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Multiple conformations of a single SNAREpin between two nanodisc membranes reveal diverse pre-fusion states

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Abstract

SNAREpins must be formed between two membranes to allow vesicle fusion, a required process for neurotransmitter release. Although its post-fusion structure has been well characterized pre-fusion conformations have been elusive. We used single molecule FRET and EPR to investigate the SNAREpin assembled between two nanodisc membranes. The SNAREpin shows at least three distinct dynamic states, which might represent pre-fusion intermediates. While the N-terminal half above the conserved ionic layer maintains a robust helical bundle structure the membrane-proximal C-terminal half shows either high FRET representing a helical bundle (45%), low FRET reflecting a frayed conformation (39%), or mid FRET revealing an yet unidentified structure (16%). It is generally thought that SNAREpins are trapped at a partially zipped conformation in the pre-fusion state, and complete SNARE assembly happens concomitantly with membrane fusion. However, our results show that the complete SNARE complex can be formed without membrane fusion, which suggests that the complete SNAREpin formation could precede membrane fusion, providing an ideal access to the fusion regulators such as complexins and synaptotagmin 1.

Keywords

trans-SNAREpin; nanodisc; EPR; single-molecule FRET

INTRODUCTION

Neurotransmitter release at the synapse requires fusion of neurotransmitter-laden vesicles with presynaptic plasma membrane. Synaptic membrane fusion is mediated by the soluble

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AUTHOR CONTRIBUTION

J.S., D.-H.K., and Y.-K.S. designed the experiment. J.S. and X.L. performed the experiments. J.S. and Y.-K.S. analyzed data and wrote the paper.

N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex [1–3]. To allow membrane fusion to happen target membrane (t-) SNAREs syntaxin-1a and SNAP-25 interact with vesicle membrane (v-) SNARE synaptobrevin-2 (or VAMP-2) to form the *trans*-SNARE complex or SNAREpin that bridges two membranes. Cognate SNARE motifs, which are conserved heptad-repeat sequences of 60–70 residues, mediate SNAREpin formation.

Structural studies have shown that SNAREs tend to spontaneously assemble into a parallel four stranded coiled coil. However, SNARE structures were determined with isolated SNARE motifs [4–7] or in a *cis* state without the repulsion force between two apposed membranes [8]. It is thus unclear whether the four stranded coiled coil represents a pre-fusion state or simply a post-fusion state. A biochemical analysis [9] as well as studies with mechanical tweezers [10, 11] raised the possibility of a partially zipped state in which membrane-distal N-terminal region is assembled while the membrane-proximal C-terminal region is frayed. It is however unknown how many intermediate states exist and what their conformational details are.

In this work, we employed a nano-sized membrane called nanodisc [12–14] and reconstituted a single SNARE complex between two separate nanodiscs. The investigation of the dye-labeled SNARE complex with single molecule (sm) FRET and site-directed spin labeling EPR reveal that there are three distinct conformational states for VAMP-2; (1) a C-terminal-frayed state, (2) a fully assembled state, and (3) a structurally uncharacterized state that appears to be in between the two two major states. Furthermore, EPR shows that the structural break occurs around the ionic layer.

MATERIALS AND METHODS

Protein expression, purification, and fluorophore- or spin-labeling

All recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) grown in LB medium at 37 °C to an A₆₀₀ of 0.7. The protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were then grown for another 5 hours at 25 °C for soluble proteins, SNAP-25 and apoA1 or 16 hours at 16 °C for proteins with the transmembrane domain, syntaxin-1a and VAMP-2, respectively. Pellets were resuspended in phosphate buffered saline (PBS) or PBS with 0.3% triton (PBST) with the protease inhibitor, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and additional dithiothreitol (DTT) for single cysteine mutants of syntaxin-1a or VAMP-2. Cells were lysed by sonication, after which lysates were centrifuged at 13,000 rpm for 30 min at 4 °C to remove insoluble fractions. Supernatants containing glutathione S-transferase (GST)-fused recombinant proteins, syntaxin-1a, SNAP-25, and VAMP-2 were then incubated with glutathione sepharose beads (GE healthcare) while 6× His-tagged SNAP-25 and apoA1 were incubated with Ni-NTA agarose beads (Qiagen) for 1 hour at 4 °C. After washing with PBS or PBST, GST-fusion proteins were eluted with thrombin (0.02 unit/μl, Sigma) in PBS or PBS with 0.8% octyl β-D-glucopyranoside (PBS-OG) for membrane proteins and 6× His-tagged proteins were eluted with 200 mM imidazole (Sigma) in PBS. The elution buffer for single cysteine mutant proteins contained 2 mM DTT. For pull down of the *trans*-SNAREpin sandwiched between two nanodiscs, His-tag-free apoA1 was obtained with

thrombin cleavage of His-tag (Figure S1). For the smFRET study of the *trans*-SNAREpin, single cysteine mutants of syntaxin-1a (I203C or V241C) and VAMP-2 (Q33C or A72C) were desalted with PD 10 column (GE healthcare) to eliminate free DTT in solution, then incubated with 10× molar excess of maleimide-derivative fluorophore, Cy5 or Cy3, respectively for overnight at 4 °C. Unreacted free fluorescence labels were removed by the PD 10 column and the labeling efficiency of each SNARE protein was measured with spectrophotometry (Beckman). Extinction coefficients of Cy5 ($250\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 650 nm) and Cy3 ($150\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 552 nm) were used to calculate the concentration of the fluorophore. The detergent compatible Lowry assay (DC assay, Bio Rad) was recruited to determine the concentration of SNAREs. The labeling efficiency for each protein was 46% and 40% for syntaxin-1a I203C and V241C and 55% and 62% for VAMP-2 Q33C and A72C, respectively. For spin labeling with MTSSL (Enzo Life Sciences) of VAMP-2 cysteine mutants, 10× molar excess of MTSSL was added to GST-VAMP-2 cysteine mutants on glutathione sepharose beads right after rapid washing with DTT free PBST before overnight incubation at 4 °C. Free MTSSL was washed out before eluting spin-labeled VAMP-2 with thrombin.

Reconstitution and purification of SNARE-incorporated nanodiscs

To generate homogeneously-sized nanodiscs, phospholipid mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS) (Avanti Polar Lipids) with the molar ratio of 85:15 were dried and then resuspended in T150 buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4), which included 50 mM sodium cholate. The SNARE protein and apoA1 protein were then added. The molar ratio for lipids:SNARE:apoA1 was 160:0.5:2. The self-assembly of SNARE-incorporated nanodiscs was initiated by a rapid removal of sodium cholate by treating the sample with SM-2 Bio-Beads. The t-SNARE or VAMP-2 incorporated nanodiscs were then purified through gel-filtration using SuperdexTM200 GL 10/30 column (Amersham Biosciences) (Figure S2)

Lipid mixing between v-discs and either t-discs or t-liposomes

To determine whether v-discs fuse with either t-discs or t-liposomes, 1.5 molar % of each 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine-PE) (Avanti Polar Lipids) were included in v-discs. Both t-discs and t-liposomes were prepared with unlabeled phospholipids to dilute NBD-PE and rhodamine-PE when fusion occurred. The molar ratios of lipid:SNARE for nanodiscs and t-liposomes were 160:0.5 and 200:1, respectively. The v-discs were then mixed with 9× lipid molar excess of either t-discs or t-liposomes at 37 °C in a fusion buffer (10 mM HEPES, 100 mM KCl, pH 7.4). The reaction solution contained total 0.5 mM lipids. The NBD fluorescence intensity was measured at excitation and emission wavelengths of 465 and 530 nm, respectively with a fluorescence spectrophotometer (Cary Eclipse model, Varian). The maximum fluorescence intensity was obtained by adding 0.1% reduced Triton X-100 (Sigma).

FRET measurements of *trans*-SNARE complex with TIR

To immobilize Cy5-labeled t-discs on the biotinylated PEG-coated quartz surface for smFRET, 0.5% of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-biotinyl (biotinyl-PE, Laysan Bio Inc.) was included into the phospholipid mixture for t-disc assembly. The quartz slide was assembled into a flow chamber and then covered with 0.2 mg/ml streptavidin. t-discs (100~200 nM) were flown into the chamber and incubated with the 2 μ M 49–96 peptide derived from VAMP-2. 5 $\times$ molar excess of Cy3-labeled v-discs were then added and incubated for 25 min to allow SNAREpin formation between two nanodiscs afterward. All TIR experiments were performed at room temperature (~25 °C) in the presence of oxygen scavenger system (0.4 % (w/v) glucose (Sigma), 4 mM Trolox (Calbiochem), 1 mg/ml glucose oxidase (Sigma), 0.04 mg/ml catalase (Calbiochem)) in T150 buffer. smFRET measurements were carried out on a prism type TIR setup, which is based on inverted microscope (Eclipse Ti, Nikon) with the 100 ms or 200ms time resolution. Each Cy3 and Cy5 fluorescence signal from the chamber were collected by a water immersion lens (PlanApo VC 60 $\times$, Nikon) and separated by a dichroic mirror (T660lpxr, chroma), which had a threshold at 635 nm wavelength. Two separated signals were then transferred to separated images on an electron multiplying charged-coupled device camera (iXon3 DU897D, Andor Technology) as donor and acceptor signals, respectively. FRET efficiencies (E) were obtained by

$$E = \frac{F_A}{(F_A + F_D)}$$

where F_A and F_D are the acceptor and donor fluorescence intensities.

Purification and measurement of spin labeled *trans*-SNARE complex in EPR spectroscopy

We used the pull down method to purify spin labeled *trans*-SNAREpin in between nanodiscs. Spin-labeled VAMP-2 was incorporated into the His-tag-free v-nanodisc and the unlabeled t-SNARE was incorporated into the 6 $\times$ His-tagged t-nanodisc, respectively. The t-nanodiscs were attached to Ni-NTA sepharose beads and equal molar quantities of S-v-nanodiscs were then added and incubated for overnight at 4 °C to form spin labeled *trans*-SNAREpin. After washing out of unbound free S-v-nanodiscs spin labeled *trans*-SNAREpins were eluted from the beads by imidazole. To obtain spin labeled *cis*-SNARE complex in the nanodisc, the mixture of syntaxin-1a, 6 $\times$ His-tagged SNAP-25, and spin-labeled VAMP-2 were incubated with molar ratio of 1:2:1. Unbound syntaxin-1a and spin-labeled VAMP-2 were washed out using the Ni-NTA column and the complex was then incorporated into the nanodisc. Each spin-labeled VAMP-2 was reconstituted into nanodiscs for the EPR measurement. EPR spectra were collected in the first-derivative mode with 1 mW microwave power using the Bruker ESP 300 spectrometer equipped with a loop-gap resonator. The modulation amplitude was set at no greater than one-fourth of the line width. All EPR measurements were performed at room temperature.

RESULTS

Reconstitution of the SNAREpin into two nanodiscs

To investigate the structure and the dynamics of the SNAREpin using smFRET and EPR, we reconstituted VAMP-2 into a population of nanodiscs (v-discs) and the 1:1 binary complex of syntaxin-1a and SNAP-25 into a separate population of nanodiscs (t-discs) using a standard assembly method [15]. Briefly, all components required for the reconstitution, phospholipids, SNAREs, and the belt-forming apoA1 protein were dissolved in the 50 mM sodium cholate solution. The rapid removal of the detergent by Bio-Beads SM-2 prompted self-assembly of proteonanodiscs (Figure 1A). Assembled nanodiscs were purified using gel-filtration chromatography and the size and the shape were examined with transmission electron microscopy (TEM) (Figure 1B). We also confirmed, using photobleaching, that most nanodiscs carry a single SNARE protein (Figure S3).

For the spectroscopic investigation of the SNAREpin the nanodisc structure is desired to be sufficiently robust to hold off the fusion activity of the SNARE complex. When t- and v-discs were mixed we observed no significant lipid mixing (Figure 1C), indicating that nanodiscs maintain the structural integrity despite SNARE complex formation. There is however the possibility that the lipid flow is restricted while the two bilayers are connected as it is the case with 'restricted hemifusion' [16]. In contrast, lipid mixing occurred when v-discs were mixed with t-SNARE reconstituted liposomes, consistent with previously reported results [13].

The t- and v-SNAREs assemble into a parallel configuration between two nanodiscs, some with the frayed C-terminal end

For smFRET we prepared an N-terminal FRET pair VAMP-2 Q33C and syntaxin-1a I203C labeled with Cy3 and Cy5, respectively. We also prepared a C-terminal FRET pair, Cy3-labeled VAMP-2 A72C and Cy5-labeled syntaxin-1a V241C (Figure 2A). We immobilized the t-disc using the biotin lipid-to-streptavidin conjugation on the polyethylene glycol-coated quartz surface in a flow cell (Figure 2B). The v-disc solution was flown in to the flow cell to allow SNAREpin formation between two nanodiscs. To promote efficient SNARE complex formation we used the recombinant peptide derived from the C-terminal region of VAMP-2 (aa. 49–96), which is known to make the binary complex more compatible for VAMP-2 [17].

When the SNAREpin with the N-terminal FRET pair I203C/Q33C (NN) was examined with total internal reflection (TIR) we found a single major FRET distribution picked at FRET efficiency $E \sim 0.8$. Meanwhile, when the SNAREpin with the C-terminal FRET pair V241C/A72C (CC) was examined we observed two distinct major FRET distributions, one centered at $E \sim 0.8$ ($45.4 \pm 6.0\%$), the other near $E \sim 0.1$ ($38.9 \pm 4.5\%$), and one minor distribution at $E \sim 0.45$ ($15.7 \pm 4.8\%$). However, when the out-of-register FRET pair I203C/A72C or V241/Q33C (NC or CN) was examined we detected mostly low FRET and the distributions were centered at $E \sim 0.1$ or 0.35 with little population at high FRET. Together, the results show that v- and t-SNAREs assemble into a parallel configuration and the antiparallel orientation is negligible [18, 19].

Given that the SNAREpin is all parallel, it is interesting that the NN pair has mostly the high FRET population ($E \sim 0.8$), while the CC pair has the two major states at high ($E \sim 0.8$) and low FRET ($E \sim 0.1$). Thus, the results imply that the CC pairs have one ordered and the other disordered and dynamic states (Figure 2C). Although minor (16%) the distinct population centered at $E \sim 0.45$ is interesting. This state appears to be neither fully extended nor fully structured, which might indicate a possible new intermediate state.

EPR confirms that both the fully assembled four-helix bundle and the half-zipped complex represent trans-SNAREpin conformations

To further characterize the structure of the SNAREpin with EPR, we prepared eleven single cysteine mutants of VAMP-2 around the conserved central ionic layer (Figure S4A), labeled them with a nitroxide spin label MTSSL (*S*-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl) methyl methanesulfonothioate spin label), and reconstituted them into nanodiscs (*S-v*-nanodiscs). Proteonanodiscs carrying the unlabeled binary complex (*t*-nanodiscs) contain 6× His-tagged apoA1 and it was used to pull down the partner *S-v*-nanodisc to purify the spin labeled *trans*-SNAREpins (Figure 3A). The fusion activity of spin-labeled VAMP-2 was analyzed by the *in vitro* proteoliposome lipid mixing assay and we confirmed that all spin labeled VAMP-2 maintains the wild-type fusion activity (Figure S4B).

Each *S-v*-nanodisc sample generated a narrow EPR spectrum reflecting unstructured conformation of VAMP-2 prior to complex formation with *t*-nanodisc [20] (Figure 3B, uncomplexed). Meanwhile, when the *cis* complex, which was prepared by premixing spin-labeled VAMP-2 with *t*-SNAREs, was reconstituted into nanodiscs, EPR spectra showed significant line-broadening due to formation of a stable four-helix bundle (Figure 3B, *cis*).

EPR spectra of spin labeled *trans*-SNAREpins showed broad line shapes for all N-terminal positions before the conserved ionic layer (residue R56). However, the EPR spectra for all C-terminal positions beyond the ionic layer exhibited two spectral components, one broad and the other sharp (Figure 3B, *trans*). The broad spectral components may represent a well-structured coiled coil while the sharp components reflect the unstructured and freely moving conformation of VAMP-2.

A spectral decomposition analysis [21, 22] was used to separate two spectral components (Figure 3B, decomposition) and the ratio of two spectral components was calculated. The ratio of unstructured component was low in the N-terminal region but reaches ~30% for C-terminal positions after position 58. The transition from the structured to unstructured conformation happens between residues 50 and 54 (Figure 3C). Therefore, our EPR results show that for *trans*-SNAREpin the N-terminal half of VAMP-2 above the conserved ionic layer is a well-structured coiled coil while the C-terminal half after the ionic layer is either fully structured (70%) or unstructured (30%), qualitatively consistent with the results from smFRET. We note that smFRET showed 39% the unstructured population, which was somewhat higher than the EPR estimate.

The real-time analysis of single SNAREpins reveals a subpopulation with highly fluctuating dynamics

Both snapshot smFRET and static EPR analysis revealed three distinct conformational states of *trans*-SNAREpins; a fully assembled helix bundle, the half-zipped SNARE complex, and a structurally unknown mid FRET state. Next, we investigated the real-time dynamics of single FRET pairs to identify yet unidentified or transient dynamic substates. For NN FRET pairs, as expected, we observed stable high FRET time traces with little fluctuation (Figure 4A). The high stability of the FRET time traces indicates that the N-terminal region of the *trans*-SNARE complex maintains a robust helical bundle.

In contrast, the analysis of single CC FRET pairs reveals a few different dynamic facets; (1) time traces maintaining stable high FRET ($E \sim 0.8$, 40.3%, Figure 4B), (2) those maintaining stable low FRET ($E \sim 0.1$, 39.7%, Figure 4C), (3) those making transition between high and low FRET (3.4%, Figure 4D), (4) those maintaining stable mid FRET ($E \sim 0.45$, 14.5%, Figure 4E), and finally, (5) traces fluctuating between multiple FRET states (2.1%, Figure 4F). Stable high FRET may correspond to a fully assembled helical bundle while stable low FRET may represent fully unstructured conformation. Fluctuating traces may reflect the equilibrium conformational change between multiple states. Stable mid FRET traces are consistent with the structural intermediate state, which can transition to either the high or the low FRET state (Figure S5). Thus, the real time analysis of smFRET pair confirmed the three conformational states identified with the static smFRET analysis without adding further intermediate state and revealed equilibrium fluctuations between three conformational states for some of them.

DISCUSSION

For SNAREpins trapped between two nanodiscs we identified two major conformational states and one minor state; a fully-assembled helical bundle (45%), a half-zipped complex in which the C-terminal half of VAMP-2 is frayed as a random coil (39%), and a structurally unknown mid FRET state (16%). EPR revealed that for VAMP-2, the structural transition from a α -helix to a random coil occurs right near the conserved ionic layer located at residue R56. It is likely that t-SNAREs syntaxin-1a and SNAP-25 maintains a complete three-stranded coiled coil in the half zipped complex [10, 11].

The real-time analysis of smFRET showed that the two major conformational states are mostly very stable with few transitions between the two within our experimental time window (2 min). The analysis however revealed a small subset of SNAREpins for which relatively fast transitions between multiple FRET states are allowed. How is it possible that a small subset exhibits fast fluctuations despite the majority do not? To understand such different dynamic behaviors we must take into account the distance between two nanodiscs holding the SNAREpin, which sets the geometric constraints to the SNARE conformation. At sufficiently large distances between t- and v-nanodiscs the random coil conformation would be preferred for the C-terminal half of VAMP-2 and reziping might not be allowed. For closely apposed nanodiscs, however, a fully assembled SNARE core would be energetically stable. We speculate that the fluctuation between two conformations is allowed only in a narrow window of reasonable inter-membrane distances.

Based on the experimental results, we propose a three-state model for *trans*-SNAREpins in the inter-membrane gap (Figure 5). The N-terminal half above the ionic layer maintains a robust parallel four stranded coiled coil for all intermediates. The C-terminal half below the ionic layer, however, may have three different states: First, the C-terminal half of VAMP-2 is unstructured in a large inter-membrane gap (Figure 5A). Second, it is structurally immobilized as it was indicated in EPR spectra but not fully assembled to the four helix bundle yet (Figure 5B). We speculate that the structure might be some kind of the out-of-register coiled coil that has been observed in other coiled coils [23]. Third, it remains as a fully assembled coiled coil between a closely apposed two membranes (Figure 5C). With EPR we found that about 30% of SNAREpins hold the fully unstructured conformation while smFRET shows 39% are unstructured. The discrepancy might arise from the differences in nanodisc concentrations in two experiments. While we used only a 100 pmole range in sm FRET we used tens of mM for EPR that would create a clouded environment for nanodiscs, which in turn might force the inter-membrane gap between two nanodiscs to decrease.

The fact that the fully assembled coiled coil is the major conformation of the SNAREpin is surprising because it is generally thought that the four-stranded coiled coil is the ‘post-fusion’ conformation and in the ‘pre-fusion’ state it might have a partially assembled structure like one shown in Figure 5A. In a Ca^{2+} -triggered exocytosis we expect that the fusion machinery enters into the ‘primed state’ that is ready to fuse upon the rise of Ca^{2+} signal [24]. Therefore, we wonder if the fully assembled coiled coil represents the true primed state. The major Ca^{2+} -sensor synaptotagmin-1 then interacts with the juxtramembrane region and/or the membrane to mediate membrane fusion [25]. We speculate that a priming agent such as complexin might play a role in holding the SNAREpins in the fully assembled coiled coil, instead of a partially assembled state [26–28].

In summary, we characterized the structure and the dynamics of SNAREpin sandwiched between two nanodisc using smFRET and EPR. We found that while the N-terminal half maintains a robust coiled coil structure the C-terminal half have multiple dynamics states including the fully zipped, the half zipped, the partially zipped, and fluctuating states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

1. Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE. SNAP receptors implicated in vesicle targeting and fusion. *Nature*. 1993; 362:318–324. [PubMed: 8455717]
2. Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Söllner TH, Rothman JE. SNAREpins: Minimal Machinery for Membrane Fusion. *Cell*. 1998; 92:759–772. [PubMed: 9529252]
3. Jahn R, Scheller RH. SNAREs [mdash] engines for membrane fusion. *Nat. Rev. Mol. Cell. Biol.* 2006; 7:631–643. [PubMed: 16912714]
4. Sutton RB, Fasshauer D, Jahn R, Brunger AT. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature*. 1998; 395:347–353. [PubMed: 9759724]
5. Poirier MA, Xiao W, Macosko JC, Chan C, Shin Y-K, Bennett MK. The synaptic SNARE complex is a parallel four-stranded helical bundle. *Nat. Struct. Mol. Biol.* 1998; 5:765–769.
6. Antonin W, Fasshauer D, Becker S, Jahn R, Schneider TR. Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. *Nat. Struct. Mol. Biol.* 2002; 9:107–111.
7. Strop P, Kaiser SE, Vrljic M, Brunger AT. The Structure of the Yeast Plasma Membrane SNARE Complex Reveals Destabilizing Water-filled Cavities. *J. Biol. Chem.* 2008; 283:1113–1119. [PubMed: 17956869]
8. Stein A, Weber G, Wahl MC, Jahn R. Helical extension of the neuronal SNARE complex into the membrane. *Nature*. 2009; 460:525–528. [PubMed: 19571812]
9. Sorensen JB, Wiederhold K, Muller EM, Milosevic I, Nagy G, de Groot BL, Grubmüller H, Fasshauer D. Sequential N- to C-terminal SNARE complex assembly drives priming and fusion of secretory vesicles. *EMBO J.* 2006; 25:955–966. [PubMed: 16498411]
10. Gao Y, Zorman S, Gundersen G, Xi Z, Ma L, Sirinakis G, Rothman JE, Zhang Y. Single Reconstituted Neuronal SNARE Complexes Zipper in Three Distinct Stages. *Science*. 2012; 337:1340–1343. [PubMed: 22903523]
11. Min D, Kim K, Hyeon C, Hoon Cho Y, Shin Y-K, Yoon T-Y. Mechanical unzipping and reziping of a single SNARE complex reveals hysteresis as a force-generating mechanism. *Nat. Commun.* 2013; 4:1705. [PubMed: 23591872]
12. Bayburt TH, Carlson JW, Sligar SG. Reconstitution and Imaging of a Membrane Protein in a Nanometer-Size Phospholipid Bilayer. *J. Struct. Biol.* 1998; 123:37–44. [PubMed: 9774543]
13. Shi L, Shen Q-T, Kiel A, Wang J, Wang H-W, Melia TJ, Rothman JE, Pincet F. SNARE Proteins: One to Fuse and Three to Keep the Nascent Fusion Pore Open. *Science*. 2012; 335:1355–1359. [PubMed: 22422984]
14. Krishnakumar, Shyam S, Kümmel D, Jones, Sunny J, Radoff, Daniel T, Reinisch, Karin M, Rothman, James E. Conformational Dynamics of Calcium-Triggered Activation of Fusion by Synaptotagmin. *Biophys. J.* 2013; 105:2507–2516. [PubMed: 24314081]
15. Bayburt TH, Sligar SG. Membrane protein assembly into Nanodiscs. *FEBS Lett.* 2009; 584:1721–1727. [PubMed: 19836392]
16. Chernomordik LV, Frolov VA, Leikina E, Bronk P, Zimmerberg J. The Pathway of Membrane Fusion Catalyzed by Influenza Hemagglutinin: Restriction of Lipids, Hemifusion, and Lipidic Fusion Pore Formation. *J. Cell. Biol.* 1998; 140:1369–1382. [PubMed: 9508770]
17. Pobbati AV, Stein A, Fasshauer D. N- to C-Terminal SNARE Complex Assembly Promotes Rapid Membrane Fusion. *Science*. 2006; 313:673–676. [PubMed: 16888141]
18. Bowen ME, Weninger K, Brunger AT, Chu S. Single Molecule Observation of Liposome-Bilayer Fusion Thermally Induced by Soluble N-Ethyl Maleimide Sensitive-Factor Attachment Protein Receptors (SNAREs). *Biophys. J.* 2004; 87:3569–3584. [PubMed: 15347585]
19. Weninger K, Bowen ME, Choi UB, Chu S, Brunger AT. Accessory Proteins Stabilize the Acceptor Complex for Synaptobrevin, the 1:1 Syntaxin/SNAP-25 Complex. *Structure*. 2008; 16:308–320. [PubMed: 18275821]

20. Brewer KD, Li W, Horne BE, Rizo J. Reluctance to membrane binding enables accessibility of the synaptobrevin SNARE motif for SNARE complex formation. *Proc. Natl. Acad. Sci.* 2011; 108:12723–12728. [PubMed: 21768342]
21. Thorgeirsson TE, Russell CJ, King DS, Shin Y-K. Direct Determination of the Membrane Affinities of Individual Amino Acids†. *Biochemistry.* 1996; 35:1803–1809. [PubMed: 8639661]
22. Zhang Y, Su Z, Zhang F, Chen Y, Shin Y-K. A Partially Zipped SNARE Complex Stabilized by the Membrane. *J. Biol. Chem.* 2005; 280:15595–15600. [PubMed: 15713667]
23. Xi Z, Gao Y, Sirinakis G, Guo H, Zhang Y. Single-molecule observation of helix staggering, sliding, and coiled coil misfolding. *Proc. Natl. Acad. Sci.* 2012; 109:5711–5716. [PubMed: 22451899]
24. Südhof TC. THE SYNAPTIC VESICLE CYCLE. *Annual Review of Neuroscience.* 2004; 27:509–547.
25. Honigsmann A, van den Bogaart G, Iraheta E, Risselada HJ, Milovanovic D, Mueller V, Müller S, Diederichsen U, Fasshauer D, Grubmüller H, Hell SW, Eggeling C, Kühnel K, Jahn R. Phosphatidylinositol 4,5-bisphosphate clusters act as molecular beacons for vesicle recruitment. *Nat. Struct. Mol. Biol.* 2013; 20:679–686. [PubMed: 23665582]
26. Schaub JR, Lu X, Doneske B, Shin Y-K, McNew JA. Hemifusion arrest by complexin is relieved by Ca²⁺-synaptotagmin I. *Nat. Struct. Mol. Biol.* 2006; 13:748–750. [PubMed: 16845390]
27. Tang J, Maximov A, Shin O-H, Dai H, Rizo J, Südhof TC. A Complexin/Synaptotagmin 1 Switch Controls Fast Synaptic Vesicle Exocytosis. *Cell.* 2006; 126:1175–1187. [PubMed: 16990140]
28. Giraudo CG, Garcia-Diaz A, Eng WS, Chen Y, Hendrickson WA, Melia TJ, Rothman JE. Alternative Zippering as an On-Off Switch for SNARE-Mediated Fusion. *Science.* 2009; 323:512–516. [PubMed: 19164750]

Summary

The detailed structure of SNAREpin at the primed state is not clear yet. The single *trans*-SNAREpin study with our new platform, the nanodisc revealed that the *trans*-SNAREpin has diverse conformations.

Figure 1. Preparation of SNARE-incorporated proteonanodisc

(A) Self-assembly of SNARE-incorporated nanodiscs. By rapid removal of sodium cholate (orange) from the mixture of the SNARE protein (blue/yellow), phospholipids (grey), and apoA1 (green) resulted in assembly of the proteonanodisc carrying the SNARE protein. (B) Purification of proteonanodiscs. SNARE-incorporated proteonanodiscs were collected from the earlier part of the elution volume (blue dashed square) than empty-nanodiscs (red spectrum) through the gel-filtration column and characterized by transmission electron microscopy (bottom). (C) Lipid mixing assays. v-nanodiscs containing NBD and rhodamine were mixed with unlabeled either t-liposomes (black trace) or t-nanodiscs (red trace).

Figure 2. Distributions of the FRET efficiency at the N- and C-terminal regions of *trans*-SNAREpin

(A) Fluorescent dye-labeled positions on VAMP-2 and syntaxin-1a, respectively. Q33C or A72C of VAMP-2 (blue ribbon) were labeled with Cy3 (light red saw-tooth circle) while I203C or V241 of syntaxin-1a (red ribbon) were labeled with Cy5 (light blue saw-tooth circle). The α -carbon-to- α -carbon distance between two cysteine residues were indicated. The flexible linker region (gray) and transmembrane domain (yellow) are also included in the structure. (B) TIR setup for the FRET detection on the SNAREpin sandwiched between two nanodiscs. t-nanodisc carrying Cy5-labeled syntaxin-1a was immobilized on the quartz

surface through biotin (dark red square) -streptavidin (purple, cross shape) conjugation and allowed to interact with v-nanodisc carrying Cy3-labeled VAMP-2. (C) Distributions of the FRET efficiencies for the NN, CC, and out-of-register NC and CN FRET pairs. The Gaussian fitting lines also are included (black line).

Figure 3. EPR analysis of spin labeled *trans*-SNAREpins

(A) Purification of nitroxide-labeled *trans*-SNAREpins sandwiched between two nanodiscs using pull-down method with imidazole elution (top). TEM images of sandwiched nanodiscs mediated by *trans*-SNAREpins (bottom, yellow dashed circle). (B) The first derivative EPR spectra of each eleven spin-labeled VAMP-2 in specific conformations. S-v-nanodisc only (uncomplexed), pre-assembled *cis*-SNAREpin in nanodisc (*cis*), purified spin labeled *trans*-SNAREpin in nanodiscs (*trans*), and complexed fraction in *trans*-SNAREpin resulted from spectral decomposition (decomposition, red). (C) Unstructured fraction of each eleven VAMP-2 in *cis*-SNAREpin (black closed circle and line) and *trans*- (red closed circle and line) conformation with the breaking point at right near the ionic layer.

Figure 4. Real time traces of FRET in *trans*-SNARE complex conformation

All traces for donor and acceptor signals under donor-exciting 532 nm laser source, unless otherwise indicated, were in the top panel and the calculated FRET traces are in the bottom panel. (A) The NN FRET pair (Q33Cy3 of VAMP-2 and I203Cy5 of syntaxin-1a) in *trans*-SNAREpin shows stable high FRET (blue open circle and line) followed by acceptor photobleaching (red open circle and line) and the recovery of the donor fluorescence (green open circle and line). The CC FRET pair (A72Cy3 of VAMP-2 and V241Cy5) show 5 different time traces; B) stable high FRET, C) stable low FRET and co-localized two fluorophores were confirmed by switching laser sources between acceptor-exciting 635 nm (red rectangle) and donor-exciting 532 nm (green rectangle), D) transition between high and low, E) stable mid FRET, and F) relatively fast fluctuation between multiple FRET states.

Figure 5. A proposed model of multiple conformations for the SNAREpin

When a synaptic vesicle with VAMP-2 (blue) is recruited into the t-SNARE complex of SNAP-25 (green) and syntaxin-1a (red) on the presynaptic plasma membranes, docking is initiated by the N-terminal half interaction between VAMP-2 (blue, rod shape) and t-SNAREs while C-terminal half of VAMP-2 (blue line) is remained as unbound (A). As the vesicle gets closer to the membrane within a certain distance, the C-terminal VAMP-2 forms hypothetical intermediate structure (distorted blue line) (B). Afterwards, in close enough contact between two membranes, SNARE proteins form fully assembled four-helical bundle structure and wait for the action of synaptotagmin-1 and Ca^{2+} for Ca^{2+} -triggered vesicle fusion (C).