Insertion of the Membrane-proximal Region of the Neuronal SNARE Coiled Coil into the Membrane*

Dae-Hyuk Kweon‡, Chang Sup Kim‡, and Yeon-Kyun Shin§

From the Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, Iowa 50011

Received for publication, October 30, 2002, and in revised form, January 12, 2003
Published, JBC Papers in Press, January 15, 2003, DOI 10.1074/jbc.M211123200

In the neuron, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins assemble into an α-helical coiled coil that bridges the synaptic vesicle to the plasma membrane and drives membrane fusion, a required process for neurotransmitter release at the nerve terminal. How does coiled coil formation drive membrane fusion? To investigate the structural and energetic coupling between the coiled coil and membrane, the recombinant SNARE complex in the phospholipid bilayer was studied using fluorescence quenching and site-directed spin labeling EPR. Fluorescence analysis revealed that two native Trp residues at the membrane-proximal region of the coiled coil are inserted into the membrane, tightly coupling the coiled coil to the membrane. The EPR results indicate that the coiled coil penetrates into the membrane with an oblique angle, providing a favorable geometry for the basic residues to interact with negatively charged lipids. The result supports the proposition that core complex formation directly leads to the apposition of two membranes, which could facilitate lipid mixing. Trp residues and basic residues are abundant at the membrane-proximal region of transmembrane SNARE proteins, suggesting the generality of the proposed mechanism for the SNARE complex-membrane coupling.

Neurotransmitter release at synapses requires the fusion of neurotransmitter-containing vesicles to the presynaptic plasma membrane. Membrane fusion is, however, an exceedingly difficult process to go through without the assistance of specific proteins, because of the protective nature of the biological membranes. In the neuron, soluble N-ethylmaleimide-sensitive factor attachment protein (SNARE) proteins play an essential role in promoting membrane fusion (1). It is proposed that assembly of the SNARE complex initially bridges two membranes, induces lipid mixing, and leads to the hemifusion state and the fusion pore, of which the detailed mechanism is largely unknown (2–5).

Progress has been made in understanding the biophysical principles of SNARE assembly. SNARE assembly starts with the interaction of vesicle-associated membrane protein 2 (VAMP2 or synaptobrevin) with target plasma membrane SNAREs Syntaxin 1A and SNAP-25. Interactions between SNARE proteins are mediated by “SNARE motifs” that are essentially coiled coil sequences and are present in all SNARE proteins (3). For the SNARE complex, one SNARE motif each from Syntaxin 1A and VAMP2 and two from SNAP-25 assemble into a 110-Å-long four-stranded coiled coil (6, 7). It is worthwhile to note that target plasma membrane SNAREs Syntaxin 1A and SNAP-25 also spontaneously assemble into a similar but less stable four-stranded coiled coil (8–10).

How does coiled coil formation lead to membrane fusion? There are two features of the SNARE coiled coil that might be important. First, the helices are all aligned parallel, suggesting the co-location of two membrane attachment points, which sets up a favorable geometry for membrane fusion (6, 7, 11–13). Second, the coiled coil is highly stable (14, 15). Therefore, coiled coil formation might have the capacity to overcome the repulsive force between two apposing membranes. Although this mechanistic model appears to be structurally and energetically attractive, there are caveats that require careful consideration. For example, if the SNARE core were tethered with flexible linkers to membrane domains, coiled coil formation might not be able to bring about membrane apposition no matter how strong the pulling force it generates because the energy would be dissipated.

To validate this model, a direct coupling between the coiled coil and membranes appears to be necessary. Previously, Brunner and co-workers proposed a hypothetical model for the coiled coil-to-membrane coupling (7). In this model, the coiled coil is linked to transmembrane domains (TMD) as continuous helices. This model arbitrarily assumes some bending flexibility of helices in short amino acid stretches at the membrane-proximal region. Furthermore, helix-disrupting mutations or amino acid insertions in the linker region have little or only moderate effect on the SNARE fusion activity, inconsistent with this model (16, 17). How then is the coiled coil energetically coupled to membranes? The answer to this fundamental question hinges on structural information of the connection of the coiled coils to the membranes.

Recent EPR investigations of intact SNAREs using site-directed spin labeling EPR have yielded new results that not only confirm the existence of coupling between the coiled coil and the membrane but also suggest a tentative mechanism of the SNARE core-membrane coupling. EPR analysis indicated that the linker region of Syntaxin 1A, enriched with basic amino acids...
acid residues, is understructed but laterally inserted into the membrane, tightly coupling the coated coil to the membrane (18, 19). Importantly, clusters of basic amino acid residues are found in the linker regions of all transmembrane SNAREs (20), raising the possibility that SNARE linker regions generally insert into the membrane. Further, this tentative model offers a plausible explanation as to why this region is tolerant to helix-disrupting mutations. However, structure and membrane topology of the VAMP2 linker region is not experimentally confirmed yet.

In this work, we report the EPR and fluorescence investigations of the membrane topology of the recombinant SNARE complex. Fluorescence quenching analysis revealed that the native Trp residues at positions 89 and 90 in VAMP2 are inserted into the acyl chain region of the bilayer. Further, the EPR results reveal that the core domain maintains the coated coil structure up to residue 92, suggesting that the SNARE coated coil is partially inserted into the head group region of the bilayer. The EPR data also suggest that the coated coil penetrates into the membrane with an oblique angle. Taken together, the new results further establish the concept of the tight SNARE core-membrane coupling, providing structural basis for the force transmission from the core region to the membrane during SNARE assembly.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-palmitoyl-2-deoxy phosphatidylcholine (POPC) and 1,2-dioleoyl phosphatidylserine (DOPS), 1-palmitoyl-2-stearoyl-(6,7)-dibromo-sn-glycero-3-phosphocholine (6,7-Br2-PC), and 1-palmitoyl-2-stearoyl-(11,12)-dibromo-sn-glycero-3-phosphocholine (11,12-Br2-PC) were purchased from Avanti Polar Lipids (Birmingham, AL). (1-Oxy-2,2,5,5-tetramethylylpyrroline-3-methyl) methanethiosulfonate spin label (MTSSL) was obtained from Toronto Research Chemicals (North York, Canada). The paramagnetic reagent, nickel (II) (Ce) hexaamine-Tetracyclene-diacetic acid (NiEDDA) was synthesized following the procedure described elsewhere (21). FuSy Turbo DNA polymerase and Escherichia coli BL21-CodonPlus RIL were purchased from Stratagene (La Jolla, CA). n-Octylglycycloside (OG) was from Roche Molecular Biochemicals. Bio-beads SM2 was obtained from Bio-Rad. Oligonucleotides for site-directed mutagenesis were obtained from Qiagen (Valencia, CA). Ultrafree Centrifugal Devices Biomax-5K for proteins was obtained from Millipore (Bedford, MA). Nickel-nitrilotriacetic acid (Ni-NTA) agarose beads in buffer A. The mixture was left equilibrated at 4 °C overnight. Free MTSSL was removed by washing with PBST-Met-Triton buffer (PBST-Met with 0.5% Triton X-100) for full-length VAMP2, Triton X-100 (0.5% (v/v) and 1-methylquinolinium chloride (MQC) were added to a final concentration of 0.1 M. After equilibration the beads were washed with an excess volume of buffer A.

The protein concentration was estimated by Bio-Rad protein assay kit using bovine serum albumin as a standard. The spin labeling efficiency was estimated by comparing the double integration with the standard Tempo sample at 100 μM. Spin labeling was nearly quantitative for all VAMP2 mutants.

**Membrane Reconstitution of Recombinant SNARE Complex**—Large unilamella vesicles with a 100-nm diameter (100 mM total lipids) were prepared in buffer B without OG using an extruder. Vesicles of POPC containing 15 mol % of DOPS were first mixed with two volumes of the SNARE complex. OG was added to the mixture to a final concentration of 0.6%. After dilution with an equal volume of buffer B, the samples were dialyzed against the buffer containing Bio-beads SM2 adsorbent for 4 °C for 40 h. During the dialysis, the buffer was changed three times. The samples were centrifuged at 100,000 × g for 5 min to remove both the protein precipitates and the fraction of large vesicles. Protein Expressions, Purification, and Spin Labeling—Recombinant GLUT-1 transfusion fusion proteins were expressed in E. coli BL21-CodonPlus RIL and purified using glutathione-agarose chromatography. The recombinant cells were grown to an OD600 of 0.6. After the addition of isopropyl-D-thiogalactopyranoside (0.2 mM), the cells were grown further for 5 h at 37 °C. For individual mutants, the power saturation curves were obtained by the EPR method. In this method, the protein concentration was calculated from the peak-to-peak amplitude of the central line (M = 0) of the first derivative EPR spectrum as a function of incident microwave power in the range 0.1–40 mW. Three power saturation curves were obtained and the data were generated by the computer program. EPR spectra were obtained using a Bruker ESP 300 spectrometer (Bruker, Germany) equipped with a low noise microwave amplifier (Miteq, Hauppauge, NY) and a loop-gap resonator (Medical Advances, Milwaukee, WI). The modulation amplitude was set at no greater than one-fourth of the line width. The spectra were collected at room temperature in LB medium. The gas atmosphere of the protein sample was achieved with the TPX EPR tube for the loop-gap resonator. For individual mutants, the power saturation curves were obtained from the peak-to-peak amplitude of the central line (M = 0) of the first derivative EPR spectrum as a function of incident microwave power in the range 0.1–40 mW. Three power saturation curves were obtained and the data were generated by the computer program. EPR spectra were obtained using a Bruker ESP 300 spectrometer (Bruker, Germany) equipped with a low noise microwave amplifier (Miteq, Hauppauge, NY) and a loop-gap resonator (Medical Advances, Milwaukee, WI). The modulation amplitude was set at no greater than one-fourth of the line width. The spectra were collected at room temperature in LB medium. The gas atmosphere of the protein sample was achieved with the TPX EPR tube for the loop-gap resonator. For individual mutants, the power saturation curves were obtained from the peak-to-peak amplitude of the central line (M = 0) of the first derivative EPR spectrum as a function of incident microwave power in the range 0.1–40 mW. Three power saturation curves were obtained and the data were generated by the computer program. EPR spectra were obtained using a Bruker ESP 300 spectrometer (Bruker, Germany) equipped with a low noise microwave amplifier (Miteq, Hauppauge, NY) and a loop-gap resonator (Medical Advances, Milwaukee, WI). The modulation amplitude was set at no greater than one-fourth of the line width. The spectra were collected at room temperature in LB medium. The gas atmosphere of the protein sample was achieved with the TPX EPR tube for the loop-gap resonator.
reagent. The $\Delta P_i$ value is proportional to the diffusion coefficient of the nitroxide to the freely diffusing reagents such as oxygen and NiEDDA. Thus, $\Delta P_i$ is considered to be equivalent to the accessibility $W$. The immersion depth is calculated based on the reference curves determined from a set of lipid molecules spin-labeled at different acyl chain positions.

**Fluorescence Quenching Experiment**—For fluorescence measurements, membrane samples were prepared from the recombinant SNARE complex with the wild-type sequences. Total lipid concentration was $-2.5$ mM, whereas the concentration of the SNARE complex was $5 \mu$M. For acrylamide quenching, appropriate amounts of the acrylamide stock solution (2 x) were added to the membrane sample to make the final concentration in the range of $0-160$ mM, whereas lipids and proteins concentrations remain constant among samples. The fluorescence measurements were carried out with PerkinElmer fluorescence spectrophotometer. The samples were excited at 285 nm, and the emission spectra were collected in the range of 300–400 nm. The total fluorescence intensity $F$ was obtained by integrating the intensity in this spectral range. The degree of quenching was analyzed according to the following Stern-Volmer equation.

$$F/F = 1 + K_{sv}[Q] \quad (Eq. 1)$$

where $F_i$ and $F$ are the fluorescence intensities in the absence and presence of acrylamide, respectively, $K_{sv}$ is the Stern-Volmer constant for collisional quenching, and $[Q]$ is the concentration of the quencher. The equation predicts a linear behavior of $F/F$ versus $[Q]$ for a homogeneous solution (24). After fluorescence measurements, the membrane samples were treated with Triton X-100 (final concentration, 1%) to subsequently measure the acrylamide quenching in a detergent-solubilized state.

To measure the membrane immersion depth of Trp residues, membrane samples containing two types of lipid quenchers were prepared. Lipid quencher, 6,7-Br$_2$-PC or 11,12-Br$_2$-PC, was added in replacement of part of POPC while maintaining the DOPC mole fraction at 15%. The reconstitution of SNARE complex was carried out by the same procedure described above. The degree of quenching was determined as a function of the mole fraction of added brominated PC.

The averaged immersion depth of two Trp residues was calculated according to the parallax analysis (25, 26). The distance of the Trp residue from the bilayer center $Z_{lip}$ is given by the following.

$$Z_{lip} = L_{c1} + [-\ln(F/F_o)/\gamma C L]/2L \quad (Eq. 2)$$

where $L_{c1}$ represents the distance from the bilayer center to the shallow quencher (11 Å for 6,7-Br$_2$-PC), $C$ is the mole fraction of the quencher divided by the area of the lipid molecule (70 A$^2$), $F_1$ and $F_0$ are the relative fluorescence intensities of the shallow (6,7-Br$_2$-PC) and deep quenchers (11,12-Br$_2$-PC), respectively, and $L$ is the difference in the depth of the two quenchers (0.9 Å/CH$_2$ or CBr$_2$ group). The thickness of the hydrophobic region was approximated to be $-28$ Å (27). For the calculation of immersion depth, the data collected for the 0.4 molar fraction quencher were used.

**RESULTS**

**Fluorescence Quenching Experiments**—For fluorescence measurement the recombinant complex was assembled from full-length VAMP2, soluble Syntaxin 1A, and two separate SNARE motifs from SNAP-25 (Fig. 1). The TMD of Syntaxin 1A was not included this time to avoid the coexistence of two TMDs in one membrane and to best mimic the “hypothetical” trans-SNARE complex in which two TMDs are separately anchored to two apposing membranes. In the recombinant SNARE complex, there are total two native Trp residues. Both of them reside at the membrane-proximal region of VAMP2 (Trp$^{89}$ and Trp$^{90}$) and belong to the coiled coil in the core structure (Fig. 1). This is ideal for the investigation of the possible coiled coil-membrane coupling using fluorescence.

First, to examine whether these residues are exposed to the solvent or not, we monitored Trp fluorescence in the presence of an added quencher acrylamide that is hydrophilic and partitions heavily into the solution phase. For the detergent-solubilized SNARE complex, the fluorescence intensity (F) decreases sharply as the acrylamide concentration increases, suggesting that Trp residues are solvent-exposed (Fig. 2a, open circles). In contrast, for the membrane-reconstituted complex, F decreases little in the presence of added acrylamide (Fig. 2a, closed circles). This result strongly implies that Trp residues in the SNARE complex are sequestered from the water phase, suggesting the possibility of insertion into the membrane.

Next, the insertion of Trp residues into the membrane is probed utilizing brominated lipids in which bromines are attached to the acyl chain of the lipid. In the presence of a brominated lipid, Trp fluorescence is effectively quenched only when Trp is in contact with the acyl chain region of the membrane. Otherwise, we would expect a negligible effect. As the mole fraction of the brominated lipid increases, a significant decrease in F was observed (Fig. 2b), suggesting that Trp residues are inserted into the membrane. The immersion depth of Trp residues was calculated based on quenching efficiencies by the shallow lipid quencher 6,7-Br$_2$-PC (the lipid with bromines at the sixth and seventh carbon positions) and the deep lipid quencher 11,12-Br$_2$-PC. Trp residues are $-8.8$ Å below the phosphate groups of lipids. Because there are two Trp residues, the immersion depth determined here must be an average depth of two residues. In conclusion, Trp$^{89}$ and Trp$^{90}$ that belong to the coiled coil are inserted in the membrane in the SNARE complex.

**Site-directed Spin Labeling EPR**—To investigate the membrane topology of the SNARE complex further using site-directed spin labeling EPR, residues of full-length VAMP2 near the membrane-water interface were replaced with cysteines, to which a nitroxide spin label was attached. We prepared 13 spin-labeled mutants (K83C-M95C) to explore the interface region with EPR (Fig. 1). For EPR measurements, the recombinant complex was assembled from spin-labeled VAMP2, soluble Syntaxin 1A, and two separate SNARE motifs from SNAP-25. All spin-labeled recombinant complexes were capable of forming the SDS-resistant complex, which is one characteristic feature of the core complex, as confirmed with SDS-PAGE (data not shown).

After reconstitution of the SNARE complex into POPC vesicles containing 15 mol % of DOPS, the EPR spectra were collected for spin-labeled mutants at room temperature. The EPR spectrum is sensitive to the tumbling rate of the nitroxide. EPR spectra shown in Fig. 3 are all relatively broad, indicating slow motion. Slow motional spectra represent motationally restricted nitroxides. There are three structural factors that might have contributed to the immobilization of the nitroxide (28–30): (i) The motional restriction of the peptide backbone, because of the a-helical secondary structure, could have re-
duced the tumbling rate of the nitroxide. (ii) Tertiary interactions with other parts of the protein would slow down the motion of the nitroxide significantly. On the basis of the crystal structure (7), we expect that the four-stranded coiled coil structure extends up to residue 92, which gives rise to many potential tertiary contacts between helices. (iii) From the fluorescence measurement it is clearly shown that Trp89 and Trp90 are inserted into the acyl chain region of the bilayer. Therefore, we expect that a significant part of the region is immersed in the membrane, which exposes nitroxides into the viscous membrane environment and the high density head group region. It is likely that the combination of all three factors contributed to the EPR spectral broadening.

Additionally, to examine the possibility of intermolecular interactions between SNARE complexes, we measured low temperature (130 K) EPR spectra in which the spectral broadening caused by the spin-spin interaction is readily identified (31). Comparison of the low temperature EPR spectra with the standard confirmed that SNARE complexes are separated from each other beyond the detectable distance range (less than 25 Å) (data not shown), eliminating the possibility of self-aggregation of the SNARE complex.

It is interesting to note that EPR spectra for positions 93–95 are less broad than others. In fact, EPR spectra for these three positions closely resemble those observed for the nitroxide attached to the membrane-inserted linker region of Syntaxin 1A (18, 19).

**EPR Accessibility Measurements**—The EPR line shape is a useful parameter for the tumbling rate of the nitroxide that, in many cases, provides a qualitative assessment of the local environment surrounding the nitroxide (29). However, the line shape alone is often not sufficient to yield information pinpointing the secondary and the tertiary structures. Furthermore, the partial insertion of the protein into the bilayer makes the matters complicated for the SNARE complex. Here, we utilized the EPR saturation method to assess the local structure and the membrane topology of the linker region. For the nitroxide, the EPR saturation method measures the accessibility to a
water-soluble paramagnetic reagent such as NiEDDA (W NiEDDA) to estimate the solvent exposure of the spin-labeled site, or the accessibility to a nonpolar paramagnetic reagent such as molecular oxygen (WO2) to probe, for example, the insertion into the membrane (32–34).

In Fig. 4 W NiEDDA and WO2 for the SNARE complex are plotted against the residue number, respectively. We observe an overall decrease of W NiEDDA, whereas we detect an overall increase of WO2. Interestingly, however, there are quite significant increases and decreases for W NiEDDA along the sequence, which might imply a secondary structure such as α-helix. Further, the WO2 values show variations that appear to be in opposite directions to those of W NiEDDA, although the trend is much more clear. Such an out-of-phase oscillatory behavior of W NiEDDA and WO2 has been previously found for α-helical peptides residing at membrane-water interface, which includes the fusion peptide of influenza hemagglutinin and a synthetic amphiphilic peptide (32, 35, 36).

Quantitatively, the ratio of W NiEDDA to WO2 has been shown to be a useful parameter to characterize the secondary structure. For a α-helix we expect a periodical behavior of this parameter along the sequence with a periodicity of 3.5. In Fig. 5, the Φ value, which is defined as the logarithm of the ratio of W NiEDDA to WO2, is plotted as a function of residue number. We observe a significant variation of the Φ value along the sequence. In particular, it appears that there is a periodic oscillation of the Φ values in the region of residues 83–92. To better represent this oscillatory behavior, we fit the data with a sine function of the 3.5 residue repeat, which represents the α-helical geometry. In this fit, we also take into account the overall decreasing trend of the Φ values along the sequence as a linear term added to the sine function. The EPR data are shown overlapped with the fit in Fig. 5. As mentioned before EPR spectra for positions 93–95 exhibit intermediate motional rates, characteristic of lipid-exposed nitroxides. It is also highly likely that these three residues are disordered when judged from the disagreement with the continued α-helical geometry. For these three positions, we compared the Φ values with those obtained from the spin-labeled lipids in the membrane of the same lipid composition containing the similar concentration of the unlabeled SNARE complex. The immersion depth analysis revealed that these three positions are inserted into the membrane as depicted to the right of the dotted line.

Combining the EPR data and the fluorescence data, we conclude that the C-terminal part of the coiled coil is inserted into the membrane at an oblique angle. Further, we propose that
residues 93–95 might be unstructured although inserted into the membrane.

**DISCUSSION**

The location of VAMP2 residues Trp89 and Trp90 in the SNARE complex inside the membrane is not surprising taking into account the fact that Trp residues are commonly found in membrane proteins near the membrane-water interface (37). It is further supported by the fact that the membrane-proximal region of VAMP2 has phospholipid binding affinity (38). The high affinity of Trp to the membrane (approximately −2.5 kcal/mol/residue) helps the stabilization of the structure and topology of membrane proteins (39). Trp residues are also found in multiple numbers in the linker region of many transmembrane SNARE proteins (20), implying potential functional roles of those Trp residues in stabilizing specific topological structures of individual SNAREs and their complexes, which is perhaps necessary to achieve membrane fusion.

The EPR results suggest that a small C-terminal portion of the SNARE coiled coil penetrates into the membrane with an oblique angle. Such a tilted topology provides a favorable geometry for six nearby basic residues (Lys86, Lys88, Arg89, Lys90, Lys91, and Lys92) to be able to effectively interact with the membrane surface charge electrostatically. According to the previous estimation, each basic residue contributes approximately −1 kcal/mol to the free energy of the membrane-peptide interactions (40). Therefore, we expect that the total electrostatic contribution would be close to −6 kcal/mol. Combining the free energy contributions from Trp residues and basic residues, we speculate that the membrane-dipped SNARE complex is highly stable with the free energy of as much as −11 kcal/mol.

We expect that the TMD of VAMP2 is nearly perpendicular to the membrane surface (41). However, the EPR results suggest that the coiled coil domain of the complex is tilted significantly with respect to the membrane, requiring bending or disorder at the linker region. The EPR results suggest that positions 93–95 are strong candidates for the disordered connection between the TMD and the core. The saturation EPR analysis suggested that those three positions are immersed in the acyl chain region. In parallel, previous EPR studies demonstrated the adhesion of the polybasic linker region (Arg262, Arg263, Lys264, and Lys265) of Syntaxin 1A onto the membrane (18, 19). It was found that this region is laterally inserted into the membrane, similarly pulling the SNARE core toward the membrane.

However, one must be very careful in interpreting the EPR immersion depth data. Because EPR measures the position of the substituted nitroxide side chain, the immersion depths do not necessarily report the actual location of the native amino acids when the native residues are charged amino acids. For instance, EPR reported the depth of 12–13 Å for K94C (Fig. 5). However, the actual location of the lysine side chain may be much shallower. The positive charge on Lys304 could snorkeled out to seek the negative changes on phosphate, opposite to what is expected for the relatively hydrophobic nitroxide side chain.

A model for the trans-SNARE complex that sums up experimental results is depicted in Fig. 6. In this model, the tight coupling between the SNARE core and two apposing membranes is achieved by (i) the electrostatic interactions between the basic residues and the negatively charged lipids in the membrane and (ii) the insertion of interfacial Trp residues into the membrane. We estimate that the total free energy of the stabilization from these two sources amounts approximately to −16 kcal/mol.

The theoretical estimation of the free energy barrier for membrane fusion is as high as 25 kcal/mol (42), a significant fraction of which arises from the deformation of bilayers, a necessary step toward the lipid mixing and the hemifusion intermediate. Evidence suggests that coiled coil formation generates sufficient force to overcome this energy barrier. The important question is, then; how is the force delivered from the coiled coil to the membrane domains? To accomplish this, the coiled coil must be structurally coupled to the membrane. Otherwise, the force generated by the coiled coil formation would be dissipated. The EPR analysis suggested that there is indeed structural coupling between the coiled coil and the membrane. Furthermore, we estimated that this coupling amounts to −16 kcal/mol. However, it appears that the coupling force (−16 kcal/mol) is somewhat smaller than the theoretically predicted membrane distortion force during fusion (25 kcal/mol). Therefore, one SNARE complex might not have the capacity to hold the membrane deformation force during fusion. This energetic insufficiency must be overcome by the coordinated effort of more than one SNARE complex (23, 43–45). In fact, it has been recently shown that three SNARE complexes cooperate for the successful fusion (46).

Oblique dipping of the SNARE coiled coil to the membrane appears to have important implications regarding the trans-SNARE complex. In the trans state, the four-helix bundle, the diameter of which is as large as 20 Å (7), is sandwiched between two membranes. If the coiled coil were not inserted into the membrane, the juxtaposition of two membranes would be inherently inhibited by the existence of the coiled coil in the middle. In fact, it has been previously thought that the SNARE complex might hinder membrane apposition closer than 20 Å (47). At this distance, two membranes would remain well hydrated on the surface and would not proceed to fusion (48). The oblique insertion of the C-terminal end of the coiled coil would provide a solution to this geometric problem.

The functional significance of Trp and the basic residues discussed above is strongly supported by the recent experiment using PC12 cells. Using the tetanus toxin-resistant VAMP2 mutant, it has been shown that double mutation W89A/W90A reduced the secretion of human growth hormone significantly. Further, similar reduction in human growth hormone secretion has been observed for the double mutation K53A/K57V (49). SNARE proteins are central to the membrane fusion machinery in the neuron. However, membrane fusion is regulated by the Ca2⁺ influx. There is evidence that the vesicle protein synaptotagmin is a Ca2⁺ sensor (50, 51). Although its function remains elusive, recent studies have shown that synaptotagmin not only binds to the membrane in a Ca2⁺-dependent manner but also interacts with SNARE complexes. Coincidentally, loops 1 and 3 of synaptotagmin inserts into the interfacial region of the bilayer, similar to the linker regions of SNARE complex (52). We speculate that the interaction between SNARE and synaptotagmin might occur in the membrane environment, warranting further investigation.

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