Investigation of the molecular mechanism of SNARE-mediated membrane fusion

Bin Lu
Iowa State University
Investigation of the molecular mechanism of SNARE-mediated membrane fusion

by

Bin Lu

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Program of Study Committee:
Yeon-Kyun Shin, Major Professor
   Linda Ambrosio
   Reuben J. Peters
   Edward W. Yu
   Alan A. DiSpirito

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Intracellular membrane fusion is essential for eukaryotic cell life. Synaptic vesicle exocytosis is an exquisitely regulated form of intracellular membrane fusion. In vivo, neurotransmitter release is triggered by the Ca\(^{2+}\) influx within submillisecond timescale. SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins are believed to be the minimal fusion machinery which serves as the energy source to overcome the energy barrier between fusion intermediates. In addition to SNAREs, many other regulatory proteins and lipids also exert their effects in this calcium-dependent regulated exocytosis.

Site-directed spin labeling (SDSL) and electron paramagnetic resonance (EPR) spectroscopy are well established techniques to study the structure and topology of membrane proteins in lipid bilayers. Fluorescent labeled lipid mixing assay is employed to test the functional activity of SNARE proteins and regulators in vitro. To characterize the specific steps in the fusion pathway, the single-vesicle lipid and content mixing assay are developed based on the total internal reflection fluorescence (TIRF) microscopy.

With the advantage of these techniques, we can investigate the interplay between SNAREs and regulatory proteins and lipids in synaptic vesicle exocytosis and the mechanisms of how the interplay between them regulates the rapid membrane fusion. In this dissertation, the conformational change of SNAP-25 along the SNARE assembly pathway has been studied and the mechanism of neurotoxins blocking neurotransmitter release has been examined. The function of the N terminus and accessory \(\alpha\)-helix of complexin has been disclosed based on the structural analysis of complexin/SNARE quaternary complex. One of key lipid components in the membrane, phosphatidylethanolamine (PE), is also involved in the study to figure out the mechanism of regulatory lipids in the arrangement of SNAREs beyond curvature effect. The information gathered from these detailed investigations will contribute to elaborate the molecular mechanism of SNARE-mediated membrane fusion.
CHAPTER 1: GENERAL INTRODUCTION

1.1 SNARE proteins and membrane fusion

The intracellular vesicle trafficking between the organelles enclosed by membranes is essential to the life of eukaryotic cells. The whole process includes vesicular budding from the donor organelle, then transport, targeting and finally fusing with the membrane of the acceptor organelle[1]. Our research focuses on the final step: the membrane fusion between the vesicles and the target membrane, which can be further divided into docking, priming, hemifusion and full fusion. During the membrane fusion, two separate membranes merge together, involving the dramatic membrane structural disturbance and remodeling. The fusion process has to overcome several high energy barriers on the fusion pathway[2]. SNAREs (soluble N-ethylmaleimide sensitive factor attachment protein receptors) have been proposed as the minimal fusion machinery in almost all the intracellular membrane fusion. The similarity of this superfamily is an evolutionarily conserved domain known as the SNARE motif, which is made up of about 60-70 amino acids arranged in heptad repeats[3]. Functionally, SNARE proteins can be classified into vesicle associated (v-) SNARE and target membrane (t-) SNARE[4]. The v-SNARE interact with the cognate t-SNAREs to form a four-helix SNARE bundle[5], which brings the two membranes into close proximity, merging them into one continuous bilayer[6]. The objective of this research is to investigate the structure and function of neuronal SNARE proteins and the interplay with the regulatory proteins and lipids during membrane fusion.

The neuronal SNAREs is a well characterized example of the SNARE superfamily, including three components (Fig. 1): synaptobrevin 2 or VAMP 2 (vesicle-associated membrane protein 2) on the synaptic vesicles[7], syntaxin 1 on the target plasma membrane[8] and a soluble protein SNAP-25 (synaptosome-associated protein 25 kDa)[9] which could be tethered to the plasma membrane by the palmitoylation[10]. VAMP 2 and syntaxin 1 are membrane integral proteins. They contain a single transmembrane domain (TMD) at the C-
terminus and one SNARE motif in the membrane proximal region. SNAP-25 has two
SNARE motifs connected by a flexible linker region, SN1 at the N-terminus and SN2 at the
C-terminus. Syntaxin 1 also contains a regulatory N-terminus, named Habc domain, which
could form a three-helix bundle and bind to its SNARE motif, thereby isolating syntaxin 1
from interactions with other SNAREs.

In vivo, neurotransmitter release is tightly controlled by calcium[11, 12]. Therefore, it is
conceivable that there should be calcium sensors in neurons that respond the Ca\(^{2+}\) influx and
promote the rapid synaptic vesicle exocytosis. Several candidates, such as synaptotagmin and
complexin, have been proposed to play the key role in the Ca\(^{2+}\)-dependent regulation[13-18].

1.2 The SNARE hypothesis: the minimal fusion machinery

The SNARE complex is associated by the SNARE motif, which is mostly unstructured when
SNAREs are monomeric and undergoes dramatic conformational change during the assembly
process[19]. The SNARE core complex formed by the soluble SNARE motifs is extremely
stable, resistant to SDS denaturation, neurotoxin cleavage and thermally stable up to 90°C
heating[20-22]. These unusual characteristics lead to the hypothesis that the energy released
from the formation of the SNARE complex might be provided to lower the energy barrier
between fusion intermediates[23].

The X-ray crystal structure suggested that the SNARE core complex is a four-helix parallel
coiled coil[24, 25](Fig. 2). The internal of the bundle contains 16 layers of interacting and
mostly hydrophobic side chains that are stacked perpendicular to the axis of the superhelix in
the middle of the bundle, an ionic “0” layer was found that is composed of three Gln residues
(contributed by syntaxin 1 and SNAP-25) and one Arg residue (contributed by VAMP2).

These residues are highly conserved throughout the entire SNARE superfamily, leading to
their classification into Q-SNAREs (syntaxin 1 and SNAP-25) and R-SNARE (VAMP2),
respectively. The grooves on the surface of the four-helix bundle can serve as the binding sites for the regulatory proteins, such as Munc18, synaptotagmin and complexin.

1.3 The SNARE assembly pathway and the zippering model

The assembly of the neuronal SNARE complex is accompanied by significant conformational changes and could be briefly dissected into three steps (Fig. 3): First, the regulatory N-terminus of syntaxin 1 is detached from its SNARE motif with the help of SM proteins, Munc18-1[26], altering syntaxin 1 from “closed” conformation to “open” conformation. Therefore syntaxin 1 becomes accessible for the other two SNAREs. Then, syntaxin 1 and SNAP-25 associate into a binary complex on the target membrane before VAMP2 on the opposing vesicular membrane approaches and finally associate with the binary complex to form the ternary complex. At this state since the ternary complex simultaneously resides on the two approaching membranes, it is named as trans-SNAREs. After the two membranes merge together, the trans complex transits to the cis-SNAREs (Fig. 4). In the case of neurotransmitter release, the cis-SNAREs complex are extremely stable and their disassembly requires ATPases: NSF (N-ethylmaleimide sensitive factor) and α-SNAP (soluble NSF attachment protein) [27-30].

There are two forms of binary complex with the different stoichiometry of syntaxin 1 and SNAP-25[31]. The 1:1 mode is the three-helix bundle, containing one copy of syntaxin 1 and one copy of SNAP-25. The 2:1 mode is the four-helix bundle with one more copy of syntaxin 1. Both of these complexes could exist in vivo[32] and recent studies[33] show that only the 1:1 complex is the active intermediate for the further trans complex assembly and the 2:1 complex is the off-pathway intermediate. The equilibrium of these two forms might be a regulatory mechanism for the SNARE complex assembly.

The formation of the trans-SNAREs complex is proposed as the rate-limiting step for the SNARE assembly, starting at the membrane-distal N-terminus, followed by the extension to the membrane-proximal C-terminus. The stepwise mechanism is called zippering model[19].
The parallel arrangement of SNARE motifs within complexes brings their transmembrane anchors, and the two membranes, into close proximity[28]. The high stability of the cis-SNAREs complex suggests that the SNARE complex assembly is also associated with energy release, which might provide the force to overcome the energy barrier for membrane fusion[21, 24].

1.4 Models for membrane fusion

How about the whole fusion process from the opposing membranes forward to the final connection of separated aqueous contents? There have been several hypotheses for the fusion process. Generally, they can be classified into two different models (Fig. 5): the protein pore model and the lipidic pore model [2, 34].

The protein pore model assumes that the transmembrane domains of t- and v-SNAREs can each form the half gap junction-like pore on the membrane. Then the two halves on the opposing membranes can be juxtaposed by the assembly of the trans-SNARE complexes. Expansion of these proteinaceous pores by lipid incorporation leads to the complete membrane fusion.

The lipidic pore model suggests a sequential merging of the membranes. First, the two outer leaflet layers of membrane merge together with the inner leaflet layers intact, forming a lipid stalk intermediate, called hemifusion state. Then, the inner leaflets start to mix and lead to the formation of a lipidic fusion pore. The metastable hemifusion state is a highly bended structure with negative curvature for the mixed proximal leaflets. Some lipids with cone-shape geometrically favor to this negative curvature, and are therefore able to regulate the fusion process, which is known as the curvature effect (Fig. 6). For example, a cone-shaped lipid molecule such as phosphatidylethanolamine (PE) matches the lipid stalk structure, whereas the inverted-cone-shaped lipid molecule such as lysophosphatidylcholine (LPC) disturbs the packing of the lipids in the stalk.
The main difference between these two models is the nature of the initial fusion pore, lipidic pore or gap-junction protein pore. Hemifusion state can be captured in both of two models, as Fig. 5 (a3) and (b3) shown. After fusion pore opening, there is no difference in the pore expansion state between these two models. However, both of two models have some limitations. For example, the hydrophobic transmembrane domain of SNARE proteins can not transfer the ionic transmitters like ion channel; the lipidic pore model can not explain the “kiss-and-run” phenomenon which is detected in vivo.

1.5 The SNARE-interacting proteins

There are many proteins involved in the Ca\(^{2+}\)-triggered neurotransmitter release. Some of them directly bind to SNARE proteins individually or the SNARE complex, the others function at the upstream and downstream of synaptic vesicle fusion. Here, three kinds of interacting proteins are listed which are related to the study course.

1.5.1 Clostridial neurotoxins

Clostridial neurotoxins (CNTs) are bacterial proteins which can induce very severe neuroparalytic diseases botulism and tetanus. There are two groups of the CNT family, botulinum neurotoxins (BoNTs) and tetanus neurotoxin (TeNT), have been discovered to date. And BoNTs have seven distinct serotypes A-G. Both BoNTs and TeNT are synthesized as single polypeptides of 150 kDa and subsequently are activated by cleaving into two chains linked via a disulfide bond. The heavy chain (100 kDa) carries the receptor binding and transmembrane domains of the toxin, and the light chain (50 kDa) contains the catalytic domain that disrupts the ability of SNAREs to promote synaptic vesicle fusion and blocks neurotransmitter release[35]. Clostridial neurotoxins are zinc endopeptidases[36], the Zn\(^{2+}\)-dependent proteolytic activity relies on the Zn\(^{2+}\)-binding motif (His-Glu-X-X-His) in the light chain.
SNAREs are the targets of the clostridial neurotoxins, including botulinum and tetanus neurotoxins[22, 37] (Fig. 7). All neurotoxin-mediated cleavage sites are located between the C-terminal membrane anchors and the ionic “0” layers. Only uncomplexed or partially assembled SNARE proteins can undergo proteolysis by neurotoxins[22]. The fully assembled SNARE complex is resistant to proteolysis because either the protease-cleavage sites or the protease-recognition sites are protected upon SNARE complex formation. Since neurotoxins cleave syntaxin 1 and VAMP2 at the membrane-proximal end, cleavage may detach either the presynaptic plasma membrane or the vesicle membrane from the assembled complex. In contrast, the result of cleavage of the SNAP-25 SN2 by neurotoxins A and E, is less direct as the C-terminus of SN2 is not directly anchored to the membrane. Cleavage by these two toxins may destabilize the four-helical bundle of the SNARE core complex, and may disrupt the ability of the complex to bridge membranes[38].

1.5.2 Synaptotagmin

Synaptotagmin (Syt) is considered the primary calcium sensor in the Ca\(^{2+}\)-triggered synaptic exocytosis[12]. By now, 13 isoforms have been identified in neurons and non-neuronal cells[39]. All synaptotagmin isoforms are composed of a short intravesicular N-terminal region, a single transmembrane domain, a lysine- and arginine-rich linker, and two tandem C2 domains, named C2A and C2B (Fig. 8). The X-ray crystal structure reveals the similar composition of a β-sandwich containing eight β-strands, with eight flexible loops emerging from the top and bottom. C2A generally binds three Ca\(^{2+}\) ions, whereas C2B binds only two Ca\(^{2+}\) ions[40]. All C2B domains contain one or two bottom helices between the 7\(^{\text{th}}\) and 8\(^{\text{th}}\) β-strands that is absent from C2A domain. In addition to synaptotagmin, the double C2-like domain containing (Doc2) proteins contain similar C2A and C2B domains but lacking the transmembrane domain (TMD)[41, 42].

Synaptotagmin I (Syt I) in synaptic vesicles triggers the Ca\(^{2+}\)-dependent rapid membrane fusion[43]. It has been shown that Syt I can interact with SNAREs and the acidic lipids, such as phosphatidylserine (PS), phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) in the plasma
membrane simultaneously in a Ca\(^{2+}\)-dependent manner[44]. The detailed studies further reveal that C2A domain interacting with syntaxin 1 and C2B domain involving in the lipid binding[45]. The stimulation effect of Syt I has been dissected in the docking stages and the late step of synaptic exocytosis. It is reported that synaptotagmin, Munc18-1 and t-SNAREs binary complex form the minimal docking machinery preceding the formation of the SNARE complex[46]. C2B domain of Syt I also closely interacts with the membrane-proximal portion of the SNARE complex when the Ca\(^{2+}\) influx at the late step of the activation of exocytosis. In addition, the C2B domain can promote Syt oligomerization in a Ca\(^{2+}\)-dependent fashion[47].

1.5.3 Complexin

Complexins (Cpxs), also known as synaptic, are small cytoplasmic proteins (15 to 18 KDa) that are mainly found in the presynaptic part of neuronal cells[48]. The crystal structure shows that the central region (amino acids 48-70) binds to the groove between syntaxin 1 and VAMP2 of the SNARE core as an antiparallel \(\alpha\)-helix, which attaches complexin to the SNARE complex[49]. Besides the central region, Complexin I has the N-terminus (amino acids 1-26) and accessory \(\alpha\)-helix (amino acids 27-47) at the membrane-proximal region, and the C-terminal region at the membrane-distal region (Fig. 9). Complexin I was originally identified as the synaptic exocytosis inhibitor in “clamping model”[15, 50]. However, recent biochemical[51-53] and biophysical[54] studies show that complexin has the dual functions in SNARE-mediated membrane fusion. The N-terminus of complexin has the stimulation effect, while the accessory \(\alpha\)-helix located between N-terminus and the central region of complexin has the inhibition effect by replacing the C-terminal of VAMP2 in the SNARE core complex. These two opposite functions work as the fine-tuned mechanism for complexin-regulated membrane fusion. At the rest state, synaptotagmin sequester the N terminus of complexin, the accessory \(\alpha\)-helix inhibits the SNARE assembly, leading to accumulate at the docking stage. After the influx of Ca\(^{2+}\), the inhibition will be removed by the N terminus function, which produces fast synchronized membrane fusion[55].
Despite there is no atomic resolution structural information about the interaction between synaptotagmin I and SNAREs due to the weak association[17], many biochemical and biophysical detailed studies show that Syt I can interact with syntaxin 1[56] and SNAP-25[57] and their binary and ternary complex via the C2A domain. Based on these findings, we propose Syt I C2A domain may bind to the groove between syntaxin 1 and SNAP-25 SN1. Combining with the X-ray crystal structure of complexin/SNARE complex, the structural organization of regulatory proteins (Syt I and Cpx I) interacting with the four-helix SNARE bundle is shown in Fig. 10.

1.6 Study methods

1.6.1 SDSL and EPR

Site-directed spin labeling (SDSL) and electron paramagnetic resonance (EPR) spectroscopy are established techniques for investigation of structures and topologies of membrane associated proteins in the phospholipids bilayer[58, 59]. In this method (Fig. 11), native residue is substituted with cysteine to provide a specific labeling site for the thiol-specific nitrooxide spin labels such as methanethiosulfonate spin label (MTSSL). Distances between the interspin in the range of 7-25Å can be determined by continuous wave EPR (CW EPR) based on the Fourier deconvolution method. For distances >25Å, pulsed dipolar EPR is used to analyze the spin-spin interaction. Pulsed dipolar spectroscopy represented by DEER (double electron–electron resonance) and DQC (double quantum coherence) is a proven technique[60] to measure the distances in the range of 10-80Å between dipolar introduced in biomolecules and the distributions in distances, $P(r)$[61]. Nitroxide-scanning EPR utilizes the power saturation method to yield information on the secondary structure and topology of membrane-bound proteins such as SNAREs[62]. Compared to solution NMR and X-ray crystallography, EPR does not require crystallization, does not have a molecular weight limit for the sample, can be performed in various conditions (aqueous solution, membrane environment, or aggregates), and requires low protein concentration (typically 10-100 μM).
1.6.2 Ensemble lipid mixing assay

The ensemble/bulk fusion assay is a powerful tool to study the molecular function of SNAREs and specialized regulators in membrane fusion[5, 6]. This method is based on the dequenching of two fluorescent dyes (NBD and Rhodamine) labeled on the head group of phospholipids (Fig. 12). When these two dye-labeled lipids are incorporated into a lipid vesicle together and excited with the donor (NBD) wavelength, the emission of NBD will be quenched by acceptor (Rhodamine) and the emission of Rhodamine will be observed. After fusion with unlabeled vesicles by ensemble of lipid mixing of proteoliposomes reconstituted with SNARE proteins, dequenching occurs due to the dilution of the two dyes in the membrane, which increases the emission of NBD. Thus, by monitoring the fluorescence intensity of NBD during the liposome fusion, we are able to track SNARE-mediated lipid mixing in real time. Also, by selectively reducing the donor (NBD) to a non-fluorescent ABD, we can distinguish the inner and outer leaflets lipid mixing[63].

1.6.3 Single-vesicle fusion assay

1.6.3.1 Lipid mixing assay

Since the observation of the ensemble lipid mixing assay over time is the sum of all the unsynchronized events, it is impossible to dissect the different stages in the whole fusion pathway, which includes docking and a series of fusion intermediates, such as hemifusion and fusion pore opening and expansion. In order to circumvent this asynchrony issue, the new generation of single-vesicle lipid mixing assay, based on total internal reflection fluorescence (TIRF) microscopy, was developed[64, 65] (Fig. 13). By this method, the fusion event could be observed on the level of the single liposome in real time through fluorescence resonance energy transfer (FRET) between the donor and acceptor fluorophores incorporated into the separate proteoliposome membranes reconstituted with target membrane SNARE (t-SNARE) or vesicle SNARE (v-SNARE) proteins. It is carried on the 2-D surface and revealed the characteristics of the lipid mixing in unprecedented details[66, 67].
1.6.3.2 Content mixing assay

To directly detect the content exchange resulting from the SNARE-mediated two vesicles fusion, one reliable and efficient single-vesicle content mixing assay was developed employing the molecular beacon as probes[68] (Fig. 14). The fluorescent dual-labeled (Cy3-Cy5) DNA hairpin molecule shows high FRET when it is enclosed inside the vesicles reconstituted with v-SNARE proteins. When the vesicles fused with the t-SNAREs reconstituted vesicles containing a complementary DNA strand, FRET decreases to a low level due to the formation of double-strand DNA. This method is good for detecting the fusion pore formation and expansion and dissecting how regulatory proteins such as complexin and synaptotagmin function during the late steps of membrane fusion.

1.7 References


1.8 Figures and Captions

Fig. 1. **Primary domain structure of neuronal SNARE proteins.** Primary structure diagram for syntaxin 1A, SNAP-25A, and synaptobrevin 2 (vesicle-associated membrane protein 2, VAMP2). TMR, transmembrane region is located at the C-terminal of syntaxin 1A and VAMP2. The SNARE motifs are defined through the 16 layers as found in the crystal structure of the neuronal SNARE core complex. The N-terminal domain of syntaxin 1A is named Habc which can bind with the SNARE core domain or Munc18. For SNAP-25A, the two SNARE motifs are named as SN1 and SN2, the four palmitoylation sites (cysteine 85, 88, 90 and 92) are indicated by lines.
Fig. 2. The crystal structure of neuronal SNARE core complex. (a). Backbone ribbon drawing of the SNARE core complex. Synaptobrevin 2 (Sb) is shown in blue; syntaxin 1 (Sx) is in red; and SNAP-25 (Sn1 and Sn2) is in green. (b). Organization of the SNARE core complex shows 15 hydrophobic layers and 1 ionic “0” layer. (c). The structure of the central ionic “0” layer.
**Fig. 3. The SNARE conformational cycle during synaptic vesicle exocytosis.** 
Qa-SNARE stands for syntaxin 1, in red; Qb- and Qc-SNARE are SNAP-25 SN1 and SN2, in green; and R-SNARE is VAMP 2, in blue.
Fig. 4. Two states of the SNARE complex: trans and cis. (a). Partially assembled trans state of two SNARE complexes that dock a vesicle to the target membrane. (b). Fully assembled cis state of the SNARE complex after membrane fusion and pore formation.
Fig. 5. Two models of membrane fusion pathway. (a). The lipidic pore model shows a fusion pore lined by lipids. (b). The protein pore model shows a fusion pore from transmembrane domains of v- and t-SNAREs. The main difference between these two models is the nature of the initial fusion pore, lipidic pore or gap-junction protein pore. Hemifusion state can be captured in both of two models, as (a3) and (b3) shown in the figure. After fusion pore opening, there is no difference between these two models.
Fig. 6. Hemifusion state and curvature effect. The structure of lipids PE (green) and LPC (red) are opposite, PE has the cone-shaped molecular structure and LPC, by contract, owns reverse cone-shaped, promoting them to adopt different spontaneous curvature, which makes PE fit well in the negative curvature of outer leaflets in hemifusion state better than LPC.
Fig. 7. The distribution of neurotoxins cleavage sites on the SNARE proteins. The red helix is syntaxin 1; the two green helices are SN1 and SN2 of SNAP-25; and the blue helix is VAMP2. All the cleavage sites are located between the “0” layer of SNARE core complex and the juxta-membranous sequences which close to the initial fusion pore opening site.
Fig. 8. The structure of synaptotagmin I (Syt I). The transmembrane (TM) domain is at the N-terminal of Syt I. The two tandem C2 domains, C2A and C2B, locate at the C terminus. C2A has three Ca$^{2+}$-binding sites and C2B has two. Ca$^{2+}$ ions are shown in red and Ca$^{2+}$ ligands are indicated in blue. The main difference between C2A and C2B is one or two extra short α-helices at the “bottom” of C2B domain. The Ca$^{2+}$-binding regions are inserted into the membrane after the activation of exocytosis.
Fig. 9. The primary structure of complexin I and the crystal structure of the complexin/SNARE quaternary complex. Complexin I (amino acids 26-83) is resolved as an anti-parallel $\alpha$-helix binding to the groove between syntaxin 1 and VAMP2. Complexin is in pink. The SNARE core complex is color coded as follows: VAMP2, red; syntaxin 1, yellow; SNAP-25 SN1, blue; SNAP-25 SN2, green.
Fig. 10. Schematic diagrams that show the interplay of synaptotagmin I (Syt I) and complexin I (Cpx I) with the SNARE core complex. Syt I, most likely the C2A domain, interacting with both syntaxin 1 and SNAP-25; while Cpx I binding to the groove between syntaxin 1 and VAMP2.
Fig. 11. A schematic description of site-directed spin labeling (SDSL) strategy. The native amino acid is substituted by cysteine by site-directed mutagenesis. Then the mutated site is specially labeled by the nitroxide spin label, (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl methanethiosulfonate (MTSSL).
Fig. 12. Lipid mixing assay based on fluorescence resonance energy transfer (FRET). The average spatial separation of the donor (NBD) and acceptor (Rhodamine) lipid probes increases upon fusion of labeled vesicles with unlabeled vesicles, resulting in decreased efficiency of proximity-dependent FRET, which is registered by increased donor and decreased acceptor fluorescence intensity. (a). Total lipid mixing assay, the distance between donor and acceptor increasing leads to increased donor fluorescence intensity. Fluorescence intensity was measured at the NBD’s excitation and emission wavelengths of 465 and 530 nm, respectively. (b). Selective reduction of fluorescent NBD in the outer leaflets to non-fluorescent ABD by dithionite. (c). Inner leaflet mixing assay, experimentally distinguishing hemifusion from full fusion. Upper: hemifusion state, no FRET change between donor and acceptor; lower: full fusion, increased fluorescence intensity of donor.
Fig. 13. Schematics of the single-vesicle lipid mixing assay of yeast SNARE-mediated membrane fusion. The v-SNARE vesicles containing membrane fluorescent acceptors DiD are tethered on a PEG-coated quartz slide by the interaction between biotin and neutravidin. And Sso1p HT-reconstituted t-SNARE vesicles doped with membrane fluorescent donors Dil are introduced together with soluble Sec9c to induce fusion. The mixing of donor and acceptor dyes caused by fusion between the cognate proteoliposomes leads to increase in FRET efficiency, which is being monitored by wide-field total internal reflection fluorescence (TIRF) microscopy. For neuronal SNARE-mediated membrane fusion, syntaxin 1A and SNAP-25, the homolog of yeast t-SNAREs, Sso1p and Sec9c respectively, first preform the binary complex and then reconstituted in the donor vesicles. VAMP2, the homolog of yeast v-SNARE Snc2p, is in the accepter vesicles.
Fig. 14. Schematics of single-vesicle content mixing assay of yeast SNARE-mediated membrane fusion. Vesicles reconstituted with Snc2p (v-vesicles) and encapsulating Cy3-Cy5 labeled DNA probes are immobilized on the surface of the flow cell by the interaction between biotin and neutravidin. Vesicles reconstituted with Sso1pHT (t-vesicles) and encapsulating poly-A DNA strands are flown in, along with soluble Sec9c, and the sample is incubated at 37 °C for 30 min. After vesicle fusion between t- and v-vesicles, the two DNA molecules mix up and form the double strand DNA. The FRET efficiency between donor Cy3 and acceptor Cy5 decreases as the separate distance increases.
CHAPTER 2: ACCESSORY α-HELIX OF COMPLEXIN I CAN DISPLACE VAMP2
LOCALLY IN THE COMPLEXIN-SNARE QUATERNARY COMPLEX*

Bin Lu†, Shuang Song† and Yeon-Kyun Shin

2.1 Abstract

The calcium-triggered neurotransmitter release requires three SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins: synaptobrevin 2 (or VAMP2) on the synaptic vesicle and syntaxin 1 and SNAP-25 at the presynaptic plasma membrane. This minimal fusion machinery is believed to drive fusion of the vesicle to the presynaptic membrane. Complexin, also known as synaphin, is a neuronal cytosolic protein that acts as a major regulator of synaptic vesicle exocytosis. Stimulatory and inhibitory effects of complexin have both been reported, suggesting the duality of its function. To shed light on the molecular basis of the complexin’s dual function, we have performed an EPR investigation of the complexin-SNARE quaternary complex. We found that the accessory α-helix (amino acids 27-48) by itself has the capacity to replace the C-terminus of the SNARE motif of VAMP2 in the four-helix bundle and makes the SNARE complex weaker when the N-terminal region of complexin I (amino acids 1-26) is removed. However, the accessory α-helix remains detached from the SNARE core when the N-terminal region of complexin I is present. Thus, our data show the possibility that the balance between the activities of the accessory α-helix and the N-terminal domain might determine the final outcome of the complexin function, either stimulatory or inhibitory.

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† Contributed equally to this work.
2.2 Introduction

Synaptic vesicle fusion is driven by assembly of the trans-SNARE complex between the vesicle and the plasma membrane, which facilitates merging of the opposite bilayers [1-7]. The physiological trigger for this process is the spike in the local concentration of calcium. As Ca\(^{2+}\) enters the presynaptic cytoplasm, the Ca\(^{2+}\)-sensor synaptotagmin I [8-11], which is localized on synaptic vesicles, interacts with the SNARE complex and phospholipids simultaneously [12-14], leading to the fast opening of the fusion pore [15-18].

In addition to synaptotagmin I, complexins also participate in the fast Ca\(^{2+}\)-dependent neuronal exocytosis [19-22]. Complexins are small soluble cytoplasmic proteins (15- to 18-kDa) that are mainly found in the presynaptic part of neuronal cells [23-25] and the central region (amino acids 48-70) binds to the SNARE core as an anti-parallel \(\alpha\)-helix, which attaches complexin to the SNARE complex [26,27].

Complexin is believed to have a dual function [20,28,29] in synaptic membrane fusion, both stimulation [19,26,27] and inhibition [21,30-35]. Although the exact molecular mechanism is unknown, the N-terminal region of complexin is shown to play an important role in promoting the neurotransmitter release [28,29,36]. It is proposed that the N-terminal region interacts with the membrane-proximal region of VAMP2 and functions as a force transducer [29]. However, under different conditions, complexin exhibits the inhibitory function, which is the basis for the recent “complexin-clamp model” [34]. Two possible molecular-level mechanistic models have been proposed to explain the inhibition by complexin. Firstly, complexin is found to have the micromolar affinity to the t-SNARE complex in addition to its submicromolar affinity to the SNARE complex [20,26]. Thus, under favorable conditions complexin could compete with the VAMP2 binding to the t-SNARE complex, which would competitively inhibit SNARE assembly and membrane fusion. Secondly, when bound to the SNARE core, the accessory \(\alpha\)-helix of complexin, which franks the central SNARE-anchor domain and the stimulatory N-terminal region, might replace the C-terminal portion of the VAMP2 helix to form an alternative four-helix bundle with the helices from t-SNAREs
Such structural replacement would prevent formation of the complete SNARE four-helix bundle, thereby inhibiting membrane fusion.

In this work, we performed the EPR investigation of the conformational changes in the v-SNARE VAMP2 after complexin binding to the SNARE complex. The results show that there is no major conformational change, such as the replacement of VAMP2 by complexin, occurring at the membrane-proximal region of VAMP2 upon binding of the full-length complexin to the SNARE complex. However, when the stimulatory N-terminal region of complexin is removed, the accessory α-helix replaces the C-terminal region of VAMP2 partially. Thus, our results show that the balance between the stimulatory and inhibitory activities of the accessory α-helix and the adjacent N-terminal region, respectively, might determine the exact function of the particular complexin.

2.3 Materials and Methods

2.3.1 Plasmid construction and site-directed mutagenesis

DNA sequences encoding full-length syntaxin 1A (amino acids 4-288), the soluble VAMP2 (amino acids 1-94), full-length complexin I (amino acids 1-134 with one native cysteine C105 replaced by alanine), and the truncated complexin I Δ26 (amino acids 27-134) were cloned into the pGEX-KG vector and expressed as the N-terminal glutathione S-transferase (GST) fusion protein. SNAP-25 (amino acids 1-206 with four native cysteines C85, 88, 90 and 92 replaced by alanines) was cloned into pET-28b vector and expressed as N-terminal His6-tagged protein. All cysteine mutants were generated by QuikChange site-directed mutagenesis kit (Stratagene), and they were confirmed by DNA sequencing (Iowa State University DNA Sequencing Facility).

2.3.2 Protein expression, purification, and spin labeling
GST fusion proteins were expressed in *E. coli* Rosetta (DE3) pLysS (Novagen) and purified using glutathione-agarose beads (Sigma) [38]. Briefly, the cells were grown at 37 °C in LB with glucose (2 g/liter), ampicillin (100 µg/ml), and chloramphenicol (50 µg/ml) until A₆₀₀ reached 0.6-0.8. After adding 0.3 mM isopropylthio-β-D-galactopyranoside (IPTG), the cells were further grown for 6 h at 22 °C for GST-soluble VAMP2, GST-complexin I, and GST-complexin I Δ26 but at 16 °C for GST-syntaxin 1A. Finally, the protein was cleaved to remove the GST tag by thrombin in the cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0). We add 1% n-octyl-glucoside (OG) in the cleavage buffer for syntaxin 1A.

His₆-tagged SNAP-25 was expressed in *E. coli* BL21 (DE3) Codon Plus RIL (Stratagene) and purified using Ni-NTA resin (Qiagen) [38]. The cells were grown at 37 °C in LB with glucose (2 g/liter), kanamycin (34 µg/ml), and chloramphenicol (50 µg/ml) until A₆₀₀ reached 0.6-0.8. Protein expression was induced by 0.5 mM IPTG, and the cells were grown for an additional 6 h at 30 °C. Finally, the protein was eluted from the Ni-NTA resin by elution buffer (25mM HEPES, 100mM KCl with 250mM imidazole, pH 7.4).

The cysteine mutants of soluble VAMP2 and complexin I were reacted with (1-oxyl-2,2,5,5-tetramethylpyrrolinyl-3-methyl) methanethiosulfonate (MTSSL) spin label at 4°C overnight while the proteins were bound to the GST-agarose beads. To remove free spin label, the beads with bound proteins were extensively washed with the cleavage buffer then cleaved by thrombin (Sigma). The spin-labeling efficiency was determined by comparing the spin concentrations with the 50 μM 2,2,6,6-tetramethyl-4-piperidine N-oxide (TEMPO) standard. For all samples, the efficiency was ~80%.

2.3.3 Preparation of the complexin-SNARE quaternary complex

Purified His₆-tagged SNAP-25 was first added to the Ni-NTA resin solution and nutated for 1 h at room temperature. After washing out the free proteins, purified syntaxin 1A and soluble VAMP2 were mixed with His₆-SNAP-25 to form the SNARE complex with the molar ratio of 2:5:1. The mixture was incubated at 4 °C overnight. After washing one time, 2-fold excess
of purified complexin I or complexin I △26 was added to the solution and incubated at 4 °C overnight again. After extensive washing to remove the unbound proteins, the complexin-SNARE quaternary complex was eluted with a buffer containing 250 mM imidazole and 1% n-octyl-glucoside (OG). The formation of the complex was confirmed with SDS-PAGE gel. We obtained identical results when the quaternary complex was formed by adding all four components simultaneously.

2.3.4 EPR data collection

EPR spectra were obtained using a Bruker ESP 300 spectrometer (Bruker, Germany) equipped with a low noise microwave amplifier and 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 in the first-derivative mode with 1mW microwave power.

As for the complexin-SNARE quaternary complex in the detergent (1% OG), the samples were concentrated to the final concentration of 50-100 μM using a 5-kDa cutoff centrifugal filter (Millipore) before EPR spectra collection. For the purified complex in the membrane, the samples were reconstituted into vesicles at about 1:200 protein-to-lipid molar ratio using the published method [39]. The large unilamellar vesicles (~100 nm in diameter) of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) containing 15% 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) were prepared in a detergent-free buffer using an extruder (Avanti). The detergent was removed by treating the sample with the dialysis buffer (25 mM HEPES, 100mM KCl, pH 7.4) at 4 °C overnight. After dialysis, the sample solution was concentrated using a 100-kDa cutoff centrifugal filter (Millipore) before taking an EPR spectrum.

2.4 Results

2.4.1 Complexin-SNARE quaternary complex formation
The recent study by Rothman et al. suggests that the accessory α-helix of complexin replace VAMP2 and form an alternative four-helix bundle with t-SNAREs near the membrane, thereby inhibiting complete SNARE complex formation and membrane fusion [34]. According to this proposed model, the displaced sequence of VAMP2 includes both the cleavage site (between residues 76 and 77) and recognition site (residues 62 to 71) for BoNT/B toxin. Therefore, our EPR investigation was focused on the structure of the C-terminal region of VAMP2. We prepared five single cysteine mutants of VAMP2 (Fig. 1a), including A69C within the recognition site of BoNT/B and Q76C on its cleavage site, and K83C, R86C, and W89C in the membrane-proximal region. The cysteine mutants were derivatized with methanethiosulfonate spin label (MTSSL) for EPR measurements. Next, we used the pull-down method (Fig. 1b) to prepare the complexin-SNARE quaternary complex that includes the spin-labeled soluble VAMP2, full-length syntaxin 1A, SNAP-25, and complexin. As the bait, His6-tagged SNAP-25 was attached to the Ni-NTA beads and the SNARE complex was first formed by flowing syntaxin 1A and soluble VAMP2 into the bead solution. Complexin was next added to the column to form the complexin-SNARE quaternary complex. The transmembrane domain (TMD) of VAMP2 was not included in order to avoid the interaction between two TMDs in one membrane and to best mimic the trans-SNARE complex. For individual mutants we analyzed formation of the complexin-SNARE quaternary complex by the SDS-PAGE gel after heating. We found that the immobilized His6-tagged SNAP-25 co-purified with stoichiometric amounts of syntaxin 1A, soluble VAMP2 and complexin (Fig. 1c).

2.4.2 Full-length complexin does not replace VAMP2

The EPR line shape is a sensitive function of the motional rate. The slower the motion of the nitroxide side chain, the broader the EPR spectrum. Therefore, if the proposed structure of the quaternary complex in Fig. 1a were to be true, we expect the narrowing of the EPR spectra for the spin labeled positions when compared with the EPR spectra from the SNARE complex only. EPR spectra of the SNARE complex before and after binding to full-length complexin I were shown in Fig. 2. The spectra for positions 69 and 76 of the complexin-
SNARE complex became much broader than those from the SNARE complex only. This tendency was more obvious at 69 indicating that there was strong tertiary interaction at this site. Our data were also consistent with the deuterium exchange results which showed that position 69 was highly protected after complexin binding [27]. Thus, the EPR results for positions 69 and 76 were not consistent with the proposed structure in Fig. 1a. Instead, the results suggest that complexin bind on the surface of the SNARE four helix bundle, as shown in the crystal structure [27].

For positions 83, 86, and 89, which are located in the membrane-proximal region, there was no obvious narrowing due to the replacement of VAMP2 by complexin either. Unlike the cases with positions 69 and 76, we did not observe extra line broadening due to the complexin binding, suggesting complexin might not even interact with the SNARE complex locally in the region. Further addition of complexin to the complexin-SNARE quaternary complex did not change the EPR lineshape either (Fig. S1), suggesting that the reason we did not observe complexin-induced line broadening was neither due to the insufficient complexin binding nor due to the intermolecular complexin-binding equilibrium. Therefore, the EPR analysis of the quaternary complex suggested that full-length complexin I does not replace VAMP2 when bound to the SNARE complex.

2.4.3 The accessory α-helix of complexin has no obvious interaction with the SNARE core

We now examined the conformation of complexin in the complexin-SNARE quaternary complex using spin-labeled complexin. We aligned the sequences of complexin with VAMP2 in an antiparallel orientation as they were shown in the crystal structure [27,34] and selected five positions R42, E35, P28, E25, and G22 in the accessory α-helix as the spin labeling sites. EPR spectra for these sites in the complexin-SNARE complex were shown in Fig. 3. The line shapes for spin labeled complexin alone were all very sharp, reflecting the freely diffusing random coil. When the spin-labeled mutants were bound to the SNARE complex, the spectra became broader, reflecting the slower motion of the nitrooxide, most likely due to formation of α-helix. However, the spectra were still not so broad with no
indication of any tertiary contact. In fact, the spectra are sharper than those for the solvent exposed positions on the surface helix [40,41], indicative of substantial motion of the accessory α-helix. Therefore, our EPR analysis shows that the accessory α-helix most likely has a little or no tertiary interaction with the SNARE core.

2.4.4 The accessory α-helix of complexin can partially displace VAMP2 locally when the N-terminal region is absent

When complexins are deleted from the neuron, the Ca\(^{2+}\)-triggered fast release is severely reduced. In contrast, the frequency of the Ca\(^{2+}\)-independent spontaneous release is dramatically enhanced. However, when the N-terminal 26 residues are removed from complexin, both the evoked and the spontaneous releases are reduced when compared with those of complexin-null mutant [29]. Thus, these results show that the shortened complexin mutant lacking the stimulatory N-terminal sequence has an inhibitory function for the spontaneous release. In this case, it is possible that the accessory α-helix displaces VAMP2, thereby inhibiting membrane fusion. To test this possibility, we prepared the shortened construct of complexin lacking the N-terminal 26 residues (amino acids 27-134), and investigate the structure of the complexin △26-SNARE quaternary complex employing the spin labeled VAMP2 mutants (Fig. 4a and b). For nitroxides attached to positions 69 and 76, the EPR spectra were very similar to those obtained from the full-length complexin–SNARE quaternary complex (Fig. 2). However, for three C-terminal spin-labeled positions 83, 86, and 89, the EPR spectra show a small but distinctly sharp spectral component (Fig. 4b, see arrows, 7–17%), which is most likely from the frayed VAMP2 chain that is freely diffusing and fast moving in solution. Thus, the EPR analysis show that the accessory α-helix of complexin has the capacity to replace VAMP2 and make the SNARE complex somewhat weaker in the absence of the N-terminal region. It appears though that the sharp spectral component for N-terminal position 83 (17%) is larger than those for C-terminal positions 86 and 89 (9% and 7%, respectively). One, however, must be cautious in interpreting these numbers when taking into account the perturbation by nitroxides and the uncertainty of the spectral subtraction analysis. Therefore, given the experimental uncertainty, we would not
want to put much weight on the order of the extent of displacement among the spin-labeled positions.

Our EPR analysis shows that the displacement of VAMP2 by complexin Δ26 happens locally in the membrane-proximal region. If true, we expect that the accessory α-helical region of complexin Δ26 would interact with the t-SNARE helices in replacement of VAMP2. Indeed, for spin-labeled complexin Δ26 mutants R37C and L41C, we observe small but distinct line broadening (Fig. 4c) most likely due to the tertiary interaction between the accessory α-helix and the t-SNARE core. Although positions 37 and 41 are not in register with VAMP2 residues 83–89, the results show that the tertiary interaction between complexin Δ26 and the t-SNARE core might occur in the part preceding this region.

2.5 Discussion

The EPR analysis of the complexin-SNARE quaternary complex revealed that the accessory α-helix has small but distinct capacity to displace the C-terminal part of VAMP2 from the SNARE four-helix bundle. However, in full-length complexin I such an inhibitory function of the accessory α-helix against SNARE complex formation is nearly completely suppressed by the presence of the N-terminal sequence. It is unclear how the N-terminal sequence might override the inhibition by the accessory α-helix. One possible scenario is that the N-terminal region directly interacts with the accessory α-helix to pull it out from its interaction with the SNARE complex. An alternative scenario is that the N-terminal region itself binds to the SNARE complex in a yet unknown site in such a way that it destabilizes the binding of accessory α-helix to the three helix bundle made of t-SNAREs syntaxin 1A and SNAP-25 non-competitively.

In the previous NMR study of complexin–SNARE quaternary complex using an N-terminally truncated fragment [27], such displacement of the membrane-proximal region of VAMP2 was not observed. A fundamental difference between EPR and NMR is in the time scales of measurement. The EPR time scale is at least 2 orders of magnitude faster than that
of NMR and it can pick up fast-exchanging events. We speculate that the equilibrium exchange between the accessory α-helix and VAMP2 is sufficiently fast for NMR not to be able to detect. Further, only ~10% insertion exacerbates the situation for the detection by NMR.

Although complexin’s ability to displace VAMP2 in the quaternary complex appears to be small, it could actually be much bigger for the trans-SNARE complex in which the interaction between membrane-anchored VAMP2 and t-SNARE on the opposite membrane is expected to be much weaker than that in our model system. In our system, the transmembrane domain of VAMP2 was deleted to avoid the situation of the cis-SNARE complex in which the transmembrane domains of v- and t-SNAREs are in the same membrane, imposing no repulsion of the bilaye-bilayer interaction to the SNARE core.

The modes of interaction between complexin and the SNARE complex appear to be quite diverse. So far, three different interaction modes are discovered. Firstly, complexin has the capacity to interact with the SNARE complex additively without changing the basic coiled coil structure of the SNARE core [27]. Such an interaction is expected to work favorably for the stabilization of the SNARE complex and believed to play a stimulatory role in SNARE-dependent membrane fusion. Secondly, the accessory α-helix displaces the C-terminal part of VAMP2 to form an alternative four-helix bundle locally while the four plus one helix bundle structure is maintained in the middle [28, 34]. Our EPR results, however, show that only a small fraction of the quaternary complex have such a structure. It is most likely that the first and the second modes are inter-convertible and in equilibrium. Third, complexin interacts with the t-SNARE complex, which acts as the competitive inhibitor for VAMP2 binding to the t-SNARE complex [20,42]. The binding constant for the complexin/t-SNARE complex is reported to be somewhat higher than its affinity to the ternary SNARE complex [20]. It is highly likely that all three interaction modes exist in neuronal cells and the delicate balance between these three modes of interaction produces the inhibitory-stimulatory control of the neurotransmitter release. For example, the knockout experiments reveals that complexin from drosophila melanogaster is highly inhibitory [22,32], while complexin from rat is stimulatory
We speculate that such species specific function of complexin stems from the balance between the functional strengths between the inhibitory accessory $\alpha$-helix and the stimulatory N-terminal sequence.

The interaction mode in which the accessory $\alpha$-helix replaces the VAMP2 while bound to the SNARE core constitutes the structural basis for the fusion clamp model [34]. Indeed, in the absence of the N-terminal sequence the accessory $\alpha$-helix has the capacity of replacing VAMP2 partially and locally from the SNARE core while the other part of the complex is fully engaged. Such local structural inhibition may be advantageous over the fully competitive inhibition relying on the t-SNARE/complexin binding because the system could react more quickly in responding to the $\text{Ca}^{2+}$ signal. Paradoxically, however, for full-length complexin, where the N-terminal sequence is present, such replacement was not seen. In pure speculation, if another protein factor such as synaptotagmin I could sequester the N-terminal sequence, the inhibition of SNARE assembly might be possible and the inhibition could be removed by the influx of $\text{Ca}^{2+}$, which might produce synchronized membrane fusion. The results from the present analysis show that such a mechanistic model is structurally possible.

2.6 References


2.7 Figures and Captions

Fig. 1. Characterization of the complexin-SNARE quaternary complex. (a) The fusion clamp model proposes that complexin can replace the C terminus of VAMP2 upon binding to the trans-SNARE complex (adapted from ref. 34). Positions 69, 76, 83, 86 and 89 are the spin-labeled sites of the soluble VAMP2. Positions 35 and 28 of complexin have also been shown in the model. Positions 25 and 22, which are located at the N-terminal of complexin, have not been shown. (b) The flow diagram of purification of the complexin-SNARE quaternary complex. (c) SDS-PAGE analysis of the purified complexin-SNARE quaternary complex.
Fig. 2. EPR spectra of the spin-labeled soluble VAMP2 in the full-length complexin-SNARE quaternary complex at room temperature. First-derivative mode EPR spectra for the complex in the detergent (a) and in the membrane (b) are shown.
Fig. 3. EPR spectra of the spin-labeled complexin in the full-length complexin-SNARE quaternary complex at room temperature. First-derivative mode EPR spectra for the complex in the detergent and in the membrane are shown. Positions 22, 25, 28, 35 and 42 of complexin are corresponding to the positions 89, 86, 83, 76 and 69 of VAMP2 respectively.
Fig. 4. (a) and (b), EPR spectra of the spin-labeled soluble VAMP2 in the truncated complexin Δ 26-SNARE quaternary complex at room temperature. First-derivative mode EPR spectra for the complex in the detergent (a) and in the membrane (b) are shown. The arrowheads indicate the sharp component from the locally displaced VAMP2. The percentage of the sharp component was calculated using the spectral subtraction method (ref. 39). (c), EPR spectra of the spin-labeled full-length and truncated complexin upon binding with the ternary SNARE complex at room temperature. The inset figures show the difference between full-length and truncated complexin involved in the quaternary complex much more clearly.
2.8 Supplementary Data

Fig. S1. EPR spectra of the spin-labeled soluble VAMP2 in the complexin-SNARE quaternary complex at room temperature when adding more complexin. First-derivative mode EPR spectra for the complex in the membrane are shown. Two-fold molar excess of complexin are added into the purified complexin-SNARE quaternary complex. There are still broad spectra at the positions 83, 86, and 89 of VAMP2.
CHAPTER 3: PHOSPHATIDYLETHANOLAMINE IN THE SYNAPTIC VESICLES ENHANCES SNARE-DEPENDENT MEMBRANE FUSION

Bin Lu, Peter P. Borbat†, Yuji Ishitsuka‡, Taekjip Ha‡, Jack H. Freed‡ and Yeon-Kyun Shin

3.1 Abstract

Membrane fusion is essential for an array of cellular functions such as neurotransmitter release, fertilization, tissue formation, and viral infection. Synaptic exocytosis requires the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins syntaxin 1, SNAP-25, and synaptobrevin 2/VAMP2, which are believed to drive two opposite membranes merged. Phosphatidylethanolamine (PE) is a major component of phospholipids in biological membranes, especially in synaptic vesicles. However, the mechanism by which PE modulate SNARE-dependent intracellular membrane fusion is not well understood. Here we report that PE significantly enhances the rate and extent of SNARE-dependent liposome fusion at a physiological concentration. Surprisingly, this stimulatory effect is asymmetric. It is more pronounced when PE in the v-SNARE vesicles than in the t-SNARE vesicles. Site-directed spin labeling EPR show that PE induces the interaction between the transmembrane domains (TMD) of v-SNARE VAMP2 and stabilizes the optimum conformation for their function. Meanwhile, single-vesicle fusion assay reveal the step-specific fusion pathway which PE mostly involved localizes at the priming stage with the transition from hemifusion to the initial fusion pore formation. All together, our results suggest curvature effect of PE accelerates hemifusion accumulation and the interplay between lipids and membrane proteins triggers the progression from the hemifused state to full fusion.

* This paper is preparing for submission. Necessary modifications are made to fit the format of this dissertation.
† Department of Chemistry and Chemical Biology and the Advanced ESR Technology Center, Cornell University
‡ Department of Physics and Center for Biophysics and Computational Biology, University of Illinois at Urbana-Champaign
3.2 Introduction

Synaptic exocytosis involves synaptic vesicles fusing with the presynaptic plasma membrane and then releases neurotransmitters into synaptic cleft[1-3]. During membrane fusion, two separate membranes merge into a continuous bilayer, along with the extremely dynamic membrane remodeling[4]. The fusion process has to overcome the high energy barrier between fusion intermediates along the fusion pathway[5]. SNAREs (soluble N-ethylmaleimide sensitive factor attachment protein receptors) are believed to act as an effective catalyst and serve as the energy source in almost all the intracellular vesicle trafficking[6-8]. Besides the SNARE fusion machinery, the cooperation between lipids and proteins at the onset of membrane fusion may also play a key role in the transition between fusion intermediates[9-11].

Phosphatidylethanolamine (trivial name Cephalin) is usually the second most abundant phospholipid of biological membranes, compared to phosphatidylcholine (PC) in eukaryotic cells and it is frequently the main lipid component of microbial membranes[12]. It can amount to 20% of liver phospholipids and as much as 45% of those of brain; higher proportions are found in mitochondria than in other organelles[13]. As such, PE is obviously a key building block of membrane bilayers. It is a neutral or zwitterionic phospholipid under physiological condition with the structure shown in Fig. 1A.

The structure of PE is crucial to its function[14]. PE has a smaller head group and relatively bigger and unsaturated acyl tails, which gives the lipid a cone shape[15]. The prompting spontaneous negative curvature makes PE fit well in the metastable hemifusion state, which is a highly bended structure with negative curvature for the mixed proximal leaflets[16, 17]. Meanwhile, the condensation of PE small head group makes it easy to overcome the repulsion of hydration pressure between two opposite membranes[18]. All these physical characteristics are believed to help PE take effect in membrane fusion.
Despite progress in understanding the PE’s effect on membrane curvature, not much is known about the manner in which PE modulates membrane proteins arrangement. PE is believed to exert a lateral pressure that induces membrane proteins in their optimum conformation for their functions[19]. There is evidence that PE acts as a molecular ‘chaperon’ during the assembly of membrane proteins to guide the folding path for the proteins and to aid in transition from the cytoplasmic to the membrane environment[20, 21]. In the absence of PE, membrane proteins may not have the correct tertiary structure and so will not function correctly.

In this work, we focus on studying the effect of PE on membrane fusion and specifically the conformation change of the transmembrane domains of SNARE proteins induced by PE. The fluorescence lipid mixing assay was employed to investigate the effect of PE on the SNARE-dependent membrane fusion. And we found that PE significantly enhanced the rate and extent of the liposome fusion when it located in the v-SNARE vesicles but not in the t-SNARE vesicles. Single-vesicle fusion assay suggested that PE mostly affect the priming stage of membrane fusion and may assist the transition from hemifusion intermediates to the initial fusion pore formation. The SDSL EPR investigation results showed that PE induces the direct interaction between the TMD of v-SNARE VAMP2. All together, our data provide the detailed structural basis for the correlation of the PE-induced conformational changes of v-SNARE TMD to the accelerated fusion activity of neuronal SNAREs.

3.3 Experimental Procedures

3.3.1 Plasmid Construction and site-directed Mutagenesis

Full-length VAMP2 (amino acids 1–116) was inserted into pGEX-KG vector to make the N-terminal glutathione S-transferase (GST) fusion protein. To introduce unique cysteine at the specific position of VAMP2, the native cysteine C103 was changed to alanine. Recombinant syntaxin 1A without the Habc domain (SyxHT, amino acids 168–288) and SNAP-25 (amino acids 1-206 with four native cysteines C85, 88, 90 and 92 replaced by alanines) were also
made as GST fusion proteins. All cysteine mutants were generated by QuikChange site-directed mutagenesis kit (Stratagene), and were confirmed by Iowa State University DNA Sequencing Facility.

3.3.2 Protein Expression, Purification, and Spin Labeling

GST fusion proteins were expressed in *E. coli* Rosetta (DE3) pLysS (Novagene) and purified using glutathione-agarose beads (Sigma). The cells were grown at 37 °C in LB with glucose (2 g/liter), ampicillin (100 µg/ml), and chloramphenicol (50 µg/ml) until A$_{600}$ reached 0.6-0.8. After adding 0.3 mM isopropylthio-β-D-galactopyranoside (IPTG), the cells were further grown for 4-6 hours at 22 °C for VAMP2 and SNAP-25 but at 16 °C for SyxHT. GST fusion protein purification was performed following the procedure described in elsewhere[22]. We add ~1% n-octyl-glucoside (OG) in the cleavage buffer for SyxHT and VAMP2.

The cysteine mutants of VAMP2 were reacted with (1-oxyl-2,2,5,5-tetramethylpyrrolinyl-3-methyl) methanethiosulfonate (MTSSL) spin label at 4°C overnight while the protein was bound to the GST-agarose beads. To remove free spin label, the beads with bound proteins were extensively washed with the cleavage buffer then cleaved by thrombin (Sigma). The spin-labeling efficiency was determined by the 50 μm 2,2,6,6-tetramethyl-4-piperidine N-oxide (TEMPO) standard. For all samples, the efficiency was ~80%.

3.3.3 Vesicle Preparation and Membrane Reconstitution

Large unilamellar vesicles (LUVs) of 100 nm diameter were prepared using an extruder as described previously[23]. The lipid-to-protein molar ratio was 100:1 or 200:1. For the lipid mixing assay, the mixture of POPC, DOPS, and POPE in a molar ratio of 85:15:0, 60:15:25 or 45:15:40 were used. Additionally, NBD-PS and rhodamine-PE (1.5 mol % each) were added to the v-SNARE vesicles for fluorescence detection of lipid mixing. For the single-vesicle fusion assay, the t-SNARE vesicles contain 2 mol% Dil, whereas the v-SNARE vesicles contain 2 mol% DiD and 0.1 mol% biotinylated lipid. The t-SNAREs were
preformed by mixing SyxHT and SNAP-25 in a molar ratio of 1:2 at room temperature for 60 min before reconstitution. In all cases proteins were reconstituted by using the dialysis method as described previously[24].

3.3.4 Fluorescence Lipid Mixing Assay

The v-SNARE vesicles were mixed with the t-SNARE vesicles in a molar ratio of 1:9 for the total lipid mixing assay. 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 measured at the NBD’s excitation and emission wavelength of 465 and 530 nm, respectively. Fluorescence signals were recorded by a Varian Cary Eclipse model fluorescence spectrophotometer using a quartz cell of 100 μl with 2 mm path length. After 3600s, 0.1% (vol/vol) reduced Trion X-100 (Sigma) was added to obtain the maximum fluorescence intensity (MFI). All measurements were performed at 35 °C. The inner leaflet mixing assay was performed following the procedure described in elsewhere[23]. After collecting the time traces of total lipid mixing and inner leaflet mixing separately, the percentage of hemifusion was calculated as $2(P_T - P_I)/(2(P_T - P_I) + P_I) \times 100$, where $P_T$ is the percentage of maximum for total lipid mixing and $P_I$ is the percentage of maximum for inner leaflet mixing. For the kinetic comparison of fusion reactions, the v-SNARE and t-SNARE vesicles were mixed in a molar ratio of 1:1 and the final reaction solution contained 0.4 mM lipids.

3.3.5 EPR Data Collection

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. The spectra were collected at low temperature (130K) in the first-derivative mode with 8 mW microwave power. To measure interspin distances from EPR line shapes, Fourier deconvolution analysis was used[25].
3.3.6 Single-vesicle Fusion assay

The single-vesicle fusion assay was performed as described elsewhere[26]. Briefly, the VAMP2-reconstituted v-SNARE vesicles were immobilized on a PEG-coated quartz surface through the biotin-neutravidin conjugation. The SyxHT and SNAP-25-reconstituted t-SNARE vesicles were flowed into the flow cell to induce fusion on the surface. The flow cell was incubated at 37°C for 20 min prior to data acquisition. The single fusion events were monitored in a prism-type total-internal reflection fluorescence microscope (IX70, Olympus) by using an electron multiplying charge-coupled device camera (iXon DV 887-BI, Andor Technology). Fluorescence data were acquired with custom Visual C++ (Microsoft) routines, and donor and corresponding acceptor intensities were obtained through running IDL (Research Systems, Boulder, CO) programs.

3.4 Results

Although dioleoyl (DO) lipids are commonly employed in studies, a 1-palmitoyl, 2-oleoyl (PO) fatty acyl chain composition is more physiological and may yield more stable bilayers[13]. We therefore employ POPE not DOPE in our study. The structure of POPE is shown in Fig. 1A. Negatively stained electron micrograph shows that most of vesicles which are made up of POPC, DOPS, and POPE are around 100 nm in diameter (Fig. 1B). The SNAREs-reconstituted vesicles used in the study are checked by the SDS-PAGE (Fig. 1C).

3.4.1 PE enhances SNARE-dependent membrane fusion

To study the PE effect on the SNARE-dependent membrane fusion, 25 mol% and 40 mol% POPE were employed in the t- or v-SNARE vesicles and both of them. We kept the negatively charged DOPS concentration at 15 mol% for all vesicles and used the lipid component of 85 mol% POPC without POPE as the control. For the fluorescence detection of lipid mixing, NBD-PS and rhodamine-PE (each of 1.5 mol%) were incorporated into the v-vesicles with reconstituted VAMP2, then mixed with excess t-SNARE vesicles, resulting in
the dilution of NBD and rhodamine. Dequenching of the NBD signal is used to detect membrane fusion.

In total lipid mixing assay (Fig. 2A), we observed that POPE strongly promotes liposome fusion at both 25 and 40 mol% POPE level, and interestingly, this effect depends on the asymmetric distribution of POPE in the vesicles. POPE which is only in the v-SNARE vesicles has the strongest effect, more than POPE in both t- and v-SNARE vesicles. And surprisingly, POPE located in the t-SNARE vesicles has no obviously stimulatory effect on membrane fusion.

We also compared the relative initial rate of the POPE promoted liposome fusion reactions (Fig. 2B). And we found that POPE only in the v-SNARE vesicles has the highest relative initial rate among all arrangements, about 6.5-fold (40 mol%) and 5.3-fold (25mol%) increase of the initial rate of lipid mixing with respect to that in the absence of POPE in the t- and v-SNARE vesicles. And both vesicles with POPE is about 4.7-fold (40 mol%) and 4-fold (25mol%) of the control, only t-SNARE vesicles containing 25 or 40 mol% POPE has no obvious stimulation, almost the same as the control. Therefore, our data show that PE enhances the rate and extent of SNARE-dependent membrane fusion, especially when PE located in the v-SNARE vesicles.

3.4.2 PE accelerates the accumulation of hemifusion

Next, we further dissect the curvature effect of PE on membrane fusion. The cone-shaped geometry is favorable for PE to match the highly negative curvature of the lipid stalk, which has been shown in hemifusion state[27]. So PE can induce the hemifusion state[10], in which just outer leaflets merged, while the inner leaflets keep intact.

To test this possibility, we performed the total lipid mixing and the inner leaflet lipid mixing at 1:200 molar protein/lipid molar ratio. We used the modified Meer’s method that uses dithionite to selectively reduce the fluorescence donor NBD on only the outer leaflet, which
allows the selective detection of inner leaflet mixing. Based on our previous study[23, 24], hemifusion state can not be trapped at this relatively high surface protein density in the vesicles with PC/PS lipids.

Indeed, when the v-SNARE vesicles with 40 mol% POPE, we can detect the hemifusion state whatever t-SNARE vesicles with (33% of lipid mixing stay at hemifusion state, shown in Fig. 3, inset) or without POPE (26% hemifusion). If only t-SNARE vesicles with POPE, there is no obvious hemifused state (7%), just like the control (3%). For the control without any POPE in the t- and v-SNARE vesicles, both total and inner leaflet lipid mixing assays showed the almost same mixing efficiency, indicating that all fusion events were at full fusion (Fig. 3). So our data show that the curvature effect of PE accelerates more hemifusion (outer leaflet mixing) than full fusion (inner leaflet mixing) formation when v-SNARE vesicles including PE.

3.4.3 Single-vesicle fusion assay supports the asymmetric effect of PE stimulation on membrane fusion

The single-vesicle fusion assay was used to further investigate which stage of membrane fusion has been mainly promoted by POPE. The v-SNARE vesicles were made by reconstituting VAMP2 into the vesicles that was doped with the fluorescence acceptor DiD (2 mol%) and the biotinylated lipid (0.1 mol%). The v-SNARE vesicles were immobilized on a PEG-coated quartz surface in a flow cell by the conjugation of biotin and neutravidin. Meanwhile, the preformed SyxHT/SNAP-25 t-SNAREs binary complex was reconstituted into a separate population of vesicles that contained the fluorescence donor DiI (2 mol%). The t-SNARE vesicles were flowed into the flow cell at low concentration of 25 pM in order to minimize the interaction of multiple t-SNARE vesicles with single v-SNARE vesicles.

Lipid mixing between t- and v-SNARE vesicles caused by fusion would lead to an increase in 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. For simplification, we set a threshold at $E=0.5$ for
deciding whether full fusion has occurred or not. The full fusion population of vesicles with varied POPE location was significantly different (Fig. 4). Only the v-vesicles with 40 mol% POPE have the highest percentage of population (58%), more than POPE in both sides (33%). This may due to some fusion events arrested at the hemifusion state ($E \approx 0.35$), no transition to the full fusion stage when both vesicles have POPE. This result is consistent with the bulk fusion assay (Fig. 3 inset), both vesicles containing PE has the higher percentage of hemifusion than PE just in v-SNARE vesicles. However, just the t-SNARE vesicles with POPE have little full fusion population (6%), even less than the control (18%) which both t- and v-SNARE vesicles without POPE. Most of fusion events are arrested at the docking stage ($E \leq 0.5$).

So it was further proven that the stimulatory effect of PE on membrane fusion depended on its uneven distribution. And the results show that PE might promote the transition from hemifusion to the initial fusion pore formation.

3.4.4 PE modulates the rearrangement of the v-SNARE transmembrane domain (TMD)

If PE just induces the hemifusion state, it seems like PE cannot promote the fusion transitions from hemifusion to the full fusion. That means it just forms the ‘off-pathway’ product, we called it non-productive hemifusion state[9]. However, in our bulk and single-vesicle fusion assay, PE actually promotes the full fusion especially when it located in the v-vesicles. So we proposed that PE might modulate the rearrangement of the v-SNARE VAMP2 TMD, and promote the recruitment of the v-SNARE complexes, which are complementary to the t-SNARE clusters on the plasma membranes. The highly ordered SNARE complexes quickly migrate toward each other through the lipid stalk (hemifusion) to the full fusion state.

To test this idea, we spin-labeled all the TMD residues of VAMP2 (aa. 96-106) individually and collected their EPR spectra in 40 mol% POPE vesicles at low temperature (130K). At low temperature, the nitroxide side chain motional broadening is effectively suppressed and the spectral broadening is exclusively due to spin-spin interaction[28], which comes from the
spin-labeled position in the TMD of VAMP2. That means the broadening EPR spectra indicate the PE-induced interactions between the TMD of VAMP2.

As the EPR spectra (Fig. 5A) showed, the position 99 of VAMP2 TMD exhibits significant spectral broadening characteristic of the strong spin-spin interaction in the 40 mol% POPE. The calculated interspin distance between VAMP2 position 99 was about 15Å (Fig. 5C). It is shown that the nitroxide is located ~7Å away from the center of the helices[29], so that indicates the two TMDs of the cognate VAMP2 are close each other at position 99. At positions 98, 102, 103, 106 and 110, we can also detect some interactions between TMDs of VAMP2, and all of them are located on one side of the helix (Fig. 5B), suggesting that this may be an interacting face of the helix.

The global arrangement of the VAMP2 TMD region in the presence of 40 mol% POPE can be determined with the Fourier deconvolution method based on the low-temperature EPR spectra (Fig. 5C). Compared to the distance profile throughout the TMD in the absence of PE, the distances at the N- and C-terminal halves of VAMP2 TMD were all <25Å in the presence of 40 mol% POPE, which indicated that all TMD residues interacted closely with residue 99 as the interfacial position. While the C-terminal halves of TMD in the absence of PE, the distances were all >25Å. That suggested that they were splayed out. All together, our results show that PE induces the C-termini of VAMP2 TMD closer.

### 3.5 Discussion

During vesicle trafficking, membrane curvature achieved extensive deformation due to the dynamic membrane remodeling driven by the interplay between lipids and membrane proteins[5]. Within the extensive membrane structural distribution, the lipid stalk and fusion pore are two key hallmarks of membrane fusion[30]. Recent studies have been suggested that regulatory lipids such as phosphatidic acid (PA), phosphatidylinositol-4,5-bisphosphate (PIP2), and cholesterol play key roles in SNARE-dependent membrane fusion[31-33]. Due to the rigid nature of its structure, cholesterol has the capacity to thicken the bilayer when
incorporated in membranes. The scissors model has been proposed for cholesterol promoting membrane fusion[34], which indicates that the TMDs of VAMP2 form an open scissors-shaped dimer in the absence of cholesterol that would fit better to the initial negatively curved bilayer; while in the presence of cholesterol, it can induce the conformational change of VAMP2 TMDs dimer from the open to the closed-scissors, driving the more favorable shape for the thicker bilayer to maintain the hydrophobic match between membranes and the TMD.

Phosphatidylethanolamine is also shown to be required in synaptotagmin C2B domain regulated Ca\(^{2+}\)-triggered fusion[35] and p97/p47-mediated mitotic Golgi membrane fusion[36]. High amount of PE has been found in both synaptic vesicles and presynaptic plasma membranes[37-39]. Most PE resides on the cytoplasmic leaflet of the plasma membrane and synaptic vesicles, so a high proportion of PE distributes on both proximal leaflets of the vesicle/plasma membrane, creates a fusion-competent surface[40]. The spontaneous negative curvature of PE promotes the two proximal leaflets merged and stabilizes the hemifusion state (lipid stalk)[41].

However, the curvature effect of PE can not fully explain the asymmetric effect of PE stimulation on membrane fusion. Our bulk fusion and single-vesicle fusion assay show the dramatic enhancement of the rate and extent of fusion reaction when addition of PE to the v-SNARE vesicles. Surprisingly, addition of PE to the t-SNARE vesicles has less of a stimulatory effect than it does for the v-SNARE vesicles. The results raise the possibility that PE enhances membrane fusion not only through its preference for specific membrane curvature but also through its impacts on the TMD conformation in the membrane. The interplay between PE and the TMD of VAMP2 facilitates the transition of fusion intermediates. Our data suggest that the latter is more pronounced for SNARE-dependent membrane fusion and it is markedly through its impacts on the structure of the v-SNARE TMD. So we conclude that PE initially stabilizes the productive hemifusion state and the cooperation between PE and VAMP2 TMD lower the free energy barrier between fusion intermediates and then transit to full fusion.
Recently, the TMD of t-SNARE syntaxin 1 are found to be critical for the formation and opening of fusion pore. The clustering of syntaxin 1 at the active zone forms a barrier lined with the fusion pore[42, 43]. For successful fusion, we believe the TMD of v-SNARE VAMP2 also form the complementary gap junction structure through the vesicle membrane, and the SNARE complex holds these two parts together[44]. The exclusive interaction between PE and VAMP2 TMD demonstrated by SDSL EPR investigation show that the TMD rearrangement induced by PE hold v-SNARE TMD in the closer conformation, which can be correlated with its fusion potency.

There is still a subject of debate on the fundamental question of whether the nascent fusion pore is lipidic or protein-lined[45]. Although our results do not provide the direct evidence to address this question, it clues the importance of the proper geometry of the protein-lipid complex at the active zone for rapid membrane fusion. Based on our data, we suggest that the proteolipidic fusion pore exits on the path to membrane fusion.

3.6 References


3.7 Figures and Captions

**Fig. 1.** A. Structure of PE and POPE which were used in study. B. Electron micrograph of the v-SNARE reconstituted vesicles containing 40 mol% POPE. The vesicles were stained with 1% phosphotungstic acid on the carbon grids. The size of the vesicles was 90±15 nm. C. SDS-PAGE of SNAREs after reconstitution into vesicles at different protein/lipid molar ratios.
Fig. 2. POPE promotes SNARE-mediated fusion reaction

A. Kinetic comparison of various fluorescence lipid mixing assay in 25 and 40 mol% POPE. Fluorescence changes normalized with respect to the maximum fluorescence intensity (MFI) are shown. The control runs with the v-SNARE vesicles and the t-SNARE vesicles without SNAP-25.

B. Graph of the relative initial rates for different kinetic traces. Error bars, s.d. of three independent measurements. The $k$ value for lipid mixing of vesicles without PE was set at 1 and those for the other kinetics were scaled accordingly.
Fig. 3. Total and inner leaflet lipid mixing assays at normal surface protein concentration (1:200 protein/lipid molar ratio). The bars represent the fluorescence intensity change at 3,600 s. The error bars are s.d. of three independent measurements. The inset figure shows the hemifusion percentage of fusion events in different PE distribution.
Fig. 4. Single-vesicle assay of the SNARE-mediated docking and membrane fusion. Histograms represent the distribution of FRET signals from v- and t-SNARE vesicles mixing after 20 min incubation. The vesicle populations showing FRET efficiency below 0.5 and above 0.5 were assigned to be unfused and fused population, respectively.
Fig. 5. The Global EPR analysis of VAMP2 TMD in 40 mol% POPE

A. The absorbance mode EPR spectra taken at 130K for all positions in the transmembrane domain (96-116) of VAMP2. Red spectra represent VAMP2 in 40 mol% POPE, while black spectra without POPE.

B. Helical wheel diagram of the VAMP2 TMD. Position 99 which shows the stronger tertiary interaction is red, and positions 98, 102, 103, 106, and 110 which also show some interactions on the same side of the helix are green.

C. The interspin distances of VAMP2 TMD determined with the Fourier deconvolution method. The distances of VAMP2 TMD in the absence of PE refer to Fig. 3C, Tong et al., 2009*.

CHAPTER 4: THE C TERMINUS OF SNAP-25 IS ESSENTIAL FOR POSTDOCKING STAGES OF SNARE-MEDIATED MEMBRANE FUSION*

Bin Lu, Yuji Ishitsuka†, Taekjip Ha† and Yeon-Kyun Shin

4.1 Abstract

Synaptic exocytosis requires the assembly of syntaxin 1A and SNAP-25 on the plasma membrane and synaptobrevin 2 (VAMP2) on the vesicular membrane to bridge the two opposite membranes. It is believed that the three SNARE proteins assemble in steps along the dynamic assembly pathway. The C terminus of SNAP-25 is known to be the target of botulinum neurotoxins (BoNT/A and BoNT/E) that block neurotransmitter release in vivo. In this study, we employed EPR technique to investigate the conformation of the SNAP-25 C terminus in binary and ternary complex. Our in vitro lipid mixing assay shows that the C terminal of SNAP-25 is essential for membrane fusion and the truncated SNAP-25 mutants cleaved by BoNT/A and BoNT/E display the different inhibition effect on membrane fusion: SNAP-25E abolishes the fusion activity of SNARE complex, while SNAP-25A just has a little function although it can still form the SDS-resistant SNARE complex as the wild-type SNAP-25. We propose that the truncated SNAP-25 mutants will disrupt the assembly of SNARE core complex and then inhibit the initial fusion pore opening. Single-vesicle lipid mixing assay reveals that the difference in neurotoxin effectiveness results from the different membrane fusion stages induced by the truncated SNAP-25 mutants on the membrane fusion pathway.

* This paper is preparing for submission. Necessary modifications are made to fit the format of this dissertation.
† Department of Physics and Center for Biophysics and Computational Biology, University of Illinois at Urbana-Champaign
4.2 Introduction

The assembly of target SNAREs (t-SNAREs): syntaxin 1A and SNAP-25 on the plasma membrane with the vesicular SNARE (v-SNARE): synaptobrevin 2 (VAMP2) is crucial for Ca\textsuperscript{2+}-triggered regulated exocytosis[1-4]. The highly-tight ternary SNARE complex is formed at the late step of membrane fusion and bridges the vesicle and plasma membrane[5]. However, preceding the formation of ternary complex, the SNARE assembly pathway is not very clear. One study suggests that the three SNARE proteins do not pre-assemble into any intermediate before the activation of exocytosis[6]. Other studies show that the t-SNARE proteins may form the binary complex first and then engage with VAMP2 to form the ternary complex[7-9]. But the composition of binary complex may different. Syntaxin 1A may interact with SNAP-25 at 1:1 or 2:1 molar ratio \textit{in vitro}[10]. In the 2:1 configuration, VAMP2 needs to display the excess syntaxin 1A in binary complex by the high affinity with syntaxin 1A/SNAP-25. If not, the 2:1 binary complex is the “dead-end” intermediate on the pathway to membrane fusion[11, 12].

SNAP-25 is also known to be the target of botulinum neurotoxins (BoNT/A and BoNT/E), which block neurotransmitter release \textit{in vivo}[13-15]. BoNT/A cleaves SNAP-25 nine amino acids from the C-terminus between Gin\textsuperscript{197} and Arg\textsuperscript{198} and BoNT/E cuts 26 amino acids from the C-terminus of SNAP-25 at Arg\textsuperscript{180} and Ile\textsuperscript{181}. Previous \textit{in vivo} study[16, 17] showed that vesicle docking still appeared to occur and unfused vesicles accumulated at the plasma membrane when syntaxin 1A and VAMP2 were inactivated by toxins in neuronal synapses due to the interaction between calcium sensor synaptotagmin and SNAP-25[18]. In contrast to SNAP-25 in the complex with syntaxin 1A and VAMP2[19], the binary complex SNAP-25/synaptotagmin is accessible to both BoNT/A and BoNT/E, the resulting cleavage of SNAP-25 can still bound to synaptotagmin. In PC12 cells, it was found that BoNT/A inhibition could be reversed by elevated Ca\textsuperscript{2+} concentration, but not for BoNT/E[20]. These results suggest that synaptotagmin could be incorporated into synapse vesicle docking[21], and the essential role of the C terminus of SNAP-25 in Ca\textsuperscript{2+}-depended interactions between synaptotagmin and SNARE core complex at the late step in regulated exocytosis.
In this study, we employ the pull-down method to prepare the spin-labeled binary and ternary complex[22] and investigate the conformation of the C-terminus of SNAP-25 in the SNARE complex assembly pathway. We also investigate the function of the C-terminal SNAP-25 in the SNARE-mediated membrane fusion by the fluorescence lipid mixing assay involved with different SNAP-25 mutants. We find that the C-terminus of SNAP-25 SN2 is essential for SNARE-reconstituted proteoliposome fusion, and although the truncated SNAP-25A can form the SDS-resistant complex with syntaxin 1A and VAMP2, like the wild-type SNAP-25, the fusion activity decrease significantly, and SNAP-25E mutant totally abolishes the fusion activity in accord of the SDS-resistant ability missing.

To address the molecular mechanism for neurotoxins inhibitory effect, single molecule FRET[23] is applied to identify the specific stage which was affected due to the loose SNARE assembly from the truncated SNAP-25 mutants by neurotoxin proteolysis. Our data suggest that compared to wild-type SNAP-25, the truncated SNAP-25A involved ternary SNARE complex cannot induce the efficient full fusion and most fusion population accumulate at the stage after docking and before full fusion step. The truncated SNAP-25E mutant completely inhibits membrane fusion, blocking the fusion event at the docking stage.

4.3 Experimental Procedures

4.3.1 Plasmid construction and site-directed mutagenesis

Full-length syntaxin 1A (amino acids 1-288) was inserted into pET-28b vector to make N-terminal His6-tagged protein. Full-length and soluble VAMP2 (amino acids 1–116 and 1-94) were inserted into pGEX-KG vector to make N-terminal glutathione S-transferase (GST) fusion proteins. Recombinant syntaxin 1A without the Habc domain (STX1A HT, amino acids 168–288) and wild-type SNAP-25, truncated SNAP-25A (△9) and SNAP-25E (△26) (amino acids 1-206, 1-197 and 1-180, respectively) were also made as GST fusion proteins. To introduce unique cysteine at the specific position of SNAP-25 and syntaxin 1A, the native cysteines C85, 88, 90 and 92 in SNAP-25 and C145, 271 and 272 in syntaxin 1A were
changed to alanines. All cysteine mutants were generated by QuikChange site-directed mutagenesis kit (Stratagene), and were confirmed by Iowa State University DNA Sequencing Facility.

4.3.2 Protein expression, purification, and spin labeling

GST fusion proteins were expressed in *E. coli* Rosetta (DE3) pLysS (Novagene) and purified by glutathione-agarose beads (Sigma). The cells were grown at 37 °C in LB with glucose (2 g/liter), ampicillin (100 µg/ml), and chloramphenicol (50 µg/ml) until $A_{600}$ reached 0.6-0.8. After adding 0.3 mM isopropylthio-β-D-galactopyranoside (IPTG), the cells were further grown for 6 h at 22 °C for VAMP2 and SNAP-25 but at 16 °C for syntaxin 1A. GST fusion protein purification was performed following the procedure described in elsewhere[24]. We add 1% n-octyl-glucoside (OG) in the cleavage buffer for syntaxin 1A and VAMP2.

His$_6$-tagged syntaxin 1A were expressed in *E. coli* BL21(DE3) Codon Plus RIL (Stratagene) and purified using Ni-NTA resin (Qiagen). The cells were grown at 37 °C in LB with glucose (2 g/liter), kanamycin (34 µg/ml), and chloramphenicol (50 µg/ml) until $A_{600}$ reached 0.6-0.8. Protein expression was induced by 0.5 mM IPTG, and the cells were grown for an additional 4-6 hours at 16 °C. His$_6$-tagged protein purification was performed following the procedure described in elsewhere[25]. Finally, the protein was eluted from the Ni-NTA resin by elution buffer (25mM HEPES, 100mM KCl with 250mM imidazole, pH 7.4).

The cysteine mutants of SNAP-25 and syntaxin 1A were reacted with (1-oxyl-2,2,5,5-tetramethylpyrrolinyl-3-methyl) methanethiosulfonate (MTSSL) spin label at 4°C overnight while the protein was bound to the GST-agarose beads. To remove free spin label, the beads with bound proteins were extensively washed with the cleavage buffer then cleaved by thrombin (Sigma). The spin-labeling efficiency was determined by the 50 μm 2,2,6,6-tetramethyl-4-piperidine N-oxide (TEMPO) standard. For all samples, the efficiency was ~80%.
4.3.3 Pull-down method preparing for the binary and ternary complex

Details of SNARE complex formation by pull-down method have been described[22]. Briefly, purified His$_6$-tagged syntaxin 1A was first added to the Ni-NTA resin solution and nutated for 1 h at room temperature. After washing out the free proteins, 2-fold excess of purified GST-SNAP-25 or 2-fold excess of GST-SNAP-25 and 4-fold excess of soluble VAMP2 were mixed with His$_6$-syntaxin 1A to form the t-SNAREs binary complex or trans-SNARE ternary complex, respectively. The mixture was incubated at 4 °C overnight. After extensive washing to remove the unbound proteins, the complex was eluted with a buffer containing 250 mM imidazole and 1% $n$-octyl-glucoside (OG). The formation of the complex was confirmed with SDS-PAGE gel.

4.3.4 Vesicle preparation and membrane reconstitution

Large unilamellar vesicles (LUVs) of 100 nm diameter were prepared using an extruder as described previously[26]. The lipid-to-protein molar ratio was 200:1 (except as noted). For the lipid mixing assay, the mixture of POPC and DOPS in a molar ratio of 85:15 was used. Additionally, NBD-PS and rhodamine-PE (1.5 mol % each) were added to the v-SNARE vesicles for fluorescence detection of lipid mixing. For the single-vesicle fusion assay, the mixture of POPC, DOPS and cholesterol in a molar ratio of 45:15:40 was used. Additionally, the t-SNARE vesicles contain 2 mol% Dil, whereas the v-SNARE vesicles contain 2 mol% DiD and 0.1 mol% biotinylated lipid. The t-SNAREs were preformed by mixing syntaxin1A HT and SNAP-25 in a molar ratio of 1:1 at room temperature for 60 min before reconstitution. In all cases proteins were reconstituted by using the dialysis method as described previously[27].

4.3.5 Fluorescence lipid mixing assay

The v-SNARE vesicles were mixed with the t-SNARE vesicles in a molar ratio of 1:9 for the total lipid mixing assay. 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 measured at the NBD’s excitation and emission wavelengths of 465 and 530 nm, respectively. Fluorescence signals were recorded by a Varian Cary Eclipse model fluorescence spectrophotometer using a quartz cell of 100 μl with 2 mm path length. After 3600 s, 0.1% (vol/vol) reduced Trion X-100 (Sigma) was added to obtain the maximum fluorescence intensity (MFI). All measurements were performed at 35 °C.

4.3.6 Single-vesicle lipid mixing assay

The single-vesicle fusion assay was performed as described elsewhere[28]. Briefly, the VAMP2-reconstituted v-SNARE vesicles were immobilized on a PEG-coated quartz surface through the biotin-neutravidin conjugation. The syntaxin1A HT and SNAP-25-reconstituted t-SNARE vesicles were flowed into the flow cell to induce fusion on the surface. The flow cell was incubated at 37°C for 20 min prior to data acquisition. The single fusion events were monitored in a prism-type total-internal reflection fluorescence microscope (IX70, Olympus) by using an electron multiplying charge-coupled device camera (iXon DV 887-BI, Andor Technology). Fluorescence data were acquired with custom Visual C++ (Microsoft) routines, and donor and corresponding acceptor intensities were obtained through running IDL (Research Systems, Boulder, CO) programs.

4.3.7 EPR data collection

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. The spectra were collected at room temperature in the first-derivative mode with 1mW microwave power.

4.4 Results
4.4.1 The conformation of the C terminus of SNAP-25 in binary and ternary complex

To compare the SN1 and SN2 conformation in the t-SNAREs binary complex, we prepare six cysteine mutants which locate in the C-terminal of SN1 and SN2, respectively (Fig. 1A). That is C63, 70 and 77 of SNAP-25 SN1; C184, 191 and 198 of SNAP-25 SN2. The selected residues are all at the predicted “g” position in the heptad repeats of SNAP-25 SNARE motifs. In order to avoid the oligomerization of syntaxin 1A, we use His$_6$-tagged full-length syntaxin 1A to pull down the spin-labeled GST-tag SNAP-25. In this case, we add 2-fold excess of purified SNAP-25 to incubate with syntaxin 1A and then wash extensively to get rid of the free SNAP-25.

EPR spectra (Fig. 1B) clearly show that in the C-terminal of SN1, each position has the broad lineshape especially in the membrane. That indicates these positions are all interacting with syntaxin 1A and form the ordered binary complex. However, at the corresponding positions located in the C-terminal of SN2, the EPR lineshape becomes much sharper, which indicates these positions may not completely involved in the binary complex. Based on these data, we propose that the SN1 of SNAP-25 and syntaxin 1A form the highly ordered structure while the C terminal of SN2 is unstructured. This kind of binary complex is much closer to the active t-SNAREs binary complex in vivo which has 1:1 composition of syntaxin 1A and SNAP-25[9].

We further investigate the C-terminal conformation of SNAP-25 SN2 in the ternary complex. His$_6$-tagged syntaxin 1A incubates with 2 times of spin-labeled GST-tag SNAP-25 and 4 times of GST-tag soluble VAMP2. At the three C terminal positions (C177, 184 and 198), when adding soluble VAMP2, EPR spectra all change to broader, which indicate the C terminal of SNAP-25 SN2 interact with VAMP2 and form the trans-SNARE ternary complex (Fig 2). However, we still detect some sharp components at these positions, that may due to the trans-SNARE complex is easy to splay out at the C-terminal end, and it also implies the complete SNARE assembly requires other regulatory proteins (such as
synaptotagmin, complexin) at the late zippering step to promote the tight SNARE bundle formation which is crucial for fusion pore opening.

4.4.2 SN2 of SNAP-25 is essential for membrane fusion

EPR lineshape analysis reveals that the conformation of the C terminus of SN2 in the binary complex is partially free, and the sharp components can be detected even in the ternary complex. One then might wonder whether the formation of the completely assembled SNARE complex is required for membrane fusion. To address this question, we examine the fusion activity of the nitroxide spin-labeled SNAP-25 by the fluorescence lipid mixing assay. The SNARE reconstituted vesicles employed in the study are shown in Fig. 3A. The size of the nitroxide side chain is relatively bulky, and it is comparable with that of tryptophane. Therefore, if the formation of the completed coiled coil is essential for membrane fusion, the alterations at the internal positions (‘a’ or ‘d’) might cause some serious perturbations. As Fig. 3B show that, we detect the distinct lipid mixing activity decreasing with the mutations as compared with that of wild-type SNAP-25. And when the position closer to the ‘0’ layer, the perturbation is stronger. SNAP-25 C188 has 78% fusion activity of wild-type SNAP-25, while SNAP-25 C181 just left 40%. So our data confirm that SN2 of SNAP-25 is required for activating membrane fusion.

4.4.3 The different detergent resistant ability of the truncated SNARE complex

Former studies show that the SNARE proteins can form a very stable complex which can resistant to SDS denaturation and high temperature (90°C)[29, 30]. So we compare the SDS resistant property of the SNARE complexes which are composed of different truncated SNAP-25, syntaxin 1A and VAMP2 (Fig. 4). We mix syntaxin 1A, wild-type SNAP-25, truncated SNAP-25A (Δ9) and SNAP-25E (Δ26) at the molar ratio of 1:2, and with four different amounts of soluble VAMP2 (1-, 2-, 4-, 6-fold of syntaxin 1A) at room temperature for 1 hour, no boiling and run the SDS-PAGE gel. As expected, the wild-type SNAP-25 can form the SDS-resistant complex with the other two SNARE proteins (red arrow, Fig 4A).
And we can also find that there are many high molecular weight complexes besides the monomeric complex, which may due to the aggregation of SNARE complexes by SNAP-25 swapping (red bracket, Fig. 4A). While SNAP-25A (\(\triangle 9\)) can also form the SDS-resistant mono-complex and small amount of the high molecular weight complexes (Fig. 4B). For SNAP-25E (\(\triangle 26\)), it cannot form any SDS-resistant complex (Fig. 4C). Therefore, these results suggest that the SNARE complexes composed of the truncated SNAP-25 display the low stability to the SDS-PAGE.

### 4.4.4 The fusion activity of the truncated SNAP-25

The less stable of the truncated SNARE complexes due to the short SNAP-25 may decrease the activity of their fusion activity. To test this idea, we use the fluorescence lipid mixing assay to detect the function of the truncated SNAP-25. As Figure 5 shows that, the truncated SNAP-25E formed SNARE complex totally abolish the function to induce membrane fusion, just as the negative control, which has no SNAP-25. The SNAP-25A mutant just has about 6% of maximum fluorescence intensity (MFI), while the wild-type SNAP-25 is about 20% of MFI. So, it is clear that the truncated SNAP-25A loss most of its function although it can also form the SDS-resistant SNARE complex (Fig. 4B). Our lipid mixing results are also in good agreement with the \textit{in vivo} studies, which show the same tendency of different membrane fusion activities in neurotoxins treated spinal cord cell cultures[31, 32].

### 4.4.5 The global structure of the truncated SNARE complex

Now we are very interested in why there is so big difference between the wild-type and truncated SNARE complexes. So we employ SDSL EPR to investigate the global structure of the truncated ternary SNARE complex. We use spin-labeled syntaxin 1A to incubate with soluble VAMP2 and the different truncated SNAP-25 mutants and then form ternary complexes. We select four positions corresponding to the upstream positions of SNAP-25 neurotoxins cleavage sites (N terminus). As Figure 6 A and B show that, in the N-terminal SNARE motif of syntaxin 1A, the spectra are all very broad, both of the wild-type SNAP-25
and the truncated mutants. This suggests that SNARE proteins form the complete coiled-coil, whatever which kind of the truncated SNAP-25 is involved. Because the BoNT/A and E cleavage sites are both far away from the spin-labeled positions, the truncated SNAP-25 do not disrupt the SNARE core formation.

However, when syntaxin 1A spin-labeled positions are corresponding to the last four positions of SNAP-25 SNARE motif (C terminus), as Fig. 6 C and D show that, EPR spectra become much sharper, which reflect fast motion of nitroxide, indicating that this region is largely unstructured. So our structural data show that the truncated SNAP-25 mutants induce the partially free conformation in the C terminus of the SNARE core. This tendency is much more obvious when the ternary complex reconstituted in the membrane, which suggests the function of membrane in the arrangement of the SNARE complex.

4.4.6 Which stage of membrane fusion will be blocked by neurotoxins?

Finally, we wonder how the conformational changes induced by neurotoxins proteolysis affect the progress of membrane fusion. The single molecule FRET fusion assay is powerful in dissecting individual fusion steps and transient intermediates along the pathway of SNARE-mediated membrane fusion[28, 33]. So we employ single-vesicle lipid mixing assay to compare the population of different fusion stages which is induced by the truncated SNARE complexes.

Lipid mixing due to SNARE-mediated membrane fusion leads to an increase in FRET efficiency, $E$. In the absence of SNAP-25, $E$ distribution was centered at 0.1, and mostly less than 0.25. The low $E$ value suggests that some t- and v-SNARE vesicle pairs make close contact like docking. The truncated SNAP-25E is mostly like the control, most population has low $E$ distribution, while the truncated SNAP-25A has some population around middle $E$ distribution ~0.5, which indicates some fusion events stop at the hemifusion stage. In contrast, the wild-type SNAP-25 has most population at the high $E$ values centered at 0.8, indicating that extensive membrane fusion occurred (Fig. 7). The wild-type SNAP-25 has the higher
full fusion population than the SNAP-25A and SNAP-25E. This tendency is consistent with the result of our ensemble fusion assay. Our data suggest that SNAP-25E mutant abolishes the fusion activity and accumulates at the docking stage, while SNAP-25A mutant, which can form the relatively stable SNARE complex, can not promote the fusion pore opening and stop at the hemifusion stage.

4.5 Discussion

Neurotransmitter release requires the complete assembly of three SNAREs, namely syntaxin 1A and SNAP-25 on the target plasma membrane and synaptobrevin 2 (VAMP2) on the synaptic vesicles at the late step of Ca\(^{2+}\)-dependent regulated exocytosis. Regulatory proteins, such as synaptatogmin and complexin may modulate the structure of SNARE complex at each step along the assembly pathway to the tight ternary SNARE complex ultimately leading to membrane fusion. How the SNAREs assemble? Although some study suggested that SNAREs may not pre-assemble into any intermediate upon the activation of exocytosis[6], it is thought that SNAREs assemble gradually into the ultimate ternary complex leading to membrane fusion[34]. The active 1:1 syntaxin 1A/SNAP-25 binary complex was stable and clustered on the plasma membrane in chromaffin cells[35]. And the nucleation of assembly process required all three N-terminal SNARE motif of syntaxin 1A and SNAP-25[36], but not VAMP2. It was reported that \textit{in vitro} high concentration of syntaxin would form the 2:1 binary complex with SNAP-25 in which the additional syntaxin occupied the position of VAMP2 in the ternary complex[25]. However, our EPR data reveal that the conformation of the C terminal of SNAP-25 SN2 in binary complex is totally free compared to the corresponding positions in the C terminal of SN1, which indicates that the dominant of the 1:1 t-SNAREs binary complex in our pull-down experiment.

In our EPR study, we also detect the sharp components at the C terminal of SNAP-25 when adding soluble VAMP2 to form the \textit{trans}-SNARE ternary complex. It may due to the tendency of SNARE complex to fray at the proximal-membrane region, which is close to the initial fusion pore. Another possible scenario is only the SNARE proteins cannot proceed the
tight C-terminal zippering, the complete SNARE assembly needs other regulatory proteins together to induce initial fusion pore opening. Complexin and synaptotagmin are two good candidates to play this role[37]. The N-terminal of complexin may bind to the juxta-membranous sequence of SNAREs to transfer the force from SNARE complexes to membranes in fusion[38]. The late step also involved Ca\(^{2+}\)-stimulated manner. Synaptotagmin, major calcium sensor in synapses, conducts the conformational changes after Ca\(^{2+}\) influx, binding to SNAREs and membrane lipids simultaneously, leading to the fast opening of fusion pore[39]. And it was also showed that BoNT/A treatment essentially increased Ca\(^{2+}\) concentration required to activate exocytosis[20]. This result shows that the C terminus of SNAP-25 mediates Ca\(^{2+}\)-dependent interaction between synaptotagmin and SNAP-25.

Recently, it is reported that the C-terminal truncated SNAP-25Δ9 less-tight complex displays smaller amperometric foot current, reduced fusion pore conductances, and lower fusion pore expansion rates in chromaffin cells[40]. Cleavage by neurotoxins may destabilize the four-helical bundle of the synaptic fusion complex in the C-terminal region, and may disrupt the ability of the complex to join membranes. Our EPR data provide the structural basis for the less-tight zippering in the C terminal of SNAP-25. Based on the single-vesicle fusion assay, we find that the truncated SNARE complexes already displayed the different fusion activity before fusion pore opening and the conformational change in the assembled SNARE complex leads to the different structure of initial fusion pore. Compared with the distinct fusion activity between SNAP-25A and SNAP-25E, we propose that the sequence between the cleavage site of BoNT/A and BoNT/E (SNAP-25 Ile\(^{181}\)-Gln\(^{197}\)) is important for the SNARE complex stability and regulatory proteins interaction.

### 4.6 References


4.7 Figures and Captions

A

SNAP-25

B

C-terminal of SN1

C-terminal of SN2

Fig. 1. EPR spectra of the C-terminal of SNAP-25 SN1 and SN2 in binary complex under room temperature

A. The diagram of the spin-labeled positions of the C-terminus SNARE motifs of SNAP-25. The red oval shows the position of the “0” layer of SN1 and SN2 of SNAP-25.

B. EPR spectra of the spin-labeled C-terminal SN1 and SN2 of SNAP-25 in the pull-down prepared His-tag syntaxin 1A full-length and spin-labeled GST-tag SNAP-25 binary complex. (a) and (c) show the EPR spectra in the detergent, (b) and (d) in the membrane.
Fig. 2. EPR spectra of the spin-labeled C-terminal SN2 of SNAP-25 under room temperature

(A) EPR spectra for three spin-labeled C-terminal SNAP-25 SN2 mutants (177C, 184C and 198C of SNAP-25) in the detergent. (B) and (C) are those in the membrane-bound syntaxin 1A full-length-SNAP-25 binary complex (B) and syntaxin 1A full-length-SNAP-25-soluble VAMP2 (1-94) ternary complex (C). All the binary and ternary complexes are prepared through His-tag syntaxin 1A full-length pull-down spin-labeled GST-SNAP-25 mutants and/or GST-soluble VAMP2 (1-94), respectively.
Fig. 3. Lipid mixing assay for the nitroxide labeled SNAP-25

A. Electron micrograph of the SNARE reconstituted vesicles used in the study. The vesicles were stained with 1% phosphotungstic acid on the carbon grids. The size of the vesicles was 90±15 nm.

B. The curves represent the lipid mixing when syntaxin 1A HT (168-288), wild-type or nitroxide labeled SNAP-25 (181C and 188C) and VAMP2 were used in the proteoliposome fusion assay (protein/lipid molar ratio 1:200). The data were normalized against the maximum fluorescence intensity (MFI) obtained by adding 0.1% reduced Triton X-100. The control runs with the v-SNARE vesicles and the t-SNARE vesicles without SNAP-25.
Fig. 4. The SDS-resistant ability of the truncated SNARE complex

One time full-length syntaxin 1A mix with two times wild-type SNAP-25 and truncated SNAP-25 $\triangle 9$ and $\triangle 26$ (which mimic the neurotoxin BoNT/A and BoNT/E cutting products, respectively) and different amount soluble VAMP2 (1, 2, 4 and 6 times of full-length syntaxin 1A).

(A) shows the wild-type SNAP-25 with syntaxin 1A full length and soluble VAMP2. (B) shows BoNT/A truncated SNAP-25A ($\triangle 9$), and (C) BoNT/E truncated SNAP-25E ($\triangle 26$). The arrows show the position of the SNARE complex and the brackets show the higher ordered SNARE complex due to SNAP-25 swapping.
Fig. 5. Lipid mixing assay for the truncated SNARE complex

Fluorescence changes normalized with respect to the maximum fluorescence intensity (MFI) are shown for the lipid: protein molar ratio of 100:1. Wild-type SNAP-25 and the truncated SNAP-25A (△9) and SNAP-25E (△26) were used in the proteoliposome fusion assay. The control runs with the v-SNARE vesicles and the t-SNARE vesicles without SNAP-25.
Fig. 6. EPR spectra for the spin-labeled syntaxin 1A full-length mutants involved in the truncated SNARE ternary complex under room temperature

A and B. First-derivative mode EPR spectra of positions 199C, 206C, 248C and 249C of syntaxin 1A. These four positions correspond to the upper-stream positions of SNAP-25 neurotoxins cutting sites (N terminus). (A) is the complexes in the detergent, (B) is in the membrane.

C and D. First-derivative mode EPR spectra of positions 256C, 257C, 258C and 259C of syntaxin 1A. These four positions correspond to the last four positions of SNAP-25 SNARE motif (C terminus). (C) is the complexes in the detergent, (D) is in the membrane.
Fig. 7. Single-vesicle lipid mixing assay for the SNARE-mediated docking and membrane fusion. Histograms represent the distribution of FRET signals from v-SNARE vesicles (VAMP2 reconstituted) and t-SNARE vesicles (syntaxin 1A HT with wild-type SNAP-25, SNAP-25A (△9) and SNAP-25E (△26) preformed binary complex reconstituted) mixing after 20 min incubation. The vesicle populations showing FRET efficiency below 0.5 and above 0.5 were assigned to be unfused and fused population, respectively. The control is v-vesicles mixing with t-vesicles without SNAP-25.
CHAPTER 5: CONCLUDING REMARKS

5.1 Conclusions

One obvious difference between yeast post-Golgi membrane traffic and the Ca\(^{2+}\)-envoked synaptic vesicle fusion is the precise timing[1]. Compared with the constitutive exocytosis in yeast, the neurotransmitter release is triggered rapidly, within 1 millisecond of Ca\(^{2+}\) influx[2]. Although the SNARE proteins are central components of the molecular machinery mediating membrane fusion, the rapid synaptic vesicle exocytosis is tightly regulated by various regulators at each step of the membrane fusion pathway[3].

In the course of study, I focus on the structural studies of the interaction between SNAREs, regulatory proteins and membrane lipids by EPR spectroscopy and the functional studies of the dynamic processes and transient intermediates during membrane fusion by fluorescence spectroscopy, including single-molecule techniques. In this dissertation, three main projects have been presented as the examples that show how the interplay between SNAREs and regulatory proteins and membrane lipids modulates neuronal SNARE-mediated membrane fusion.

Complexin is one of key regulators in the SNARE-mediated membrane fusion[4]. At the first beginning, “clamping” model indicates that complexin works as an inhibitor for membrane fusion[5, 6]. Recent studies show that it has dual-function at the N-terminal region including the N-terminal domain and accessory \(\alpha\)-helix[7, 8]. With the binding to the SNARE core, complexin can transfer the force from the membrane distal to proximal region which closes to the onset of fusion pore opening[9]. With the correlation of the primary Ca\(^{2+}\) sensor synaptotagmin, complexin is coupled to the calcium activation of exocytosis. The structural analysis of the complexin-SNARE quaternary complex indicates that the accessory \(\alpha\)-helix of complexin can partially replace the C-terminal SNARE motif of VAMP2 and its N-terminal domain can suppress this replacement. So these two adjacent domains work as the fine-
turned switch for complexin-regulated membrane fusion. Our EPR data provide the structural basis for a relative shift in the balance of stimulatory and inhibitory functions of complexin.

Recently, it has been suggested that regulatory lipids such as sterols, diacylglycerol, phosphoinositides and cholesterol play critical roles in SNARE-dependent membrane fusion as essential cofactors[10-12]. Phosphatidylethanolamine (PE) has a high proportion in intracellular membranes. And the cone-shaped structure of PE prompts the spontaneous negative curvature and makes PE fit well in the metastable hemifusion state[13]. The fluorescence lipid assay reveals that PE in the synaptic vesicles can enhance the rate and extent of liposome fusion reaction in vitro. And structural studies indicate the stimulation effect of PE is not just due to the curvature effect, but also related to the rearrangement of transmembrane domain (TMD) of v-SNARE VAMP2 by lipid molecules. Our studies dissect the effect of the regulatory lipids in the remodeling of SNARE proteins.

Membrane fusion involves extensive bilayer rearrangement, which requires the energy source or effective catalysts that lowers the fusion energy barrier[14, 15]. The SNARE complex, composed of the plasma membrane t-SNAREs syntaxin 1A and SNAP-25 and the vesicle v-SNARE synaptobrevin 2 (VAMP2), is believed to be the fusion machinery that bridges two opposite bilayers and facilitates membrane fusion[16, 17]. The SNARE complex assembly from the N-terminal to the C-terminal of SNARE motifs drives membrane merged together. As the transition from “loose” to “tight” trans-SNARE complex and finally formation of cis-SNARE complex, SNARE proteins undergo dramatic conformational changes. The conformation of the C-terminal SNAP-25 motifs of SNAP-25 along the SNARE assembly pathway has been investigated by EPR spectroscopy. And botulinum neurotoxins (BoNT/A and BoNT/E) cleaved the C-terminal truncated mutants of SNAP-25 display the low stability of SDS-resistant and significantly reduced membrane fusion activity. The single vesicle fusion assay indicates the inhibition steps located at the postdocking stages in the membrane fusion pathway.
Although some studies cast doubt on the SNARE hypothesis[18, 19], the notion that SNARE proteins play a central role in membrane fusion is widely accepted[20]. However, besides SNAREs, additional factors are required to facilitate the extreme speed and exquisite Ca^{2+} sensitivity of synaptic vesicle fusion. In this fast and sophisticated regulation, the SNARE fusion machinery is highly controlled by the temporally and spatially regulated mechanisms of specialized regulators involving the exquisite modulation. The information gathered from above three detailed investigations will contribute to elaborate the molecular mechanism of SNARE-mediated membrane fusion.

5.2 References


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