munc18a.

Recent studies have shown that Munc18a could specifically stimulate SNARE-mediated *in vitro* membrane fusion with neuronal SNAREs (Shen *et al.*, 2007), which extended our previous observation that yeast Sec1p stimulates membrane fusion *in vitro* (Scott *et al.*, 2004). Although a larger magnitude of stimulation was observed in this recent study (Shen *et al.*, 2007), the degree of stimulation by Munc18a can be largely attributed to differences in the concentration of SNAREs in the proteoliposomes. We confirm and extend

SNARE-only reactions. All reactions contained 45 μ l Syntaxin1a/SNAP25b proteoliposomes and 5 μ l VAMP2 proteoliposomes, and wild-type or mutant Munc18a was added to final concentration of 20 μ M. All reactions were mixed to a final volume of 60 μ l. T-SNARE proteoliposome protein:lipid ratios ranged from 1:100:1:200, and v-SNARE proteoliposome protein:lipid ratios ranged from 1:30 to 1:80 as determined by Amido Black assays and lipid recovery after reconstitution as determined by scintillation count of 3 H incorporated into the lipid. Wild-type Munc18a produced a 2.02-fold stimulation of net fusion, whereas Munc18a E59R showed an ~25% impairment (1.56-fold stimulation). An ~10% deficit in stimulation was observed with Munc18a R39E (1.81-fold stimulation), whereas the phosphomimetic Munc18a T574D showed slightly increased endpoint fusion (2.16-fold stimulation). Results with Munc18a E59R were statistically significant as determined by a paired Student's *t* test with *p* < 0.0001. Although small differences in initial rate and endpoint fusion were consistently observed with Munc18a R39E and Munc18a T574D, these differences were not statistically significant by a paired Student's *t* test. Error bars, SEM. Bottom, histogram of differences in initial rate (6–10 min) of fusion among the Munc18a mutants. Net fusion (% max fusion) for the 6-, 8-, and 10-min time points was calculated in each fusion reaction in the top panel, and the slope from linear curve fits was calculated for each reaction. The slopes were then averaged and compared with curve fits of the same time points taken from SNARE-only reactions. More dramatic increases in fold stimulation were observed with initial rates than endpoint fusion, as wild-type Munc18a produced a 4.48-fold increase in initial rate of fusion compared with a twofold increase in net fusion at 120 min. The impairment in fusion caused by the Munc18a E59R mutation was more noticeable in initial rate, as Munc18a E59R stimulated the initial rate of fusion by 2.83-fold, an ~40% reduction from the initial rate of wild-type Munc18a. Little deviation in rate was observed with Munc18a R39E compared with wild-type (4.29- vs. 4.48-fold), whereas the initial rate of fusion of the phosphomimetic was ~20% faster than wild-type (5.56- vs. 4.48-fold). Error bars, SEM.

Figure 5. Stimulation by Munc18a is specific to neuronal SNAREs and requires the Syntaxin1a H3 domain but not SNAP25b. (A) Munc18a stimulates fusion of Syntaxin1a/Sec9c and VAMP2. Representative kinetic fusion graph of stimulation of Syntaxin1a/H₈-Sec9c by Munc18a compared with fusion reactions containing equivalent amounts of Syntaxin1a/H₈-SNAP25b. Difficulty in expression of Syntaxin1a/H₈-Sec9c necessitated the use of liposomes with ~1:500 protein:lipid ratios as determined by Amido Black protein quantitation and lipid recovery as measured by scintillation count of ³H in the lipid. Wild-type Syntaxin1a/H₈-SNAP25b liposomes were reconstituted in matching protein:lipid ratios. Soluble wild-type Munc18a was added to a final concentration of 20 μ M in both cases, and the final volume of all reactions was 60 μ l. The same v-SNARE population (1:30 protein:lipid ratios) was used in each experiment. Each fusion reaction was conducted as described in Figure 1A. Background signal was measured by inhibiting fusion reactions with 20 μ M CDV (solid line, top and bottom). Munc18a stimulates endpoint fusion of Syntaxin1a/H₈-Sec9c (bottom, ●) compared with SNARE-only reactions (bottom, ○). Munc18a also stimulates equivalent reactions with wild-type Syntaxin1a/H₈-SNAP25b (top, ●) compared with SNARE-only reactions (top, ○). However, the initial rate of fusion is significantly impaired when Munc18a is added to Syntaxin1a/H₈-Sec9c compared with addition of Munc18a to Syntaxin1a/H₈-SNAP25b as shown in Figure 7. (B) Stimulation of fusion by Munc18a is specific to neuronal SNAREs. Representative kinetic fusion graph of the effect of 20 μ M Munc18a on fusion of the yeast t-SNAREs Sso1p/Sec9c and the neuronal v-SNARE VAMP2 (top) versus the yeast v-SNARE Snc1p (bottom). Data are reported as % maximum fluorescence. The fusion assay was performed as described in Figure 1A. Background signal was measured by inhibiting a fusion reaction with 20 μ M CDV (solid line). An ~1.2-fold stimulation of fusion resulted from addition of 20 μ M Munc18a to fusion of Sso1p/Sec9c and VAMP2 (top, ●) compared with SNARE-only Sso1p/Sec9c and VAMP2 (top, ○). However, stimulation of fusion was completely abolished when 20 μ M Munc18a was added to fusion of Sso1p/Sec9c and Snc1p (bottom, ●) compared with SNARE-only Sso1p/Sec9c and Snc1p reactions (bottom, ○). (C) The Syntaxin1a H3 domain is required for stimulation of fusion. Representative kinetic fusion graph of the effect of 20 μ M Munc18a on fusion of the Syntaxin1a NRD/Sso1 H3 domain/SNAP25b chimera fused with VAMP2. Fusion reactions were conducted as described in Figure 1A, and data are reported as % of maximum fluorescence. Background signal was measured by inhibiting a fusion reaction with 20 μ M CDV (solid line). Addition of 20 μ M Munc18a had no observable effect on fusion of Syntaxin1a NRD/Sso1 H3 domain/SNAP25b and VAMP2 (●) compared with SNARE-only reactions (○).

Figure 6. Deletion of the Syntaxin1a linker region largely abolishes stimulation of fusion. Kinetic fusion graph of Syntaxin1a Δ 159-182/H₆-SNAP25b acceptor proteoliposomes fused with VAMP2 donor proteoliposomes in the presence (●) or absence of 20 μ M Munc18a (○). Fusion of Syntaxin1a Δ 159-182/H₆-SNAP25b and VAMP2 without Munc18a was compared with equivalent wild-type Syntaxin1a/H₆-SNAP25b and VAMP2 reactions (◆). The fusion assay was performed as described in Figure 1A, with final volumes of 60 μ l/reaction. The same population of VAMP2 donor proteoliposomes was used in all reactions. Results are reported as % maximum fluorescence. Background signal was measured by inhibiting a fusion reaction with 20 μ M CDV. Fusion of Syntaxin1a Δ 159-182/H₆-SNAP25b was not impaired compared with wild-type Syntaxin1a/H₆-SNAP25b. However, Munc18a was only able to stimulate fusion of Syntaxin1a Δ 159-182/H₆-SNAP25b by ~20%, very similar to the effect of Munc18a on Sso1p/Sec9c and VAMP2.

these results by showing that Munc18a stimulates fusion by neuronal SNAREs (Figure 1). Furthermore, we see that the rate enhancement that Munc18a provides is limited to early time points in the reaction and is largely complete within the first 20 min (Supplemental Figure S1), an effect similar to that seen previously for Munc18a (Shen *et al.*, 2007). The underlying basis for this time-limited effect remains to be determined. We also find that fusion stimulation by Munc18a requires that all SNARE participants be present during preincubation of the reaction (Figure 2) and define Munc18a bound to the t-SNARE complex as a functional intermediate (Figure 3).

Given that the binary Munc18a/Syntaxin1a complex appears incompatible with further SNARE complex formation, we probed the associations required for Munc18a interactions with the assembled t-SNARE complex. The main goal was to determine if molecular interactions important for the binary Munc18a/Syntaxin1a were preserved when SNAP25 was present. We approached this task by mutational analysis of key residues within Munc18a (Figure 4) observed in the Munc18a/Syntaxin1a crystal structure as well as by interchanging SNARE domains with the homologous yeast SNARE complex (Figure 5).

We examined the effect of disrupting ionic interactions between the NRD or the H3 core domain of Syntaxin1a and Munc18a (Figure 4). We found that mutation of Munc18a E59 to arginine, which is predicted to disrupt an ionic interaction in Helix C (Syn1a R114-Munc18a E59) within the NRD, was much more detrimental to the ability of Munc18a to stimulate fusion than a similar charge reversal (R39E) that interacts with a central region of the H3 domain (Syn1a E234). These results suggest that the R114-E59 interaction is maintained and functionally relevant in the ternary complex between Syntaxin1a/SNAP25 and Munc18a, whereas the

E234-R39 interaction is less important for t-SNARE complex-bound Munc18a. Consistent with our results, mutation of Munc18a R39 to Cys had little or no impact on stimulation of fusion (Shen *et al.*, 2007).

Next, we addressed the effects of individual SNARE domains by changing the composition of the assembled t-SNARE complex. Munc18a fails to stimulate fusion with plasma membrane yeast SNAREs (Figure 5B), which suggests that systematic replacement of neuronal t-SNARE complex domains with their yeast homologues would effectively negate interaction of Munc18a with the replaced domains. We began by expressing a novel “mixed t-SNARE complex” composed of Syntaxin1a and Sec9c designed to analyze the contribution of the SNAP25 SNARE chains. Not only was this hybrid t-SNARE complex functional, it was stimulated by Munc18a to a similar extent as the fully neuronal SNARE complex (Figure 5A). This result indicates that Munc18a does not associate significantly with SNAP25b in the assembled t-SNARE complex and highlights the importance of Munc18a/Syntaxin1a interactions even within the assembled t-SNARE complex.

Because replacing SNAP25 with Sec9c had only minor effects, we modified the t-SNARE complex further by substituting the Syntaxin1a H3 domain within the mixed t-SNARE complex with the corresponding yeast Sso1p H3 domain, leaving the Syntaxin1a NRD as the sole neuronal piece of the t-SNARE complex (Figure 5C). When this Syntaxin1a NRD/Sso1p H3 chimera was paired with Sec9c, stimulation of fusion by Munc18a was abolished entirely, indicating that the Syntaxin1a H3 domain is a crucial determinant of the ability of Munc18a to stimulate fusion. Although the interaction of the Syntaxin1a H3 domain and Munc18a is required for *in vitro* fusion stimulation, it is unlikely that the configuration of the H3 domain seen in the crystal structure is maintained in the Munc18a bound t-SNARE complex considering the small detrimental effect caused by the Munc18a R39E mutant.

Overall, the results of mutations in Munc18a and systematic domain replacements within the t-SNARE complex indicate that Munc18a binds the assembled t-SNARE complex in a fundamentally different manner than free Syntaxin1a, although some of the same contacts found in the binary Munc18a/Syntaxin1a complex may be retained.

Our results, paired with the recent appreciation that the extreme N-terminus of Syntaxin1a also interacts with Munc18a, suggests a possible path describing the transition from the binary Munc18a/Syntaxin1a complex to Munc18a bound t-SNARE complex through a Munc18a bound *trans*-SNARE complex. The ability of Munc18a to stimulate fusion *in vitro* absolutely requires an intact Syntaxin1a N-terminus. Stimulation is lost when the entire NRD is removed (data not shown), when the N-terminal 19 amino acids are truncated and even when a single point mutation (L8A) is introduced (Shen *et al.*, 2007). We suggest that this point of interaction is critical for the binding of Munc18a to the assembled t-SNARE complex and likely part of the mechanism that allows t-SNARE complex formation to occur while Syntaxin1a remains bound to Munc18a. We found that the H3 core domain of Syntaxin1a was absolutely required for Munc18a stimulation, yet the SNARE domains of SNAP25 have minimal effect on the ability of Munc18a to stimulate fusion (Figure 5). We also determined that the linkage between the NRD and the H3 core helix is critically important for Munc18a stimulation (Figure 6). These data fit a model in which movement of the Syntaxin1a NRD away from the H3 core domain within the binary Munc18a/Syntaxin1a complex allows SNAP25 binding, but requires an extreme N-

Figure 7. Summary of contribution of individual SNARE complex domains to Munc18a-mediated stimulation of fusion. Histogram of fold stimulation of net fusion at 120min (top) and of initial rate of fusion (6–10 min; bottom) by addition of 20 μ M Munc18a to fusion reactions in which neuronal SNARE domains have been replaced with their homologous yeast SNARE domains. A maximum 2.2-fold stimulation in endpoint fusion was observed by addition of 20 μ M Munc18a to fusion reactions of wild-type Syntaxin1a/SNAP25b and VAMP2, whereas the initial rate was stimulated 4.64-fold. Replacement of the t-SNARE light-chain SNAP25b with the yeast t-SNARE Sec9c resulted in an $\sim$ 20% decrease in net endpoint fusion, but entailed a dramatic 60% decrease in stimulation of initial rate from 4.64- to 1.96-fold. Deletion of the Syntaxin1a linker region (residues 159-182) resulted in an $\sim$ 40% reduction in stimulation of endpoint fusion (1.27-fold stimulation vs. 2.03-fold for wild-type), whereas stimulation of initial rate decreased by $\sim$ 65% (1.65- vs. 4.64-fold for wild-type). Replacement of the SNARE domains of the t-SNARE complex light chain with equivalent helices from Sec9c combined with replacement of the Syntaxin1a SNARE (H3) domain with the Sso1 SNARE domain, leaving the Syntaxin1a NRD as the only native piece of the Syntaxin1a/SNAP25b t-SNARE complex resulted in essential abrogation of stimulation of endpoint fusion as well as initial rate. Surprisingly, a 1.3-fold stimulation of endpoint fusion of Sso1/Sec9c and VAMP2 was observed, with an equivalent increase in initial rate. Munc18a was without effect on endpoint fusion or initial rate of Sso1p/Sec9c and Snc1p.

terminal attachment to maintain contact with Munc18a during and after this transition. This hypothetical movement of the NRD would also require that the linkage between the NRD and the H3 core helix be flexible enough to permit this transition. Removal of this flexible linker region should prevent the NRD from being moved or stretched by Munc18a, preventing productive association.

Although the Munc18a/t-SNARE complex interaction clearly is necessary for stimulation of fusion, order of addition experiments indicate that accelerated fusion cannot occur unless all components, including the v-SNARE, are incubated together (Figure 2). A precedent for SM/v-SNARE interaction was established by work on Vps45 and Snc1p (Carpp *et al.*, 2006). Although we were able to detect weak Munc18a/VAMP2 association biochemically, a functional Munc18a/VAMP2 interaction is suggested by our *in vitro* fusion results. Munc18a has absolutely no effect on the full yeast SNARE complex (Sso1p/Sec9c/Snc1p Figure 5B, bottom) but shows a reproducible $\sim$ 30% stimulation with the yeast t-SNARE complex (Sso1p/Sec9c) and VAMP2. The most straightforward interpretation of these data are that VAMP2 interacts with Munc18a, whereas Snc1p does not. This interpretation is bolstered by mutations in VAMP2 that diminished the ability of Munc18a to stimulate fusion (Shen *et al.*, 2007).

The end result of these proposed structural changes would be that Munc18a binding actively positions the

Syntaxin1a NRD in a conformation favorable to fusion, first for t-SNARE complex formation and ultimately for *trans*-SNARE complex formation. Hypothetical models indicate that Munc18a could effectively “roll” the Syntaxin1a NRD so that it allows SNAP25 binding and exposes the VAMP2-binding groove in the t-SNARE complex, thereby facilitating VAMP2 binding and consequent membrane fusion (McNew, 2008).

Taken together, these results place Munc18a as an assembly platform that may orchestrate t-SNARE complex formation and trans-SNARE complex assembly that stimulates membrane fusion by placing the Syntaxin1a NRD in a position that facilitates t-SNARE complex/v-SNARE interaction. In addition, Munc18a may actively recruit and position VAMP2 to facilitate fusion. These *in vitro* fusion results support the idea that Munc18a facilitates post-Golgi vesicle fusion with the plasma membrane in neurons.

ACKNOWLEDGMENTS

The authors thank Drs. Kirilee Wilson and Brenton Scott for preliminary observations and Dr. Blair Doneske, Joseph Faust, and Tyler Moss (All three from Rice University) for kind gifts of protein and liposomes, as well as for advice. The authors also thank other members of the McNew Lab for close reading of the manuscript. Support for this work was provided by National Institutes of Health Grant GM71832 and the G. Harold and Leila Mathers Charitable Foundation.

REFERENCES

- Aalto, M. K., Ruohonen, L., Hosono, K., and Keranen, S. (1991). Cloning and sequencing of the yeast *Saccharomyces cerevisiae* SEC1 gene localized on chromosome IV. *Yeast* 7, 643–650.
- Arunachalam, L. *et al.* (2008). Munc18-1 is critical for plasma membrane localization of Syntaxin1 but not of SNAP-25 in PC12 cells. *Mol. Biol. Cell* 19, 722–734.
- Assaad, F. F., Huet, Y., Mayer, U., and Jurgens, G. (2001). The cytokinesis gene KEULE encodes a Sec1 protein that binds the syntaxin KNOLLE. *J. Cell Biol.* 152, 531–543.
- Banta, L. M., Vida, T. A., Herman, P. K., and Emr, S. D. (1990). Characterization of yeast Vps33p, a protein required for vacuolar protein sorting and vacuole biogenesis. *Mol. Cell. Biol.* 10, 4638–4649.
- Bracher, A., and Weissenhorn, W. (2002). Structural basis for the Golgi membrane recruitment of Sly1p by Sed5p. *EMBO J.* 21, 6114–6124.
- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71–94.
- Burgoyne, R. D., and Morgan, A. (2007). Membrane trafficking: three steps to fusion. *Curr. Biol.* 17, R255–258.
- Burkhardt, P., Hattendorf, D. A., Weis, W. I., and Fasshauer, D. (2008). Munc18a controls SNARE assembly through its interaction with the syntaxin N-peptide. *EMBO J.* 27, 923–933.
- Carpp, L. N., Ciuffo, L. F., Shanks, S. G., Boyd, A., and Bryant, N. J. (2006). The Sec1p/Munc18 protein Vps45p binds its cognate SNARE proteins via two distinct modes. *J. Cell Biol.* 173, 927–936.
- Carr, C. M., Grote, E., Munson, M., Hughson, F. M., and Novick, P. J. (1999). Sec1p binds to SNARE complexes and concentrates at sites of secretion. *J. Cell Biol.* 146, 333–344.
- Ciuffo, L. F., Barclay, J. W., Burgoyne, R. D., and Morgan, A. (2005). Munc18-1 regulates early and late stages of exocytosis via syntaxin-independent protein interactions. *Mol. Biol. Cell* 16, 470–482.
- Connell, E., Darios, F., Broersen, K., Gatsby, N., Peak-Chew, S. Y., Rickman, C., and Davletov, B. (2007). Mechanism of arachidonic acid action on syntaxin-Munc18. *EMBO Rep.* 8, 414–419.
- Cowles, C. R., Emr, S. D., and Horazdovsky, B. F. (1994). Mutations in the VPS45 gene, a SEC1 homologue, result in vacuolar protein sorting defects and accumulation of membrane vesicles. *J. Cell Sci.* 107(Pt 12), 3449–3459.
- D'Andrea-Merrins, M., Chang, L., Lam, A. D., Ernst, S. A., and Stuenkel, E. L. (2007). Munc18c interaction with syntaxin 4 monomers and SNARE complex intermediates in GLUT4 vesicle trafficking. *J. Biol. Chem.* 282, 16553–16566.
- Dresbach, T., Burns, M. E., O'Connor, V., DeBello, W. M., Betz, H., and Augustine, G. J. (1998). A neuronal Sec1 homolog regulates neurotransmitter release at the squid giant synapse. *J. Neurosci.* 18, 2923–2932.
- 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. USA* 104, 2697–2702.
- 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.
- Dulubova, I., Yamaguchi, T., Gao, Y., Min, S. W., Huryeva, I., Sudhof, T. C., and Rizo, J. (2002). How Tlg2p/syntaxin 16 'snares' Vps45. *EMBO J.* 21, 3620–3631.
- Fisher, R. J., Pevsner, J., and Burgoyne, R. D. (2001). Control of fusion pore dynamics during exocytosis by Munc18. *Science* 291, 875–878.
- Fletcher, A. I., Shuang, R., Giovannucci, D. R., Zhang, L., Bittner, M. A., and Stuenkel, E. L. (1999). Regulation of exocytosis by cyclin-dependent kinase 5 via phosphorylation of Munc18. *J. Biol. Chem.* 274, 4027–4035.
- Gallwitz, D., and Jahn, R. (2003). The riddle of the Sec1/Munc-18 proteins—new twists added to their interactions with SNAREs. *Trends Biochem. Sci.* 28, 113–116.
- Garcia, E. P., Gatti, E., Butler, M., Burton, J., and De Camilli, P. (1994). A rat brain Sec1 homologue related to Rop and UNC18 interacts with syntaxin. *Proc. Natl. Acad. Sci. USA* 91, 2003–2007.
- Graham, M. E., Barclay, J. W., and Burgoyne, R. D. (2004). Syntaxin/Munc18 interactions in the late events during vesicle fusion and release in exocytosis. *J. Biol. Chem.* 279, 32751–32760.
- Guan, R., Dai, H., and Rizo, J. (2008). Binding of the Munc13-1 MUN Domain to Membrane-Anchored SNARE Complexes. *Biochemistry* 47, 1474–1481.
- Hammarlund, M., Palfreyman, M. T., Watanabe, S., Olsen, S., and Jorgensen, E. M. (2007). Open syntaxin docks synaptic vesicles. *PLoS Biol.* 5, e198.
- Harrison, S. D., Broadie, K., van de Goor, J., and Rubin, G. M. (1994). Mutations in the *Drosophila* Rop gene suggest a function in general secretion and synaptic transmission. *Neuron* 13, 555–566.
- Hata, Y., Slaughter, C. A., and Sudhof, T. C. (1993). Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin. *Nature* 366, 347–351.
- Jahn, R., and Scheller, R. H. (2006). SNAREs—engines for membrane fusion. *Nat. Rev. Mol. Cell. Biol.* 7, 631–643.
- Khvotchev, M., Dulubova, I., Sun, J., Dai, H., Rizo, J., and Sudhof, T. C. (2007). Dual modes of Munc18-1/SNARE interactions are coupled by functionally critical binding to syntaxin-1 N terminus. *J. Neurosci.* 27, 12147–12155.
- Latham, C. F. *et al.* (2006). Molecular dissection of the Munc18c/syntaxin4 interaction: implications for regulation of membrane trafficking. *Traffic* 7, 1408–1419.
- Liu, S., Wilson, K. A., Rice-Stitt, T., Neiman, A. M., and McNew, J. A. (2007a). In vitro fusion catalyzed by the sporulation-specific t-SNARE light-chain Spo20p is stimulated by phosphatidic acid. *Traffic* 8, 1630–1643.
- Liu, Y. *et al.* (2007b). A mechanism of Munc18b-syntaxin 3-SNAP25 complex assembly in regulated epithelial secretion. *FEBS Lett.* 581, 4318–4324.
- Margittai, M., Fasshauer, D., Jahn, R., and Langen, R. (2003). The Habc domain and the SNARE core complex are connected by a highly flexible linker. *Biochemistry* 42, 4009–4014.
- McNew, J. A. (2008). Regulation of SNARE-mediated membrane fusion during Exocytosis. *Chem. Rev.* 108, 1669–1686.
- McNew, J. A., Parlati, F., Fukuda, R., Johnston, R. J., Paz, K., Paumet, F., Sollner, T. H., and Rothman, J. E. (2000). Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature* 407, 153–159.
- Medine, C. N., Rickman, C., Chamberlain, L. H., and Duncan, R. R. (2007). Munc18-1 prevents the formation of ectopic SNARE complexes in living cells. *J. Cell Sci.* 120, 4407–4415.
- Melia, T. J., Weber, T., McNew, J. A., Fisher, L. E., Johnston, R. J., Parlati, F., Mahal, L. K., Sollner, T. H., and Rothman, J. E. (2002). Regulation of membrane fusion by the membrane-proximal coil of the t-SNARE during zippering of SNAREpins. *J. Cell Biol.* 158, 929–940.
- 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.
- Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* 21, 205–215.
- Novick, P., and Schekman, R. (1979). Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 76, 1858–1862.
- Ossig, R., Dascher, C., Trepte, H. H., Schmitt, H. D., and Gallwitz, D. (1991). The yeast SLY gene products, suppressors of defects in the essential GTP-binding Ypt1 protein, may act in endoplasmic reticulum-to-Golgi transport. *Mol. Cell. Biol.* 11, 2980–2993.
- Parlati, F., Weber, T., McNew, J. A., Westermann, B., Sollner, T. H., and Rothman, J. E. (1999). Rapid and efficient fusion of phospholipid vesicles by the alpha-helical core of a SNARE complex in the absence of an N-terminal regulatory domain. *Proc. Natl. Acad. Sci. USA* 96, 12565–12570.
- Peng, R., and Gallwitz, D. (2004). Multiple SNARE interactions of an SM protein: Sed5p/Sly1p binding is dispensable for transport. *EMBO J.* 23, 3939–3949.
- Pevsner, J., Hsu, S. C., Braun, J. E., Calakos, N., Ting, A. E., Bennett, M. K., and Scheller, R. H. (1994a). Specificity and regulation of a synaptic vesicle docking complex. *Neuron* 13, 353–361.
- Pevsner, J., Hsu, S. C., and Scheller, R. H. (1994b). n-Sec1, a neural-specific syntaxin-binding protein. *Proc. Natl. Acad. Sci. USA* 91, 1445–1449.
- Rodkey, T. L., and McNew, J. A. (2008). SNARE Effector Proteins, Austin, TX: Landes Bioscience.
- Salzberg, A., Cohen, N., Halachmi, N., Kimchie, Z., and Lev, Z. (1993). The *Drosophila* Ras2 and Rop gene pair: a dual homology with a yeast Ras-like gene and a suppressor of its loss-of-function phenotype. *Development* 117, 1309–1319.
- Schaffner, W., and Weissmann, C. (1973). A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* 56, 502–514.
- Schulze, K. L., Littleton, J. T., Salzberg, A., Halachmi, N., Stern, M., Lev, Z., and Bellen, H. J. (1994). rop, a *Drosophila* homolog of yeast Sec1 and vertebrate n-Sec1/Munc-18 proteins, is a negative regulator of neurotransmitter release in vivo. *Neuron* 13, 1099–1108.

- Schutz, D., Zilly, F., Lang, T., Jahn, R., and Bruns, D. (2005). A dual function for Munc-18 in exocytosis of PC12 cells. *Eur. J. Neurosci.* 21, 2419–2432.
- Scott, B. L., Van Komen, J. S., Irshad, H., Liu, S., Wilson, K. A., and McNew, J. A. (2004). Sec1p directly stimulates SNARE-mediated membrane fusion in vitro. *J. Cell Biol.* 167, 75–85.
- Scott, B. L., Van Komen, J. S., Liu, S., Weber, T., Melia, T. J., and McNew, J. A. (2003). Liposome fusion assay to monitor intracellular membrane fusion machines. *Methods Enzymol.* 372, 274–300.
- 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.
- Shuang, R., Zhang, L., Fletcher, A., Groblewski, G. E., Pevsner, J., and Stuenkel, E. L. (1998). Regulation of Munc-18/syntaxin 1A interaction by cyclin-dependent kinase 5 in nerve endings. *J. Biol. Chem.* 273, 4957–4966.
- Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362, 318–324.
- 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.
- Toonen, R. F., and Verhage, M. (2003). Vesicle trafficking: pleasure and pain from SM genes. *Trends Cell Biol.* 13, 177–186.
- Verhage, M. *et al.* (2000). Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science* 287, 864–869.
- Voets, T., Toonen, R. F., Brian, E. C., de Wit, H., Moser, T., Rettig, J., Sudhof, T. C., Neher, E., and Verhage, M. (2001). Munc18-1 promotes large dense-core vesicle docking. *Neuron* 31, 581–591.
- 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.
- Weimbs, T., Low, S. H., Chapin, S. J., Mostov, K. E., Bucher, P., and Hofmann, K. (1997). A conserved domain is present in different families of vesicular fusion proteins: a new superfamily. *Proc. Natl. Acad. Sci. USA* 94, 3046–3051.
- Weimer, R. M., and Richmond, J. E. (2005). Synaptic vesicle docking: a putative role for the Munc18/Sec1 protein family. *Curr. Top. Dev. Biol.* 65, 83–113.
- Weimer, R. M., Richmond, J. E., Davis, W. S., Hadwiger, G., Nonet, M. L., and Jorgensen, E. M. (2003). Defects in synaptic vesicle docking in unc-18 mutants. *Nat. Neurosci.* 6, 1023–1030.
- 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* 16, 308–320.
- Wu, M. N., Littleton, J. T., Bhat, M. A., Prokop, A., and Bellen, H. J. (1998). ROP, the *Drosophila* Sec1 homolog, interacts with syntaxin and regulates neurotransmitter release in a dosage-dependent manner. *EMBO J.* 17, 127–139.
- Wu, M. N., Schulze, K. L., Lloyd, T. E., and Bellen, H. J. (2001). The ROP-syntaxin interaction inhibits neurotransmitter release. *Eur. J. Cell Biol.* 80, 196–199.
- Yamaguchi, T., Dulubova, I., Min, S. W., Chen, X., Rizo, J., and Sudhof, T. C. (2002). Sly1 binds to Golgi and ER syntaxins via a conserved N-terminal peptide motif. *Dev. Cell* 2, 295–305.
- Yang, B., Steegmaier, M., Gonzalez, L. C., Jr., and Scheller, R. H. (2000). nSec1 binds a closed conformation of syntaxin1A. *J. Cell Biol.* 148, 247–252.
- 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, e330.