Membrane Fusion Induced by Neuronal SNAREs Transits through Hemifusion*

Xiaobing Lu‡§, Fan Zhang‡§, James A. McNew¶ and Yeon-Kyun Shin‡

From the ‡Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA 50011 and the ¶Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77251-1892

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Address correspondence to: Yeon-Kyun Shin, Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA 50011, Tel. 515 294-2530; Fax. 515 294-0453; E-Mail: colishin@iastate.edu

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

In the neuron, SNARE assembly plays a critical role in promoting the fusion of the synaptic vesicles with the plasma membrane (1-7). Cognate SNAREs pair to form a coiled coil structure that bridges two membranes (8,9). The subsequent steps yielding one common phospholipid bilayer remain a matter of debate. It has been proposed that SNAREs involved in neurotransmitter release at synapses may promote membrane fusion by the formation of two juxtaposed transmembrane pores preassembled by the transmembrane domains of SNAREs in respective membranes (10). In sharp contrast, recent evidence for SNAREs involved in trafficking in yeast has indicated that hemifusion might be involved in the SNARE fusion pathway (11,12), analogous to the lipid-protein stalk model generally accepted for viral membrane fusion proteins (13-17). However, hemifusion has only been observed as an outcome rather than as an intermediate, raising some concerns if hemifusion is an off-pathway product in SNARE-mediated membrane fusion (16). Alternatively, the mechanism by which neuronal SNAREs induce membrane fusion might be entirely different from those for other systems including yeast SNAREs.

In this work, we examine the kinetics of membrane fusion of liposomes mediated by neuronal SNAREs Syntaxin 1A, SNAP-25, and Synaptobrevin using fluorescence assays (18) that monitor both total lipid mixing and inner leaflet mixing. Our results demonstrate that hemifusion is the main event at the early stage of the fusion reaction. Over time, hemifusion converts to the complete fusion, strongly supporting that hemifusion is a true fusion intermediate.

MATERIALS AND METHODS

Protein sample preparation - Plasmid construction, protein expression, and purification for neuronal SNAREs were described elsewhere (19). Briefly, v-SNARE synaptobrevin (amino acids 1–116) and a truncated version of t-SNARE syntaxin...
(amino acids 168–288), for which the N-terminal α-helical Habc domain was deleted, were expressed as N-terminal glutathione S-transferase (GST) fusion proteins. Another t-SNARE SNAP-25 was also expressed as GST fusion protein, in which the four cysteines were replaced with alanines. Recombinant proteins were expressed in E. coli Rosetta (DE3) pLysS (Novagene). GST fusion proteins were purified by affinity chromatography using glutathione-agarose beads (Sigma). The protein was cleaved by thrombin in cleavage buffer (50 mM Tris–HCl, 150 mM NaCl, pH 8.0, 1 % OG only for syntaxin and synaptobrevin). Purified proteins were examined with 15 % SDS–PAGE and the purity was at least 90 % for all proteins (data not shown).

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

The reconstitution efficiency was determined using SDS-PAGE, visualized by Coomassie blue staining. The amount of protein in liposomes was estimated by comparing the band in the gel with that of the same protein of known concentration. The reconstitution efficiency was more than 90 % for both t- and v-SNAREs (Fig. 1B). The orientation of the SNAREs in the liposomes was examined with the trypsin digestion experiments(19). The SNAREs-reconstituted vesicles were treated with trypsin (0.5 mg/ml) under room temperature for 1 hour. Nearly all SNARE proteins were digested by trypsin, indicating that SNARE molecules are oriented inside-out, exposing the soluble domain to the solution phase (Fig. 1B).

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

Inner leaflet mixing assay - The inner leaflet mixing assay was modified from the method developed by Meers (20). The method is based on the fact that the sodium dithionite reacts more rapidly with NBDs in the outer leaflet than those in the inner leaflet. By controlling the time and amount of dithionite, the reaction can be limited to the outer leaflet. Small aliquots (~0.7 µl) of 100 mM sodium dithionite in 50 mM Tris buffer (pH 10) were added to the v-SNARE liposomes (100 µl, 0.2 mM lipid) until a desired reduction of NBD was achieved. The reaction was monitored at room temperature by scanning the fluorescence signal for 15 minutes from 500 to 700 nm with the excitation at 460nm. Typically, in 10 minutes the reduction was complete and no more change of the spectrum was observed. The liposomes without NBDs in the outer leaflets were subject to the lipid mixing assay described above. To make sure that the percent of MFI was independent of the extent of the NBD reduction, the inner leaflet mixing assay was performed at the 55 % reduced and the 65 % reduced conditions. We found that the percents of MFI were identical for both conditions (Supplementary Information).

RESULTS AND DISCUSSION

We investigated the fusion of liposomes induced by neuronal SNAREs utilizing a well-characterized fluorescence lipid mixing assay. Recombinant target membrane (t-) SNARE complexes containing the H3 ‘core’ domain of Syntaxin 1A as the t-SNARE heavy chain and SNAP-25 as the t-SNARE light chains, were reconstituted into the liposomes containing POPC/DOPS (65/35 mol/mol) (Fig. 1A). Additionally, the vesicle-associated (v-) SNARE Synaptobrevin was also reconstituted into a separate population of the same POPC/DOPS liposomes containing fluorescent lipids, NBD-PS and rhodamine-PE (1.5 mol % each). For both t- and v-SNAREs, the initial lipid/protein ratio was set at 50:1 (Fig. 1B). When the t-SNARE liposomes were mixed with the v-SNARE liposomes at 35 °C, an increase of the fluorescence intensity was observed, indicating that the fusion occurred (Fig. 2A, red trace). While both v- and t-SNAREs have roughly equal surface density, the absolute amount of t-SNARE liposomes in the cuvette was about 10 times greater than the v-SNARE liposome. These amounts of protein and lipid were used to better insure first order kinetics. Following an initial rapid rise, the fluorescence signal approached a plateau of roughly 40 % of maximum (Fig. 2A). The half time of the fusion reaction was approximately 9 ± 1 min (~540 ± 60 sec), consistent with the previous reported half-time of 10 min with a similar truncated Syntaxin 1A (21).

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

Since we collected the time traces of total lipid mixing and inner leaflet mixing separately, it was straightforward to calculate the percent of hemifusion (defined as \( \frac{2(P_T-P_I)}{2(P_T-P_I)+P_I} \times 100 \), where \( P_T \) is the percent of maximum for total lipid mixing and \( P_I \) is that for inner leaflet mixing (Fig. 2A)) as a function of time. As expected, at the beginning of the fusion reaction, the fluorescence change was mainly due to outer leaflet mixing (Fig. 2B), indicating that hemifusion was the dominant event. As time progressed, however, the percent of hemifusion decreased dramatically. Hemifusion was about 90% at one minute and was extrapolated to be nearly 100% at the start of the reaction. The percentage steadily declined to roughly 20% at 40 minutes (Fig. 2B) and asymptotically approached 12% at 150 min. It should be noted that these estimates are from the ensemble of \( \sim 7 \times 10^{11} \) liposomes in the reaction and that each individual event is likely to be very fast (18, 21). These results provide strong kinetic evidence for the conversion of hemifusion to complete fusion over time and therefore for the sequential mechanism in which hemifusion is an on-pathway intermediate (Fig. 2C).

We analyzed outer and inner leaflet mixing separately on the basis of the sequential mechanism (Fig. 2C). For this purpose, net outer leaflet mixing was obtained by subtracting the kinetics of inner leaflet mixing from that of total lipid mixing (Fig. 2E). The time trace of outer leaflet mixing fitted well to a simple exponential function representing the first-order kinetics with the first-order rate constant \( k_1 = 1.5 \times 10^{-3} \) sec\(^{-1} \) (Fig. 2E). The kinetics of inner leaflet mixing was analyzed with the first-order kinetics theory for the sequential mechanism depicted in Fig. 2C (22). The theory fitted the data very well with first order rate constant \( k_2 = 3.7 \times 10^{-3} \) sec\(^{-1} \) and the backward rate constant \( k_{-1} = 6.3 \times 10^{-3} \) sec\(^{-1} \). It is quite interesting to find that \( k_{-1} \) is almost twice as big as \( k_2 \). Thus, once hemifusion is formed it is twice as much likely to go back to the two separate liposomes than advancing towards the complete fusion.

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

Work with viral fusion proteins as well as SNARE proteins has suggested that the surface protein density of the fusion proteins may be an important parameter that determines the outcomes of the fusion event (12, 23). To gain further insights into the role of the protein surface density, we reduced the input lipid/protein (L/P) ratios to 100:1 and 200:1, from the original 50:1. Liposomes generated with a 100:1 L/P ratio yielded qualitatively similar results to the previous 50:1 L/P ratio (Fig. 3A and B), although the overall fusion efficiency was lower and the conversion from hemifusion to complete fusion was slower. With this surface density, ~45% full fusion occurred during the 75 minute reaction. However, at the L/P ratio of 200:1 we did not observe the time-dependent shift from hemifusion to complete fusion (Fig. 3C and D). This result suggests that the majority (>60%) of hemifusion intermediate remained without transitioning to complete fusion. Thus, the surface density of SNARE proteins is indeed a determining factor for the outcome of SNARE-induced membrane fusion.

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

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§ These authors contributed equally to this work.

1 The abbreviations used are: SNARE, soluble NSF-attachment protein; POPC, 1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphatidylcholine; DOPS, 1,2-dioleoyl-sn-glycero-3-phosphatidylserine; PE, phosphoethanolamine; OG, n-octyl-D-glucopyranoside;

FIGURE LEGENDS

Fig. 1. Characterization of reconstituted proteins and liposomes. A, Electron micrograph of SNARE-reconstituted liposomes. The diameter of the vesicles was approximately 80-100 nm. B, SDS-PAGE of v-SNARE synaptobrevin before (lane 1) and after the reconstitution into liposomes (lane 2). The reconstitution efficiency was approximately 90 %. Lane 3 represents the sample after treating with trypsin. The high molecular weight band was from trypsin itself. C, SDS-PAGE of SNAREs after reconstitution into liposomes at different protein/lipid ratios.

Fig. 2. Fluorescence assay for total lipid mixing and inner leaflet mixing. A, Fluorescence changes for total lipid mixing (red traces) and inner leaflet mixing (blue traces), normalized with respect to the maximal fluorescence intensity (MFI), are shown for the lipid/protein ratio of 50:1. MFI was obtained by adding 0.2 % n-dodecylmaltoside (sudden jumps at the end). The black trace is a control run with the t-SNARE liposomes reconstituted with Syntaxin 1A only (without SNAP-25). Inset: Residual fluorescence changes for total lipid and inner leaflet mixing recorded at the longer period of time (8,500-9100 s); the former was 43 % while the latter was 40 %. B, The percent of hemifusion versus time. The percent of hemifusion was calculated using the equation 2(PT-PI)/[2(PT-PI)+PI] x 100, where PT is the percent of maximum for total lipid mixing and PI is that for inner leaflet mixing. C, A schematic diagram for the sequential mechanism in which hemifusion is an on-pathway intermediate. D, A schematic diagram for the parallel mechanism in which hemifusion is an off-pathway product. E, Analysis of outer and inner leaflet mixing based on the sequential mechanism in C and the first-order kinetics. The solid lines are the best fits to the first order kinetics. The data were fitted with program DYNAFIT (22).

Fig. 3. Fluorescence assays for total lipid mixing and inner leaflet mixing at different lipid/protein ratios. A and B are for 100:1, and C and D are for 200:1. Fluorescence changes for total lipid mixing (red traces) and inner leaflet mixing (blue traces) were normalized with respect to the maximal fluorescence intensity (MFI). The black trace is the control with the t-SNARE liposomes reconstituted with Syntaxin 1A only (without SNAP-25).
Figure 1

A.

B.

- synaptobrevin
  - 1
  - 2
  - 3
  1 in detergent
  2 in liposome
  3 + trypsin

C.

- t-SNAREs
  - SNAP-25
  - syntaxin

- synaptobrevin
  - 1:200
  - 1:100
  - 1:50
  - 1:200
  - 1:100
  - 1:50
Figure 2

A. Lipid/protein ratio = 50:1

Fluorescence Intensity (Percent of maximum)

Time (s)

B. Percent of hemifusion

Time (s)

C. Sequential mechanism

\[ \text{Outer leaflet mixing} \rightarrow \text{Inner leaflet mixing} \rightarrow \text{Control} \]

D. Parallel mechanism

\[ \text{Outer leaflet mixing} \rightarrow \text{Inner leaflet mixing} \]

E. Fluorescence Intensity (Percent of maximum)

Time (s)
Figure 3

A.

Lipid/protein ratio = 100:1

Fluorescence Intensity (Percent of maximum)

Time (s)

B.

Percent of Hemifusion

Time (s)

C.

Lipid/protein ratio = 200:1

Fluorescence Intensity (Percent of maximum)

Time (s)

D.

Percent of Hemifusion

Time (s)
Supplementary Figure. A) Total lipid mixing assay with different amounts of NBD -PS in the v-SNARE liposomes. The red line is for 1.5 mole% NBD-PS and blue one for 0.7 mole % NBD-PS. In both cases, Rhodamin-PE remained at 1.5 mole %. The two time traces overlap very well one another, indicating that the fusion kinetics is not affected by the amount of NBD-PS which uniformly distributed over the outer and inner leaflet of the liposome. B) Lipid mixing assays with different extents of dithionite reduction. The blue trace was with 55% reduction of NBD-PS and the pink trace was for 65% reduction. The red trace which is total lipid mixing without dithionite treatment is shown as a reference. The results indicate that the kinetics of inner leaflet mixing is largely unaffected by the reduction extent. The inset shows the raw data.
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