Single-Vesicle Fusion Assay Reveals Munc18-1 Binding to the SNARE Core Is Sufficient for Stimulating Membrane Fusion

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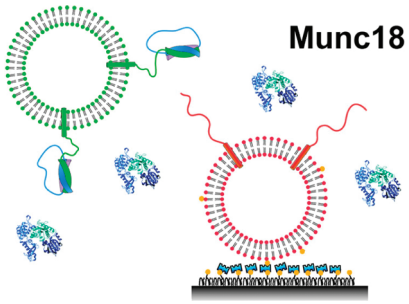
Abstract



Munc18, an essential regulatory protein for intracellular membrane fusion mediated by SNAREs, is known for stabilizing the closed conformation of syntaxin through the interaction with the N-terminal Habc domain (amino acids 28−146) of syntaxin. In addition, Munc18 accelerates membrane fusion and its interaction with SNARE core and the N-peptide (amino acids 1−24) of syntaxin is thought to be necessary for this function. Using the recently developed fluorescence resonance energy transfer assay to detect the fusion between two individual vesicles harboring cognate SNARE proteins, we studied the effect of Munc18 on the fusion induced by neuronal SNARE proteins by following the mixing of lipid molecules between the two vesicles. We found that Munc18-1 stimulates neuronal SNARE-mediated fusion not only with full-length syntaxin 1A but also with a truncated syntaxin 1A that is missing both the Habc domain and the N-peptide. The electron paramagnetic resonance analysis indicates that the SNARE core/Munc18 interaction is responsible for this stimulatory function and the membrane plays a role for establishing this interaction.

Membrane fusion is a ubiquitous process involved in a wide variety of cellular activities, such as exocytosis, viral infection, vesicle trafficking, and egg fertilization. A protein family called SNARE (soluble *N*-ethyl maleimide sensitive-factor attachment protein receptors), consisting of three components, plays a critical role in mediating fusion processes of eukaryote cells (*1*, *2*). According to their distribution in the cell, they are classified into v-SNARE, which includes synaptobrevin (VAMP), and t-SNAREs, which are composed of SNAP-25 and syntaxin for neuronal proteins. Sec1/Munc18 (SM) proteins are a family of cytoplasmic proteins with a conserved arch-shaped structure and play an essential role in intracellular membrane fusion. Along with SNARE proteins, which primarily mediate fusion of cellular transport vesicles with the target membrane, the SM proteins are thought to be central components of the exocytotic apparatus, which are required for membrane fusion (*3*−*6*). A gene knockout study has indicated that the SM proteins are crucial for synaptic membrane fusion, which is required for neurotransmitter release (*7*).

Munc18 interacts with SNARE proteins at least in two different modes, namely, binding to Habc domain (amino acids 28−146) of syntanxin and binding to the SNARE core complex (*8*, *9*). In the first mode, Munc18 interacts with monomeric syntaxin through the N-terminal helical segment of syntaxin called the Habc domain to form a Munc18/syxntaxin binary complex (*3*−*6*). This Habc binding mode stabilizes the closed conformation of syntaxin during its transportation to the plasma membrane *in vivo*. Without this stabilization effect, the syntaxin level in sensory neurons was reduced by 70% in Munc18 knockout mice (*10*). In the second mode, Munc18 also binds to the SNARE core, a four helical bundle formed by syntaxin, VAMP, and SNAP25 (*8*, *11*, *12*). Ensemble proteoliposome fusion experiments showed that Munc18 binds preassembled SNARE complexes, and effectively promotes SNARE-mediated fusion with full-length syntaxin (*11*). An interaction called N-peptide binding between Munc18 and N-terminal

Received Date: November 17, 2009
Accepted Date: January 6, 2010
Published on Web Date: January 14, 2010

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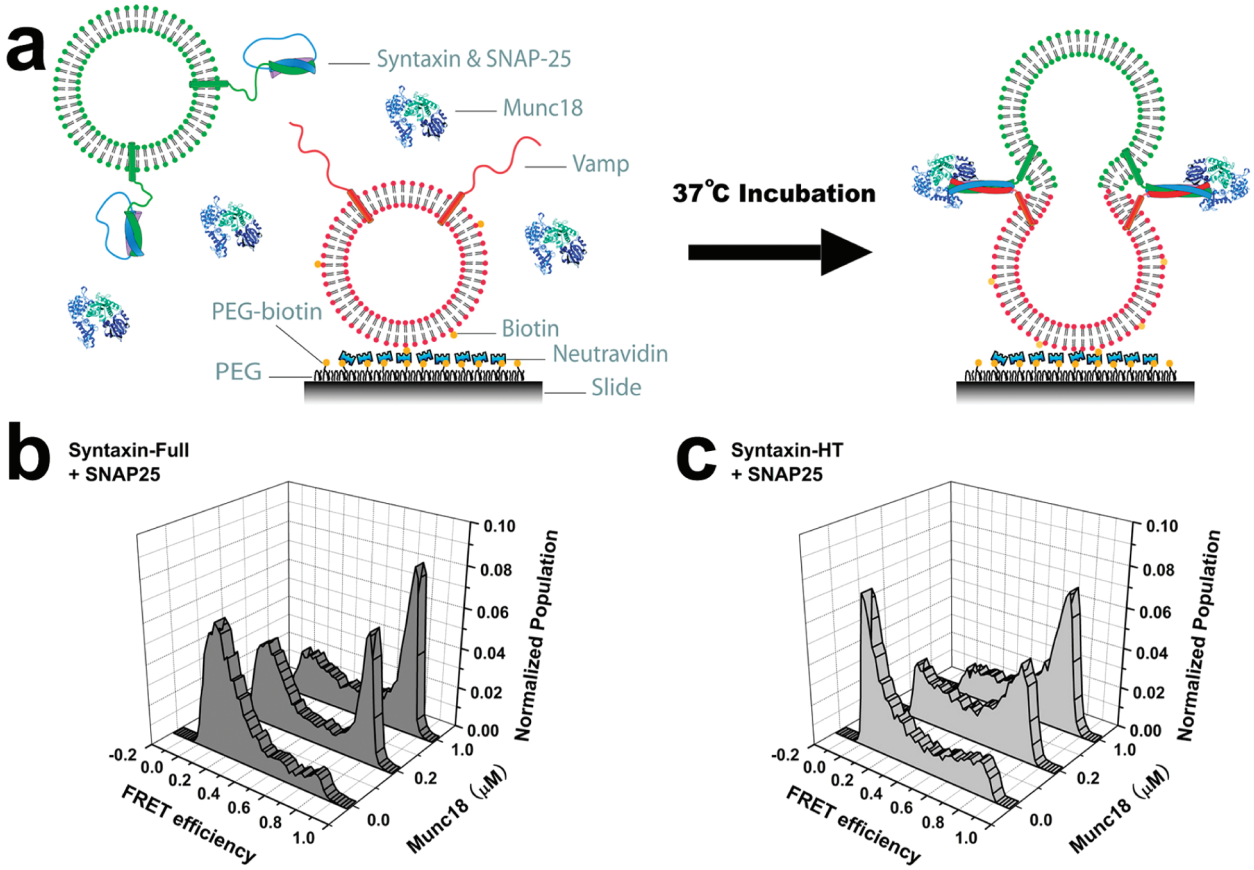

Figure 1. Single-vesicle FRET assay for Munc18-1 in neuronal SNARE-mediated fusion. (a), Schematics of the single-vesicle assay. (left) Acceptor-labeled v-SNARE vesicles are immobilized on a bottom quartz surface of a flow chamber. Donor-labeled t-SNARE vesicles, mixed with preset amount of Munc18-1, are introduced to the chamber space using a flow system. (right) Some t-SNARE vesicles dock to single v-SNARE vesicles through formation of *trans*-SNARE complexes, and Munc18-1 binds to the *trans*-SNARE complexes. Membrane fusion between t- and v-SNARE vesicles and resultant lipid mixing will cause an increase in the FRET efficiency. (b, c) FRET efficiency, E, distributions of single-vesicle complexes for various concentrations of Munc18-1 (0, 0.2, and 1 μM) in neuronal SNARE-mediated fusion with (b) syntaxin-full and (c) syntaxin-HT. Docking (or early fusion steps) shows low E values that are smaller than 0.25, and the full fusion state gives $E \approx 0.8$ (*13*, *18*). To make the comparison clearer, we normalized histograms by the total number of liposomes per experiment, which is more than one thousand for all experiments (*13*, *18*).

peptides (amino acids 1−24) of syntaxin besides this SNARE core/Munc18 interaction is believed to be critical for this fusion promotion effect (*8*, *11*). However, how Munc18 activates fusion remains unclear in part due to the inability of the ensemble in vitro fusion assay to dissect different steps of the fusion reaction.

For an unambiguous dissection of the protein-mediated membrane fusion mechanism, *in vitro* characterizations of reconstituted fusion machinery and regulators are required. Traditionally, *in vitro* studies rely on ensemble lipid mixing of proteoliposomes reconstituted with SNARE proteins, which cannot distinguish different stages of fusion such as docking, hemifusion and full fusion (*13*). Recently, new techniques have been developed for observing membrane fusion processes at the single-vesicle level (*13*−*17*). The single-vesicle fusion assay we developed could distinguish between different stages of docking, hemifusion, and full fusion via fluorescence resonance energy transfer (FRET) between the donor and acceptor fluorophores incorporated into the separate proteoliposomes reconstituted with t- and v-SNARE proteins, respectively. In addition, the single-vesicle fusion assay also allows us to describe the kinetics of transitions between different stages of fusion and postfusion pathways such as the kiss-and-run event (*13*) and to discover the dual functions of fusion regulator protein complexin I that inhibits SNARE complex formation and docking but enhances the fusion of docked vesicles together with calcium ions (*18*).

Figure 1a illustrates our single-vesicle lipid-mixing assay. The v-SNARE vesicles carrying vesicle-associated membrane protein (VAMP) and the acceptor fluorophores were immobilized on a polymer-coated quartz surface via biotinylated lipids. The t-SNARE vesicles containing syntaxin and SNAP-25 and doped with the donor fluorophores were added together with Munc18, and the sample was incubated at 37 °C. After a 12-min

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DOI: 10.1021/cn900034p | *ACS Chem. Neurosci.* (2010), *1*, 168–174

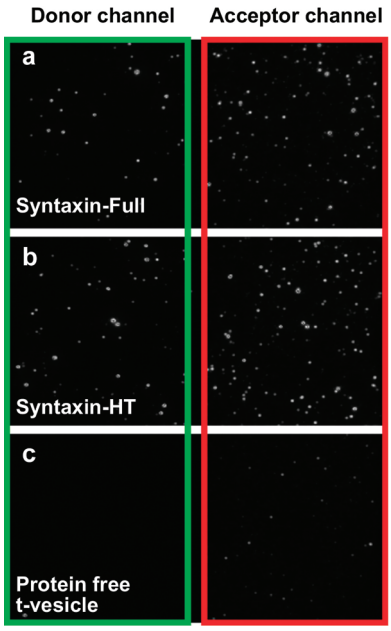

Figure 2. Laser-excited (532 nm) images of single-vesicle fusion experiments with Munc18-1. Acceptor-labeled v-vesicles are directly tethered to the surface via biotin−neutravidin linker and the donor-labeled t-vesicles are added. Because the laser excites the acceptor only very weakly, bright fluorescent spots are seen only when the t-vesicles are present: (a) t-vesicles containing syntaxin-full and SNAP-25, (b) t-vesicles containing syntaxin-HT and SNAP-25, and (c) protein-free t-vesicles. Green and red rectangles denote the donor and acceptor emission detection channels, respectively. Panels a and b show docked t-SNARE vesicles in the donor channel and bright v-SNARE vesicles through FRET in the acceptor channel. Strong FRET signal demonstrates that binding of t-SNARE vesicles to the surface is specially achieved via interaction with the surface-immobilized v-SNARE vesicles. Panel c only shows dim v-SNARE vesicles in the acceptor channel without docking of t-vesicles, demonstrating that the nonspecific adhesion of the t-SNARE vesicles to the surface is minimal. In all experiments, 1 μM Munc18-1 was used.

incubation, the sample was transferred to a dual-color total internal reflection (TIR) fluorescence microscope (*19*), and FRET measurements of individual vesicles at 37 °C were performed 20 min after the reaction began. Passivation of quartz slides via coating with poly-(ethylene glycol) (*20*) was essential in minimizing non-specific binding of the vesicles to the surface and in keeping the proteins functional (Figure 2) (*13*). The multiple intermediate states of fusion are classified according to their different FRET efficiency values as characterized previously (*13*). A finite but low-efficiency distribution ≤0.25 suggests close contact or docking between the donor and the acceptor vesicles without a high degree of lipid mixing. The final efficiency distribution around 0.35 indicates a hemifusion state. FRET efficiency distribution ≥0.5 is assigned as full fusion where both inner and outer leaflets have been mixed (*13*). The lipid composition of vesicles used in this study, 15 mol % PS (phospho-L-serine), 45 mol % PC (phosphocholine), and 40 mol % cholesterol, and the 200:1 lipid/protein ratio were chosen to emulate the composition of the native synaptic vesicles (*18*, *21*).

The resulting single-vesicle FRET efficiency histograms of the reaction product showed that Munc18-1 promotes full fusion represented at the FRET efficiency ≥0.5 in a concentration-dependent manner whether the full-length syntaxin 1A (syntaxin-full, amino acids 1−288) or the truncated syntaxin 1A lacking the N-peptide and Habc domain (syntaxin-HT (Habc-truncated), amino acids 168−288) was used (Figure 1b,c). After 20 min reaction, we observed more than 50% full fusion populations (Figure 3), which is much faster than the previous report of several hours (*11*). The fusion promotion activity of Munc18-1 is dependent on SNAP-25 for both syntaxin-full and syntaxin-HT cases because omitting SNAP-25 led to a significant reduction in full fusion population

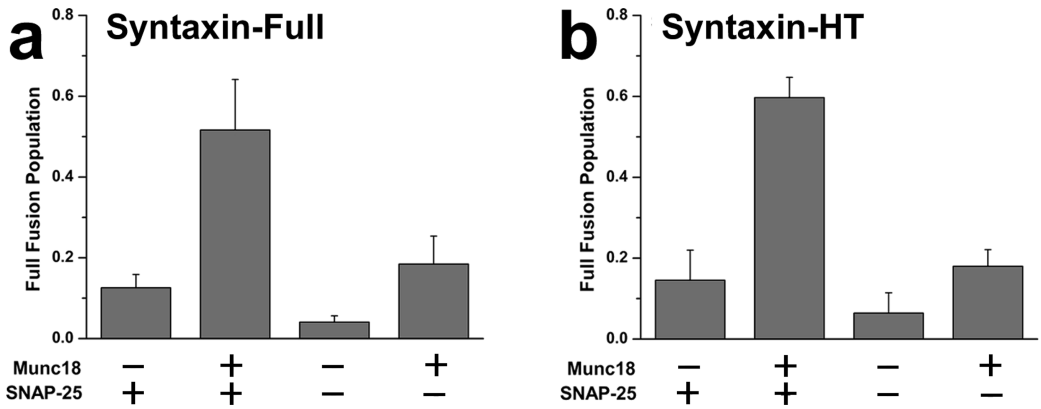

Figure 3. Fraction of vesicle complexes showing full fusion for various reaction conditions. One micromolar Munc18-1 was used. (a) Results obtained with the full-length syntaxin. (b) Results obtained with a syntanxin-HT. Controls without SNAP-25 led to a significant reduction in full fusion, likely due to the formation of incomplete SNARE complexes, which cannot mediate efficient fusion (*18*). The full fusion population is calculated by summing all normalized populations with E values >0.5 (*13*, *18*). Error bars denote the SD of three to five independent experiments with different batches of SNARE and Munc18 proteins.

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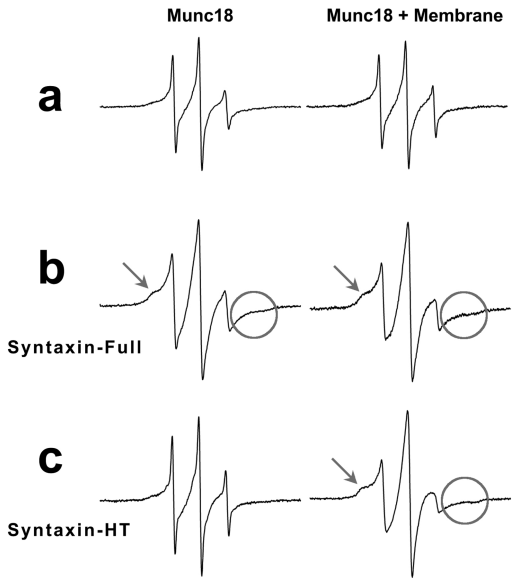

Figure 4. Electron paramagnetic resonance (EPR) spectra of Munc18-1. (a) Munc18-1 only (left panel) and Munc18-1with protein-free membrane in the fusion buffer (right panel). The same EPR sharp spectra indicate that there is no measurable interaction between Munc18-1 and membrane. (b) Munc18-1 with syntaxin-full protein (left panel) and Munc18-1with membrane-associated SNARE complex containing syxtaxin-full (right panel). Both EPR spectra are broadened in comparison to the spectrum of Munc18-1 only (a, left panel) indicating interactions. (c) Munc18-1 with syntaxin-HT protein (left panel) and Munc18-1 with membrane-associated SNARE complex containing syntaxin-HT (right panel). The sharp EPR spectrum on the left indicates that there is no interaction between Munc18-1 and syntaxin-HT, while the broadened EPR spectrum on the right suggests interactions. Arrows indicate the quaternary interaction between Munc18 and SNAREs, and circles show the spin−spin interaction due to the clustering of spin-labeled Munc18.

(Figure 3a,b). Because SNAP-25 is required for the formation of the complete SNARE complex, it is likely that the interaction between Munc18-1 and the SNARE core complex promotes fusion. Furthermore, this fusion promotion activity of Munc18-1 does not seem to require additional interactions with the N-peptide of syntaxin 1A.

Spin labeling electron paramagnetic resonance (EPR) has proven effective in detecting the intermolecular interaction such the one between syntaxin 1A and Munc18-1. The seven native cysteines in wild-type Munc18 hamper the site-specific attachment of the nitroxide spin labels. However, we could at least nonselectively spin-label native cysteines to primitively probe the putative intermolecular interaction. For this purpose, wild-type Munc18-1 was labeled with the methanethiosulfonate spin label. The EPR spectra for spin labeled Munc18-1 in solution, with syntaxin 1A (full-length and HT), and with the SNARE complex in membrane were measured at room temperature (Figure 4). The broadened line shape of the EPR spectrum indicates that the Munc18-1 binds to full-length syntaxin directly (Figure 4b, left panel). The EPR spectrum did not show any change when incubated with syntaxin-HT only (Figure 4c, left panel). These data are consistent with the previous conclusion that the Habc domain of syntaxin plays an important role in the interaction between monomeric syntaxin and Munc18 in solution.

We found a different result when the EPR spectrum from Munc18 was measured with the SNARE complex in the context of membrane. After full-length syntaxin or syntaxin-HT was reconstituted into membrane and formed a ternary complex with SNAP-25 and soluble VAMP 2 (amino acids 1−89), spin-labeled Munc18-1 was added to the complex. The EPR spectra of Munc18-1 were broadened for SNARE complexes containing both full-length syntaxin and syntaxin-HT, indicating that the N-peptide of syntaxin 1A is not necessary for Munc18-1 interaction with the membrane-associated SNARE complexes (Figure 4b,c, right panels). Interestingly, Rothman's group (*11*) also found that Munc18-1 still binds to the membrane-associated SNARE complex even with the Habc domain removed (syntaxin-HT) or the N-peptide mutated (L8A). Overall, our work shows that Munc18-1 could bind to the SNARE core complex reconstituted into the lipid membrane even in the absence of the N-peptide interaction, and this SNARE core/Munc18 binding mode is likely to be responsible for fusion acceleration by Munc18-1. Meanwhile, the EPR analysis showed that there is no direct interaction between the membrane and Munc18-1 (Figure 4a, right panel), which rules out the possibility that the spectral broadening observed in the presence of SNARE complex (Figure 4c, right panel) was caused by lipid molecules. We note here that we not only have the EPR line broadening (Figure 4, indicated by arrows) due to the quaternary interaction between Munc18 and SNAREs, but we also see some extra line broadening (Figure 4, indicated by circles) due to the spin−spin interaction. The spin−spin interaction is most likely due to the clustering of spin-labeled Munc18, perhaps reflecting the binding of several Munc18 molecules to the oligomeric supramolecular SNARE complex (*22*).

It has been debated whether the main function of Munc18 is for vesicle docking on the plasma membrane or whether it also assists membrane fusion (*5*, *23*). The role in vesicle docking is supported by impaired dense-core granule docking in adrenal chromaffin cells of Munc18-1 knockout mice (*24*). Other observations, however, suggest that SM proteins function also at a late, postdocking stage of membrane fusion (*11*, *25*). Our data on single-vesicle lipid mixing and EPR spectroscopy highlight the importance of Munc18 binding to the SNARE core complex in stimulating membrane fusion. For both roles, the syntaxin N-terminal domain containing N-peptide and Habc domain is regarded as an essential component for

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DOI: 10.1021/cn900034p | *ACS Chem. Neurosci.* (2010), *1*, 168–174

Munc18's dual interactions with syntaxin for vesicle docking as well as with the SNARE complex for fusion stimulation (*3*−*6*, *8*, *11*, *12*).

Our EPR data support the requirement of syntaxin N-terminal domain in the interaction between monomeric syntaxin and Munc18. However, the interaction between SNARE complex and Munc18 to promote fusion, in the context of membranes, is not dependent upon the N-terminal domain of syntaxin. Recently, Fasshauer's group (*26*) found that the interaction between Munc18 and the syntaxin N-terminal domain blocks the SNARE complex formation. However, a truncated syntaxin 1A (amino acids 25−262) could bind to Munc18-1 with high affinity and a SDS-resistant SNARE complex together with SNAP-25 and VAMP could be formed. Based on our data and other groups' findings (*9*, *26*, *27*), we propose that for the stimulation of membrane fusion, Munc18-1 interacts with the SNARE complex in a mode where the Habc-domain is not required.

In conclusion, Munc18-1 promotes neuronal SNARE-mediated fusion not only with the full-length syntaxin 1A but also with Habc-truncated syntaxin 1A. The SNARE complex/Munc18 interaction is mainly responsible for this effect. Furthermore, Munc18-1 accelerates vesicle fusion significantly more rapidly than previously observed. With the advent of new single-molecule imaging technologies, these protein−protein interactions critical for fusion may also become observable.

Methods

Munc18-1 Protein Expression and Purification

C-terminal His6-tagged Munc18-1 plasmid is a kind gift from Dr. James McNew at Rice University. The Munc18-1 protein was expressed in *Escherichia coli* Rosetta (DE3) pLysS (Novagene). The cells were grown at 37 °C in LB medium with 30 μg/mL of kanamycin until the A_{600} reached 0.6−0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. The cells were grown further for three more hours at 37 °C and then collected by centrifugation at 6000 rpm for 10 min. The cell pellets were resuspended in lysis buffer (25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)/KOH, 100 mM KCl, 20 mM immidazole, 2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), and 2 mM dithiothreitol (DTT), pH 7.4). After sonication on ice, the cell lysate was centrifuged at 15 000*g* for 20 min at 4 °C. The supernatant was mixed with nickel−nitrilotriacetic acid−agarose beads (Qiagen) and nutated for binding at 4 °C for 1 h. After binding, the beads were washed with washing buffer (25 mM HEPES/KOH, 100 mM KCl, 20 mM immidazole, pH 7.4). Then the protein was eluted with elution buffer (25 mM HEPES/KOH, 100 mM KCl, 150 mM immidazole, pH 7.4). Purified proteins were dialyzed overnight against dialysis buffer (25 mM HEPES/KOH, 200 mM KCl, 10% glycerol, and 1 mM DTT, pH 7.4). Recombinant SNARE proteins from rat brain, syntaxin, SNAP-25, and VAMP 2, were expressed and purified according to a detailed protocol in ref 18.

Munc18-1 Protein Spin Labeling and Electron Paramagnetic Resonance (EPR) Data Collection

The seven cysteines in the wild-type Munc18-1 were spin-labeled for EPR spectrum detection. After the cell lysate was incubated with beads and washed with HEPES buffer (25 mM HEPES/KOH, 100 mM KCl, pH 7.4) three times, DTT was added to a final concentration of 2 mM, and nutation was continued at 4 °C for another 30 min. The beads were then washed eight times with an excess volume of washing buffer to remove DTT. An approximately 20-fold excess of (1-oxy-2,2,5,5-tetramethylpyrrolinyl-3-methyl) methanethiosulfonate spin label (MTSSL) was added into the column, and nutation at room temperature was continued for 40 min. The spin-labeled protein was eluted by elution buffer and dialyzed overnight against dialysis buffer (25 mM HEPES/KOH, 200 mM KCl, 10% glycerol, pH 7.4).

EPR spectra were obtained using a Bruker ESP 300 spectrometer (Bruker) equipped with a low-noise microwave amplifier (Miteq) and a loop-gap resonator (Medical Advances). The modulation amplitude was set to be no greater than one-fourth of the line width. Spectral data were collected at room temperature in first-derivative mode with 1 mM microwave power.

Protein Reconstitution

Unilamellar vesicles containing 45:15:40 (mol/mol/mol) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS)/cholesterol (Avanti Polar Lipids) and 2 mol % DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Molecular Probes/Invitrogen, Carlsbad, California) were formed by the extrusion method (Mini-Extruder, Avanti Polar Lipids). The t-SNARE protein complexes, 1:1 (mol/mol) syntaxin/SNAP-25, were then reconstituted in the unilamellar vesicles through dialysis. The v-SNARE protein (VAMP 2) was reconstituted in a different population of unilamellar vesicles that were doped with 2 mol % DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate, Molecular Probes) and 0.1 mol % biotinylated lipids, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(biotinyl) (Avanti Polar Lipids). In both t- and v-SNARE vesicles, the lipid to protein ratio was kept at 200:1.

Single-Vesicle Lipid-Mixing Assay

Details of the single-vesicle lipid-mixing FRET assay were previously reported (*13*). A quartz slide is first processed with poly(ethylene glycol) (PEG) molecules in the ratio of 99:1 (mol/mol) PEG/biotin-PEG (Laysan Bio) to eliminate nonspecific binding of vesicles. The slide is then assembled into a flow chamber and coated with neutravidin in the concentration of 0.2 mg/mL. Through the specific interaction between biotin and neutravidin, the v-SNARE vesicles are immobilized on the PEG-coated surface by an incubation at vesicle concentration of 160 pM for 15 min. After washing the free v-SNARE vesicles in the chamber, the t-SNARE vesicles are diluted to a final vesicle concentration of 200 pM with preset amounts of Munc18-1 and injected into the flow chamber for the reaction at 37 ± 2 °C in the buffer (25 mM HEPES and 100 mM KCl, pH 7.4) for 12 min. After washing, the free

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DOI: 10.1021/cn900034p | *ACS Chem. Neurosci.* (2010), *1*, 168–174

t-SNARE vesicles and Munc18-1 proteins, the FRET measurements by a dual-color total internal reflection (TIR) fluorescence microscope (*19*) are performed at 20 min from injection of t-SNARE vesicles with the surrounding temperature of 37 ± 2 °C. Fusion events were monitored in a wide-field TIR fluorescence microscope (IX70, Olympus) using an electron multiplying charge-coupled device camera (iXon DV 887-BI, Andor Technology). Single fusion events were visually identified by an IDL program (Research Systems). A program written in Matlab (Mathworks) generated the time trajectories of the donor and the acceptor fluorescence intensities and calculated the corresponding FRET efficiency by using the equation, $I_A/(I_D + I_A)$, where I_D and I_A are the donor and the acceptor fluorescence intensities, respectively. The average donor and acceptor fluorescence intensities measured before docking were considered as the background fluorescence for each fusion event and subtracted uniformly from the fluorescence signals. The leakage of donor fluorescence into the acceptor channel (~17.5% of the total intensity) was then taken into account.

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Author Contributions

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Funding Sources

This work was supported by National Institutes of Health Grants (R01 GM067629 to Y.-K.S. and R21 GM074526 to T.H.). T.H. is an investigator with the Howard Hughes Medical Institute.

Acknowledgment

We thank Chirlmin Joo for help with preparing illustrations and Salman Syed for timely provision of logistics that made this work possible.

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