SNARE bundle and syntaxin N-peptide constitute a minimal complement for Munc18-1 activation of membrane fusion

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Sec1/Munc18 (SM) proteins activate intracellular membrane fusion through binding to cognate SNAP receptor (SNARE) complexes. The synaptic target membrane SNARE syntaxin 1 contains a highly conserved Habc domain, which connects an N-peptide motif to the SNARE core domain and is thought to participate in the binding of Munc18-1 (the neuronal SM protein) to the SNARE complex. Unexpectedly, we found that mutation or complete removal of the Habc domain had no effect on Munc18-1 stimulation of fusion. The central cavity region of Munc18-1 is required to stimulate fusion but not through its binding to the syntaxin Habc domain. SNAP-25, another synaptic SNARE subunit, contains a flexible linker and exhibits an atypical conjoined Qbc configuration. We found that neither the linker nor the Qbc configuration is necessary for Munc18-1 promotion of fusion. As a result, Munc18-1 activates a SNARE complex with the typical configuration, in which each of the SNARE core domains is individually rooted in the membrane bilayer. Thus, the SNARE four-helix bundle and syntaxin N-peptide constitute a minimal complement for Munc18-1 activation of fusion.

Introduction

Intracellular membrane fusion is mediated by two conserved families of proteins, the SNAREs and the Sec1/Munc18 (SM) proteins (Südhof and Rothman, 2009). Fusion is initiated when the v-SNARE pairs with its cognate t-SNAREs to form a thermostable trans-SNARE complex (SNAREpin) that brings two membranes into close apposition to fuse (Söllner et al., 1993; Sutton et al., 1998; Weber et al., 1998; Hu et al., 2003; Wickner and Schekman, 2008). One universal feature of the trans-SNARE complex is a four-helix coiled-coil bundle assembled from the core motifs of individual t- and v-SNAREs. N to C zippering of this SNARE core bundle is believed to provide the driving force required to merge two membrane bilayers (Melia et al., 2002; Pobbati et al., 2006; Walter et al., 2010). SM proteins are soluble factors of 60–70 kD that control vesicle fusion by directly interacting with specific subsets of SNAREs (Novick and Schekman, 1979). One critical outstanding question in cell biology is how SNAREs and SM proteins act in concert to fuse membrane bilayers.

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Abbreviations used in this paper: PDGFR, PDGF receptor; SM, Sec1/Munc18; SUMO, small ubiquitin-like modifier; WT, wild type.

The most intensely studied form of intracellular vesicle fusion is the calcium-triggered neurotransmitter release at chemical synapses, which serves as the brain’s major form of cell–cell communication (Koh and Bellen, 2003; Jahn, 2004; Südhof, 2004; Brunger, 2006; Jackson and Chapman, 2006). Neurotransmitter secretion is mediated by the fusion of exocytic vesicles with the plasma membrane and requires three SNARE proteins, syntaxin 1 (t-SNARE heavy chain or Qa), SNAP-25 (t-SNARE light chains or Qb and Qc) and VAMP2/synaptobrevin (v- or R-SNARE; Söllner et al., 1993). Munc18-1, a neuron-specific SM protein, is required for synaptic vesicle fusion, as demonstrated in a variety of organisms, including nematodes (unc-18), flies (ROP), and mice (Munc18-1; Harrison et al., 1994; Wu et al., 1998; Verhage et al., 2000; Voets et al., 2001; Weimer et al., 2003). Munc18-1 binds to the synaptic SNARE complex and promotes the speed and specificity of vesicle fusion (Shen et al., 2007). This conserved positive role of Munc18-1

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requires its specific interactions with multiple epitopes on the SNARE complex, including SNARE core domains and a short N-terminal peptide motif (N-peptide) of syntaxin 1 (Dulubova et al., 2007; Rickman et al., 2007; Shen et al., 2007). N-peptide is found in most, but not all, syntaxin subunits (Dulubova et al., 2007; Shen et al., 2007; Tareste et al., 2008). It is characterized by two or three charged residues followed by a hydrophobic leucine or phenylalanine residue inserting into a peripheral pocket on its cognate SM protein (Bracher and Weissenhorn, 2002; Dulubova et al., 2002, 2003; Hu et al., 2007). Mutations disrupting Munc18-1’s interaction with either the core bundle or the N-peptide are known to reduce synaptic release in vivo (Sørensen et al., 2002; Borisovska et al., 2005; McEwen and Kaplan, 2008; Johnson et al., 2009; Rickman and Duncan, 2010).

A prototypic SNARE protein contains a characteristic core SNARE motif and a C-terminal transmembrane domain separated by a short linker (~10 aa long; Fukuda et al., 2000). Most SNAREs also harbor regulatory sequences outside the core motifs and some adopt alternative structures. As a result, assembled SNARE complexes display a significant degree of heterogeneity across pathways or organisms (Jahn and Scheller, 2006). In synaptic vesicle fusion, for example, the t-SNARE subunit SNAP-25 lacks a transmembrane domain and contains two core motifs joined together by a flexible linker. The other synaptic t-SNARE subunit syntaxin 1 contains a highly conserved four-helix Habc domain, which connects syntaxin N-peptide to the core motif (Misura et al., 2000; Burkhardt et al., 2008). The Habc domain is critical for the inhibitory interaction of Munc18-1 with the “closed” syntaxin 1 monomer, although formation of this high-affinity syntaxin–SM dimer appears to be restricted to regulated exocytosis (Gerber et al., 2008). Whether and how Munc18-1 interacts with these regulatory sequences during fusion remain unknown.

**Results and discussion**

*Munc18-1 stimulation of fusion does not require a direct interaction with the Habc domain of syntaxin 1*

The role of syntaxin Habc domain in Munc18-1 stimulation of fusion is not known. The highly conserved Habc domain connects the N-peptide motif to the SNARE core bundle (Fig. 1 A; Fernandez et al., 1998) and may coordinate those two Munc18-1–binding modes. Indeed, it was previously suggested that the Habc domain directly regulates Munc18-1 binding to the SNARE complex (Deák et al., 2009). Figure 1. *Munc18-1 stimulation of fusion does not require a direct interaction with the Habc domain of syntaxin 1.* (A) Diagrams of WT syntaxin 1, a ΔN-peptide syntaxin 1 mutant lacking the N-peptide motif, an Habc7 mutant in which the Habc domain of syntaxin 1 was replaced with the corresponding Habc domain from syntaxin 7, and a SUMO-syntaxin mutant in which the Habc domain was replaced with the SUMO/Smt3p protein from Saccharomyces cerevisiae. The location of each syntaxin 1 domain is indicated. TMD, transmembrane domain. (B) Fusion of t-SNARE liposomes containing WT or mutant syntaxin 1 with VAMP2 liposomes in the absence or presence of 5 µM Munc18-1. (C) Fold activation of the fusion reactions in B. The dashed line indicates basal fusion level (with no Munc18-1 activation). Error bars indicate standard deviation.
Munc18-1–associated SNAREs. Reconstituted t- and v-SNAREs drove a basal fusion reaction that was strongly accelerated by Munc18-1. The stimulation was abolished when the N-peptide of syntaxin 1 was removed (Fig. 1, B and C). The potent effect of Munc18-1 on SNARE-mediated membrane fusion (approximately ninefold increase in initial rate) was in line with the severe defects in neuronal release (80% to >90% reduction) observed in Munc18-1–null neurons (Verhage et al., 2000; Voets et al., 2001; Weimer et al., 2003).

To explore whether Munc18-1 stimulation of fusion involves a specific interaction with the syntaxin Habc domain, we replaced the latter with the corresponding Habc domain of syntaxin 7, an endosomal SNARE exhibiting limited sequence homology with syntaxin 1 (Fig. 1 A; Antonin et al., 2002). This Habc7 chimeric mutant is expected to retain the overall structure of syntaxin. Unexpectedly, the SNARE complex containing this mutant syntaxin was activated by Munc18-1 at a similar level to that of wild-type (WT) SNAREs (Fig. 1, B and C). We next replaced the Habc domain with the yeast small ubiquitin-like modifier (SUMO; SUMO/Smt3p) protein that has no sequence similarity to syntaxin 1 (Fig. 1 A). SUMO contains a β-barrel structure flanked by α-helices (Bergink and Jentsch, 2009), which is markedly distinct from the three-helix conformation of the syntaxin Habc domain (Fernandez et al., 1998). Again, Munc18-1 robustly activated the fusion of liposomes reconstituted with SNAREs containing this syntaxin-SUMO chimera (Fig. 1, B and C). Importantly, all of the mutants tested in this study drove comparable basal reactions, suggesting that SNARE assembly remained intact (Fig. 1 B). These data indicate that Munc18-1 does not require a specific interaction with the syntaxin Habc domain to activate SNARE-dependent membrane fusion.

Munc18-1 activates a mutant SNARE complex in which the syntaxin N-peptide is directly attached to the core bundle without the Habc domain.

We next considered the possibility that the binding of Munc18-1 to the SNARE complex requires only a physical hinge between syntaxin N-peptide and the core bundle, rather than engaging a specific interaction with the Habc domain. The Habc domain of syntaxin 7 or even an unrelated SUMO protein may serve this hinge role as adequately as the native Habc domain of syntaxin 1. Indeed, all of these chimeric proteins have similar linker lengths (~9 nm) between the N-peptide and SNARE bundle. To this end, we removed the entire Habc domain from syntaxin 1 such that N-peptide became directly attached to the core motif without the Habc hinge (Fig. 2 A). The SNAREs lacking the Habc domain (ΔHabc mutant) drove a basal fusion reaction comparable with that of WT SNAREs and, strikingly, were fully activated by Munc18-1 (Fig. 2, B and C). The maximum stimulation was reached with 5 µM Munc18-1, similar to the dose response of WT SNAREs (Fig. S1). Munc18-1 acceleration of fusion was abolished when VAMP2 was substituted with VAMP8.

Figure 2. Munc18-1 activates a mutant SNARE complex in which the syntaxin N-peptide is directly attached to the core bundle without the Habc domain. (A) Diagrams of WT syntaxin 1, a ΔN syntaxin 1 mutant lacking the N terminus, and a ΔHabc syntaxin 1 mutant in which the Habc domain was removed (while the N-peptide was left intact). TMD, transmembrane domain. (B) Fusion of t-SNARE liposomes containing WT or mutant syntaxin 1 with VAMP2 (V2) or VAMP8 (V8) liposomes in the absence or presence of 5 µM Munc18-1. (C) Fold activation of the fusion reactions in B. The dashed line indicates basal fusion level (with no Munc18-1 activation). Error bars indicate standard deviation.
a noncognate v-SNARE involved in lysosomal/late endosomal fusion. In addition, mutation of a previously identified Munc18-1 binding motif (S75E/Q76H; Shen et al., 2007) on VAMP2 abrogated Munc18-1 activation of fusion (Fig. 2, B and C). These data suggest that the ΔHabc mutant and WT SNAREs confer the same fusion specificity and binding requirements and thus are likely activated by Munc18-1 through the same mechanism. Therefore, Munc18-1 promotion of fusion does not need the Habc domain as a physical hinge between the syntaxin N-peptide and the SNARE core bundle.

We also tested for a direct interaction between Munc18-1 and SNAREs in a copurification assay. Results obtained from this system agree well with those from other binding assays such as gel filtration and nuclear magnetic resonance (Dulubova et al., 2007; Shen et al., 2007). We found that Munc18-1 was associated with stoichiometric amounts of SNARE complexes containing both WT and ΔHabc syntaxin 1 but not with a mutant complex lacking the entire N terminus of syntaxin (Fig. 3 A). The identities of the components in the complex were confirmed by Western blotting (Fig. 3 B). Together, these results demonstrate that Munc18-1 does not require the syntaxin 1 Habc domain to interact with the SNARE complex or to activate fusion. Thus, addition of a short N-peptide to the SNARE bundle, which otherwise could not be stimulated by Munc18-1 (Fig. 2, B and C), resulted in a full activation of membrane fusion.

The central cavity region of Munc18-1 is critical for stimulating fusion but not through binding to the syntaxin Habc domain. The dispensability of the syntaxin Habc domain in Munc18-1 stimulation of fusion is a surprising finding because Munc18-1 was previously suggested to require the Habc domain for activating vesicle fusion (Rodkey et al., 2008; Deák et al., 2009). In particular, the glutamate 59 residue (E59) of Munc18-1 was found to be critical for the association of Munc18-1 with the SNARE complex (Deák et al., 2009). Mutation of E59 into lysine (E59K) strongly reduced SNARE–Munc18-1 binding affinity and caused severe defects in neuronal release (Deák et al., 2009). The E59 residue is located within the central cavity of Munc18-1 and interacts with arginine 114 (R114) of syntaxin 1 in the syntaxin–Munc18-1 binary complex (Fig. 4 B; Misura et al., 2000).Because the R114 residue is within the Habc domain of syntaxin 1 (Fig. 4 A), it was suggested that Munc18-1 E59 also interacts with the syntaxin Habc domain when activating the SNARE complex (Deák et al., 2009). Consistent with the in vivo observation, we found that the E59K mutation dramatically...
Munc18-1 activation of fusion does not require the linker or the Qbc configuration of SNAP-25

Although the interactions of Munc18-1 with syntaxin 1 and VAMP2 have been demonstrated (Figs. 1–3; Shen et al., 2007), little is known about Munc18-1 binding to SNAP-25. SNAP-25, lacking a transmembrane domain, contains two core motifs (Qb and Qc) joined together by a flexible linker (Fig. 5A). This Qbc organization is an exception to the typical configuration of the SNARE fusion machinery, in which each of the four SNARE core domains (Qa, Qb, Qc, and R) is individually rooted to the lipid bilayer through C-terminal transmembrane domains (Jahn and Scheller, 2006).

We examined whether Munc18-1 stimulation of fusion requires the flexible linker or the Qbc configuration of SNAP-25. Although the interactions of Munc18-1 with syntaxin 1 and VAMP2 have been demonstrated (Figs. 1–3; Shen et al., 2007), little is known about Munc18-1 binding to SNAP-25. SNAP-25, lacking a transmembrane domain, contains two core motifs (Qb and Qc) joined together by a flexible linker (Fig. 5A). This Qbc organization is an exception to the typical configuration of the SNARE fusion machinery, in which each of the four SNARE core domains (Qa, Qb, Qc, and R) is individually rooted to the lipid bilayer through C-terminal transmembrane domains (Jahn and Scheller, 2006).

We examined whether Munc18-1 stimulation of fusion requires the flexible linker or the conjoined Qbc configuration of SNAP-25. To this end, each of the core domains of SNAP-25 (25N or 25C) was fused to a generic transmembrane domain derived from the PDGF receptor (PDGFR; Fig. 5A). These two chimeric SNAREs, 25N-PDGFR (Qb) and 25C-PDGFR (Qc), were individually expressed and purified without the linker region or lipid modification found on the native SNAP-25 protein. 25N-PDGFR and 25C-PDGFR proteins were mixed with syntaxin 1 and VAMP2 liposomes in the absence or presence of 5 µM WT or E59K mutant Munc18-1.

Figure 4. The central cavity domain of Munc18-1 is critical for stimulating fusion but not through binding to the syntaxin Habc domain. (A) Diagram of syntaxin 1 showing the location of arginine 114 (R114). TMD, transmembrane domain. (B) Crystal structure of syntaxin 1–Munc18-1 dimer showing the interaction of syntaxin R114 with Munc18-1 glutamate 59 (E59; Misura et al., 2000). The structure was derived from reference (derived from Misura et al. [2000]) and edited in PyMOL (DeLano Scientific LLC). (C) Fusion of t-SNARE liposomes containing WT or mutant syntaxin 1 with VAMP2 liposomes in the absence or presence of 5 µM WT or E59K mutant Munc18-1. (D) Fold activation of the fusion reactions in C. The dashed line indicates basal fusion level (with no Munc18-1 activation). Error bars indicate standard deviation.
efficiently with VAMP2 liposomes, and the fusion was activated by Munc18-1 to a level comparable with that of WT SNAREs (Fig. 5, B and C). Substitution of VAMP2 with the noncognate v-SNARE VAMP8 abolished the ability of Munc18-1 to promote vesicle fusion (Fig. 5, B and C). These data establish that Munc18-1 requires neither the linker region nor the conjoined Qbc configuration of SNAP-25 to stimulate fusion.

Thus, through a series of modifications, we engineered a fully active SNARE fusion machinery that contained only the SNARE bundle and a short syntaxin N-peptide (Fig. 5 D). In this SNARE complex, each of the core domains was individually rooted in the membrane according to the typical configuration of the SNARE fusion machinery. Based on these results, we conclude that the syntaxin N-peptide and SNARE bundle constitute a minimal complement for Munc18-1 activation of SNARE-mediated membrane fusion.

**Conclusion**

In this study, we demonstrate that the syntaxin N-peptide motif and the SNARE four-helix bundle are sufficient for Munc18-1 activation of fusion, whereas all other SNARE sequences are dispensable (Fig. 5 D). We propose that this reduced SNARE complex represents the minimal complement required for Munc18-1 acceleration of fusion. Previous studies suggested that the syntaxin Habc domain can interact with Munc18-1 (Dulubova et al., 2007; Rodkey et al., 2008). However, our data clearly show that this interaction, if existent, is dispensable for Munc18-1 activation of the SNARE complex. SM proteins exhibit conserved structures and similar loss of function phenotypes (abrogation of fusion), implying that we have likely uncovered a conserved regulatory mechanism of SM proteins. This conclusion is further supported by our observation that Munc18-1 activates an engineered SNARE complex with the typical configuration.

At the center of the vesicle fusion machinery is the SNARE four-helix bundle. The bundle not only supplies the energy required for the merging of two membranes, but it is also the primary binding target of SM proteins, which are fundamentally designed to grasp four-helix bundles using their central cavity regions (Scott et al., 2004; Latham et al., 2006; Mima et al., 2008; Toonen and Verhage, 2007; Rickman and Duncan, 2010). Although individual SNARE subunits exhibit diverse conformations, the structures of assembled four-helix bundles are highly conserved across pathways (Ungar and Hughson, 2003).
C-terminal transmembrane domains of SNAREs form continuous α-helices with the core motifs and thus are integral parts of the SNARE bundle (Stein et al., 2009). Because N-peptide binding is not found among certain SNARE–SM pairs, the SNARE bundle is likely the conserved target of SM proteins.

Structural modeling and biochemical analysis demonstrate that the SNARE bundle competes with the closed syntaxin 1 monomer to occupy the central cavity domain of Munc18-1 such that the binding to this region by one bundle is mutually exclusive of the other (Xu et al., 2010; unpublished data). This agrees well with our discovery that the syntaxin Habc domain is dispensable for Munc18-1 association with the SNARE complex: once occupied by the SNARE bundle, the central cavity region of Munc18-1 will not have room to accommodate the three-helix Habc domain of syntaxin. Our data also imply that the same residues lining the Munc18-1 cavity region such as E59 may engage a dual mode of SNARE interaction, with the SNARE core bundle in the context of SNARE complex binding or with the Habc domain in the context of closed syntaxin 1 monomer binding. In contrast to the closed syntaxin monomer that displays a relatively static conformation, the SNARE bundle is highly dynamic, and Munc18-1 may recognize multiple intermediates of the assembling SNARE bundle.

It should be noted that the regulatory sequences outside the minimal SNARE complement, albeit dispensable for Munc18-1 activation, may play important roles in membrane trafficking in the cell. For instance, the flexible linker and Qbc configuration of SNAP-25 may be necessary for the proper subcellular localization of the protein. In contrast, the Habc domain of syntaxin 1 may fine-tune synaptic transmission in higher organisms when it folds back onto syntaxin’s own core motif to present a closed conformation (Misura et al., 2000; Gerber et al., 2008). The Habc domain may also serve as an organizing center allowing additional regulatory factors such as Munc13, CAPS/Unc-31, synaptotagmin, and complexin to be superimposed on the SNARE–SM fusion machinery to achieve temporal and spatial control of synaptic release (Basu et al., 2005; Giraudo et al., 2006; Jackson and Chapman, 2006; Tang et al., 2006; Martens et al., 2007; Zhou et al., 2007; Yoon et al., 2008; de Wit et al., 2009; James et al., 2009).

Materials and methods

Protein expression and purification
To produce recombinant VAMP2/synaptobrevin proteins with no extra residues, a full-length mouse VAMP2 gene was subcloned into a pET28a-based SUMO/SmGp cloning vector. The sequence between the SUMO cleavage site and the first residue of VAMP2 was removed by site-directed mutagenesis. After binding to a nickel column (GE Healthcare) through an N-terminal His6 tag, VAMP2 protein was released from SUMO by overnight on-column cleavage with SUMO protease at 4°C. The t-SNARE complex of mouse His6-SNAP-25 and rat syntaxin 1A (p1V434) was expressed and purified as previously described (Weber et al., 2000; Melia et al., 2002). Recombinant untagged Munc18-1 protein was produced in E. coli as previously described (Shen et al., 2007). SNARE and Munc18-1 mutants were generated by site-directed mutagenesis and standard molecular cloning and purified similarly to WT proteins.

Proteoliposome reconstitution
All lipids were obtained from Avanti Polar Lipids, Inc. For t-SNARE reconstitution, 1-palmityl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmityl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmityl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), and cholesterol were mixed in a molar ratio of 60:20:10:10. For v-SNARE reconstitution, POPC, POPE, POPS, cholesterol, N-[7-nitro-2,1,3-benzoazolizol-4-yl]-1,2-dipalmitoyl phosphatidyl ethanolamine (NBD-DPPE), and N-[lissamine rhodamine B sulfonyl]DPPE (rhodamine-DPPE) were mixed at a molar ratio of 60:17:10:10:1.5. SNARE proteoliposomes were prepared by detergent dilution and isolated on an Accudenz density gradient flotation (Weber et al., 1998). SNARE proteins were kept at physiologically relevant densities, with protein/lipid ratios at 1:200 for v-SNAREs (similar to VAMP2 densities reported for native synaptic vesicles; John and Sudhof, 1994; Walch-Solimena et al., 1995) and at 1:500 for t-SNARE liposomes. Reconstituted liposomes were routinely monitored by dynamic light scattering and electron microscopy with negative staining.

Liposome fusion assay
Fusion reactions and data analysis were performed as previously described (Shen et al., 2007). A standard fusion reaction contained 45 µl unlabeled t-SNARE liposomes and 5 µl labeled v-SNARE liposomes and was conducted in a 96-well Nunc plate at 37°C. Fusion was followed by measuring the increase in NBD fluorescence at 538 nm (excitation 460 nm) every 2 min. At the end of the 2h reaction, 10 µl of 2.5% dodecyl-maltoside was added to the liposomes. The raw NBD fluorescence data were converted to rounds of fusion using an equation as previously described (Parlati et al., 1999). To assess the regulatory activity of Munc18-1, v- and t-SNARE liposomes were incubated with or without 5 µM Munc18-1 on ice for 1 h before the temperature was elevated to 37°C to initiate fusion. The maximum fusion rate within the first 20 min of liposome fusion was used to represent the initial rate of a fusion reaction. Full accounting of statistical significance was included for each figure based on at least three independent experiments.

Protein interactions in a copurification assay
SNARE–Munc18-1 interactions were probed in a copurification assay in which bacterial cells expressing both GST–Munc18-1 and SNAREs were lysed in protein-binding buffer (25 mM Hepes, pH 7.4, 150 mM KCl, 10% glycerol, 1% CHAPS, and 1 mM DTT). Glutathione Sepharose (GE Healthcare) was added to the lysate to purify GST–Munc18-1 and associated proteins. After washing three times with binding buffer, protein complexes bound to the beads were resolved on SDS-PAGE and stained with a Coomassie blue staining kit (Thermo Fisher Scientific). Identities of the proteins in the complexes were confirmed with Western blotting with polyclonal anti–Munc18-1 (Sigma-Aldrich), monoclonal anti–SNAP-25 (Cl 71.2; Synaptic Systems GmbH), or anti-VAMP2 (Cl 69.1; Synaptic Systems GmbH) antibodies.

Online supplemental material
Fig. S1 shows the dose dependence of the stimulatory effects of Munc18-1 on the fusion reactions mediated by WT or ΔHis6 SNAREs. Online supplemental material is available at http://jcb.cbi.org/cgi/content/full/jcb.201003148/DC1.

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Figure S1.  **Dose dependence of the stimulatory activity of Munc18-1 on membrane fusion.** Liposomes bearing either WT or ΔH_{abc} SNAREs were incubated with the indicated amounts of Munc18-1 for 1 h at 4°C before the temperature was raised to 37°C to initiate fusion. The plots depict the fold activation of the initial liposome fusion rates with varying Munc18-1 concentration. Error bars equal standard deviation.