Studies of intermediates and regulation in SNARE-mediated membrane fusion

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Studies of intermediates and regulation in SNARE-mediated membrane fusion

by

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ABSTRACT

At the synapse, neurotransmitters are released via Ca\(^{2+}\)-triggered exocytotic fusion of synaptic vesicles with the presynaptic plasma membrane. The whole process is controlled by various proteins. SNAREs have been recognized as the key components that drive membrane fusion. In addition, many other proteins, such as Munc18/nSec1, Mun13, synaptotagmin, complexin, etc. are characterized to regulate synaptic transmission temporally and spatially.

The in vitro bulk fluorescence assay was applied to examine the kinetics of membrane fusion of liposomes mediated by recombinant neuronal SNAREs and led to the demonstration of hemifusion as an intermediate in the pathway. In order to monitor the fusion process in more sophisticated level, we developed the Single-liposome FRET assay and combined site-directed spin labeling (SDSL) and electron paramagnetic resonance (EPR) techniques to study the function of two SNARE regulators, synaptotagmin and complexin.

The EPR and fluorescence assay were also applied for the study of the SNAREs mediating the trafficking in yeast. It was found that supermolecular SNARE assembly precedes hemifusion, which was subsequently followed by distal leaflet mixing and formation of the cis-SNARE complex.
INTRODUCTION

SNAREs and membrane fusion

In eukaryotic cells, the transport vesicles play a central role in the trafficking of molecules between different membrane-bounded compartments in the secretary pathway\(^1\,^2\). Vesicles deliver proteins and lipids to their proper destinations by fusing with the plasma membrane in a process called exocytosis. The core protein families involved in the fusion are conserved from yeast to human, including soluble \(N\)-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), ATPase \(N\)-ethylmaleimide-sensitive factor (NSF) (the yeast counterpart is known as Sec18), soluble NSF attachment proteins (SNAPs) (the yeast counterpart is known as Sec17), and Sec1/Munc18. It was proposed that SNAREs are the central machinery in all membrane fusion events\(^3\,^4\).

SNAREs are a superfamily of membrane-associated proteins characterized by an \(\alpha\)-helical SNARE motif\(^8\,^9\), which contains about 8 heptad repeats. These proteins were initially categorized as v-SNAREs and t-SNAREs according to their preferential localization on the vesicle or the target membrane\(^10\), and later reclassified as R-SNAREs and Q-SNAREs according to the conserved arginine (R) or glutamine (Q) residue located in the center of the SNARE motif\(^11\). The best characterized SNARE proteins are the ones involved in the trafficking from Golgi to the plasma membrane in yeast and the ones involved in the synaptic exocytosis. In yeast system, Sso1p and Sec9 are t-SNAREs and Snc2p is v-SNARE. The synaptic SNARE complex consists of t-SNAREs syntaxin1A and SNAP-25, and v-SNARE synaptobrevin 2 (also called VAMP2). In this work, we focus on these two systems.

Structure of individual SNAREs and SNARE complex

Sequence analysis reveals that all SNAREs share a homologous ~60-70 amino acids
sequence. In addition to this SNARE motif, most of the SNAREs contain a variable N-terminal sequence and a C-terminal transmembrane domain (Fig. 1). Syntaxin contains a three-helix bundle at the N-terminal called Habc domain, which may regulate the SNARE complex assembly. SNAP-25 and Sec9 are soluble proteins and contain two SNARE motifs, and SNAP-25 is attached to the membrane surface by palmitoylation. The NMR experiments showed that the cytoplasmic domains of syntaxin and Sso1p are partially folded, whereas those of synaptobrevin and Snc2p are unstructured, characterized by circular dichroism (CD) and NMR measurement.

The NMR experiments showed that the cytoplasmic domains of syntaxin and Sso1p are partially folded, whereas those of synaptobrevin and Snc2p are unstructured, characterized by circular dichroism (CD) and NMR measurement.

The structure of the core synaptic fusion complex was determined by x-ray at 2.4Å resolution and confirmed independently by the site-directed spin-labeling EPR in 1998. As mentioned above, individual SNAREs are largely unfolded. Upon association of the three SNARE proteins in the ratio of 1:1:1 by their coil domains, the dramatic structural changes occur in order to form the ternary SNARE complex. In this complex, three SNARE motifs from t-SNAREs and one from v-SNARE assemble into a four-helix bundle composed of 16 primarily hydrophobic layers (Fig. 2). A conserved ionic layer is present at the center of the core complex, which consists of an arginine and three glutamine residues contributed from each of the four α-helices.

The SNARE assembly and the intermediates of membrane fusion induced by SNAREs

The fusion of two membranes into one continuous entity is an event leading to the unification of the lipid and protein components of the two membranes and the intermixing of the contents initially bound by them. It was proposed that at least three stages exist in the process of SNARE assembly. Firstly, the Habc domain of syntaxin/Sso1p folds back and binds to its SNARE motif to form the close conformation. Secondly, the t-SNARE complex assembled between SNAP25 and syntaxin (in neuron) or between Sec9 and Sso1p (in yeast) on target membrane. And finally, the v-SNARE on the synaptic vesicle interacts with the t-SNARE complex to form a trans-SNARE complex prior to fusion (Fig. 3). After fusion, all three proteins are anchored to the same bilayer to form a
cis-SNARE complex (Fig. 4). The SNARE core complex exhibits high stability and it needs to be heated up to 80-90°C for melting\textsuperscript{20,21}. The widely accepted scenario is that the SNARE assembly might begin at the membrane-distal N termini and zipper towards the membrane-proximal C-terminal transmembrane domains\textsuperscript{22-25}. However, this hypothesis has not been convincingly supported by the experiments yet.

Two scenarios are invoked in the studies of the process of membrane fusion\textsuperscript{26} (Fig. 5). The first one assumes that fusion proteins bring the membranes together then the proteins arrange into supermolecular complexes to establish the ‘protein pore’, which allows content mixing. The fusion pore dilation is accompanied by lipid mixing. This model is also called the ‘fusion pore’ model\textsuperscript{27}. An alternative model suggests that the bilayer mixing proceeds via sequential steps. The first step is that the outer layer leaflets merge together while leaving the inner layer leaflets intact. This intermediate stage is called hemifusion\textsuperscript{26,28-30}. The next step is the merging of the inner layer leaflets accompanying by the formation of a fusion pore. This model is referred to as fusion-through-hemifusion.

Several studies suggest the universality of the fusion-through-hemifusion pathway, and a hemifusion intermediate is an on-pathway intermediate of SNARE-driven membrane fusion. J. White and colleagues provided the first evidence for this model in viral systems\textsuperscript{30}. In their work transmembrane domain of influenza hemagglutinin was replaced by a GPI anchor to catch the hemifusion. Further work has documented that hemifusion could be arrested by shortening the transmembrane domain of the yeast v-SNARE protein to about half of its original length or using low protein-to-lipid ratios in the in vitro fusion assay\textsuperscript{32}.

**SNAREs regulators**

Many researchers have accepted that the SNAREs alone could be fusogenic, whereas multiple papers also proposed that additional proteins are essential for the fast neurotransmitter release. Several proteins have been identified as SNAREs regulators and play critical roles in the functionality of SNAREs or SNARE complexes both in vivo and in vitro. These regulators include Sec1/Muc18, synaptotagmins, Munc13, complexin,
synaptophysin, amisyn, etc.

An essential SNAREs regulator is the Sec1/Munc18 (SM) protein family. SM proteins interact with SNAREs and this interaction seems to be different across pathways and organisms. However, the precise function of SM proteins in regulation SNARE-dependent membrane fusion needs to be investigated by further studies.

Synaptotagmin family members are found exclusively in higher eukaryotes, which have been proposed to mediate stimulus-coupled exocytosis as the calcium sensors. They are characterized by an N-terminal transmembrane region, a variable linker, and two C-terminal C2-domains (C2A and C2B), which have specialized calcium binding sites (Fig. 6). Synaptotagmin I is the best characterized member of the synaptotagmin protein family, which binds t-SNARE, SNARE complex, and interacts with negatively charged phospholipids via its C2 domains. Recent research shows that it is highly likely that synaptotagmin I interacts with SNARE complex and the membrane simultaneously in a Ca\(^{2+}\)-dependent manner, thereby triggering the burst of the fusion. However, its precise roles are still in debate. Genetic and biochemical studies have indicated several possible mechanisms for synaptotagmin in vesicle trafficking and fusion. One interesting suggestion from the in vitro assays is that synaptotagmin may function at early steps to mediate vesicle docking to the plasma membrane due to its interaction with SNAP-25. Littleton et al. proposed another mechanism, in which the oligomerization of synaptotagmin and its association with partially assembled SNARE complexes could drive final assembly of the complex, thereby accelerating SNARE-mediated membrane fusion. The third possibility is to modulate the fusion pores based on the amperometry studies.

Complexins constitute a small family of soluble, highly charged neuronal protein with a molecular mass of 15-16 kDa, and they are colocalized with the neuronal SNAREs. There are four isoforms of complexin from I to IV. Complexin III and IV are yet to be characterized and most of the research has focused on complexin I and II. The x-ray structure of complexin/SNARE complex showed that complexin binds in an antiparallel \(\alpha\)-helical conformation to the groove between the synaptobrevin and syntaxin helices, and
that the binding is calcium independent\(^4\) (Fig. 7). The exact function of complexin in exocytosis is controversial. In 1998, one group found that the injection of complexin II into the nerve terminal suppressed neurotransmitter release, and the injection of anticomplexin antibody stimulated transmitter release\(^5\). And then another group found that the overexpression of complexin in PC12 reduced exocytotic release\(^6\). These results suggest complexin acts as a negative regulator for the membrane fusion. However, Reim \textit{et al} found that the double knockout of the complexin I and II genes in mice is lethal, which shows reduced neurotransmitter release\(^7\). Also in the squid synapse, Tokumaru \textit{et al} reported that the peptides which prevent complexin from binding to the SNARE complex could inhibit the evoked transmitter release\(^8\). Therefore these findings indicates that complexin could act as a positive regulator\(^9\).

\textbf{in vitro lipid mixing assay and single-liposome FRET assay}

Rothman’s group first utilized the fluorescence dequenching strategy to study the lipid mixing \textit{in vitro}\(^7\), which applies resonance energy transfer between the fluorescence donor, NBD, and the fluorescence acceptor, rhodamine. The v-SNARE is reconstituted to the vesicles containing the quenching pair of fluorescent phospholipids, NBD-PS and rhodamine-PE; whereas the t-SNAREs are reconstituted to the unlabeled liposomes. When these two types of liposomes fused together, the dilution of quencher due to the surface area expanding results in a decrease in quenching efficiency and consequently an increase in NBD fluorescence intensity. In order to selectively measure inner leaflet mixing, the method developed by Meers \textit{et al} is applied\(^54,55\). Under controlled conditions, sodium dithionite turns NBD fluorescence in the outer leaflet into a nonfluorescent derivative ABD, while leaving NBD in the inner leaflet largely unaffected because dithionite could not easily penetrate lipid bilayers due to its polarity (Fig. 8). Therefore when the dithionite-treated v-SNARE liposomes are mixed with the t-SNARE liposomes, only inner leaflet mixing is monitored.

The \textit{in vitro} lipid mixing assay employing the SNARE-reconstituted liposomes has been proven very useful; however, as an ensemble technique this method faces limitations
in detecting the behavior of individual liposomes since the observation of the bulk assay is the sum of all the unsynchronized events.

Progress has been made in developing the single fusion assays based on total internal reflection FRET (TIRF) microscopy\textsuperscript{56,57}. The basic idea has been realized to observe the fusion of the liposomes with the supported planer bilayer on the surface. The fast lipid mixing of single liposome was observed, however, SNAP-25 was found not required in this system, which is contrary to the necessity of this protein for the neurotransmitter release\textsuperscript{58}. Recently, a new single-liposome FRET assay has been carried out on monitoring liposome-to-liposome fusion on the surface, which successfully dissected and characterized the different intermediates in yeast SNAREs mediated membrane fusion\textsuperscript{59}.

**Structural study by EPR method**

Site-directed spin labeling (SDSL) Electron Paramagnetic Resonance (EPR)\textsuperscript{60,61} is proven a powerful method for exploring membrane proteins in the near native environments. In this method, native residues are substituted with cysteines that could covalently link with a chemical reactive nitrooxide spin label (Fig. 9a). Nitrooxide-scanning EPR spectrum analyses could provide the information about the secondary structure and topology of the membrane-bound protein\textsuperscript{60,62,63}.

Generally, the lineshape of the EPR spectrum is sensitive to the rate of rotation (or tumbling) of the spin label. Therefore, the EPR signal of a spin label covalently attached to a biomolecule can provide the information about its local structural environment. Broadening of the spectrum is indicative of immobilization of the spin label, whereas sharpening points to an increase in the label mobility. For example, for the nitrooxide spin label freely tumbling in solution or is attached to a molecule that is sufficiently small or flexible, the EPR spectrum is isotropic (Fig. 9b). In contrast, the restriction of the mobility of the nitrooxide spin label will exhibit a broad EPR spectrum (typical of a completely immobilized sample shown in Fig. 9d). Partially broadened spectra may also be observed, indicating mobility on an intermediate time scale depending on the degree of spin label
immobilization (Fig. 9c). Therefore, EPR could be utilized to detect the conformation change and complex assembly due to their effect on the tumbling rate of the spin label.

**THESIS ORGANIZATION**

Chapter one provides the general background information about the membrane fusion, SNAREs and SNARE regulators. Chapter two describes hemifusion intermediate in the membrane fusion induced by the neuronal SNAREs and has been published in Journal of Biological Chemistry. Chapter three discusses the function of synaptotagmin I and the effect of calcium on SNARE-mediated membrane fusion and has been published in FEBS Letters. Chapter four discussed the complexin and synaptotagmin function on the membrane fusion induced by the neuronal SNAREs, which has been published in Nature Structure and Molecular Biology. Chapter five further the discussion of the function of complexin by using single-liposome FRET assay and those results have been published in Nature Structure and Molecular Biology. Chapter six describes the intermediates in the yeast SNAREs mediated fusion pathway, which has been published in Nature Structure and Molecular Biology. Finally, Chapter seven summarizes the results and suggests the further directions.

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Figure 1 Primary domain structure of yeast and neuronal SNAREs.¹

TM, transmembrane domain. There are two SNARE core domains sn1 and sn2 in SNAP-25, and the green curves indicate the palmitoylation sites (which are absent in Sec9). In our constructs, all the N-terminal domains of Syntaxin1A, Sso1p and Sec9 were deleted.

Figure 2 Crystal structure of neuronal SNARE complex.²

(a) The four-helix bundle. Sn1 and Sn2 are two ‘SNARE motifs’ of SNAP25. Sx is Syntaxin and Sb is synaptobrevin. (b) The 16 hydrophobic layers of the SNAREs complex.

Figure 3 Schematic assembly of SNAREs.³

(a) Syntaxin1A/Sso1p in the ‘close’ conformation. The N-terminal Habc domain folds back and binds to H3 domain. (b) SNAP25/Sec9 engages in the formation of the binary complex. Habc domain was released from binding to H3 domain. (c) v-SNARE from apposed membrane comes in and assembly of four helical ternary complexes begins from N-terminal. (d) The assembly extends to C-terminal, bringing the membrane proximal and initiating the fusion.

Figure 4 Cis- and trans-SNARE complex.\(^4\)

(a) Partially assembled trans-SNARE complex. (b) Fully assembled cis-SNARE complex.

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Figure 5 Two models for membrane fusion.⁵

(a1-a5) The ‘fusion-through-hemifusion’ model. The outer layer leaflets merge together first and leave the inner layer leaflets intact, establishing the hemifusion state. Then the fusion pore lined by lipids is formed and dilates, leading to complete fusion. (b1-b5) The ‘fusion pore’ model. The fusion proteins bring the membranes together and then a fusion pore arranged by the fusion proteins is built up. b3 shows the protein-lined pore surrounded by the hemifused proximal leaflet.

Figure 6 Structure of synaptotagmin I.\textsuperscript{6}

The top panel demonstrates the primary domains structure of synaptotagmin I, and the bottom panel shows the NMR structure of the domains C2A and C2B. synaptotagmin I is anchored to the vesicle membrane via a single transmembrane domain (TM) near its N terminus. Its cytoplasmic domain is composed of two conserved motifs C2A (shown in yellow) and C2B (shown in dark blue). Blue spheres represent multiple calcium ions bound to loops 1 and 3.

Figure 7 Structure of the complexin/SNARE Complex. 

(a) Ribbon diagram of the complexin/SNARE complex with the colors representing: yellow, syntaxin; red, synaptobrevin; blue, SNAP-25 N-terminal SNARE motif; green, SNAP-25 C-terminal SNARE motif; pink, complexin. (b) Superposition of the structures of the isolated SNARE complex (Sutton et al., 1998) and the complexin/SNARE complex. (c,d) Space filling models of the complexin/ SNARE complex in two different views rotated approximately 90 degrees around the horizontal axis.

Figure 8 Schematic *in vitro* fusion assay based on fluorescence dequenching.

(a) The dilution of the surface concentration of the quencher after fusion leads to the fluorescence intensity increase. (b) Dithionite selectively reduces NBD into fluorescent silent ABD. (c) For hemifusion, the fluorescence intensity doesn’t change since there is no lipid mixing for the inner leaflets. For full fusion, the fluorescence intensity increases as without the reduction. The blue spot represents NBD and the red one rhodamin.
(a) The site-directed spin labeling (SDSL) strategy. EPR spectra of a nitroxide spin label in aqueous solution at room temperature (b), in glycerol at room temperature (c), and frozen in aqueous solution (d).\(^8\)

\(^8\) Biswas, R., Kuhne, H., Brudvig, G.W. & Gopalan, V. (2001) Use of EPR spectroscopy to study macromolecular structure and function. Sci Prog. 84, 45-67
ABSTRACT

Synaptic transmission requires the controlled release of neurotransmitter from synaptic vesicles by membrane fusion with the presynaptic plasma membrane. SNAREs are the core constituents of the protein machinery responsible for synaptic membrane fusion. The mechanism by which SNAREs drive membrane fusion is thought to involve a hemifusion intermediate, a condition in which the outer leaflets of two bilayers are combined and the inner leaflets remain intact; however, hemifusion has been observed only as an end point rather than as an intermediate. Here, we examined the kinetics of membrane fusion of liposomes mediated by recombinant neuronal SNAREs using fluorescence assays that monitor both total lipid mixing and inner leaflet mixing. Our results demonstrate that hemifusion is dominant at the early stage of the fusion reaction. Over time, hemifusion transitioned to complete fusion, showing that hemifusion is a true intermediate. We also show that hemifusion intermediates can be trapped, likely as unproductive outcomes, by modulating the surface concentration of the SNARE proteins.
INTRODUCTION

In the neuron, SNARE assembly plays a critical role in promoting the fusion of the synaptic vesicles with the plasma membrane\textsuperscript{1-7}. Cognate SNAREs pair to form a coiled coil structure that bridges two membranes\textsuperscript{8,9}. The subsequent steps yielding one common phospholipids bilayer remain a matter of debate. It has been proposed that SNAREs involved in neurotransmitter release at synapses may promote membrane fusion by the formation of two juxtaposed transmembrane pores preassembled by the transmembrane domains of SNAREs in respective membranes\textsuperscript{10}. In sharp contrast, recent evidence for SNAREs involved in trafficking in yeast has indicated that hemifusion might be involved in the SNARE fusion pathway\textsuperscript{11,12}, analogous to the lipid-protein stalk model generally accepted for viral membrane fusion proteins\textsuperscript{13-17}. However, hemifusion has been observed only as an outcome rather than as an intermediate, raising some concerns as to whether hemifusion is an off-pathway product in SNARE-mediated membrane fusion\textsuperscript{13}. Alternatively, the mechanism by which neuronal SNAREs induce membrane fusion might be entirely different from those for other systems including yeast SNAREs.

In this work, we examined the kinetics of membrane fusion of liposomes mediated by neuronal SNAREs syntaxin 1A, SNAP-25, and synaptobrevin using fluorescence assays\textsuperscript{18} that monitored both total lipid mixing and inner leaflet mixing. Our results demonstrate that hemifusion is the main event at the early stage of the fusion reaction. Over time, hemifusion converts to the complete fusion, supporting strongly the theory that hemifusion is a true fusion intermediate.

RESULTS AND DISCUSSION

We investigated the fusion of liposomes induced by neuronal SNAREs with a well characterized fluorescence lipid mixing assay. Recombinant t-SNARE complexes containing the H3 "core" domain of syntaxin 1A as the t-SNARE heavy chain and SNAP-25 as the t-SNARE light chain were reconstituted into the liposomes containing POPC (65 mol)/DOPS (35 mol) (Fig. 1A). Additionally, the v-SNARE synaptobrevin was also reconstituted into a separate population of the same POPC/DOPS liposomes.
containing fluorescent lipids, NBD-PS, and rhodamine-PE (1.5 mol % each). For both t- and v-SNAREs, the initial lipid/protein ratio was set at 50:1 (Fig. 1B). When the t-SNARE liposomes were mixed with the v-SNARE liposomes at 35 °C, an increase of the fluorescence intensity was observed, indicating that the fusion occurred (Fig. 2A, red trace). Although both v- and t-SNAREs have roughly equal surface density, the absolute amount of t-SNARE liposomes in the cuvette was about 10 times greater than the v-SNARE liposomes. These amounts of protein and lipid were used to better ensure first order kinetics. Following an initial rapid rise, the fluorescence signal approached a plateau of roughly 40% of maximum (Fig. 2A). The half-time of the fusion reaction was ~9 ± 1 min (mean ± S.D.) (~540 ± 60 s), consistent with the previous reported half-time of 10 min with a similarly truncated syntaxin 1A19.

Because the fluorescent lipids were distributed equally in the inner leaflet and the outer leaflet, the observed total lipid mixing should be the sum of outer leaflet mixing and inner leaflet mixing. To selectively measure inner leaflet mixing separately, we treated the v-SNARE liposomes with sodium dithionite. Under controlled conditions, sodium dithionite reduces NBD attached to the lipid head group in the outer leaflet to a nonfluorescent derivative while leaving NBD in the inner leaflet largely unaffected. When we mixed the dithionite-treated v-SNARE liposomes with the t-SNARE liposomes, inner leaflet mixing was observed (Fig. 2A, blue trace). The extent of the NBD reduction did not affect the kinetics of inner leaflet mixing (supplemental information). Interestingly, the half-time of inner leaflet mixing was ~20 ± 2 min (~1,200 ± 120 s), which was about twice the half-time of total lipid mixing. The kinetic difference in the half-times of the two processes suggests that outer leaflet mixing and inner leaflet mixing were not simultaneous but sequential in time. These results suggest that outer leaflet mixing likely occurred faster than inner leaflet mixing.

Because we collected the time traces of total lipid mixing and inner leaflet mixing separately, it was straightforward to calculate the percentage of hemifusion (defined as $2(PT - PI)/[2(PT - PI) + PI] \times 100$, where $PT$ is the percentage of maximum for total lipid mixing and $PI$ is the percentage of maximum for inner leaflet mixing (Fig. 2A) as a
function of time. As expected, at the beginning of the fusion reaction, the fluorescence change was mainly due to outer leaflet mixing (Fig. 2B), indicating that hemifusion was the dominant event. As time progressed, however, the percentage of hemifusion decreased dramatically. Hemifusion was about 90% at 1 min and was extrapolated to be nearly 100% at the start of the reaction. The percentage steadily declined to roughly 20% at 40 min (Fig. 2B) and asymptotically approached 12% at 150 min. It should be noted that these estimates are from the ensemble of ~7 × 1011 liposomes in the reaction and that each individual event is likely to be very fast. These results provide strong kinetic evidence for the conversion of hemifusion to complete fusion over time and therefore for the sequential mechanism in which hemifusion is an on-pathway intermediate (Fig. 2C).

We analyzed outer and inner leaflet mixing separately on the basis of the sequential mechanism (Fig. 2C). For this purpose, net outer leaflet mixing was obtained by subtracting the kinetics of inner leaflet mixing from that of total lipid mixing (Fig. 2E). The time trace of outer leaflet mixing fitted well to a simple exponential function representing the first order kinetics with the first order rate constant $k_1 = 1.5 \times 10^{-3}$ s$^{-1}$ (Fig. 2E). The kinetics of inner leaflet mixing was analyzed with the first order kinetics theory for the sequential mechanism depicted in Fig. 2C. The theory fitted the data very well with first order rate constant $k_2 = 3.7 \times 10^{-3}$ s$^{-1}$ and the backward rate constant $k_{-1} = 6.3 \times 10^{-3}$ s$^{-1}$. It is quite interesting to find that $k_{-1}$ is almost twice as big as $k_2$. Thus, once hemifusion is formed it is twice as likely to go back to the two separate liposomes than to advance toward the complete fusion.

One might argue that these results are equally consistent with a parallel mechanism as well (Fig. 2D). In this alternative mechanism, hemifusion is an off-pathway product in equilibrium with the unfused liposomes. However, the data argue against the parallel mechanism. At the beginning of the reaction, the fusion events were almost exclusively hemifusion (Fig. 2B), which is a clear indicator for a sequential mechanism. Thus, the results favor the sequential mechanism and establish that SNARE-mediated membrane fusion transitions through hemifusion.

Work with viral fusion proteins as well as SNARE proteins has suggested that the
surface protein density of the fusion proteins may be an important parameter that determines the outcome of the fusion events\textsuperscript{12,21}. To gain further insight into the role of the protein surface density, we reduced the input L/P ratios to 100:1 and 200:1, from the original 50:1. Liposomes generated with a 100:1 L/P ratio yielded results that were qualitatively similar to those for the previous 50:1 L/P ratio (Fig. 3, A and B), although the overall fusion efficiency was lower and the conversion from hemifusion to complete fusion was slower. With this surface density, \textasciitilde45\% of full fusion occurred during the 75-min reaction. However, at the L/P ratio of 200:1 we did not observe the time-dependent shift from hemifusion to complete fusion (Fig. 3, C and D). This result suggests that the majority (>60\%) of hemifusion intermediate remained without transitioning to complete fusion. Thus, the surface density of SNARE proteins is indeed a determining factor for the outcome of SNARE-induced membrane fusion.

In summary, we have shown that membrane fusion induced by neuronal SNAREs transitions from hemifusion to complete fusion in a kinetically resolvable manner, establishing that hemifusion is a true intermediate along the SNARE-induced membrane fusion pathway. We also have shown that, under low surface protein density, hemifusion can be trapped as an outcome of SNARE-induced membrane fusion.

MATERIALS AND METHODS

Protein Sample Preparation

Plasmid construction, protein expression, and purification for neuronal SNAREs were described elsewhere\textsuperscript{22}. Briefly, vesicle-associated (v-) SNARE synaptobrevin (amino acids 1–116) and a truncated version of target membrane (t-) SNARE syntaxin (amino acids 168–288), for which the N-terminal \(\alpha\)-helical Habc domain was deleted, were expressed as N-terminal glutathione S-transferase fusion proteins. Another t-SNARE, SNAP-25, was also expressed as a glutathione S-transferase fusion protein in which the four cysteines were replaced with alanines. Recombinant proteins were expressed in \textit{Escherichia coli} Rosetta (DE3) pLysS (Novagene). Glutathione S-transferase fusion
proteins were purified by affinity chromatography using glutathione-agarose beads (Sigma). The protein was cleaved by thrombin in cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0). We added 1% OG for syntaxin and synaptobrevin). Purified proteins were examined with 15% SDS-PAGE, and the purity was at least 90% for all of the proteins (data not shown).

**Membrane Reconstitution**

The mixture of POPC (1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphatidylcholine) and DOPS (1,2-dioleoyl-sn-glycero-3-phosphatidylserine) (molar ratio of 65:35) in chloroform was dried in a vacuum and was resuspended in a buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0) to make the total lipid concentration about 50 mM. Protein-free large unilamellar liposomes (~100 nm in diameter) were prepared by extrusion through polycarbonate filters (Avanti Polar Lipids). Syntaxin (480 µl, 21 µM) and SNAP-25 (630 µl, 16 µM) were mixed at room temperature for about 60 min to allow the formation of t-SNAREs. The preformed t-SNAREs were concentrated down to 90 µl using a 5-kDa cutoff centrifugal filter (Millipore) and were mixed with 10 µl of liposomes for about 15 min at room temperature, resulting in a 50:1 lipid/protein molar ratio. The fluorescent liposomes containing POPC, DOPS, NBD-PS (1,2-dioleoyl-sn-glycero-3-phosphoserine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)), and rhodamine-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)) in the molar ratio of 62:35:1.5:1.5 were prepared following the procedure described above, and the final lipid concentration was ~10 mM. Synaptobrevin (80 µl, 50 µM) was mixed with 20 µl of fluorescent liposomes for about 15 min at room temperature. The liposome/protein mixture was diluted two times, which makes the concentration of OG below the critical micelle concentration. After dialyzing against 2 liters of dialysis buffer (25 mM HEPES, 100 mM KCl, 5% (w/v) glycerin, pH 7.4) at 4 °C overnight to remove detergent, the sample was treated with Bio-Beads (Bio-Rad) to get rid of any remaining trace amount of detergent. The solution was then centrifuged at 10,000 g to remove protein and lipid aggregates. The final t-SNAREs liposome solution contained
~2.5 mM lipids and 1.9 mg/ml protein, and the v-SNARE liposome solution contained ~1 mM lipids and 0.25 mg/ml protein. The integrity and size of SNARE-reconstituted liposomes were examined with negative staining electron microscopy. The sample was stained with 1% phosphotungstic acid, pH 6.7, after the liposome sample was spread on the 200-mesh Formvar with the carbon-coated grids. The micrograph was taken on a JEOL 1200 EX electron microscope.

The reconstitution efficiency was determined using SDS-PAGE and visualized by Coomassie Blue staining. The amount of protein in liposomes was estimated by comparing the band in the gel with that of the same protein of known concentration. The reconstitution efficiency was more than 90% for both t- and v-SNAREs (Fig. 1B). The orientation of the SNAREs in the liposomes was examined with the trypsin digestion experiments [22]. The SNAREs-reconstituted vesicles were treated with trypsin (0.5 mg/ml) under room temperature for 1 h. Nearly all SNARE proteins were digested by trypsin, indicating that SNARE molecules are oriented inside out, exposing the soluble domain to the solution phase (Fig. 1B).

Total Lipid Mixing Fluorescence Assay

To measure the lipid mixing, v-SNARE liposomes were mixed with t-SNARE liposomes in the ratio of 1:9. The final solution contained ~1 mM lipids with a total volume of 100 µl. Fluorescence was measured at excitation and emission wavelengths of 465 and 530 nm, respectively. Fluorescence changes were recorded with a Varian Cary Eclipse model fluorescence spectrophotometer using a quartz cell of 100 µl with a 2-mm path length. The maximum fluorescence intensity (MFI) was obtained by adding 0.2% n-dodecylmaltoside. All of the lipid mixing experiments were carried out at 35 °C. For each lipid/protein (L/P) ratio, the experiments were performed at least three times with newly prepared samples. To make sure that the percentage of MFI was independent of the probe concentrations, we measured the total lipid mixing at 1.5 and 0.7 mol % NBD-PS while keeping the rhodamine-PE concentration at 1.5 mol %. We found that the percentages of MFI were identical for both NBD-PS concentrations (supplemental
Inner Leaflet Mixing Assay

The inner leaflet mixing assay was modified from the method developed by Meers et al.\(^\text{23}\). The method is based on the fact that sodium dithionite reacts more rapidly with NBDs in the outer leaflet than those in the inner leaflet. By controlling the time and amount of dithionite, the reaction can be limited to the outer leaflet. Small aliquots (~0.7 µl) of 100 mM sodium dithionite in 50 mM Tris buffer, pH 10, were added to the v-SNARE liposomes (100 µl, 0.2 mM lipid) until a desired reduction of NBD was achieved. The reaction was monitored at room temperature by scanning the fluorescence signal for 15 min from 500 to 700 nm with the excitation at 460 nm. Typically, in 10 min the reduction was complete, and no more change of the spectrum was observed. The liposomes without NBDs in the outer leaflets were subjected to the lipid mixing assay described above. To make sure that the percentage of MFI was independent of the extent of the NBD reduction, the inner leaflet mixing assay was performed at the 55 and 65% reduced conditions. We found that the percentages of MFI were identical for both conditions (supplemental information).

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Figure 1 Characterization of reconstituted proteins and liposomes.

A, electron micrograph of SNARE-reconstituted liposomes. The diameter of the vesicles was ~80-100 nm. B, SDS-PAGE of v-SNARE synaptobrevin before (lane 1) and after (lane 2) the reconstitution into liposomes. The reconstitution efficiency was ~90%. Lane 3 represents the sample after treating with trypsin. The high molecular weight band was from trypsin itself. C, SDS-PAGE of SNAREs after reconstitution into liposomes at different P/L ratios.
Figure 2 Fluorescence assay for total lipid mixing and inner leaflet mixing.

A, fluorescence changes for total lipid mixing (red traces) and inner leaflet mixing (blue traces), normalized with respect to the MFI, are shown for the L/P ratio of 50:1. MFI was obtained by adding 0.2% n-dodecylmaltoside (sudden increase at the end). The black trace is a control run with the t-SNARE liposomes reconstituted with syntaxin 1A only (without SNAP-25). Inset, residual fluorescence changes for total lipid and inner leaflet mixing recorded at the longer period of time (8,500-9,100 s); the former was 43%, and the latter was 40%. B, the percentage of hemifusion versus time. The percentage of hemifusion was calculated using the equation $2(PT - PI) / [2(PT - PI) + PI] \times 100$, where
PT is the percentage of maximum for total lipid mixing and PI is the percentage of maximum for inner leaflet mixing. C, a schematic diagram for the sequential mechanism in which hemifusion is an on-pathway intermediate. $k_1$, first order constant for time trace of outer leaflet mixing; $k^{-1}$, backward rate constant; $k_2$, first order constant for time trace of inner leaflet mixing. $D$, a schematic diagram for the parallel mechanism in which hemifusion is an off-pathway product. $E$, analysis of outer and inner leaflet mixing based on the sequential mechanism in $C$ and the first order kinetics. The solid lines are the best fits to the first order kinetics. The data were fitted with the program DYNAFIT\textsuperscript{22}. 
**Figure 3 Fluorescence assays for total lipid mixing and inner leaflet mixing at different L/P ratios.**

A and B, ratio = 100:1; C and D, ratio = 200:1. Fluorescence changes for total lipid mixing (*red traces*) and inner leaflet mixing (*blue traces*) were normalized with respect to the MFI. The *black trace* is the control with the t-SNARE liposomes reconstituted with syntaxin 1A only (without SNAP-25).
**Supplementary figure**

*A*, Total lipid mixing assay with different amounts of NBD-PS in the v-SNARE liposomes. The red line is for 1.5 mole% NBD-PS and blue one for 0.7 mole% NBD-PS. In both cases, Rhodamin-PE remained at 1.5 mole%. The two time traces overlap very well one another, indicating that the fusion kinetics is not affected by the amount of NBD-PS which uniformly distributed over the outer and inner leaflet of the liposome. *B*, Lipid mixing assays with different extents of dithionite reduction. The blue trace was with 55% reduction of NBD-PS and the pink trace was for 65% reduction. The red trace which is total lipid mixing without dithionite treatment is shown as a reference. The results indicate that the kinetics of inner leaflet mixing is largely unaffected by the reduction extent. The inset shows the raw data.
CHAPTER 3: SYNAPTOTAGMIN I AND Ca\textsuperscript{2+} PROMOTE HALF FUSION MORE THAN FULL FUSION IN SNARE-MEDIATED BILAYER FUSION

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ABSTRACT

Synaptic membrane fusion, which is necessary for neurotransmitter release, may be mediated by SNAREs and regulated by synaptotagmin (Syt) and Ca\textsuperscript{2+}. Fusion of liposomes mediated by reconstituted SNAREs produces full fusion and hemifusion, a membrane structure in which outer leaflets are mixed but the inner leaflets remain intact. Here, using the liposome fusion assay, it is shown that Syt promoted both hemifusion and full fusion in a Ca\textsuperscript{2+}-dependent manner. Syt \cdot Ca\textsuperscript{2+} increased hemifusion more than full fusion, modulating the ratio of hemifusion to full fusion. Unlike the case of neuronal SNAREs, stimulation of fusion by Syt \cdot Ca\textsuperscript{2+} was not seen for other SNAREs involved in trafficking in yeast, indicating that the Syt \cdot Ca\textsuperscript{2+} stimulation was SNARE-specific. We constructed hybrid SNAREs in which transmembrane domains were swapped between neuronal and yeast SNAREs. With these hybrid SNAREs, we demonstrated that the interaction between the SNARE motifs of neuronal proteins and Syt \cdot Ca\textsuperscript{2+} was required for the stimulation of fusion.

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INTRODUCTION

Communication between neurons relies on neurotransmitter release at the synapse by way of membrane fusion\(^1\)\(^{-3}\). The influx of Ca\(^{2+}\) into the neuron triggers membrane fusion of the synaptic vesicles that store the neurotransmitters with the plasma membrane\(^4\). Synaptic membrane fusion requires vesicle-associated v-SNARE VAMP2 and its partners on the plasma membrane syntaxin and SNAP-25 (or t-SNAREs)\(^5\)\(^{-8}\).

The soluble domains of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) function as molecular Velcro: the coiled coil motifs from v- and t-SNAREs associate with each other to form a parallel four-stranded helix bundle\(^9\)\(^,\)\(^10\), which brings the two membranes into close proximity. The fusogenic capacity of the SNARE complex has been demonstrated using an in vitro fusion assay employing reconstituted SNAREs\(^11\). It is thought that SNARE complex formation provides the driving force for membrane fusion\(^12\), although a simple catalytic role of the complex can not be ruled out\(^13\)\(^,\)\(^14\).

SNARE assembly is followed by the mixing of the outer leaflets while the integrity of the inner leaflets is maintained\(^15\)\(^{-18}\). This half fusion state called hemifusion is likely to be a common intermediate shared by type I and type II viruses\(^19\)\(^{-24}\). Transition from hemifusion to a fusion pore establishes the connection between two aqueous contents. However, under certain limiting conditions, such as low surface protein density, hemifusion intermediates accumulate as a terminal fusion product\(^25\). The biological implications of such terminal hemifusion are yet to be understood. It is noteworthy though that the “kiss-and-run” event in which the exocytosis is carried out without the full fusion between the vesicle and the plasma membrane is observed in the neuron\(^26\)\(^{-28}\).

Neuronal exocytosis is tightly regulated by Ca\(^{2+}\)\(^4\). A vesicular membrane protein synaptotagmin (Syt), which has two Ca\(^{2+}\) binding C2A and C2B domains (C2AB)\(^29\)\(^{-31}\), is believed to be a major Ca\(^{2+}\) sensor\(^32\)\(^{-35}\). Recent studies indicate that Syt · Ca\(^{2+}\) might stimulate SNARE-mediated membrane fusion\(^8\)\(^,\)\(^36\), promote the kiss-and-run events in vivo\(^37\), and regulate the fusion pore opening\(^38\)\(^,\)\(^39\).

In this work, we investigated the effects of soluble Syt (C2AB) and Ca\(^{2+}\) on
hemifusion using reconstituted fusion assay. We found that although C2AB · Ca\(^{2+}\) stimulates both inner and outer leaflet mixings, it stimulates significantly more outer leaflet mixing than inner leaflet mixing, resulting in the accumulation of hemifusion. Further, our results employing the hybrid proteins derived from neuronal and yeast SNAREs demonstrate that the interaction between the neuronal SNARE motif and C2AB · Ca\(^{2+}\) is necessary for the stimulation of fusion.

**RESULTS**

**C2AB · Ca\(^{2+}\) stimulates membrane fusion**

We investigated the fusion of liposomes induced by neuronal SNAREs using a fluorescence lipid mixing assay. The t-SNARE complex, which contains syntaxin and SNAP-25, was reconstituted into the liposomes made of POPC/DOPS (65/35 mol/mol). The v-SNARE VAMP2 was also reconstituted into a separate stock of the POPC/DOPS liposomes (62/35 mol/mol) containing fluorescent lipids, NBD-PS and rhodamine-PE (1.5 mol\% each). For both t-and v-SNAREs, the lipid/protein ratio was set at 200:1. When the t-SNARE liposomes were mixed with the v-SNARE liposomes at 35 °C, an increase of the fluorescence intensity was observed (Fig. 2A), indicating that the fusion occurred. Since the fluorescent lipids were distributed equally in the inner leaflet and the outer leaflet, the fluorescence change reflected the sum of outer leaflet mixing and inner leaflet mixing. To determine the effect of Syt and Ca\(^{2+}\) on total lipid mixing, we added soluble synaptotagmin C2AB that lacked the transmembrane domain (TMD) into the v-SNARE vesicles before the fusion reaction. C2AB reduced lipid mixing by about 20\% (Fig. 2A). However, when we added Ca\(^{2+}\) with C2AB, we observed a two- to threefold increase of lipid mixing (Fig. 2A and B). The results are quantitatively consistent with the previous studies by Chapman and coworkers\(^{36}\).

**C2AB · Ca\(^{2+}\) promotes half fusion more than full fusion for neuronal SNAREs**

To measure inner leaflet mixing separately, we treated the v-SNARE liposomes with
sodium dithionite. Under controlled conditions, sodium dithionite reduces NBD attached to the lipid head group in the outer leaflet to a non-fluorescent derivative while leaving NBD in the inner leaflet unaffected. When we mixed the dithionite-treated v-SNARE liposomes with the t-SNARE liposomes, inner leaflet mixing was observed. The extent of inner leaflet mixing was less than that of total lipid mixing (Fig. 2A). The difference between total lipid mixing and inner leaflet mixing indicated that some fusion events were half fusion, the sum of “kiss-and-runs” and terminal hemifusion. The fluorescence fusion assay is not capable of distinguishing these two “half-fusion” events. The percentage of half fusion was approximately 45% of all fusion events after 80 min (Fig. 2B). C2AB itself decreased inner leaflet lipid mixing slightly. The percentage of half fusion was approximately 30% (Fig. 2B). When Ca\(^{2+}\) was added with C2AB, however, there was some stimulation of inner leaflet mixing, but not as much as outer leaflet mixing (Fig. 2B). Such uneven stimulation of total lipid mixing and inner leaflet mixing resulted in the increase of the percentage of half-fusion to 60%, a twofold enhancement by Ca\(^{2+}\) (Fig. 2B). Thus, although C2AB · Ca\(^{2+}\) stimulates membrane fusion on the whole, it does more for half fusion than for full fusion.

C2AB · Ca\(^{2+}\) interacts with soluble SNARE motifs for stimulation of fusion

Recent studies have shown that the TMDs of SNAREs play an essential role in promoting the transition from hemifusion to full fusion: lipid-anchored SNAREs\(^{17,40}\) or SNAREs with a short TMD\(^{15}\) produce only half fusion. One might ask whether the increase of half fusion by C2AB · Ca\(^{2+}\) implicates its interaction with the SNARE TMDs. To test this idea, we constructed hybrid proteins in which the transmembrane domains were swapped between neuronal and yeast SNAREs (Fig. 1), with the assumption that C2AB · Ca\(^{2+}\) is orthogonal to the parts derived from yeast SNAREs.

First, as a control, we tested whether C2AB · Ca\(^{2+}\) influences fusion mediated by yeast SNAREs Sso1p, Sec9c, and Snc2p, which are involved in post-Golgi trafficking without Ca\(^{2+}\) regulation. Also, Syt has not been found on this particular secretory pathway. Sso1p and Sec9c are syntaxin and SNAP-25 analogs and Snc2p is the yeast counterpart of
neuronal VAMP2. The fusion activity of yeast SNAREs was about three times higher than that of neuronal SNAREs (Fig. 3A), under similar conditions. The extent of inner leaflet mixing was comparable to that of outer leaflet mixing (Fig. 3A), showing little accumulation of half fusion. C2AB decreased total lipid mixing and inner leaflet mixing by 20–30% (Fig. 3A and B). Ca\(^{2+}\) did not change both lipid mixings further (Fig. 3A and B), showing no Ca\(^{2+}\) effect for yeast SNAREs. One might argue that no stimulation of fusion by Ca\(^{2+}\) is due to the higher fusion activity of yeast SNAREs. In order to rule out this possibility, we deliberately decreased the fusion activity a half by lowering the protein surface density to 1:400. As expected, we did not observe stimulation of fusion by C2AB · Ca\(^{2+}\) with this lower lipid/protein ratio either (data not shown).

We first examined the hybrid SNAREs made of the yeast cytoplasmic domains (CDs) and the neuronal linker plus TMDs (Fig. 1C). For this set of mutants, the overall fusion activity was reduced to one half of the activity of yeast SNAREs. The response to C2AB and C2AB · Ca\(^{2+}\) is similar to that of yeast SNAREs: there was no stimulation of fusion by C2AB · Ca\(^{2+}\). Instead, C2AB decreased the total lipid mixing by 20% and the inner leaflet mixing by 30% (Fig. 4A), which results in some half fusion (10–20%). Thus, the results showed that the linker regions and TMDs of neuronal SNAREs were not the effectors of C2AB · Ca\(^{2+}\) action.

Next, we tested the hybrid SNAREs made of the CD plus linker of neuronal proteins and the TMD of yeast proteins (Fig. 1C). The fusion activity of this set of mutants was somewhat reduced when compared to that of neuronal SNAREs. C2AB alone decreased the fusion activity slightly, but C2AB · Ca\(^{2+}\) stimulated total lipid mixing significantly, as much as factors of 2–3 (Fig. 4B). In contrast, C2AB · Ca\(^{2+}\) stimulated inner leaflet mixing only slightly (Fig. 4B), resulting in the significant accumulation of half fusion, similar to what had been observed for neuronal SNAREs. We also tested similar hybrid SNAREs in which the amino acid sequence from yeast proteins were extended to cover the linker region. These proteins behaved nearly the same as the aforementioned hybrid SNAREs except for a slight increase of inner leaflet mixing by C2AB · Ca\(^{2+}\) (Fig. 4C).

In summary, the results with hybrid SNAREs suggest that the interaction between
C2AB · Ca$^{2+}$ and the soluble SNARE motifs of neuronal proteins was responsible for both the overall stimulation of membrane fusion and the increase of half fusion.

**DISCUSSION**

There is overwhelming evidence that the synaptotagmin acts as a calcium sensor for neurotransmitter release at synapses$^{33,41,42}$, although the exact mechanism by which it stimulates membrane fusion is elusive. The difficulty arises partly from its promiscuous interactions: Syt binds to t-SNAREs and the SNARE complex in a Ca$^{2+}$-dependent or independent manner$^{38,43-46}$. Ca$^{2+}$ also promotes the Syt binding to the negatively charged lipids$^{33,41,47,48}$, perhaps assisting the membrane apposition.

The in vitro fusion assay using reconstituted SNAREs and soluble C2AB provides an opportunity for the characterization of the Syt function in a relatively isolated situation. In this assay, C2AB did not stimulate membrane fusion induced by yeast SNAREs, in contrast to the case with neuronal SNAREs. Thus, a specific interaction between Syt and neuronal SNAREs appears to be essential for the stimulation of the fusion.

The assay results with the hybrid SNAREs excluded the linker regions and the TMDs of neuronal SNAREs as a target for C2AB · Ca$^{2+}$. We also found that the negatively charged lipid (DOPS) on the membrane is required for the stimulation for neuronal SNAREs (data not shown), which is similar to the results from a previous report$^{36}$. However, it is unknown how the stimulation of fusion is orchestrated by the C2AB · Ca$^{2+}$ binding itself both to SNARE motifs and the membrane. Interestingly, the slight inhibition of the fusion activity of yeast SNAREs by C2AB · Ca$^{2+}$ were not seen when the neutral PC liposomes were used (Fig. S1). This implies that the membrane binding alone causes the inhibition rather than the promotion of fusion.

Our results showed that C2AB · Ca$^{2+}$ stimulated both half fusion and full fusion, but more the former than the latter, increasing the net ratio of half fusion to full fusion. We predict that a significant fraction of the half-fusion events was “hemi-and-run”. At present, it is not clear whether hemi-and-run is related to kiss-and-run.

A current mechanistic model for viral membrane fusion may be summarized as$^{25}$:
Two separate membranes $\leftrightarrow$ hemifusion $\leftrightarrow$ small pore $\rightarrow$ large pore $\rightarrow$ full fusion

The initial two steps involving hemifusion and small pore are all reversible. The transition from the small pore to the large pore is the rate-determining step and it is irreversible. It was previously shown that hemifusion can coexist with the small pore\textsuperscript{25,49}, which might allow the passage of small molecules. For SNAREs, we speculate that such small pores might serve as pores for neurotransmitters. The small pore might not be exclusively lined by the TMDs of SNAREs\textsuperscript{50} but perhaps by the mixture of the TMDs and lipids\textsuperscript{51}.

In neuronal exocytosis, the “kiss-and-run” plays an important role in supporting efficient recycling of synaptic vesicles\textsuperscript{3}. Furthermore, it was demonstrated by Jackson and coworkers that the ratio between the kiss-and-run and full fusion can be modulated by Syt \cdot Ca\textsuperscript{2+} \textsuperscript{37}. However, it was Syt IV, but not Syt I, that increased the ratio of the kiss-and-run to full fusion, which was somewhat inconsistent with our results. The absence of other regulatory factors in our simplified in vitro assay system could have altered the outcome. Nevertheless, it is important to note that the two studies agree upon the role of Syt \cdot Ca\textsuperscript{2+} in modulating the ratio between the kiss-and-run (or hemi-and-run) and full fusion.

MATERIALS AND METHODS

Plasmid construction

Neuronal SNAREs, VAMP2 (amino acids 1–116), syntaxinHT (amino acids 168–288 of syntaxin 1 A) and SNAP-25 (amino acids 1–206) were all inserted into pGEX-KG (between EcoRI and HindIII sites) as glutathione S-transferase (GST) fusion proteins. Four native cysteines of SNAP-25 were replaced with alanines.

Yeast SNAREs, Sso1pHT (amino acids 185–290 of Sso1p) and full-length Snc2p (amino acids 1–115) were inserted into pGEX-KG between EcoRI and HindIII sites, as GST fusion proteins. Sec9c (amino acid 401–651 of sec9) was inserted into pET-24b(+) between NdeI and XhoI sites as a His-tagged protein.
To obtain hybrid SNARE Syn-TMY (Fig. 1) that is made of syntaxinHT cytoplasmic domain (CD) plus the linker region and the TMD of Sso1p, an *Xho*I site was introduced between the linker region and the TMD for syntaxinHT as well as for Sso1pHT. The *Xho*I site mutants were digested by *Xho*I and *Hin*dIII (New England Biolabs) to generate the TMD fragments and the remaining plasmid DNA that includes the vector, the CD and the linker region. The fragments were purified from agarose gel using QIAquick gel extraction kit (QIAGEN). The syntaxinHT CD plus linker fragment and Sso1pHT TMD fragment were ligated by Quick DNA ligation kit (New England Biolabs) to make the Syn-TMY hybrid mutant. The *Xho*I site was converted to its original sequence by mutagenesis.

The other t- or v-SNARE hybrids – VAMP-TMY, Syn-LTMY, Snc-TMN and Sso-LTMN (Fig. 1) – were generated using the same strategy (Fig. 1C). The DNA sequences for all these SNARE hybrids were confirmed by DNA sequencing (Iowa State University DNA Sequencing Facility). The plasmid for C2AB of synaptotagmin (amino acids 140–421) was kindly provided by Dr. Rizo (University of Texas Southwestern Medical Center).

**Protein expression and purification**

The details of protein expression and purification were described elsewhere\textsuperscript{15,52}. Briefly, recombinant proteins were expressed in *Escherichia coli* Rosetta (DE3) pLysS (Novagen). The His-tagged protein Sec9c was purified by Ni-NTA agarose beads (Qiagen). After binding and washing, the protein was eluted in elution buffer (25 mM Hepes, 100 mM KCl with 250 mM imidazole, pH 8.0). GST fusion proteins were purified by affinity chromatography using glutathione-agarose beads (Sigma). The proteins were cleaved by bovine thrombin (CALBIOCHEM) in a cleavage buffer (50 mM Tris–HCl, 150 mM NaCl, pH 8.0). syntaxinHT, VAMP2, Snc2p, Sso1pHT and all hybrid proteins contained 1% *n*-octyl-D-glucopyranoside (OG). Purified proteins were examined with 15% SDS–PAGE, and the purity was at least 90% for all proteins (Fig. 1A).

To purify C2AB, cell pellet was resuspended in 10 mL PBS buffer
(Phosphate-buffered saline, PH 7.4, with 0.5% (v/v) TritonX-100) with the final concentrations of 1 mM AEBSF, 1 mM EGTA, and 5 mM DTT. The cell was broken by sonication on the ice bath and centrifuged at 13 000 × g for 20 min at 4 °C. The supernatant was mixed with 5 ml glutathione-agarose beads (50%) in PBS buffer and nutated in cold room for 2 h. After nutation, the beads were washed with a high salt buffer (50 mM Hapes, 1 M NaCl, PH 7.4) five times. In the high salt buffer 1 mM MgCl2, DNase (20 µg/ml) and RNase (4 µg/ml) were added and incubated for 6 h at 4 °C53,54. After washing by high salt buffer twice and low salt buffer (50 mM Hapes, 0.1 M NaCl, PH 7.4) five times, the protein was cleaved from GST beads by thrombin in low salt buffer.

Membrane reconstitution

The procedure was described elsewhere18. Briefly, syntaxinHT, Syn-TMY and Syn-LTMY were incubated with SNAP-25 for 1 h under room temperature to allow for the formation of target membrane SNARE (t-SNARE) complex. The 50 mM liposomes containing 1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphatidylcholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) (molar ratio 65:35) were reconstituted with the preformed t-SNARE complex in a lipid/protein ratio of 200:1. The 10 mM fluorescent liposomes containing POPC, DOPS, NBD-PS (1,2-dioleoyl-sn-glycero-3-phosphoserine-N-(7-nitro-2-1,3-benzoazadiol-4-yl)), and rhodamine-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)) in the molar ratio of 62:35:1.5:1.5 were reconstituted with v-SNARE in 200:1 lipid/protein ratio. To remove OG, samples were diluted two times with dialysis buffer (25 mM Hapes, 100 mM KCl, pH 7.4), and then dialyzed against 2 L of dialysis buffer at 4 °C overnight. After dialysis, the solution was centrifuged at 10 000 × g to remove protein and lipid aggregates. For yeast SNAREs, Sso1p and its derivatives were reconstituted directly into the POPC/DOPS liposomes without performing the t-SNARE complex with Sec9c. Sec9c was later added to the Sso1p liposomes before the fusion reaction. Snc2p and its derivatives were reconstituted to the fluorescent liposomes. The
reconstitution efficiencies were determined using SDS–PAGE and were at least 70%.

**Total fluorescence lipid mixing assay**

To measure the lipid mixing, v-SNARE liposomes were mixed with t-SNAREs liposomes in the ratio of 1:9. The final solution for each reaction contained about 1 mM lipids in Hepes buffer (25 mM Hepes, 100 mM KCl, PH 7.4) with a total volume of 100 µl. Fluorescence intensity was monitored with the excitation and emission wavelengths of 465 and 530 nm, respectively. The fluorescence signal was recorded by a Varian Cary Eclipse model fluorescence spectrophotometer using a quartz cell of 100 µl with a 2-mm path length. After 5000 s, 0.25% n-dodecylmaltoside was added to obtain the maximum fluorescence intensity (MFI). All of the lipids mixing experiments were carried out at 35 °C.

**Inner leaflet mixing assay**

The inner leaflet mixing assay was modified from the method developed by Meers et al.\(^{55}\). The details of the method had been described elsewhere\(^{18}\). Simply by controlling the time and amount of dithionite, the NBD reduction can be limited to the outer leaflet. After a small amount (about 0.7 µl) of 100 mM sodium dithionite was added to the v-SNARE liposomes several times, the desired reduction of NBD could be achieved. When the outer leaflet NBD reduction was completed, the liposomes were subjected to the lipid mixing assay described above. v-SNARE liposomes without NBDs in the outer leaflets for inner leaflet mixing assay was consumed in 2 h to minimize the flip-flop between the bilayer of the liposomes. After collecting the time traces of total lipid mixing and inner leaflet mixing separately, the percentage of hemifusion was calculated as \(2(PT - PI)/(2(PT - PI) + PI) \times 100\), where \(PT\) is the percentage of maximum for total lipid mixing and \(PI\) is the percentage of maximum for inner leaflet mixing\(^{18}\).
REFERENCES


Figure 1 Hybrid SNAREs made from yeast proteins and neuronal proteins.

(A) SDS–PAGE of recombinant SNARE proteins. C2AB, soluble synaptotagmin lacking the TMD; Snc-LTMN, the v-SNARE hybrid made from the yeast Snc2p cytoplasmic domain (CD) and the neuronal VAMP2 linker plus TMD; Sso-LTMN, the t-SNARE hybrid made from the yeast Sso1pHT CD and the neuronal syntaxinHT linker plus TMD; VAMP-TMY, the v-SNARE hybrid made from the VAMP2 CD plus linker and the
Snc2p TMD; Syn-TMY, the t-SNARE hybrid made from the syntaxinHT CD plus linker and the yeast Sso TMD; VAMP-LTMY, the v-SNARE hybrid with the VMAP2 CD and the Snc2p linker plus TMD; Syn-LTMY, the t-SNARE hybrid with the syntaxinHT CD and the Sso linker plus TMD. (B) Sequence alignment of v-SNAREs Snc2p and VAMP2, and t-SNARE Sso1p and syntaxinHT in the linker and TMD region. The diagram was generated by Clustal X (1.83). (C) Schematic diagram for the arrangement of hybrid SNAREs.
Figure 2 C2AB · Ca$^{2+}$ stimulates membrane fusion mediated by neuronal SNAREs. 

(A) Fluorescence intensity changes for total lipid mixing and inner leaflet mixing for neuronal SNAREs (blue traces), in the presence of 4.5 μM C2AB and 50 μM Ca$^{2+}$ (red traces), in the presence of 4.5 μM C2AB and 100 μM EGTA (cyan traces), and in the
presence of 50 μM Ca$^{2+}$ (green traces) are shown. The lipid/protein ratio was 200:1. Raw data were normalized with respect to the maximum fluorescence intensity obtained by adding 0.25% n-dodecylmaltoside$^{15}$. The black trace is the control using the t-SNARE liposome reconstituted with syntaxinHT only (without SNAP-25). (B) The bar graph representing the efficiencies of total lipid mixing and inner leaflet mixing 80 min after the start of the reaction. The error bars represent the standard deviations from three independent measurements. The addition of Ca$^{2+}$ alone did not alter the fusion trace significantly (data not shown).
Figure 3 C2AB · Ca\(^{2+}\) does not stimulate membrane fusion mediated by yeast SNAREs.

(A) Fluorescence intensity changes for total lipid mixing and inner leaflet mixing for yeast SNAREs (blue traces), in the presence of 4.5 µM C2AB and 50 µM Ca\(^{2+}\) (red traces), in the presence of 4.5 µM C2AB and 100 µM EGTA (cyan traces), and in the
presence of 50 µM Ca^{2+} (green traces) are shown. The lipid/protein ratio was 200:1. The data were normalized with respect to the maximum fluorescence intensity obtained by adding 0.25% n-dodecylmaltoside. The black trace is the control in which Sec9c was not added to the fusion reaction. (B) The bar graph representing the efficiencies of total lipid mixing and inner leaflet mixing 80 min after the start of the reaction. The error bars represent the standard deviations from three different measurements. The addition of Ca^{2+} alone did not alter the fusion trace significantly (data not shown).
Figure 4 Fusion activities of hybrid SNAREs.

(A) The bar graph of total lipid mixing and inner leaflet mixing for hybrid SNAREs made of the yeast CD and the neuronal linker plus TMD (Snc-TMN, Sso-TMN and Sec9c). (B) The bar graph of total lipid mixing and inner leaflet mixing for hybrid SNAREs made of the neuronal CD plus linker and the yeast TMD (VAMP-TMY, Syn-TMY and SNAP-25).
(C) The bar graph of total lipid mixing and inner leaflet mixing for hybrid SNAREs made of the yeast CD and the neuronal linker plus TMD (VAMP-LTMY, Syn-LTMY and SNAP-25). The data represent the fusion efficiencies 80 min after the initiation of the fusion reaction. The error bars represent the standard deviations from three different measurements.
Supplementary figure 1 Negatively charged lipid PS is required for the reduction of the fusion activity of yeast SNAREs by C2AB · Ca\(^{2+}\).

Fluorescence intensity changes for total lipid mixing and inner leaflet mixing for yeast SNAREs (blue traces), in the presence of 4.5 μM C2AB and 50μM Ca\(^{2+}\) (red traces) and in the presence of 4.5 μM C2AB and 100 μM EGTA (cyan traces) are shown. The lipid/protein ratio was 200:1. The vesicles used in this experiment contain no DOPS, and the POPC was 100% for unlabeled liposomes and 97% for labeled liposomes. The black trace is the control in which Sec9c was not added to the reaction.
CHAPTER 4: HEMIFUSION ARREST BY COMPLEXIN IS RELIEVED BY Ca\textsuperscript{2+}-SYNAPTOTAGMIN I

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ABSTRACT

Synaptic transmission relies on an exquisitely orchestrated series of protein-protein interactions. Here we show that fusion driven by neuronal SNAREs is inhibited by the regulatory protein complexin. Furthermore, inner-leaflet mixing is strongly impaired relative to total lipid mixing, indicating that inhibition by complexin arrests fusion at hemifusion. When the calcium sensor synaptotagmin is added in the presence of calcium, inhibition by complexin is relieved and full fusion rapidly proceeds.

INTRODUCTION

The final step of neurotransmitter release into the synaptic cleft is membrane fusion of the synaptic vesicle with the presynaptic plasma membrane, catalyzed by SNARE proteins\textsuperscript{1,2}. Several extrinsic protein factors, including complexin\textsuperscript{3} and synaptotagmin\textsuperscript{4}, impinge on SNARE assembly to regulate vesicle fusion. The complexes (Cpx) are a family of small (14–20 kDa) proteins found in all multicellular eukaryotes. Complexins are primarily restricted to the nervous system and are known to interact with the neuronal SNARE core complex\textsuperscript{3}. Biophysical studies have shown that a central ~58-residue \(\alpha\)-helical segment of complexin binds in an antiparallel orientation within the groove formed by syntaxin-1A and VAMP2 in the four-helix bundle and stabilizes the overall SNARE complex\textsuperscript{5,7}. Currently, the role of complexin in synaptic transmission is unclear.

\textsuperscript{1} These authors contributed equally to this work.
The results of overexpression in chromaffin cells\textsuperscript{8} and PC12 cells\textsuperscript{9} suggest that it may inhibit fusion, yet exocytosis is somewhat impaired in hippocampal neurons from CpxI- and CpxII-deficient mice, suggesting that complexins have a positive role\textsuperscript{10}. However, analysis of the CpxI-CpxII double knockout is complicated by the recent discovery of two additional complexin isoforms: CpxIII, which is also expressed in the hippocampus, and CpxIV\textsuperscript{11}. CpxI and CpxII are soluble proteins, but CpxIII and CpxIV are attached to membranes by isoprenylation. Membrane and soluble forms of Cpx are also predicted in Drosophila melanogaster\textsuperscript{12}.

**RESULTS AND DISCUSSION**

Conflicting in vivo data regarding the function of complexins prompted us to examine their potential effects on SNARE-mediated membrane fusion in a more defined in vitro setting. For this, we used a well-characterized neuronal SNARE complex, composed of syntaxin-1A, SNAP25b and VAMP\textsubscript{2,13}. We expressed Drosophila Cpx and mouse CpxIV in Escherichia coli (Supplementary Fig. 1 and Supplementary Methods online) and examined them in fusion assays using full-length syntaxin-1A (Supplementary Figs. 2 and 3 online) and syntaxin-1A lacking its N-terminal regulatory domain (Fig. 1). Both Drosophila Cpx and mouse CpxIV markedly inhibited fusion driven by the neuronal SNAREs (Fig. 1 and Supplementary Fig. 2). Drosophila Cpx inhibited total lipid mixing by \(~46\%\), on average (Fig. 1). The inhibitory effects of Drosophila Cpx and mouse CpxIV were specific for neuronal SNAREs, as liposomes containing compartmentally analogous yeast SNARE proteins (Sso1p–Sec9p–Snc1p) were unaffected (Supplementary Figs. 2 and 3).

SNARE-mediated membrane fusion follows an ordered series of lipid rearrangements that ultimately leads to aqueous content mixing. These steps include mixing of the outer monolayer of lipids (hemifusion), normally followed by mixing of the inner monolayer of lipids to yield full fusion. The persistent \(~50\%–60\%\) total lipid mixing in the presence of large amounts of Drosophila Cpx suggested that complexin may differentially affect outer- and inner-leaflet mixing. To test this possibility, we selectively monitored
inner-leaflet mixing as a proxy for full fusion using selective reduction of the 7-nitrobenz-2-oxa-1,3-diazole (NBD) fluorophores in the outer leaflet by dithionite, a membrane-impermeant reductant. In the absence of Drosophila Cpx, inner-leaflet mixing was approximately 50% of total lipid mixing, consistent with the amount of fluorescent lipid in the inner and outer leaflets of these ~100-nm liposomes (Fig. 1a, compare red and blue traces). However, little or no inner-leaflet mixing was detected in the presence of excess Cpx (Fig. 1a, black trace), suggesting that complexin arrested fusion at hemifusion.

Similar results were seen when the total number of SNARE proteins was doubled to give a 100:1 lipid to protein ratio and the relative ratio of vesicle (v)- to target (t)-SNAREs was reduced to 1:1 (Fig. 1b,d,e). Under these conditions, a small amount of inner-leaflet mixing was observed relative to the control (Fig. 1b, compare black and gray traces), although inner-leaflet mixing was substantially reduced (~75%) compared to reactions without complexin (Fig. 1c).

Our results suggest that Drosophila Cpx selectively inhibits neuronal SNARE fusion (Fig. 1a–c). In contrast, the synaptic regulator synaptotagmin I (sytI) stimulates in vitro fusion mediated by neuronal SNAREs in a calcium-dependent manner. Although sytI alone was moderately stimulatory at a concentration of 1 mM (Fig. 1d, light blue trace), when sytI was added with Drosophila Cpx in the presence of EGTA, fusion proceeded with kinetics similar to reactions with Cpx alone (Fig. 1d, compare black and red traces). However, activation of sytI by the addition of calcium after ~13 min (800 s, green arrow) rapidly relieved inhibition by Cpx of both total (Fig. 1d) and inner-leaflet mixing (Fig. 1e). When calcium was added to reactions that contained complexin but lacked synaptotagmin, no increase in fusion was seen (data not shown).

Coimmunoprecipitation experiments suggest that both complexin and synaptotagmin can bind SNARE complexes. Given the functional cooperation demonstrated in Figure 1, we asked whether the addition of synaptotagmin influences the binding of complexin to SNARE complexes. We observed simultaneous association of both complexin and synaptotagmin I with reconstituted SNARE complexes (Fig. 2). The
syntaxin-1A–SNAP25 t-SNARE complex in liposomes was mixed with soluble VAMP2 (VAMP2ΔTMD) to complete ternary SNARE complex assembly, and complexin was added in the presence (Fig. 2, lanes 2 and 3) or absence (Fig. 2, lane 1) of synaptotagmin with EGTA (lane 2) or calcium (lane 3). Bound complexes were recovered by flotation in a Nycodenz density gradient\textsuperscript{18}. In all cases, stoichiometric levels of complexin binding were seen. Synaptotagmin binding is calcium dependent (compare lanes 2 and 3) but does not appear to displace complexin or prevent it from binding, suggesting that a more subtle mechanism, such as a small conformational change, may be sufficient to release the hemifusion block.

These observations strongly suggest that complexin and sytI cooperate to inhibit SNARE-mediated fusion in the absence of calcium, in preparation for immediate release after calcium addition. The rapid increase in membrane fusion after calcium addition occurs within 10 s, the data-acquisition interval in this kinetic assay. It is likely that the reaction is much faster than this, warranting further investigation.

We and others have recently demonstrated that SNARE-mediated fusion transitions through a hemifusion intermediate\textsuperscript{13,19,20}, like the reactions of other well-known membrane fusogens\textsuperscript{14}. The observation that sytI, in the presence of calcium, can reverse complexin inhibition suggests that the hemifusion arrest mediated by complexin is a bona fide intermediate on the pathway to full fusion. Here, we suggest that not only does SNARE-mediated fusion transition through a hemifusion intermediate, this intermediate may be used mechanistically to increase the speed of fusion in response to calcium during synaptic transmission. On the basis of our data, we propose that ‘primed’ synaptic vesicles are hemifused and contain fully assembled, metastable trans-SNARE complexes, held in check or clamped by a combination of complexin and aposynaptotagmin. This brake is released by the influx of calcium, allowing inner-leaflet mixing and aqueous continuity between the vesicle lumen and the synaptic cleft (Fig. 3).
MATERIALS AND METHODS

DNA manipulation and plasmid construction

DNA constructs were made using standard genetic manipulations. Tgo polymerase (Roche) was used for all polymerase chain reaction procedures. Restriction enzymes and ligases were from New England Biolabs and the Escherichia coli strain DH5α (EMB Biosciences) was used for standard cloning.

Plasmid pJM361 (Drosophila complexin, His6-Dm_Cpx) was generated by overlap PCR amplification using Research Genetics EST clones RH45716 (N-term) and RH31085 (C-term) as templates. The 5’ fragment used oligos Nde1-Dm_Cpx (#225) and Dm_Cpx-2 (#226) and the 3’ fragment used Dm_Cpx-3 (#227) and Dm_Cpx-Bam (#228). The full-length fragment was made using oligos #225 and #228 and the previous PCR products as templates. The Dm_Cpx gene was excised with NdeI and BamHI and ligated into pET15b (Novagen).

Plasmid pJM435 (human complexin I, GST-Hs_CpxI) was generated by PCR amplification with oligos Nde-Hs_CpxI (#269) and Hs_CpxI-Bam (#270) using Invitrogen cDNA clone MGC:3097 (clone ID3349779) as a template. The fragment was excised with NdeI and BamHI and ligated into a modified pGEX2-T containing an NdeI restriction site (pJM12).

pJM392 (human complexin I, His6-Hs_CpxI) was produced with an NdeI and BamHI fragment cut from pJM435 and ligated into pET15b.

Plasmid pJM421 (mouse complexin IV, GST-Mm_CpxIV) was generated by PCR using oligos Nde-Mm_CpxIV (#267) and Mm_CpxIV-Bam (#268) and Invitrogen cDNA clone MGC:38680 (clone ID5357047) as the template. The fragment was cut with NdeI and BamHI and ligated into a modified pGEX2-T containing an NdeI restriction site (pJM12).

pJM393 (mouse complexin IV, His6-Mm_CpxIV) was made with an NdeI and BamHI fragment from pJM421 ligated into pET15b.
Protein Expression

We produced His6-Dm_Cpx by expression of pJM361 in Rosetta(DE3)pLysS E. coli strain (Novagen). Cells were grown to OD600 0.6-0.8 in 4 L SuperBroth with 100 µg/ml ampicillin, 34 µg/ml chloramphenicol. Expression was induced with 0.3 mM IPTG for 4 hrs at 37°C. The cells were collected using centrifugation and stored at -20°C until purification. Cells were resuspended in breaking buffer (25 mM HEPES-KOH, pH 7.4, 100 mM KCl, 10% (w/v) glycerol, 1 mM TCEP (Tris(2-Carboxyethyl) Phosphine, Fluka) and EDTA-free protease inhibitor tablets). Cells were disrupted by passage through an Emulsiflex C5 cell disrupter (Avestin). Insoluble material was cleared from the extract by centrifugation and the soluble fraction was purified by metal chelate chromatography on an ÄKTApriime liquid chromatography system (Amersham). The sample was injected onto a Hitrap Chelating HP 1mL column (Amersham) and washed in low imidazole wash buffer (25 mM HEPES-KOH, pH 7.4, 100 mM KCl, 10% (w/v) glycerol, 1 mM TCEP, 20 mM Imidazole). The protein was eluted using the His-tag purification template (20-column-volume gradient elution 20 to 500 mM imidazole in 25 mM HEPES-KOH, pH 7.4, 100 mM KCl, 10% (w/v) glycerol, 1 mM TCEP, with 20-column-volume elution wash out). Peak fractions were pooled, aliquoted and stored at -80°C or further purified by anion exchange. For further purification the pooled fractions were dialyzed overnight against 4 L A50 buffer (25 mM HEPES-KOH, pH 7.4, 50 mM KCl, 10% (w/v) glycerol, 1 mM TCEP). The protein was injected onto a HiTrap Q 1mL column (Amersham) and eluted using the Anion Exchange purification template (20-column-volume gradient elution from 50 mM to 500 mM KCl in 25 mM HEPES-KOH, pH 7.4, 1 mM TCEP, with a 20-column-volume elution wash out) on the AKTApriime system. Peak fractions were pooled, aliquoted, and stored at -80°C. His6-Hs_CpxI and His6-Mm_CpxIV were expressed and purified similarly with these exceptions: 1 mM TCEP was replaced with 2 mM βME for the His-tag purification step of His6-Hs_CpxI and overnight dialysis before anion exchange was replaced by dilution for His6-Mm_CpxIV purification, due to protein degradation.

Full length syntaxin1a (rat) and SNAP25b (mouse) were co-expressed from pTW34.
VAMP2-His6 (mouse) (pTW38) and VAMP2∆TMD (pJM51-3) were expressed and purified as previously described²¹. His8-Sso1p (pJM88), GST-Sec9c (BB442), Snc1p-His6 (pJM90), and GST-Snc2∆TMD (BB464a) were expressed and purified as previously described²². The GST-SytIC2AB plasmid [rat, amino acids 96-421, a kind gift of Dr. Ed Chapman) was expressed as described¹⁵ and used in Figure 3. GST- SytIC2AB (amino acids 140-421) as expressed as previously described¹⁶ and used in Figure 2. (GSTVAMP2 (amino acids 1-116), GST-Syntaxin1A (168-288), a truncated version lacking the N-terminal HABC regulatory domain, and GST-SNAP25 (four cysteines were replaced with alanines) (all from rat) were expressed in E. coli Rosetta (DE3) pLysS (Novagen) and used for liposome reconstitution by detergent assisted insertion as previously described²⁰. Briefly, glutathione S-transferase fusion proteins were purified by affinity chromatography using glutathione-agarose beads (Sigma). The protein was cleaved by thrombin in cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0). We added 1% n-octyl-β-D-glucopyranoside (OG) for syntaxin and VAMP2. Purified proteins were examined with 15% SDS-PAGE, and the purity was at least 90% for all proteins.

Reconstitution by detergent dilution and dialysis

The neuronal t-SNARE complexes were reconstituted from co-expressed Syntaxin 1A/SNAP25 (Syn1a/SN25) protein. The neuronal t-SNARE complex (250µl, ~1,268 µg protein) was mixed with 250µl A100 buffer (25mM HEPES-KOH, pH 7.4, 100mM KCl, 10% (w/v) glycerol), 1% OG) and used to resuspend a lipid film of 1.5 µmole 1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphatidylcholine: (1,2-dioleoyl-sn-glycero-3-phosphatidylserine (POPC:DOPS) in an 85:15 mole ratio. The neuronal v-SNARE VAMP2 (20 µl, ~81 µg protein) was mixed with 80µl A100 buffer, 1% OG and used to resuspend a lipid film of 300 n mole POPC:DOPS:Rh-DPPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) :NBD-DPPE (1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-(7-nitro-2-1,3-benzoazadiazol-4-yl)) in a 82:15:1.5:1.5 mole ratio. The yeast t-SNARE complex was formed in 1% OG detergent solution by mixing 250 µl (~385 µg) of His8-
Sso1p and 250 µl (2,300 µg) GST-Sec9c for ~15 hours at 4°C. This 500 µl solution was used to resuspend a lipid film of 1.5µmole POPC:DOPS in an 85:15 mole ratio. The yeast v-SNARE Snc1p (20µl with 80µl A100, 1% OG) was used to resuspend a lipid film of 300 n mole POPC:DOPS:Rh-DPPE:NBD-DPPE in a 82:15:1.5:1.5 mole ratio. Liposomes were formed by detergent dilution and dialysis and isolated by flotation in a discontinuous Accudenz step gradient (Accurate Chemicals) in A100 buffer with 1 mM DTT as previously described.2,23 Proteoliposomes were harvested from the 30-0% interface (400 µl for t-SNARE liposomes and 150µl for v-SNARE liposomes). Protein concentration in liposomes was determined by an amido black protein assay and ranged from 0.73 to 0.87 mg/ml for syntaxin1a/SNAP25 liposome, 0.44 mg/ml for Sso1p/Sec9c liposomes, 0.2-0.25 mg/ml for VAMP2 liposomes, and 0.45 mg/ml for Snc1p liposomes. Lipid recovery was determined by tracer ³H-DPPC following flotation and was ~50-69% for t-SNARE liposomes and ~44-50% for v-SNARE liposomes.

**Reconstitution by detergent assisted insertion**

A mixture of POPC:DOPS (65:35 mole ratio) in chloroform was dried in a vacuum and resuspended in buffer (50 mM Tris- HCl, 150 mM NaCl, pH 8.0) to make the total lipid concentration about 50 mM. Proteinfree large unilamellar liposomes (100 nm in diameter) were prepared by extrusion through polycarbonate filters (Avanti Polar Lipids). Syntaxin and SNAP-25 were mixed at room temperature for about 60 min to allow the formation of t-SNAREs. The preformed t-SNAREs were concentrated down to 90 µl using a 5-kDa cut off centrifugal filter (Millipore) and mixed with 10 µl liposomes for about 15 min at room temperature, resulting in a 100:1 or 200:1 lipid/protein molar ratio. The fluorescent liposomes containing POPC, DOPS, NBD-PS (1,2-dioleoyl-sn-glycero-3-phosphoserine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl)), and rhodamine-PE ) in the molar ratio of 62:35:1.5:1.5 were prepared following the procedure described above, and the final lipid concentration was 10 mM. VAMP2, (80 µl, 50 µM) was mixed with 20 µl of fluorescent liposomes for about 15 min at room temperature. The liposome/protein mixture was diluted two times, lowering the concentration of OG below
the critical micelle concentration. After dialyzing against 2 liters of dialysis buffer (25 mM HEPES, 100 mM KCl, 5% (w/v) glycerin, pH 7.4) at 4 °C overnight to remove detergent, the sample was treated with Bio-Beads (Bio-Rad) to remove any remaining detergent. The solution was then centrifuged at 10,000 x g to remove protein and lipid aggregates. The final t-SNARE liposome solution contained 2.5 mM lipids and 1.9 mg/ml protein, and the v-SNARE liposome solution contained 1 mM lipids and 0.25 mg/ml protein. The integrity and size of SNARE-reconstituted liposomes were examined with negative staining electron microscopy.

**Total lipid mixing assay with detergent dilution and dialysis liposomes**

Standard fusion assays were run as previously described with modifications. All assays included 45 µl t-SNARE liposomes and 5 µl v-SNARE liposomes with an additional 10 µl of buffer, complexin, or soluble v-SNARE as indicated. All components were mixed in a 96-well Fluororunc polysorp plate (Nunc) on ice and incubated overnight at 4°C. The plate was then removed from 4°C and immediately placed in a 37°C fluorescent plate reader (Floroskan II, Labsystems). NBD fluorescence was measured (excitation 460 nm, emission 538 nm) at 2 min intervals for 120 min at which time 10 µl of 2.5% (w/v) ndodecylmaltoside (Roche) was added to determine absolute NBD fluorescence. The kinetic data was normalized as a percent of total fluorescence then converted to rounds of fusion as previously described.

**Total lipid mixing with detergent assisted insertion liposomes**

To measure lipid mixing, v-SNARE liposomes were mixed with t-SNARE liposomes in the ratio of 1:1 or 1:9 as indicated. The final solution contained 0.5 mM lipid for 1:1 (0.25 mM v-SNARE: 0.25 mM t-SNARE) ratio or 1mM lipid for 1:9 (0.1 mM v-SNARE : 0.1 mM t-SNARE) ratio with a total volume of 100 µl. Fluorescence was measured at excitation and emission wavelengths of 465 and 530 nm, respectively. Fluorescence changes were recorded at 5-10 second intervals with a Varian Cary Eclipse model fluorescence spectrophotometer using a 100 µl quartz cell with a 2-mm path length. The
maximum fluorescence intensity (MFI) was obtained by adding 0.2% (w/v) n-dodecylmaltoside. All of the lipid mixing experiments were carried out at 35°C.

**Inner leaflet mixing with detergent assisted insertion liposomes**

The inner leaflet mixing assay was modified from the method developed by Meers et al$^{20,24}$. The method is based on the fact that sodium dithionite reacts more rapidly with NBDs in the outer leaflet than those in the inner leaflet. By controlling the time and amount of dithionite, the reaction can be limited to the outer leaflet. Small aliquots (0.7 µl) of 100 mM sodium dithionite in 50 mM Tris buffer, pH 10, were added to the v-SNARE liposomes (100 µl, 0.2 mM lipid) until a desired reduction of NBD was achieved. The reaction was monitored at room temperature by scanning the fluorescence signal for 15 min from 500 to 700 nm with the excitation at 460 nm. Typically, the reduction was complete in 10 min, and no additional change of the spectrum was observed. The liposomes without NBD in the outer leaflets were subjected to the lipid mixing assay described above.

**Complexin binding to SNARE liposomes**

Proteoliposomes (45µl) containing the syntaxin1a/SNAP25 t-SNARE complex (16 µg, 0.28 nmole) were mixed with 10µl (47 µg, 3.7 nmole) VAMP2∆TMD and 20µl (48 µg, 2.5 nmole) Dm_Cpx. Synaptotagmin (25 µl, 40 µg, 1.1 nmole) was added in the presence of 1 mM CaCl$_2$ or 0.2 mM EGTA. The reactions were brought to a final volume of 100 µl with Buffer A100 and incubated 12-15 hrs at 4 °C. Bound protein was separated from unbound protein by flotation through a nycodenz gradient. Nycodenz (100 µl of 80% in Buffer A100) was added to each reaction, mixed, and loaded into a 5x51mm SW55Ti tube then overlaid with layers of 35% (150 µl), 30% (150 µl), and 0% (50 µl) each containing 1 mM CaCl$_2$ or 0.2 mM EGTA as appropriate. These samples were then centrifuged at 48,000 rpm (218,500xgave) in an SW55Ti rotor with adapters for 2.5 hours. Liposomes were harvested at the 30%/0% interface and analyzed by SDS-PAGE and scintillation counting.
REFERENCES


Figure 1 Ca$^{2+}$-sytI releases complexin-mediated hemifusion arrest.

(a–f) Total and inner-leaflet mixing was performed with proteoliposomes generated with a 200:1 (a) or 100:1 (b,d,e) lipid/protein ratio. Syntaxin-1A (residues 168–288)–SNAP25 liposomes were mixed with fluorescent VAMP2 liposomes in a 9:1 (a) or 1:1 (b,d,e) liposome ratio in the presence or absence of 20 mM Drosophila Cpx (DmCpx). Additionally, in d, the soluble sytIC2AB domain (1 mM) was added in the absence (black trace) or presence of 0.5 mM calcium (light blue and green traces). For green trace in d and e, calcium (1 mM) was added after B13 min (800 s, indicated by green arrow). Inner-leaflet mixing (red and black traces) was selectively monitored by pretreating the
VAMP2 liposomes with sodium dithionite. The extent of fusion at 50 min (c) or 60 min (f) was compared to results with buffer control and charted as percent of the control measurement. Averages of four (c) or three (f) independent experiments are shown. Error bars show s.e.m.
Figure 2 Drosophila Cpx (DmCpx) and sytI bind simultaneously to SNARE complexes.

Membrane-incorporated syntaxin-1A (syn1a)–SNAP25 (SN25) was mixed with excess soluble VAMP2 (VAMP2ΔTMD) and complexin, with (lanes 2 and 3) or without (lane 1) sytI, for 12–14 h at 4 °C. Calcium (1 mM) was added to the mixture in lane 3, whereas lane 2 contains 0.2 mM EGTA. SNARE-bound protein was isolated by flotation in a density gradient and analyzed by SDS-PAGE and Coomassie staining.
Figure 3 SNARE complex formation, hemifusion arrest by complexin and relief by calcium-synaptotagmin.

(a) A synaptic vesicle approaches the presynaptic plasma membrane and the v-SNARE VAMP2 (blue) engages the t-SNARE complex of syntaxin-1A (black) and SNAP25 (green). (b) Membrane contact is achieved as the SNARE complex fully assembles into a four-helical bundle. (c) Force generated by SNARE complex formation merges the outer
leaflets of the vesicle and the plasma membrane. (d) Complexin (purple) associates in an antiparallel orientation with the fully formed SNARE complex. Complexin binding arrests fusion at the normally unstable hemifusion intermediate. (e) Synaptotagmin (yellow) associates or changes its association with the SNARE complex and lipids to accommodate the presence of complexin. Synaptotagmin may also have earlier roles in t-SNARE complex formation16. (f) Calcium influx (brown) is detected by synaptotagmin, changing the assembled complex of SNAREs, complexin and synaptotagmin and resulting in the completion of inner-leaflet mixing and full fusion.
The protein sequences of Drosophila complexin, human complexin I, and mouse complexin IV were aligned using Pileup and similar amino acids shaded by Boxshade 3.2. The percent similarity and identity was determined by the Gap algorithm in the GCG software package.

**Supplementary Figure 1 Complexin alignment.**
Supplementary Figure 2 Complexins inhibit SNARE mediated fusion in vitro.

Syntaxin1a/SNAP25 liposomes (45 µl, ~33 µg protein, ~115 nmol lipid) were mixed with fluorescently labeled VAMP2 liposomes (5 µl, ~1.25 µg protein, ~5 nmol lipid) on ice and incubated overnight (12-15 hrs) at 4 °C in the presence or absence of 20 µM recombinant complexin, Dm_Cpx (open circles), or Mm_CpxIV (open squares) in a total reaction volume of 60 µl. Buffer (10µl) was added to the positive control (filled black circles) and soluble VAMP2 (lacking a TMD, VAMP2ΔTMD, 1.8 µl, ~8.5 µg, ~10x molar excess) was added to the negative control (solid black line, no symbol). NBD fluorescence was monitored at 37 °C in a fluorescent plate reader at 2 min intervals for 120 min at which time detergent (10µl of 2.5% n-dodecyl maltoside) was added to determine maximal fluorescence. (a) The raw fluorescence data was normalized as a percent of maximal fluorescence and converted to rounds of fusion using a calibration curve as previously described. (b) Kinetic fusion of yeast SNARE proteoliposomes and Dm_Cpx. Sso1p/Sec9c liposomes (45µl, ~19.8 µg protein, ~86 nmol lipid) were mixed
with fluorescently labeled Snclp liposomes (5 µl, ~2.25 µg protein, ~2.9 nmol lipid) on ice and incubated overnight (12-15 hrs) at 4 °C ± Dm_Cpx (20 µM). Fusion data were collected and analyzed as described above. (c) Coomassie blue stained gel of SNARE-containing proteoliposomes and recombinant complexin proteins. Lanes 1(t-SNARE) and 2 (v-SNARE) are yeast SNARE proteoliposomes (Fig. 1c, and Fig. 2b), lanes 3 (t-SNARE) and 4 (v-SNARE) are neuronal SNARE proteoliposomes (Fig. 1a,b, 2a,b) lane 5 is Dm_Cpx (~3 µg), lane 6 is Mm_CpxIV (~6 µg). Positions of molecular weightmarkers are shown on the left (in kDa).
Supplementary Figure 3 Complexin inhibition is specific for neuronal SNAREs.

(a) Drosophila complexin inhibition is concentration dependent. Neuronal SNARE fusion reactions containing increasing amounts of Dm_Cpx were analyzed as described in Figure 1. Endpoint fusion at 120 minutes was normalized to a control reaction containing buffer for 4 independent reactions representing two liposome preparations and two Dm_Cpx protein preparations. The mean ± standard error of the mean is plotted versus Dm_Cpx concentration in µM. Control fusion (100%) was 1.10 rounds of fusion (following background subtraction). (b) Fusion inhibition as a percent of control at 120 min for all complexins at 20 µM with neuronal SNARE proteoliposomes or yeast SNARE
proteoliposomes. The mean ± standard error of the mean is represented for each complexin protein. The number of replicates is indicated at the bottom of each bar. The data for the 20 µM in panel a is included in the Dm_Cpx histogram. A dashed line is drawn at 60% to represent the theoretical maximum degree of inhibition with the ~45 nm liposomes. Fusion data was collected and analyzed as described in Supplemental Figure 1. Fusion with neuronal SNAREs ranged from 1.10-1.44 rounds of fusion (background subtracted) while yeast SNAREs ranged from 0.93-1.16 rounds of fusion following background subtraction.
CHAPTER 5: SINGLE-VESICLE ANALYSIS REVEALS STRONG STIMULATION OF SNARE-MEDIATED MEMBRANE FUSION BY COMPLEXIN AND Ca\(^{2+}\)

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ABSTRACT

Ca\(^{2+}\)-triggered, synchronized synaptic vesicle fusion underlies inter-neuronal communication. Complexin is a major binding partner of the SNARE complex, the core fusion machinery at the presynapse. The physiological data on complexin, however, have been at odds with each other, making delineation of its molecular function difficult. Here we report on direct observation of two-faceted functions of complexin using the single-vesicle fluorescence fusion assay and electron paramagnetic resonance. We show that complexin I has two opposing effects on trans-SNARE assembly: Inhibition of SNARE complex formation and stabilization of assembled SNARE complexes. Remarkably, SNARE-mediated fusion is markedly stimulated by complexin, and further accelerated by two orders of magnitude in response to an externally applied Ca\(^{2+}\) wave. We suggest that SNARE complexes, complexins, and phospholipids collectively form a complex substrate for Ca\(^{2+}\) and Ca\(^{2+}\)-sensing fusion effectors in the neurotransmitter release.

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INTRODUCTION

Complexins are small proteins (~15 kDa) mainly found in the presynaptic part of neuronal cells\(^1\) and show a \(\alpha\)-helical propensity near the N-terminus but no apparent tertiary structure\(^4,5\). The \(\alpha\)-helical structure is probably responsible for complexin binding to the groove between the synaptobrevin and the syntaxin helices in the neuronal SNARE complex\(^4,5\), which itself is a tight bundle of four \(\alpha\) helices\(^6\)\(-\)\(^{13}\).

Despite the seemingly simple structure and binding mode of complexin, physiological studies suggested diverse functions. Overexpression of complexin\(^14\)\(-\)\(^{16}\) in chromaffin\(^14\) and PC12\(^15\) cells or expression as a fusion protein to synaptobrevin\(^16\) substantially diminishes the neurotransmitter release, suggesting an inhibitory role of complexin. In contrast, knock-out of complexin isoforms from hippocampal neurons selectively impairs the synchronous component of exocytosis\(^17,18\) indicating a stimulatory role of complexin in late fusion steps. Biophysical studies\(^4,5,16,19,20\) have also reported either stimulatory\(^4,5\) or inhibitory effects of complexin, although the recent results primarily support the inhibitory role of complexin as a clamp for SNARE-driven membrane fusion\(^19\)\(-\)\(^{21}\). Such a knowledge gap between these contrasting viewpoints in physiological and molecular analyses remains largely unfilled up to this point, making it difficult to gain further insights into precisely how complexins function in the neurotransmitter release.

In this work, we used the single vesicle fluorescence resonance energy transfer (FRET) fusion assay and the electron paramagnetic resonance (EPR) spectroscopy that have proven powerful in dissecting individual fusion steps along the pathway of SNARE-mediated membrane fusion\(^22,23\). The combined analyses show that complexin I promotes membrane fusion by directly interacting with the \textit{trans} neuronal SNARE complex (complexin I and SNAREs both from rat brain). Strikingly, complexin further accelerates fusion by two orders of magnitude upon introduction of the \(\text{Ca}^{2+}\) ion under physiologically relevant conditions, resulting in synchronization of fusion on the seconds time scale. On the other hand, high micromolar complexin inhibits vesicle docking through a weak interaction with the target membrane (t-) SNARE complex, providing reconciliation of previous conflicting findings.
RESULTS

Single vesicle FRET fusion assay

In the single vesicle fusion assay, vesicles containing v-SNARE proteins (synaptobrevin) and membrane-specific acceptor fluorophores (2 mol% DiD) are tethered to a quartz imaging surface coated with poly-ethyleneglycol (Fig. 1a, left). Then, vesicles containing t-SNARE proteins (syntaxin and SNAP-25) and donor fluorophores (2 mol% DiI) are added in solution to induce SNARE complex formation and membrane fusion between a pair of single t- and v-SNARE vesicles (Fig. 1a, middle and right). A single-vesicle fusion event leads to an increase in the FRET efficiency, \( E \), defined as \( I_A/(I_D+I_A) \) where \( I_D \) and \( I_A \) are the donor and the acceptor fluorescence intensities respectively. Docking (or early fusion steps) shows low \( E \) values that are smaller than 0.25 and the full fusion state gives \( E \approx 0.7 \) (Ref. 22). By imaging over 100 fusion events in single vesicle complexes in parallel, we can probe how individual fusion steps are modulated by the regulatory factors present such as complexin.

We first imaged fusion mediated by the SNARE proteins only, i.e., in the absence of complexin and Ca\(^{2+}\). FRET efficiency histogram of single vesicle complexes, formed by a pair of t- and v-SNARE vesicles, were obtained 15 min after addition of t-SNARE vesicles (37 °C; Fig. 1b, blue symbols) and showed a small full fusion population (\( E \approx 0.7 \)) with the vast majority remaining at the docked state (\( E \approx 0.1 \)). In the absence of SNAP-25, we did not observe any full fusion population indicating that the ternary SNARE complex is required for fusion (Supplementary Fig. 1a). The full fusion peak grew with time (Fig. 1b, red and yellow symbols) but on a slow time scale of tens of minutes. Addition of 1 mM Ca\(^{2+}\) to preformed neuronal SNARE complexes did not change the fusion kinetics (Fig. 1c and Supplementary Fig. 1b). Since the neurotransmitter release occurs on a much faster millisecond time scale, the slow fusion by SNAREs suggests that the combined effect of fusion effectors should be primarily in accelerating fusion.

Complexin inhibits docking between v- and t-SNARE vesicles

Next, we included recombinant full-length complexin I (Cpx I, residues 2-134) and 1 mM EGTA in the t-SNARE vesicle solution (Fig. 2a, flow chart). As illustrated in Fig. 1a, we mixed t-SNARE vesicles with different amounts of complexin I (0.2, 2, and 20 \( \mu \)M) and then injected the mixture into a flow chamber with surface-immobilized v-
SNARE vesicles ($t = 0$). After 12 min incubation at 37 °C, unbound t-SNARE vesicles and complexins in solution were removed by flow washing. We then counted the average number of t-SNARE vesicles per imaging area ($50 \times 100 \, \mu m^2$) that docked to individual v-SNARE vesicles (Fig. 2a and Supplementary Fig. 2a). By dividing this average number of docked t-SNARE vesicles by the average number of v-SNARE vesicles per imaging area (Supplementary Fig. 2b), we obtained the probability that a surface-tethered v-SNARE vesicle is docked by a t-SNARE vesicle (Fig. 2b). No docking was observed in the absence of v-SNARE vesicles or without SNARE proteins. Without complexin, the docking probability was 0.27. This probability was maintained up to the complexin concentration of 2 µM, but notably reduced to ~0.1 when 20 µM complexin was used (Fig. 2a,b). Therefore, complexin at this high concentration inhibits SNARE complex formation, which may explain the phenotype of complexin overexpression14-16.

**Complexin binding to SNARE complex stimulates fusion**

Comparison of the FRET histograms of single vesicle complexes, however, shows that an increasing amount of complexin I enhances the full fusion population at $E \approx 0.7$ (Fig. 2c-e). The normalized full fusion population, defined as the number of vesicle complexes with $E > 0.5$ divided by the total number of vesicle complexes imaged, was 0.18 at [Cpx I] = 0.2 µM and increased to 0.32 at 2 µM and 0.45 at 20 µM after 15 min reaction each. To probe whether the complexin/SNARE complex interaction is directly involved in the fusion reaction, we engineered double-point mutation in complexin I (C105A/R48C) to attach a nitroxide spin label at position 48, which is critically involved in complexin binding to the SNARE complex4,5. This spin labeled R48C mutant showed a minimal level of binding to the ternary SNARE complex (Fig. 2f, EPR spectra; see Supplementary Fig. 3 for enlarged spectra). Coincident with this diminished binding, the stimulation effect was largely reduced (Fig. 2f) indicating complexin binding to the SNARE complex is essential for stimulation of fusion. Furthermore, the stimulatory effect of complexin I is specific to the complete SNARE complex because drastically reduced full fusion population was observed without SNAP-25, even with reaction time of 60 min and 1 mM Ca$^{2+}$ (Fig. 2g). Therefore, stimulation of full fusion here must arise from the direct interaction between SNARE complexes and complexin I.
Data so far demonstrate two opposing effects of complexin on the SNARE complex. Complexin I at high micromolar concentrations inhibits trans-SNARE complex formation, thereby limiting docking but once SNARE complexes are formed, complexin I helps to drive the fusion process to later stages. The stimulation effect of complexin is visible at \( \sim 1 \, \mu M \) concentration, about an order of magnitude lower than what is necessary for the inhibitory effect. Ca\(^{2+}\)-independent stimulation of full fusion by complexin I continued beyond 15 min (Fig. 2h and Supplementary Fig. 4).

**Complexin-assisted, Ca\(^{2+}\)-triggered fusion events**

Synaptotagmin I is thought to be the major Ca\(^{2+}\) sensor for neurotransmitter release. However, the complexin I and II deletion mutant exhibits a phenotype similar to that of the synaptotagmin I-knocked out neuron, severely hampering the fast neurotransmitter release\(^{17,18}\). We therefore asked if Ca\(^{2+}\) can trigger SNARE-mediated fusion in the presence of complexin I but without synaptotagmin I. To probe potential Ca\(^{2+}\) effects, we used real-time tracking of single-vesicle fusion events\(^{22}\). We incubated a solution of t-SNARE vesicles, complexin I (1.2 \( \mu M \)) and 10 \( \mu M \) EGTA with surface-immobilized v-SNARE vesicles for 12 min and introduced 1 mM Ca\(^{2+}\) in the imaging area at \( t = 4.4 \) sec (at the speed of 2 ml min\(^{-1}\), \( t = 0 \) corresponds to the start of real-time tracking) while donor and acceptor signals from single vesicle complexes were being recorded with the time resolution of 150 or 200 ms (flow chart of Fig. 3a). Remarkably, we observed a substantial fraction of vesicle complexes showing fusion synchronized with the Ca\(^{2+}\) flow (Fig. 3).

In one typical real-time trace of Ca\(^{2+}\)-evoked fusion (Fig. 3a), the vesicle complex begins with the initial \( E \) value of \( \sim 0.14 \) and starts lipid mixing at \( t = 4.8 \) sec (orange arrow) that reaches the full fusion state at \( E \approx 0.72 \). Likewise, many vesicle complexes in the same imaging area start to show FRET increase (Fig. 3c, orange arrows) following the arrival of the Ca\(^{2+}\) wave (see Supplementary Fig. 5 for more traces). As a result, the cumulative time histogram of these instances of initial FRET increase shows a rapid increase in number (Fig. 3b, blue symbols). With the first-order kinetics assumed, we used two exponentials, \( A_1 (1 - \exp^{-t/t_1}) + A_2(1 - \exp^{-t/t_2}) \), to fit the plot after the Ca\(^{2+}\) arrival at 4.4 sec (Fig. 3b, red curve). The time constants \( (t_1, t_2) \) and the corresponding normalized population \( (A_1, A_2) \) are (8.87 sec, 0.95 sec) and (0.37, 0.01) respectively,
indicating that the cumulative plot exhibits mainly one time constant of ~8.9 sec. In the first 5 second window (between $t = 4.4$ and 9.4 sec), 37 out of total 217 vesicle complexes of the imaging area show the initiation of fusion. The cumulative time histogram based on five independent flow experiments (total 1,240 vesicle complexes imaged) shows the time constant of 7.9 sec (Fig. 3e, blue symbols and black curve). Overall, an appreciable fraction (~25%) of total vesicle complexes undergoes Ca$^{2+}$-induced membrane fusion events that are synchronized on the seconds time scale. Control experiments of flowing a buffer without Ca$^{2+}$ show negligible fusion (Fig. 3b, black symbols; 1,900 vesicle complexes). Furthermore, Mg$^{2+}$ is ineffective in inducing membrane fusion in complexin-primed vesicle complexes (Fig. 3b, red symbols; 1,161 vesicle complexes).

Cholesterol is a major lipid species in the synaptic membranes whose content reaches as high as 40 mole% of total lipids. Inclusion of 40 mole% cholesterol while reducing PS (phospho-L-serine) to 15 mole% produced a cumulative time histogram, nearly identical to that for vesicle complexes of PC:PS in molar ratio of 65:35 (Fig. 3e, red symbols; see Fig. 3f and Supplementary Fig. 5 for real-time traces). With this lipid composition, even when the Ca$^{2+}$ concentration is reduced to 20 µM, which represents a typical peak Ca$^{2+}$ concentration near the fusion active zone during neuronal stimulation$^{24}$, more than 10% of the vesicle complexes show Ca$^{2+}$-triggered fusion (Supplementary Fig. 6). But simple reduction of negatively charged PS lipids from 35 mol% to 15 mol% in the absence of cholesterol largely abolishes Ca$^{2+}$-evoked fusion (Fig. 3e, black symbols), suggesting that high cholesterol in synaptic vesicles$^{25}$ is essential for the Ca$^{2+}$-responsiveness. Furthermore, such delicate dependence on the lipid composition suggests that our single-vesicle fusion system may be a good mimic of the synaptic vesicle fusion. In addition, our results show that the hemifusion state is a substrate for rapid fusion induced by Ca$^{2+}$ (Fig. 3d), well in line with recent in vivo imaging data$^{26,27}$.

The percentages of Ca$^{2+}$-responding vesicle complexes, 25% and 10%, are largely underestimated values because we note that only ~30% of total vesicle complexes are available for Ca$^{2+}$-triggered fusion at the moment of the Ca$^{2+}$ addition. In the in vitro fusion assay, we find that on average 35% of total vesicle complexes are fusion-inactive (Fig. 2h and Supplementary Fig. 7) probably due to the absence of the auxiliary proteins, and at the same time, 36% of total vesicle complexes become already fully fused before Ca$^{2+}$ is added (Supplementary Fig. 6b). Therefore, it appears that nearly 90 and 35% of
the “fusable” pool of vesicle complexes respond to 1 mM and 20 µM Ca\(^{2+}\), respectively, within the first 25-second window.

**Fusion step-specific switching of complexin function**

Our single-vesicle fusion analysis revealed the two-faceted functions of complexin. At high concentrations, complexin reduces the probability of \(\text{trans-SNARE} \) complex formation. Once assembled into SNARE complexes, however, complexin strongly stimulates membrane fusion, which becomes accelerated about 100 fold via the addition of Ca\(^{2+}\). To gain further insight into the dual functions of complexin, we produced double mutation (C105A/R56C) in complexin I and attached a spin label now at position 56. The EPR spectrum of this construct, when mixed with t-SNARE complexes, shows a broad spectral component reflecting the slow motion of the nitroxide side chain arising from direct interaction between complexin and the t-SNARE complex (Fig. 4a, blue arrow). The EPR spectra are shown in the absorbance mode instead of the derivative mode because the spectral broadening due to the tertiary interaction is better represented in the former than the latter\(^{11}\). For negative control, we attached a spin label at the native cysteine at position 105 of complexin I. The EPR spectra of the C105-spin label mutant do not show any change when incubated with t-SNARE vesicles (Fig. 4a, right inset), indicating the broadening observed for the R56C-spin label mutant specifically reflects the interaction between complexin I and t-SNARE proteins. The spectral subtraction analysis\(^{28}\) gives the dissociation constant of ~50 µM, much weaker than the complexin binding to the ternary SNARE complex. This weak interaction would lead to competition between v-SNARE and complexin for binding to t-SNAREs (Fig. 4b, inside grey circle). When highly expressed, complexin outnumbers v-SNARE thereby inhibiting SNARE complex assembly. Such an inhibitory effect may have physiological function in some species including *Drosophila*\(^{29}\).

However, the primary function of complexin appears to be binding to \(\text{trans-SNARE} \) complexes and stimulating membrane fusion (Fig. 4c,d). Earlier structural studies\(^{4,5}\) positioned complexin as a potential fusion stimulator because complexin was thought to stabilize assembled SNARE complexes. Without complexin I, the number of docked t-SNARE vesicles considerably decreases with time (Fig. 4e, blue circles), reflecting disassembly of \(\text{trans-SNARE} \) complexes probably due to repulsion between t- and v-SNARE vesicles. In contrast, we observe even a moderate concentration of complexin I
(2 µM) has a noticeable effect of keeping trans-SNARE complexes from being disassembled (Fig. 4e, black triangle; see also Supplementary Fig. 8).

**DISCUSSION**

It is remarkable that Ca\(^{2+}\) can trigger the fusion burst in the presence of complexin alone without synaptotagmin I\(^30\). Evidence indicates the observed Ca\(^{2+}\)-triggered fusion should be attributed to a concerted action of complexin, SNARE complexes, and Ca\(^{2+}\) ions. The distribution of the initial E values for the vesicle complexes showing Ca\(^{2+}\)-induced fusion has a major population between 0.2 and 0.5 (Fig. 3d), and this FRET range approximately corresponds to the hemifusion state\(^{19,22}\). The same FRET range can also be obtained using only SNARE complexes (Fig. 1b,c) but these FRET states do not show notable progression to full fusion in response to 1 mM Ca\(^{2+}\) (Fig. 1c and Supplementary Fig. 1b). Therefore, complexin bound to trans-SNARE complexes lowers the fusion energy barrier of two membranes. Then, further action of Ca\(^{2+}\) ions enables many vesicle complexes to finally overcome the fusion energy barrier, accelerating the fusion kinetics by additional two orders of magnitudes (Fig. 3b, blue vs. black symbols) that corresponds to reduction of the energy barrier by 2.8 kcal/mole.

Several recent reports\(^{31,32}\) have detected the existence of an independent Ca\(^{2+}\)-sensing mechanism for the asynchronous exocytosis mode. The molecular identity of this second pathway does not include any synaptotagmin isoforms and is yet to be identified. The time scale of the asynchronous mode is found to be hundreds of milliseconds, only one order of magnitude different from the time scale that we observe with SNAREs and complexin. Therefore, we speculate that the supramolecular complex consisting of SNARE complexes, phospholipids, and complexin works as a substrate of Ca\(^{2+}\) for asynchronous release. The action of synaptotagmin I may be superimposable on the same vesicle pool to further lower the fusion activation energy (Fig. 4f). Such an action of synaptotagmin I\(^33\) may be needed to push the synchrony level down to milliseconds, a requirement for synchronous neural communication.

Although observation of the inhibitory effect of complexin is reminiscent of the fusion clamp model\(^{19-21}\), the underlying fusion energetics suggested by our results is fundamentally different from that of the clamp model. In the fusion clamp model, SNARE complex assembly is thought to release sufficient free energy to overcome all the fusion energy barriers, so its energy release needs to be spring-loaded by a ‘clamp’ to
gain control over the timing of fusion. Our data, however, suggest that the SNARE complexes should be intimately helped by complexin and lipid molecules to accelerate fusion and to increase the Ca\(^{2+}\)-sensitivity (Fig. 2 and Fig. 3).

Very recently, the dual function of complexin, both as a stimulator and an inhibitor, has been proposed by Xue et al based on their physiological experiments\(^{17}\). Our results provide the molecular mechanism underlying the dual function as well as switching between the two. The EPR analyses find that complexin has two divergent interaction modes with SNAREs: a low affinity interaction with the t-SNARE complex and a high affinity binding to the ternary SNARE complex\(^4\). Using these different interaction modes, complexin switches between different functions, inhibitory before but stimulatory after SNARE assembly as our single vesicle fusion data demonstrate.

Additionally, we note that many of our findings were made possible by the single-vesicle fusion assay. In the proteoliposome fusion systems, docking requires much longer time than the fusion process itself\(^2\). Bulk ensemble assays therefore primarily detect changes in the docking kinetics and thus the inhibitory effect of complexin (Supplementary Fig. 9), while the subsequent fusion kinetics and the role of complexin as a fusion stimulator would be very difficult to observe in bulk. In contrast, in the single-vesicle FRET assay, docking and fusion steps are clearly distinguished since undocked t-SNARE vesicles are invisible in our imaging system. As a result, we were able to observe real-time ‘triggering’ of fusion process by Ca\(^{2+}\) flow and to study the synchrony level of fusion events in a given pool of single-vesicle complexes. Our assay should be applicable to the studies of other fusion regulators of the synaptic membrane fusion including synaptotagmin I. However, we note that studying syntatotagmin I offers many challenges due to its ability to induce vesicle clustering\(^3\) as well as the difficulty to steer the vectorial interaction with the target membrane\(^3\).

**MATERIALS AND METHODS**

**Protein expression and purification**

Recombinant neuronal SNARE proteins of rat brain, syntaxin HT (amino acids 168-288 of syntaxin 1A, lacking the Habc domain), SNAP-25 (amino acids 1-206, four native cysteines of SNAP-25 were replaced with alanines) and VAMP2 (amino acids 1-116) were expressed as N-terminal glutathione S-transferase fusion proteins. Recombinant
proteins were expressed in Escherichia coli Rosetta (DE3) pLysS (Novagene, Madison, Wisconsin). The cells were grown at 37 °C in LB with 100 g ml\(^{-1}\) ampicillin until OD600 reached 0.6-0.8. The cells were further grown for 4-6 hours after adding Isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) (0.5 mM final concentration) at 16 °C for syntaxin HT and VAMP2 or at 24 °C for SNAP-25 and complexin I. We purified the proteins using glutathione-agarose chromatography. Cell pellets were resuspended in 10 mL PBS buffer (phosphate-buffered saline with 0.5 % (v/v) TritonX-100, pH 7.4) with the final concentrations of 1 mM AEBSF, 5 mM DTT. Cells were broken by sonication on the ice bath and centrifuged at 13,000×g for 20 min at 4 °C. The supernatant was mixed with 2 mL glutathione-agarose beads in PBS and mutat ed in cold room for 2 hours. The proteins were then cleaved by thrombin in cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0) for syntaxin, SNAP-25 and VAMP2, or cleaved by thrombin in HEPES buffer (25 mM HEPES, 100 mM KCl, pH 7.4) for complexin I. We added \(n\)-octyl-\(\beta\)-glucopyranoside (OG) of 0.8 g per 100 ml for syntaxin and VAMP2. Purified proteins were examined with 15 % SDS-PAGE, and the purity was at least 90% for all of the proteins.

**Membrane Reconstitution**

Unilamellar vesicles containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-[phospho-l-serine] (DOPS) and cholesterol (all purchased from Avanti Polar Lipids, Birmingham, Alabama) and doped with 2 mol% DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes/Invitrogen, Carlsbad, California) were formed by using the extrusion method (MiniExtruder, Avanti Polar Lipids). Syntaxin HT and SNAP-25 were mixed at room temperature for about 1 hour to allow the formation of t-SNARE complexes. The preformed t-SNARE proteins were then reconstituted into the unilamellar vesicles through dialysis. The v-SNARE protein VAMP2 was reconstituted into a different population of unilamellar vesicles that were doped with 2 mol% DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate;Molecular Probes) and 0.1 mol% biotinylated lipids, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl) (Avanti Polar Lipids). The lipid composition for each experiment is specified in the corresponding figure legend. For membrane reconstitution, proteins were mixed with vesicles at the protein-to-lipid molar ratio of 1:200 with ~0.8 g per 100 ml OG in buffer at
room temperature for 20 min. The mixture was diluted two times with dialysis buffer (25 mM Hepes, 100 mM KCl, 3 % (w/v) glycerol, pH 7.4) and then was dialysed against 2 liters of dialysis buffer at 4 °C overnight. After dialysis, the vesicle was treated with SM-2 beads once and centrifuged at 10,000×g for 5 min to remove protein and lipid aggregates.

**Single vesicle fusion assay**

Details of the single-vesicle FRET imaging were previously reported\(^2\). Briefly, a quartz slide was coated with 99:1 (mol/mol) PEG:biotin-PEG (Laysan Bio, Arab, Alabama) to eliminate non-specific binding of vesicles. The slide was then placed at the bottom of a flow chamber and coated with neutravidin. The v-SNARE vesicles were immobilized on this PEG-treated surface through incubation at 160 pM [vesicle] for 15 minutes via specific biotin-neutravidin binding. The t-SNARE vesicles were diluted to a final vesicle concentration of 200 pM, mixed with preset amounts of complexin I, and injected into the flow chamber for reaction. Such a low concentration of t-SNARE vesicle minimizes interaction of multiple t-SNARE vesicles with a single v-SNARE vesicle. Fusion events on surface were monitored in a wide-field TIR fluorescence microscope (IX-71, Olympus, Melville, New York) using an electron multiplying charge-coupled device camera (iXon DV 887, Andor Technology, South Windsor, Connecticut). All measurements were made at 37 (±2) °C in fusion buffer (25 mM Hepes, 100 mM KCl, pH 7.4). Single fusion events were visually identified using an IDL program (Research Systems, Boulder, Colorado), and the FRET distribution and the time trajectories of fusion dynamics were analyzed with MATLAB programs (programs available upon request). For the real-time tracking experiments of Fig. 3e and Fig. 3f, the oxygen scavenging system consisting of 1 mg ml\(^{-1}\) glucose oxidase (Sigma, St. Louis, Missouri), 0.4% (w/v)D glucose (Sigma), 0.04 mg ml\(^{-1}\) catalase (Roche, Indianapolis, Indiana), and 1% v/v 2-mercaptoethanol (Acros Organics, Morris Plains, New Jersey) was used when flowing the fusion buffer with Ca\(^{2+}\). The use of the oxygen scavenger system slowed down photobleaching of the DiI and DiD dyes (Supplementary Fig. 5) without affecting the fusion reaction.
Spin labelling and EPR measurements

We prepared two cysteine mutants of complexin I, R48C/C105A and R56C/C105A, using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, California). The DNA sequences were confirmed by the DNA facility in Iowa State University. The Complexin I mutants were labelled with (1-oxyl-2,2,5,5-tetramethyl-pyrrolinyl-3-methyl) methanethiosulfonate spin label (MTSSL) at 4 °C while the protein was bound to the GST-agarose beads. To remove unreacted free spin label, the beads containing complexin I were extensively washed with cleavage buffer. The labelled complexin I was cleaved by thrombin. The protein concentration and the spin labelling efficiency were determined by a DC protein assay kit (Biorad, Hercules, California) and the 50 µM TEMPO standard, respectively. The spin labelling efficiency was more than 75%. EPR spectra were obtained using a Bruker ESP 300 spectrometer (Bruker, Germany) equipped with a low noise microwave amplifier (Miteq, Hauppauge, New York) and a loop-gap resonator (Medical Advances, Milwaukee, Wisconsin). The modulation amplitude was set to be no greater than one-fourth of the line width. Spectral data were collected at room temperature in the first-derivative mode with the 1 mM microwave power.

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Figure 1 Single-vesicle FRET analysis of neuronal SNARE-induced membrane fusion. (a) Schematics of the single-vesicle FRET assay. Some t-SNARE vesicles dock to single v-SNARE vesicles immobilized on the surface through formation of trans-SNARE complexes, and complexin I binds to the trans-SNARE complexes. Membrane fusion between t- and v-SNARE vesicles and resultant lipid mixing will entail an increase in the FRET efficiency. (b,c) FRET distribution of basal, neuronal SNARE-mediated membrane fusion. As in a, t-SNARE vesicles were flowed into surface-immobilized v-SNARE vesicles. After incubation for 12 minutes at 37 °C, residual, undocked t-SNARE vesicles were removed by fusion buffer either with 1 mM EGTA (b) or 1 mM Ca\(^{2+}\) (c). A lipid composition of 65:35 (mol/mol) POPC:DOPS was used. FRET values of single-vesicle complexes formed on the surface were measured at \(t = 15\) (blue symbol), 30 (red), and 60 min (yellow) while the temperature of the flow chamber was maintained at 37 °C. The numbers of vesicle complexes included in statistics (n) are 9,602 (blue), 7,410 (red), and 6,032 (yellow) for b and 11,037 (blue), 12,324 (red), and 9,479 (yellow) for c.
Figure 2 Observation of dual functions of complexin I in the absence of Ca$^{2+}$.

FRET values of single-vesicle complexes and number of docked t-SNARE vesicles were measured at $t = 15$, 30, and 60 min at 37 °C. A lipid composition of 65:35 (mol/mol) POPC:DOPS (see Methods) was used for all the data in Fig. 2. See Supplementary Fig. 8b for the number of vesicle complexes studied in each case. (a) Exemplary donor-channel images directly obtained from TIR imaging. Each fluorescence spot represents a single t-SNARE vesicle docked to a v-SNARE vesicle, and the number of fluorescence spots in one imaging area can be unambiguously counted. (b) Docking probability of a single v-SNARE vesicle measured at $t = 15$ min as a function of the Cpx I concentration. Error bars represent SD. (c-e) FRET histograms of single-vesicle complexes measured at $t = 15$ min at [Cpx I] = 0.2 (c), 2 (d), and 20 µM (e). (f) Control data using C105A/R48C-spin label complexin I mutant. (Inset) Room temperature EPR spectra of the spin-labeled mutant when incubated in simple fusion buffer (black trace) or in a solution of vesicles carrying cis-SNARE complexes (red trace). Nearly complete overlap of two curves indicates binding to the ternary SNARE complex is largely abolished for this complexin I mutant (Supplementary Fig. 3), and the FRET population assay (blue symbol: without complexin I, red: with 2 µM mutant; $t = 15$ min) shows the stimulation effect is largely diminished. (g) Control data without SNAP-25. t-SNARE vesicles harboring only syntaxin HT (lipid to protein ratio kept at 200:1) and [Cpx I] = 2 µM were used for
incubation with v-SNARE vesicles. Blue symbols show the FRET distribution measured at $t = 15$ min. (Red symbol) 1 mM Ca$^{2+}$ was injected subsequent to flow washing of t-SNARE vesicles and complexin I, and FRET values were imaged at $t = 60$ min. (h) Time-course change of the single-vesicle FRET histogram at [Cpx I] = 20 µM. Three FRET histograms, measured at $t = 15$ (same data as e, revisited as blue symbols), 30 (red), 60 min (yellow), are shown. See Supplementary Fig. 4 for the time-course changes of c and d.
Figure 3 Complexin-assisted, Ca\(^{2+}\)-triggered single vesicle fusion events. 

(a) Exemplary real-time fusion trace of a single-vesicle complex. (Upper panel) Shown are the changes in the donor (green) and the acceptor (red) fluorescence intensities. (Lower panel) The corresponding changes in the FRET efficiency are shown in blue. The initial and the final FRET states are marked by black bars and the instance of initial FRET increase is marked by an orange arrow. The data in a-d were measured using the lipid composition of 65:35 (mol/mol) POPC:DOPS. (b) (Blue symbol) Cumulative plot of the instances of initial FRET increase for 217 vesicle complexes in one imaging area. (Red and Black symbols) Cumulative plots of the instances of initial FRET increase upon injection of 1 mM Mg\(^{2+}\) (Red) or fusion buffer without Ca\(^{2+}\) (Black). Both cumulative plots are based on multiple numbers of flow experiments; Total number of vesicle complexes studied are 1,161 (Red) and 1,900 (Black). (c) Exemplary real-time traces showing synchronized FRET jumps. The instances of initial FRET increase, marked by orange arrows, are 5.8 and 6.2 sec (from upper to lower). (d) Distribution of the initial $E$ values for the single-vesicle complexes that show an FRET increase within the first 30 second window. Fitting with a Gaussian distribution gives the center at $E = 0.35$ and the
standard deviation of 0.15 (red curve). Total 1,240 vesicles from five independent flow experiments are included in statistics, and the corresponding cumulative time plot is shown as blue symbols in e. (e) Cumulative plots of the instances of initial FRET increase for different lipid compositions: (blue symbol) 65:35 (mol/mol) POPC:DOPS, (red) 45:15:40 (mol/mol/mol) POPC:DOPS:cholesterol, and (black) 85:15 (mol/mol) POPC:DOPS. \(n = 1,240\) (blue), 835 (red), and 1,003 (black). (f) Exemplary real-time traces showing synchronized FRET jumps. The lipid composition of 45:15:40 (mol/mol/mol) POPC:DOPS:cholesterol was used. The instances of initial FRET increase, marked by orange arrows, are 4.8 and 6.2 sec (from upper to lower).
Figure 4 Molecular model of complexin function for Ca\(^{2+}\)-triggered neurotransmitter release.

(a) Room temperature EPR spectra of a spin-labeled complexin I mutant, C105A/R56C-spin label, when incubated in fusion buffer (black trace) or mixed with solution of t-SNARE vesicles (red trace). The spectra are normalized with respect to the maximum heights of the central line, but not with respect to the number of spins. (Inset) EPR spectra of the complexin I mutant having the spin label attached to its native cysteine at position 105. (b) Pre-docking stage: Complexin competes with v-SNAREs for binding to t-SNAREs. At a high expression level, complexin has the effect of suppressing formation of trans-SNARE complexes. (c) Docking stage: Once trans-SNARE complexes are formed, synaptic vesicles are more tightly associated with the plasma membrane, and complexin binds to trans-SNARE complexes. (d) Priming stage: Complexin lowers energy barriers for synaptic vesicle fusion in cooperation with neuronal SNAREs in part due to stabilization of SNARE complexes. (e) Time-course change of the number of docked t-SNARE vesicles. The sequence of experiments is the same as that for Fig. 2. The numbers of docked t-SNARE vesicles at [Cpx I] = 0 (blue circle), 0.2 (red square), and 2 µM (black triangle) were measured at \( t = 15, 30, 60 \) min. Each case was normalized by the docking number measured at \( t = 15 \) min and error bars represent SD (see also Supplementary Fig. 8). (f) Fusion pore opening stage: Triggered by Ca\(^{2+}\) influxes, action of Ca\(^{2+}\)-sensing synaptotagmin I further lowers fusion energy barriers to achieve fast fusion pore opening.
Supplementary Figure 1 SNAP-25 negative control and Ca\textsuperscript{2+}-response of neuronal SNARE-mediated membrane fusion.

(a) t-SNARE vesicles carrying only syntaxin HT (without SNAP-25) were injected using a flow \((t = 0)\) and incubated with surface-tethered v-SNARE vesicles for 12 minutes at 37 °C. After flow washing, FRET values of single vesicle complexes were measured at \(t = 15\) (blue symbol) and 60 min (red). \(n = 4,401\) (blue) and 3,433 (red).

(b) Ca\textsuperscript{2+}-response of neuronal SNARE-mediated fusion. Fusion products are imaged at \(t = 15\) min. \(n = 9,602\) (blue) and 11,037 (red). A lipid composition of 65:35 (mol/mol) POPC:DOPS was used for both (a) and (b).
Supplementary Figure 2 Measurements of the docking probability for a single v-SNARE vesicle.

(a) Number of docked t-SNARE vesicles in one imaging area (50 × 100 µm²), measured at $t = 15$ min. (b) (Left) The number of v-SNARE vesicles in an imaging area were counted by selectively exciting v-SNARE vesicles using a 633 nm red laser. An examplary image, directly obtained from TIR imaging, shows excited v-SNARE vesicles in the acceptor channel. (Right) The measured average is 1,052 ± 39 vesicles/imaging area. Such a uniform surface density of v-SNARE vesicle was obtained through incubation of 160 pM v-SNARE vesicle for 15 minutes. The docking probability of a v-SNARE vesicle is obtained by dividing the number of docked t-SNARE vesicles (a) with the average number of v-SNARE vesicles (b). All error bars represent SD.
**Supplementary Figure 3** EPR spectral analysis on binding of the R48C-spin label mutant to the ternary SNARE complex.

Room temperature EPR spectra of the C105A/R48C-spin label complexin I mutant were measured after the spin label mutant was incubated in buffer (black trace) or in a solution of vesicles with *cis*-SNARE complexes (red trace). Near complete overlap between two traces indicates that the binding to the ternary SNARE complex is largely abolished for the R48C-spin label mutant.
Supplementary Figure 4 Time course changes of the single-vesicle FRET histograms of Fig. 2c, d.

A mixture of t-SNARE vesicles and different concentrations of complexin I: 0.2 (a) and 2 µM (b) was introduced to surface-immobilized v-SNARE vesicles (t = 0). After incubation (12 min, 37 °C) and flow washing, FRET distributions of single vesicle complexes measured at t = 15 (same as Fig. 2c, d, revisited here as blue symbols), 30 (red), and 60 min (yellow) are shown.
PC:PS = 65:35

PC:PS:Chol = 45:15:40
Supplementary Figure 5 Single-vesicle time traces of Ca\(^{2+}\)-triggered fusion events.

(Upper panel) Shown are the changes in the donor (green) and the acceptor (red) fluorescence intensities. (Lower panel) The corresponding changes in the FRET efficiency (blue) are shown. The initial FRET states are marked by black bars, and the 1 mM Ca\(^{2+}\) wave arrives at the imaging area at \(t = 4.4\) sec. (a,b) Single-vesicle fusion traces of Fig. 3c with now the donor and acceptor fluorescence intensities shown. (c-e) Additional single-vesicle fusion traces showing Ca\(^{2+}\)-triggered fusion. The real-time traces of a-e were obtained in one imaging area, of which cumulative time histogram is shown in Fig. 3b (blue symbol). The lipid composition of POPC:DOPS (mol/mol) = 65:35 was used. (f,g) Single-vesicle fusion traces of Fig. 3f with now the donor and acceptor fluorescence intensities shown. (h-j) Additional single-vesicle fusion traces showing Ca\(^{2+}\)-triggered fusion. The real-time traces of f-j were obtained using the lipid composition of POPC:DOPS:cholesterol (mol/mol/mol) = 45:15:40 and the oxygen scavenger system. The use of the oxygen scavenging molecules greatly improved the imaging quality. For example, the real-time traces of f-j show significantly stabilized fluorescence intensity signals in both the donor and the acceptor channels, reflecting much reduced photobleaching in the system. Thus, we were able to increase the laser excitation and the fluorescence intensity levels, which correspondingly gave an increased signal to noise ratio and reduced fluctuations in FRET signals.
Supplementary Figure 6 Ca\textsuperscript{2+}-concentration dependence of Ca\textsuperscript{2+}-triggered fusion events.  
(a) Using the experimental design of Fig. 3, the Ca\textsuperscript{2+}-response of single-vesicle complexes was studied when the Ca\textsuperscript{2+} concentration was reduced to 20 µM. The lipid composition of POPC:DOPS:cholesterol (mol/mol/mol) = 45:15:40 was used for the t- and v-SNARE vesicles (n = 835 (blue) and 326 (red)). The cumulative time plot shows that even with reduced 20 µM Ca\textsuperscript{2+}, more than 10 % of single-vesicle complexes start Ca\textsuperscript{2+}-triggered fusion within the first 30 second window (red symbol). Furthermore, a small population of vesicle complexes, about 5 % (inside blue circle), shows fusion triggering as fast as the 1 mM Ca\textsuperscript{2+} case (blue symbol), indicating dynamic heterogeneity that this small population is much more receptive than other vesicle complexes.  
(b) FRET value distribution of the single vesicle complexes at the moment of the Ca\textsuperscript{2+} addition. 36 % of the total vesicle complexes are already fully fused \((E > 0.5)\) when Ca\textsuperscript{2+} is added.
Supplementary Figure 7 Observation of dual functions of complexin I in the absence of Ca\(^{2+}\) for the PC:PS = 85:15 and the PC:PS:Chol = 45:15:40 cases.

The dual effects of complexin I on neuronal SNARE-mediated fusion (in the absence of Ca\(^{2+}\)) was examined for different lipid compositions: POPC:DOPS (mol/mol) = 85:15 (a-d) and POPC:DOPS:cholesterol (mol/mol/mol) 45:15:40 (e-g). The same experimental design as that for Figure 2 was used. Both the inhibitory effect on docking (a) and the stimulation effect on fusion process (b-g) were preserved regardless of reduced PS concentration or addition of high cholesterol. Error bars represents SD. \(n = 4,508\) (b), 4,167 (c), 2,350 (d), 14,226 (e), 10,202 (f) and 8,988 (g).
Supplementary Figure 8 Time courses of the docking probability as a function of complexin concentration.

(a) We plotted the docking probability at time points of 15, 30, and 60 min as a function of complexin concentration. Error bars represent SD. This plot shows our single-vesicle docking data in a collective way. For example, the docking probability at $t = 15$ min (inside red box) corresponds to the data shown in Fig. 2b, and the time courses of the docking probabilities (inside blue box) are the same as those shown in Fig. 4e. We also emphasize that the same set of vesicles were used for the FRET histogram analysis (Figs. 1 and 2) and the docking analysis (Fig. 4e and Supplementary Fig. 8a), indicating one vesicle pool simultaneously exhibits all the features described in this work, such as slow fusion by SNAREs, dual functions of complexin, and stabilization of SNARE complexes.

(b) Number of vesicle complexes studied for each case.
Supplementary Figure 9 Bulk ensemble measurements of complexin I effects on neuronal SNARE-mediated membrane fusion.

For the bulk ensemble measurement, t-SNARE vesicles are mixed with v-SNARE vesicles with a molar ratio of 9:1, giving the final vesicle concentration of 1 mM. We used the same t- and v-SNARE vesicles and complexins as those for the single-vesicle measurements. Basal fusion kinetics mediated by neuronal SNAREs (black curve) becomes slowed down with increasing concentration of complexin I. Such slowing down of the fusion kinetics by complexin was also observed in Ref. 19. However, it should be noted that Drosophila complexin was used in Ref. 19 while complexin I from rat brain was used throughout our study. Fluorescence intensities of the donor (DiI) and the acceptor (DiD) were monitored (Cary Eclipse, Varian) with the excitation/emission wavelengths pairs of 530/570 nm and 530/700 nm, respectively. The grey control curve shows the fusion kinetics of the negative control without SNAP-25.
CHAPTER 6: SUPRAMOLECULAR SNARE ASSEMBLY PRECEDES HEMIFUSION IN SNARE-MEDIATED MEMBRANE FUSION

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ABSTRACT

Formation of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex facilitates intracellular membrane fusion. A single SNARE complex is thought to be insufficient, and multiple copies of SNARE complexes must work cooperatively. However, the mechanism by which such a high order SNARE structure is assembled is unknown. The EPR and fluorescence analyses show that at least three copies of target membrane t-SNARE self-assemble through the interaction between the transmembrane domains (TMDs) and this multimeric structure serves as scaffolding for trans-SNARE assembly. SNARE core formation in solution induces oligomerization of the TMDs of vesicle associated v-SNAREs in the apposing membrane, transiently forming a supramolecular protein structure spanning two membranes. This high order protein intermediate evolves, by involving lipid molecules, to the hemifusion state. Hemifusion is subsequently followed by distal leaflet mixing and formation of the cis-SNARE complex.

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INTRODUCTION

SNARE-mediated membrane fusion underlies intracellular protein trafficking and intercellular communication such as neurotransmission\(^1\text{-}^4\). Molecular recognition between vesicle-associated v-SNARE and target membrane t-SNARE leads to formation of a coiled coil that bridges two membranes\(^5\text{-}^{11}\). Successful membrane fusion however requires cooperative action of multiple copies of SNARE complexes\(^12\text{-}^{16}\). One obvious reason for such cooperative action is to overcome the repulsion between two apposing membranes\(^17\). The TMDs are thought to play a role in driving oligomerization of SNARE complexes\(^18\text{-}^{22}\). Although the past work revealed information about the likely stoichiometric number of the SNARE oligomers, the process whereby SNARE oligomerization is brought about remains to be understood.

Oligomerization of SNARE complexes at the onset of membrane fusion may have functional implications for some exocytotic events such as neurotransmitter release. A gap junction-like arrangement of multiple SNARE complexes may allow a narrow protein pore traversing two membranes, which is possibly the source of a small foot current observed in the electrophysiological measurements\(^21,23,24\).

Recently, progress has been made in understanding the transitions that the lipids experience during membrane fusion. There is compelling evidence that SNARE-mediated fusion transits through the hemifusion state in which proximal leaflets are merged while the distal leaflets remain unchanged\(^25\text{-}^{31}\). The hemifusion state progresses to full fusion possibly after a few flicker between the hemifusion and the small pore states\(^25\). Recently, it is shown that hemifusion may have important biological implications. For example, in sea urchin the cortical granules are hemifused prior to fusion with the egg plasma membrane\(^31\). In neurons, majority of synaptic vesicles are found to be at the hemifused state on the presynaptic plasma membrane\(^34\).

It is often argued that the gap junction-like protein intermediate is incompatible with the hemifusion intermediate in the fusion pathway for some energetic reasons\(^17\). Therefore, two seemingly mutually exclusive mechanistic models, one based on the hemifusion intermediate\(^25,28,29,35,36\) and the other built around the protein pore\(^37\text{-}^{39}\), have
been developed for SNARE-mediated membrane fusion. In this work, we show that both the two membrane-spanning protein intermediate and the hemifused state do exist sequentially along the fusion pathway. We also investigated the mechanism by which the supramolecular SNARE structure is formed for yeast SNAREs.

RESULTS

TMDs of t-SNARE self-assemble to form an oligomer

In this work, we studied SNAREs that play a role in trafficking in yeast. Yeast SNAREs share functional and structural similarities with neuronal SNAREs that are involved in synaptic membrane fusion. Sso1p and Sec9 are t-SNAREs, analogous to neuronal Syntaxin 1A and SNAP-25, respectively. Sso1p has a transmembrane domain that anchors t-SNARE to the membrane (Fig. 1a). Snc2p is the yeast counterpart of neuronal v-SNARE synaptobrevin (VAMP). For simplicity, we used shorter versions of t-SNAREs Sso1pHT and Sec9c. Sso1pHT lacks the N-terminal regulatory Habc domain and Sec9c contains the homologous region to SNAP-25 (see Method section).

Previously, it was shown that there was a weak interaction among Sso1pHT TMDs in the membrane, which renders equilibrium between the monomeric form and the multimeric form. Here, we used site-directed spin labeling (SDSL) and EPR to analyze equilibrium quantitatively. In SDSL, native amino acid residues are replaced one by one with cysteine to which the nitroxide side chain is attached. The EPR line shape is sensitive to the motional rates of the nitroxide. For monomeric Sso1pHT, no tertiary interaction is expected for residues in the TMD, which would result in relatively narrow EPR spectra reflecting fast motion of the nitroxide side chain. On the other hand, for homooligomeric Sso1pHT we expect the tertiary contacts for several TMD positions, which would instead result in broad EPR spectra reflecting retarded motion of the nitroxide side chain.

In equilibrium, the monomers coexist with the multimers, which, for those positions with the tertiary contacts, gives rise to composite EPR spectra composed of a sharp
component representing the monomer and a broad component representing the multimer (Fig. 1b). As the first approximation, we assume that equilibrium is between the monomeric species and a single multimeric form with the stoichiometric number n:

\[ nM \rightleftharpoons K \rightarrow Mn \]  

(1)

where \( K \) represents the equilibrium constant. Based on this simple two state model, the fraction of the homomultimer \( f \) and that of the monomer \( (1-f) \) are readily obtained using a standard EPR spectral subtraction analysis (Fig. 1b).

The EPR spectral analysis was performed for four different TMD positions (269, 272, 273, 284), which showed clear tertiary interactions in the EPR spectra\(^{40} \). The \( f \) values were calculated at four different surface protein densities (L/P ratio \( C = 400:1, 200:1, 100:1, 50:1 \)), for each spin labeled mutant reconstituted into the POPC vesicle containing 15 mol\% negatively charged DOPS. The variation of \( f \) as a function of \( C \) was collectively fitted to the equation representing equilibrium (1) (see the caption of Fig. 1c) to determine the best fitting values of \( n \) and \( K \). The best fitting \( n \) value was four (Fig. 1c). However, other \( n \) values except \( n=2 \) also fitted the data reasonably well although the corresponding statistical tests were somewhat worse (Supplementary Table 1). Therefore, we conclude that at least three Sso1pHT molecules self-associate to form the oligomer. The composite EPR spectra were not altered at all by the addition of Sec9c at all L/P ratios for each mutant, indicating that the equilibrium properties observed for Sso1pHT alone are virtually the same for t-SNARE.

**SNARE assembly vs. oligomerization of the v-SNARE TMDs**

Our EPR results showed that at least three copies of the Sso1pHT TMD cluster together to form a homooligomeric structure in the membrane. In contrast, it was previously shown that the TMDs of v-SNARE Snc2p exist as monomers\(^{28} \). Therefore, we wonder if multimeric Sso1pHT provides scaffolding for formation of the initial fusion complex at the fusion active zone. Here, we use site-specific fluorescence labeling to investigate the dynamic changes occurring during SNARE assembly and membrane
fusion. As it was the case with EPR, cysteine-free versions of Sso1pHT and Snc2p were used to generate two N-terminal mutants Sso1pHT E185C (Nt) and Snc2p P13C (Nv), and two C-terminal mutants Sso1pHT R290C (Ct) and Snc2p S115C (Cv). The Sso1pHT mutants were derivatized with the fluorescence donor Cy3 maleimide, while the Snc2p mutants were reacted with the acceptor Cy5 maleimide. The labeled proteins were reconstituted into the POPC vesicle containing 35 mol% negatively charged DOPS.

The fusion reaction was conveniently initiated by adding Sec9c to the mixture of the t-SNARE vesicles and the v-SNARE vesicles. We first monitored SNARE complex formation employing Nt-Cy3 and Nv-Cy5 (N-N). Upon addition of Sec9c a rapid increase of fluorescence was observed for the acceptor Cy5, while rapid decrease was observed for the donor Cy3, indicating that fluorescence resonance energy transfer (FRET) happened due to formation of the SNARE core (Fig. 2a). As controls, when the t-SNARE vesicles with Nt-Cy3 were mixed with the unlabeled v-SNARE vesicles or vice versa, we did not observe any substantial spectral changes in time (Fig. 2b, pink and cyan traces), suggesting that the observed fluorescence changes were mainly due to the energy transfer from the fluorescence donor to the acceptor. Likewise, when the t-SNARE vesicles with Ct-Cy3 were reacted with the unlabeled v-SNARE vesicles by adding Sec9c, no fluorescence change was detected (Fig. 2b, blue trace), suggesting that the TMDs of Sso1pHT remained unchanged during SNARE core formation. In contrast, when the v-SNARE vesicles carrying Cv-Cy5 was reacted with unlabeled t-SNARE vesicles, the fluorescence intensity decreased rapidly (Fig. 2b, red trace), suggesting the possibility of self-quenching due to the self-association of the v-SNARE TMDs in the membrane. Most interestingly, the kinetics of this particular fluorescence change matched with that of SNARE assembly detected at the N-terminal tip of the SNARE core, showing that the change reported by Cv-Cy5 at the C-terminal end occurs concurrently with SNARE complex formation.

To make sure that the observed fluorescence change is due to the change in the inter TMD interactions, but not due to the environmental changes surrounding individual TMDs, Cv-Cy5 was diluted with the three-fold excess of wild-type Snc2p before
reconstitution into vesicles. No fluorescence change was observed for this diluted sample (Supplementary Fig. 1), supporting that the fluorescence change here is most likely due to self-quenching and that the TMDs of Snc2p self-associate during SNARE assembly. Therefore, as self-quenching is not observed either on N- or C-termini of t-SNARE Sso1pHT but the fluorescence label attached to the C-terminus of v-SNARE Snc2p, the results strongly support the idea that the Sso1pHT oligomer recruits Snc2p to the fusion active zone to form a supramolecular SNARE complex that traverses two closely apposed membranes.

Supramolecular SNARE assembly is followed by hemifusion

How do the changes in SNARE structure correlate with the changes in membrane structure? There is evidence that SNARE-mediated fusion transits through the hemifusion intermediate. We asked if hemifusion was concurrent with or sequential to formation of the initial supramolecular complex. Lipid mixing was measured by a well established method employing fluorescent lipids. Briefly, the v-SNARE vesicles were doped with 2 mol% each of the fluorescent acceptor rhodamine-PE and the fluorescence donor NBD-PS. Membrane fusion causes the dilution of the fluorescent probes, which can be measured as the recovery of the donor fluorescence signal. To detect hemifusion, it is necessary to separate outer leaflet mixing from inner leaflet mixing. Such separation can be achieved by measuring total lipid mixing which is the sum of inner and outer leaflet mixing and inner leaflet mixing independently. Inner leaflet mixing was measured using the modified Meers’ method that employs the dithionite reduction of NBD on the outer leaflet to non-fluorescence ABD. The results show that outer leaflet mixing occurred faster than inner leaflet mixing (Fig. 3), confirming the existence of the hemifusion intermediate. Further, when the kinetics of outer leaflet mixing is compared with that of clustering of v-SNARE TMDs, the former occurs slower than the latter (Fig. 4). The quantitative analysis of the data based on the second order kinetics (Fig. 4a, solid lines) showed that the second order rate constant $k_2$ for supramolecular SNARE assembly is
about 4 times faster than that of hemifusion (**Fig. 4b**). Thus, the results show that hemifusion follows formation of the initial fusion complex sequentially.

**cis-SNARE complex formation vs. inner leaflet mixing**

In the membrane fusion pathway, hemifusion is followed by opening of the fusion pore. Pore opening accompanies inner leaflet mixing. Further, the v- and t-SNARE TMDs merge into the same bilayer to form the *cis*-SNARE complex. We expect that formation of the *cis*-SNARE complex will occur concurrently with inner leaflet mixing. To verify this, we used C-terminus labeled t- and v-SNAREs (C-C) in our FRET fusion assay. We first reconstituted the mixture of Cv-Cy5 and wild-type Snc2p in the ratio of 1:3 into one population of vesicles. These vesicles were then reacted with another population of vesicles containing Ct-Cy3 and wild-type Sso1pHT in the ratio of 1:3. In this experiment, we diluted the labeled proteins with the corresponding wild-types to avoid the potential complications arising from self-quenching. When Sec9c was added to the reaction, an increase of the fluorescence intensity in the acceptor channel (Cy5) and a decrease of the fluorescence intensity in the donor channel (Cy3) were observed (**Fig. 4c**). The results show that the distance between Cy5 and Cy3 was decreased, most likely due to the co-localization of v- and t-SNARE TMDs in the newly merged single bilayer. The time scale of the fluorescence change was consistent with that of inner leaflet mixing (**Fig. 4**). Therefore, we conclude that formation of the *cis*-SNARE complex occurs concurrently with inner leaflet mixing, but slower than hemifusion. The data analysis (**Fig. 4a**, solid lines) showed that the $k_2$ value for *cis*-SNARE complex formation is about 3 times slower than that of hemifusion (**Fig. 4b**). The data are however insufficient to tell if there was association between the v- and t-SNARE TMDs because FRET can be observed in several nm range in the absence of physical contacts.

**DISCUSSION**

SNARE complex formation may drive membrane fusion. A recent force measurement however showed that the energy from a single SNARE complex might not be sufficient to
overcome the full fusion energy barrier\textsuperscript{42}. Therefore, multimerization of SNAREs appears to be essential for successful fusion. Consistently, the existence of high order SNARE complexes was confirmed in squid synaptosomes and brain by extraction\textsuperscript{43} as well as in artificial membrane by atomic force microscopy\textsuperscript{44}.

Quantitatively, a kinetic analysis of exocytosis in cracked PC12 cells showed that at least three SNARE complexes work together for membrane fusion\textsuperscript{12}. Based on the EPR analysis of equilibrium between the Sso1pHT TMD monomers and the oligomers, we estimated the likely stoichiometry of the TMD oligomer for yeast SNAREs is three or higher, which is fully consistent with the previous results for neuronal SNAREs by Scheller and coworkers\textsuperscript{12}.

We used fluorescence self-quenching between identical probes to assess the clustering of the v-SNARE TMDs, which is driven by SNARE core formation. Self-quenching is photophysically different from homo-FRET between two identical dyes. While homo-FRET can measure distances up to 3-6 nm by analyzing fluorescence polarization\textsuperscript{45}, self-quenching requires close physical proximity between dyes and is only visible when the dyes are within ~1 nm\textsuperscript{46,47}. Therefore, self-quenching observed at the C-terminal end of Snc2p is a strong indicator for self-association of the v-SNARE TMDs in the membrane.

The combined EPR and fluorescence analysis of SNARE assembly showed that the very early fusion intermediate might be a protein-only supramolecular SNARE complex that spans two membranes (Fig. 5a,b). Such a protein intermediate is assembled through a dynamic process in which the preformed t-SNARE homooligomer recruits v-SNAREs via SNARE complex formation (Fig. 5a,b). Previously, Chapman and Jackson predicted, with little direct evidence, formation of a gap junction-like protein pore for neuronal SNAREs at the onset of membrane fusion\textsuperscript{21,37}. They envisioned that this hypothetical transient protein pore harbors early aqueous passage through which small amount of neurotransmitters can be released from the synaptic vesicles to the synapse. It is unknown, however, if the initial fusion complex formed by yeast SNAREs harbors such a pore within the supramolecular structure.
Our results clearly demonstrate that hemifusion follows formation of the transient protein intermediate sequentially (Fig. 5c). How does it go from the protein-only supramolecular structure to the lipid-rich hemifusion state? It is possible that the lipid stalk-like state emerges at the center of the protein complex (Fig. 5c), which requires the lateral diffusion of the individual TMDs into the lipid matrix. In this process, the lipid molecules would be fed into the central zone to establish a stalk-like structure in the middle. Favorable to this model, the Sso1pHT TMDs are held together by weak interactions\(^{40}\), and therefore, such lateral diffusion of the TMDs can occur rather naturally as part of equilibrium fluctuation between the monomeric TMD and the oligomeric form. We speculate that this hypothetical stalk corralled by the SNARE TMDs is lower in free energy than the lipid-only stalk. Alternatively, outer leaflet mixing can occur through the hydrophobic surface of the transient protein complex\(^{37}\). Even in this case, the lipid molecules must be fed into the central zone during the lateral expansion of the TMDs. It is hard to imagine, for energetic reasons, that the lipids in the central region form the lipid-lined pore directly without forming an initial stalk-like structure\(^{17}\). At minimum, for SNARE-mediated fusion, the lipid-only stalk model must be revised to incorporate the presence of the TMDs that are concentrated in high numbers in the fusion active zone.

The hemifusion state transitions to the fusion pore, which completes the merging of the two membranes (Fig. 5d). It is reasonable to expect the disruption of the SNARE TMD oligemer in this stage. In our EPR data, several positions in the Sso1pHT TMD including 269, 273, 276, 281, 282, 286, 287 and 288 showed a reduction of the broad components in the \(cis\)-SNARE complex compare to those for Sso1pHT alone (Supplementary Fig. 2). The decrease of the broad components may reflect the dissociation of the Sso1pHT TMD oligemer. In our fluorescence experiment, however, we did not observe self-dequenching from C-terminus-labeled Sso1pHT reflecting such dissociation of TMD oligomer during fusion. One possible explanation for this is that in the bulk fusion assay, most of the vesicles remain at docked state. For docked vesicles, only about 10% of them progresses to the hemifused or full fused states while 90% of them remained to be docked without fusion. In the docked state, we predict that the
SNARE complex maintains the oligomeric form. Since only a small fraction of vesicles would show dissociation due to fusion, the change may be too small to be detectable in our bulk fusion assay. The recent developed single fusion assay\textsuperscript{25} may be used to resolve this issue. In this method, the docking and subsequent fusion steps are clearly resolved in a single fusion level and the expected dissociation of the TMDs can be readily detected.

Using FRET we found that inner leaflet mixing is concurrent with formation of \textit{cis}-SNARE complex, indicating that the \textit{trans}-to-\textit{cis} transition of the SNARE TMDs may be closely correlated with fusion pore opening. Our results also suggest that during inner leaflet mixing the fluorescent dyes in the two separate contents cross the aqueous pore so that they could be co-located on a newly merged single membrane.

The analysis of the kinetic data (\textbf{Fig. 4b}) showed the second order rate constant $k_2$ for inner leaflet mixing is three times slower than that of outer leaflet mixing. The result implies that the activation energy for the former is $\sim 0.7$ kcal/mole higher than that of latter. SNARE complex formation must provide the energy required to overcome the activation energy barriers for both hemifusion and pore opening. Supposing that SNARE core formation drives primarily hemifusion\textsuperscript{42}, where does then the extra energy for pore opening come after hemifusion? Hypothetically, the energy from the SNARE complex may be released in two steps, initially, for hemifusion and later, for pore opening. A single molecule force measurement revealed the possibility of such stepwise release of the energy via two-step assembly of the SNARE core complex, the membrane-distal region first and the membrane-proximal region later\textsuperscript{42}.

\textbf{MATERIALS AND METHODS}

\textbf{Plasmids and site-directed mutagenesis}

DNAs encoding Sso1pHT (amino acids 185–290 of Sso1p) and Snc2p (amino acids 1–115) were inserted into the pGEX-KG vector between \textit{Eco}RI and \textit{Hind}III sites as N-terminal glutathione \textit{S}-transferase (GST) fusion proteins. Sec9c (amino acids 401-651 of Sec9) was inserted into pET-24b(+) between \textit{Nde}I and \textit{Xho}I sites as a C-terminal
His_{6}-tagged protein. To introduce a unique cysteine residue for the specific nitroxide attachment, native cysteine 266 of Sso1pHT was mutated to alanine. We used the Quick Change site-directed mutagenesis kit (Stratagene) to generate all cysteine mutants; DNA sequences were confirmed by the Iowa State University DNA Sequencing Facility.

**Protein expression, purification, and labeling**

Expression of recombinant GST fusion proteins was conducted in *Escherichia coli* Rosetta (DE3) pLysS (Novagene). Cells were grown at 37°C in the LB medium with glucose (2 g/l), ampicillin (100 µg/ml), and chloramphenicol (25 µg/ml) until $A_{600}$ reached 0.6-0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM final concentration) was then added to induce protein expression. Cells were grown for additional 4 hours at 16°C. Cell pellets were then harvested by centrifugation at 5,524 g for 10 min.

Purification of GST fusion proteins was achieved with affinity chromatography using glutathione-agarose beads (Sigma). The frozen cell pellets were resuspended in PBS buffer (phosphate-buffered saline, pH 7.4, with 0.2% (v/v) Triton X-100, PBST) with 2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), and 5 mM dithiothreitol (DTT). Cells were broken by sonication in an ice bath and then centrifuged at 14,500 g for 20 min at 4°C. The supernatant was mixed with the glutathione-agarose beads in the same PBS buffer and nutated at 4°C for 120 min. The protein was cleaved from the resin by thrombin (Sigma) at room temperature for 40 min. The protein was stored at -80°C with 10% (v/v) glycerol.

Cysteine mutants of Sso1pHT were spin labeled while the proteins were bound to the beads. About 20-fold excess of (1-oxy-l-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate spin label (MTSSL) was added to the column. The reaction mixture was left overnight at 4°C. Free MTSSL was removed by washing with excess PBS buffer with 0.2% (v/v) Triton X-100. The protein was cleaved by thrombin in the cleavage with 0.2% (v/v) Triton X-100 or 0.8% (w/v) OG.

Labeling of the Sso1pHT and SnC2p cysteine mutants with fluorescent probes was carried out after thrombin cleavage. Sso1pHT E185C and Sso1pHT R290C proteins were
labeled with Cy3 maleimide, Snc2p P13C and Snc2p S115C were labeled with Cy5 maleimide (Amersham). Free dyes were removed from the proteins by using PD-10 desalting columns (Amersham).

The His₆-tagged protein Sec9c was expressed in E. coli Rosetta (DE3) pLysS. For purification, the frozen cell pellet was resuspended in lysis buffer (PBS buffer with 20 mM imidazole, 0.2% (v/v) Triton X-100, 2 mM AEBSF, pH 8.0). After sonication, the supernatant was mixed with nickel-nitrilotriacetic acid-agarose beads (Qiagen) in the lysis buffer. The mixture was nutated for binding at 4°C for 120 min. After binding, the beads were washed with washing buffer (PBS buffer with 50 mM imidazole, pH 8.0). Then the protein was eluted by elution buffer (PBS buffer with 250 mM imidazole, pH 8.0). The proteins were stored at −80°C with 10% (v/v) glycerol. All purified proteins were examined with 15% SDS-PAGE.

**Membrane reconstitution**

Large unilamellar vesicles (~100 nm in diameter) of 1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphatidylcholine (POPC) containing 15 or 35 mol% 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) were prepared in a detergent-free Hepes buffer (25 mM Hepes, 100 mM KCl, pH 7.4) using an extruder. The total lipid concentration was 100 mM. To study the stoichiometry of the Sso1pHT TMD oligomer, the proteins were reconstituted into the vesicles by the Bio-Beads method. The proteins were mixed with the vesicles at a series of the molar L/P ratios (400:1, 200:1, 100:1, 50:1). The detergent was removed by treating the sample with Bio-Beads SM2 (Bio-Rad), which was directly added to the sample in the ratio of 200 mg/ml. After 45 min of nutation, the Bio-Beads were removed from the sample by centrifugation at 10,000 g for 1 min. The Bio-Beads treatment was repeated three times. The reconstitution efficiency was estimated by determining the protein concentration using EPR before and after reconstitution. The reconstitution efficiency was nearly quantitative.
EPR data collection

The EPR spectra were collected using the Bruker ESP 300 spectrometer equipped with a loop-gap resonator. The modulation amplitude was set at no greater than one-fourth of the line width. Spectra were collected at room temperature.

Lipid mixing assay

Sso1pHT was reconstituted into the vesicles containing POPC and DOPS (molar ratio 65:35) in an L/P ratio of 200:1. Snc2p was reconstituted into the vesicles containing POPC, DOPS, NBD-PS (1,2-dioleoyl-sn-glycero-3-phosphoserine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)), and rhodamine-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyle)) in the molar ratio of 62:35:1.5:1.5. To measure lipid mixing, the v-SNARE (Snc2p) vesicles were mixed with the t-SNARE (Sso1pHT) vesicles and Sec9c in the ratio of 1:1:1. The final lipid concentration was 1 mM. The fluorescence intensity was monitored with the excitation and emission wavelengths of 465 and 530 nm, respectively. The fluorescence signal was recorded by a Varian Cary Eclipse model fluorescence spectrophotometer using a quartz cell of 100 µl with a 2-mm path length. After 3,600 s, 0.1% (v/v) reduced-triton was added to obtain the maximum fluorescence intensity (MFI). The lipid mixing assay was carried out at 35°C. The inner leaflet mixing assay was modified from the method developed by Meers et al. The details of the method was described elsewhere. After collecting the time traces of total lipid mixing (\( P_T \)) and inner leaflet mixing (\( P_I \)) separately, the time trace for outer leaflet mixing was calculated as \( 2P_T - P_I \), where \( P_T \) is the percentage of maximum for total lipid mixing and \( P_I \) is the percentage of maximum for inner leaflet mixing.

Fluorescent assay for SNARE assembly and self-quenching

Preparation for the fusion reaction was nearly identical to that described above for the lipid mixing assay except that the vesicles did not contain lipid dyes. The L/P ratio was 200:1 and the final lipid concentration was 1 mM. The fluorescence intensity was
monitored in two channels with the excitation wavelength of 555 nm and emission wavelengths of 570 and 668 nm, respectively. To measure self-quenching, fluorescence intensity was monitored with the excitation and emission wavelengths of 555 and 570 nm for the cy3 labeled proteins and with the excitation and emission wavelengths of 625 and 668 nm for the cy5 labeled proteins.

REFERENCES


Figure 1 Determination of stoichiometry of the Sso1pHT TMD oligomer using EPR.

(a). The primary structure of Sso1pHT. Sso1pHT contains the amino acids 185-290 of Sso1p. This polypeptide includes both the SNARE motif and the transmembrane domain, which are represented by the black and gray rectangle, respectively. The amino acid sequence of part of the linker region and the transmembrane domain is shown. Positions selected for the EPR analysis are labeled by asterisks.

(b). EPR spectra of I273C collected at the several L/P ratios are shown. Each spectrum is decomposed into a sharp EPR component (black trace) and a broad component (blue trace).

(c). Natural log of the L/P ratios (C) is plotted vs. fraction of oligomeric Sso1pHT f for each spin-labeled mutant. The data was fitted with the equation, \[ \ln C = \frac{\ln K + \ln n + n \times \ln(1 - f) - \ln f}{1 - n} \]. The variable K represents the equilibrium constant. The best fitting values of n and lnK were 4 and 18.4 (black trace). For comparison, the curves representing n = 2 through n = 5 are shown.
Figure 2 Fluorescence detection of SANRE assembly at different locations.

(a). SNARE complex formation was monitored at the N-termini. Fluorescence changes are shown for the acceptor channel (blue trace) and for the donor channel (red trace). The fusion reaction was initiated by adding Sec9c to the mixture of the t-SNARE vesicles containing Cy3 labeled Sso1pHT E185C (Nt-Cy3) and the v-SNARE vesicles carrying Cy5 labeled Snc2p P13C (Nv-Cy5) (upper panel). (b). Self-quenching occurs for C-terminus labeled Snc2p due to clustering of the v-SNARE TMDs in the membrane. Self-quenching was detected by the fluorescence changes when vesicles reconstituted with Cy5 labeled Snc2p S115C (Cv-Cy5) was mixed with vesicles carrying unlabeled Sso1pHT and Sec9c (red trace). In contrast, we did not observe fluorescence changes for other three labeled proteins Sso1pHT E185C-Cy3 (Nt-Cy3, pink trace), Sso1pHT R290C-Cy3 (Ct-Cy3, blue trace) and Snc2p P13C-Cy5 (Nv-Cy5, cyan trace) in the same assays.
Figure 3 Fluorescence lipid mixing assay.

Fluorescence changes for total lipid mixing (red trace), inner leaflet mixing (black trace) and outer leaflet mixing (blue trace), normalized with respect to the maximum fluorescence intensity (MFI) obtained by adding 0.1% reduced-triton, are shown for the L/P ratio of 200:1. Outer leaflet mixing $P_O$ was calculated using the equation $P_O = 2P_T - P_I$, where $P_T$ is the percentage of maximum for total lipid mixing and $P_I$ is the percentage of maximum for inner leaflet mixing. The gray trace is a control run with the t-SNARE vesicles and the v-SNARE vesicles without Sec9c.
Figure 4 Kinetic comparison of various fluorescence assays.

(a). Analysis of various fluorescence assays based on second order kinetics. Red trace, the Snc2p115C-Cy5 (Cv-Cy5) self-quenching kinetics; blue trace, the N-N assembly kinetics; pink trace, outer leaflet mixing; black trace, inner leaflet mixing; cyan trace, the \textit{cis}-SNARE complex formation kinetics. The solid line in each trace is the best fit to the equation, \( F(t) = C_0^2 k_2 t / (1 + C_0 k_2 t) \) representing the second order kinetics, where \( F \) is the fluorescence change, \( C_0 \) is the initial vesicle concentration, and \( k_2 \) is the second order rate constant. For the individual time traces, the fluorescence intensities at 3,000 sec were normalized to 100 in arbitrary units. The symbols shown here are the same as in figure 2.

(b). A bar graph for the relative \( k_2 \) values for different kinetic traces. The error bars represent the s.d. from three independent measurements. The \( k_2 \) value for inner leaflet mixing was set at one and those for the other kinetics were scaled accordingly. (c). Measurement of \textit{cis}-SNARE complex formation using fluorescence. The vesicles reconstituted with the mixture of Snc2p S115C-Cy5 (Cv-Cy5) and unlabeled Snc2p in the ratio of 1:3 were reacted with the vesicles containing Sso1pHT R290C-Cy3 (Ct-Cy3) and unlabeled Sso1pHT in the ratio of 1:3 by adding Sec9c (upper panel). Fluorescence changes were observed for acceptor channel (blue trace) and donor channel (red trace).
Figure 5 A mechanistic model for SNARE assembly and membrane fusion.

(a). v-SNARE on the vesicle (red) and t-SNARE on target membrane (blue) interact to facilitate the apposition of two membranes. The t-SNARE TMDs form an oligomeric (most likely tetrameric) structure in the membrane. (b). The Sso1pHT homooligomer provides scaffolding for formation of the supramolecular SNARE complex at the fusion site. (c). Hemifusion. (d). Fusion pore opening coincides with cis-SNARE complex formation. Cyan dots and red dots on membrane represent NBD and rhodamine, respectively.
Supplementary figure 1 Self-quenching measurement for Cy5 labeled Snc2p S115C (Cv-Cy5) at different conditions. The red trace represents the fluorescence change when the v-SNARE vesicles with Cv-Cy5 were reacted with the t-SNARE vesicles with wild-type Sso1pHT by adding Sec9c. The blue trace represents the fluorescence change when the v-SNARE vesicles with the mixture of Cv-Cy5 diluted with the three-fold excess of wild type Snc2p were reacted with the t-SNARE vesicles with wild-type Sso1pHT by adding Sec9c.
Supplementary figure 2 EPR spectra of the spin labeled Sso1pHT mutants in the self-assembled tetramer and those in the cis-SNARE complex.

The nitroxides were attached to the indicated positions in the TMD. Spin labeled Sso1pHT and the cis-SNARE complex were reconstituted into the vesicles at the lipid-to-protein ratio of 50:1, a condition in which Sso1pHT exists predominantly as the tetramer. The EPR spectra corresponding to the various Sso1pHT TMD positions within the homotetramer are shown in column a, while those of the cis-SNARE complex are shown in column b.
**Supplementary table 1** Statistical analysis of the fits in Fig. 1c

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*DoF: degree of freedom
CHAPTER 7: GENERAL CONCLUSIONS

SUMMARY

In this dissertation, the kinetics of membrane fusion of liposomes mediated by neuronal SNAREs syntaxin1A, SNAP-25, and synaptobrevin was examined using fluorescence assays that monitored both the total lipid mixing and the inner leaflet mixing. The kinetics difference in the inner and outer leaflet mixing suggested that they might occur sequentially. Outer leaflets likely mix faster than inner leaflet. At the beginning of the fusion reaction, the fluorescence change was mainly due to outer leaflet mixing, indicating the hemifusion was the dominant event. As time progressed, however, the percentage of hemifusion decreased dramatically. The results provide strong kinetic evidence for the conversion of hemifusion to complete fusion over time and therefore support the sequential mechanism in which hemifusion is an on-pathway intermediate, instead of the parallel mechanism in which hemifusion is an off-pathway product.

The effects of soluble synaptotagmin (C2AB) and calcium on hemifusion was investigated using reconstituted fusion assay. Although C2AB•Ca\(^{2+}\) stimulates both inner and outer leaflet mixings in neuronal SNARE induced fusion, it stimulates significantly more outer leaflet mixing than inner leaflet mixing, resulting in the accumulation of hemifusion. Unlike the case of neuronal SNAREs, the stimulation of the fusion by C2AB•Ca\(^{2+}\) was not seen for other SNAREs involved in the trafficking in yeast, indicating that the C2AB•Ca\(^{2+}\) stimulation was SNARE-specific.

The fluorescence fusion assay was then employed to study the function of complexin derived from Drosophila on SNARE mediated membrane fusion. The fusion driven by neuronal SNAREs, but not by their yeast equivalents, was inhibited by complexin. Total lipid mixing is significantly inhibited by about 46% on average, and little or no inner leaflet mixing was detected in the presence of the excess complexin. These data are consistent with complexin permitting mixing of the outer leaflet, but inhibiting mixing of the inner leaflet, which suggests that complexin may arrest fusion at hemifusion. When synaptotagmin I is added in the presence of EGTA with Drosophila complexin, fusion
proceeded with kinetics similar to complexin alone. However, when synaptotagmin I is activated by the addition of calcium at ~13 minutes, a rapid relief of inhibition by complexin is observed for both total lipid mixing and, more importantly, inner leaflet mixing. This result suggests that complexin and synaptotagmin I may cooperate to inhibit SNARE mediated fusion in the absence of calcium, which could serve as a preparation stage for the immediate release following calcium addition.

Considering the limitation of bulk fusion assay in dissecting the different stages of the membrane fusion such as docking, hemifusion and complete fusion, a new fusion assay method based on total internal reflection fluorescence (TIRF) microscopy has been developed to study the function of complexin I derived from rat. The results show that complexin I has two opposing effects on trans-SNARE assembly: Inhibition of SNARE complex formation and stabilization of the assembled SNARE complexes. Remarkably, SNARE-mediated fusion is markedly stimulated by complexin I, and further accelerated by two orders of magnitude in response to an externally applied Ca\(^{2+}\) wave. In single-liposome FRET assay, docking and fusion steps are clearly distinguished since undocked t-SNARE liposomes are invisible in this imaging system. As a result, it is able to observe real-time ‘triggering’ of fusion process by Ca\(^{2+}\) flow and to study the synchrony level of fusion events in a given pool of single-liposome complexes.

Our assay should be applicable to the studies of other fusion regulators of the synaptic membrane fusion including synaptotagmin I. Since the Ca\(^{2+}\)-triggered fusion events in the presence of complexin I are synchronized on the seconds time scale, additional lowering of the fusion energy barrier, most likely by the action of synaptotagmin I, is required to push the synchrony level down to milliseconds.

REFERENCES

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