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A stable interaction between syntaxin 1a and synaptobrevin 2 mediated by their transmembrane domains

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Abstract The proteins synaptobrevin (VAMP), SNAP-25 and syntaxin 1 are essential for neuronal exocytosis. They assemble into a stable ternary complex which is thought to initiate membrane fusion. In vitro, the transmembrane domains of syntaxin and synaptobrevin are not required for association. Here we report a novel interaction between synaptobrevin and syntaxin that requires the presence of the transmembrane domains. When co-reconstituted into liposomes, the proteins form a stable binary complex that cannot be disassembled by NSF and that is resistant to denaturation by SDS. Cleavage of synaptobrevin with tetanus toxin does not affect the interaction. Furthermore, the complex is formed when a truncated version of syntaxin is used that contains only 12 additional amino acid residues outside the membrane anchor. We conclude that the interaction is mediated by the transmembrane domains.

Key words: Syntaxin; Synaptobrevin; SNAP-25; Proteoliposome; Membrane fusion

1. Introduction

Neurotransmitter release is mediated by exocytosis of synaptic vesicles. In recent years, major progress has been made in identifying the proteins responsible for vesicle docking and membrane fusion [1–3]. Three membrane proteins, the vesicle protein synaptobrevin 2 (also referred to as VAMP) and the plasma membrane proteins syntaxin 1 and SNAP-25, play a key role in membrane fusion. Each of them is a member of a growing family of fusion proteins that are highly conserved in evolution and that are thought to mediate intracellular fusion events in all eukaryotic cells [4–6].

In vitro, synaptobrevin 2, syntaxin 1a and SNAP-25 form a stable ternary complex that can be reversibly disassembled by the chaperone-like ATPase NSF in conjunction with cofactors termed SNAPs (soluble NSF attachment proteins). Since SNAPs and NSF appear to operate on most fusion proteins, these are collectively termed SNAREs (SNAP-receptors). It is generally accepted that cyclic assembly-disassembly of SNARE proteins is intimately associated with membrane fusion although the precise mechanism of the fusion reaction is not yet understood. Assembly of the neuronal SNARE complex is associated with an increase in α-helical content and the release of energy [7]. Furthermore, all transmembrane anchors are positioned on the same side of the complex [8,9]. The crystal structure of the soluble core fragment of the complex has revealed a highly twisted and extended four-helix bundle with all helices aligned in parallel (N-termini distal, C-termini proximal to the transmembrane domains) [10]. Together, these findings led to the proposal that the assembly reaction is initiated by contact between the N-terminal tips of vesicular synaptobrevin and plasma membrane-bound syntaxin 1a and SNAP-25. According to this view, assembly would pull the membranes destined to fuse closely together, thus initiating the fusion reaction [8,11].

The structural requirements for the interactions between syntaxin 1, synaptobrevin 2 and SNAP-25 were studied in detail by several laboratories using recombinant proteins lacking their transmembrane domains. Binding is mediated by the C-terminal domain of syntaxin, both C- and N-terminal domains of SNAP-25 (excluding the cysteine-rich 'loop' in the center), and the entire cytoplasmic domain of synaptobrevin [12–21]. Amino acid substitutions and deletions shown to weaken the interactions can mostly be explained by interference with helix packing in the extended four-helix bundle [10,22]. However, due to the length of the helix bundle and the numbers of interacting side chains in the complex it is not surprising that interactions can still be observed with fragments smaller than the binding domains or with one or the other binding partner lacking. Of all possible binary complexes only the complex between SNAP-25 and syntaxin is stable and is associated with major structural changes [21]. Furthermore, complexes including synaptobrevin form when only one of the two binding domains of SNAP-25 is present [20]. The interaction between syntaxin 1 and synaptobrevin is weak [19], and it is therefore thought that synaptobrevin can only interact with a preformed syntaxin-SNAP-25 complex.

Recent evidence suggests that the transmembrane domains of synaptobrevin and its close homologue cellubrevin, are involved in additional interactions. Binding of synaptobrevin and cellubrevin to synaptophysin and BAP-31, respectively, is dependent on the presence of the transmembrane domains [23,24]. Furthermore, the transmembrane domain of synaptobrevin mediates dimerization that is dependent on sequence specific residues in the hydrophobic domain [25]. We therefore investigated whether the transmembrane domain of synaptobrevin participates in other interactions between members of the fusion complex. We found that synaptobrevin forms a binary complex with syntaxin that is partially resistant to SDS and that is not disassembled by NSF.

2. Materials and methods

2.1. Materials

The recombinant protein fragments were derived from cDNAs encoding rat synaptobrevin 2 and rat syntaxin 1a (kindly provided by R.H. Scheller). The recombinant light chain of tetanus toxin (TeNT) was a generous gift of H. Niemann. The NSF and α-SNAP constructs in pQE-9 vectors were kindly provided by S. Whiteheart and J.E. Rothman.

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2.2. Plasmid construction

*Nde*I/*Eco*R1 constructs encoding synaptobrevin 2 (amino acid positions (aa) 1–116 and 1–94) and syntaxin 1a (aa 1–288) were generated by PCR and subcloned into pHO2d (derived from pET11d [7]). Following start and stop primers were used for construct amplification: synaptobrevin 2 (aa 1–116): gggattccatatgtcggctaccgctgcc and gcgaattcccagtgctgaagtaaacgat, synaptobrevin 2 (aa 1–94): gggattccatatgtcggctaccgctgc and gcgaattccccttgaggtttttccacca, syntaxin 1a (aa 1–288): gggattccatatgaaggaccgaacccag and gcgaattccctccaaagatgccccgat.

The coding sequence for a truncated syntaxin 1a construct (aa 254–288) was amplified using the syntaxin stop primer from above and ccacgccatggccgtcaagtaccagagc as a start primer. The PCR product was subcloned into pHO4d. This vector was built by inserting DNA coding for a C-terminal His_6-tag followed by a c-myc epitope and a stop codon into the *Eco*R1/*Bam*H1 sites of pHO2d [7]. The sequence of this insert was: gcgaattcgggccaccatcaccaccatcacggcgaacagaaactgatcagcgaagaagatctgaactaggatccg.

2.3. Purification of recombinant proteins

Recombinant syntaxin 1a (aa 1–265), full-length SNAP-25, NSF and α-SNAP were expressed and purified as described previously [7,16]. All other proteins were purified using Ni-NTA-agarose according to [7] except that 1.5% Na-cholate (w/v) was included in all buffers when transmembrane proteins were used. All amino acid numbering refers to the rat sequences.

2.4. Preparation of proteoliposomes

All lipids were purchased from Avanti Polar Lipids and were solubilized in chloroform/methanol, 2:1 (v:v) immediately before use. The following mixture was prepared for reconstitution (molar ratios): cholesterol (1), phosphatidylserine (1), phosphatydylinositol (1), phosphatidylethanolamine (2), phosphatidylcholine (5), and rhodamine phosphatidylethanolamine as tracer (0.05). The lipid mixture was dried on a rotary evaporator and resuspended in cholate buffer (20 mM Tris (pH 7.4), 120 mM NaCl, 1 mM dithiothreitol (DTT) 5% cholate (w/v)) at a detergent to lipid molar ratio of 8:1. Where indicated, equal volumes of SNARE proteins (containing 1.5% cholate) were added, with a final lipid:protein molar ratio of approximately 1000:1. Proteoliposomes were formed by detergent removal using size-exclusion chromatography on Sephadex G-50 S [26] (volume ratio sample:column 1:35).

2.5. Detergent assisted insertion

Proteoliposomes containing recombinant synaptobrevin were incubated for 45 min at room temperature with constant concentrations of recombinant syntaxin and increasing concentrations of octylglucoside. The approximate molar ratios of synaptobrevin:syntaxin were 2:1.

Synaptobrevin proteoliposomes that were cleaved with TeNT light chain were sedimented by ultracentrifugation (85 000 rpm for 30 min in a Beckman TlA 100.3-rotor) and subsequently resuspended in standard buffer (20 mM Tris (pH 7.4), 120 mM NaCl, 1 mM DTT).

2.6. Other methods

Disassembly of ternary complexes and cleavage by TeNT light chain was performed as described previously [27]. SDS-PAGE and immunoblotting was performed according to standard procedures [28,29]. Monoclonal antibodies used for detection include HPC-1 for syntaxin [30], Cl 69.1 for synaptobrevin 2 [31], commercially available from Synaptic Systems (Göttingen/Germany) and monoclonal antibody for c-myc (cell line obtained from American Tissue Culture Company). Secondary antibodies coupled to horse-radish peroxidase or alkaline phosphatase were purchased from Sigma. Immunoblots were developed using either an Enhanced Chemiluminescence Kit from Pierce (Super Signal), or where indicated, a combination of nitro blue tetrazolium (0.33 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.17 mg/ml).

3. Results

Recombinant full-length syntaxin 1a and synaptobrevin 2 containing a C-terminally added His_6-tag were expressed in *E. coli* and purified by Ni-NTA affinity chromatography in the presence of 1.5% (w/v) cholate as detergent. After addition of cholate-solubilized phospholipids, the proteins were co-reconstituted in liposomes by detergent removal using size-exclusion chromatography. The proteoliposomes eluted at the void volume and were dialyzed overnight to remove any residual detergent. When the proteins in the proteoliposome fraction were analyzed by SDS-PAGE and immunoblotting, several protein bands with an apparent M_r higher than that of the monomers were detected (Fig. 1). In addition to a syntaxin dimer, a complex between syntaxin and synaptobrevin was observable that migrated at an apparent M_r of 45 000 (Fig. 1). This complex was also detectable when the blot was incubated separately with either anti-syntaxin or anti-synaptobrevin antibodies (not shown). Digestion of the proteoliposomes with tetanus toxin (TeNT) light chain, which resulted in a partial cleavage of synaptobrevin (not shown, [32]), led to the appearance of an additional band of lower M_r (Fig. 1) that probably consists of an adduct between syntaxin and the C-terminal membrane-anchored fragment of synaptobrevin.

These data indicate that synaptobrevin and syntaxin form a 1:1 complex in proteoliposomes that is at least partially resistant to SDS and that involves the C-terminal end of synaptobrevin. To investigate whether the transmembrane domain of syntaxin is participating in this interaction, we expressed a truncated, epitope(myc)-tagged version of syntaxin that corresponds to the C-terminal cleavage product of botulinum neurotoxin C1 [33] and that contains only 12 amino acid residues in addition to the transmembrane domain. As shown in Fig. 2, co-reconstitution of this fragment with full-length synaptobrevin resulted in the formation of a complex that was detectable with both anti-synaptobrevin and anti-

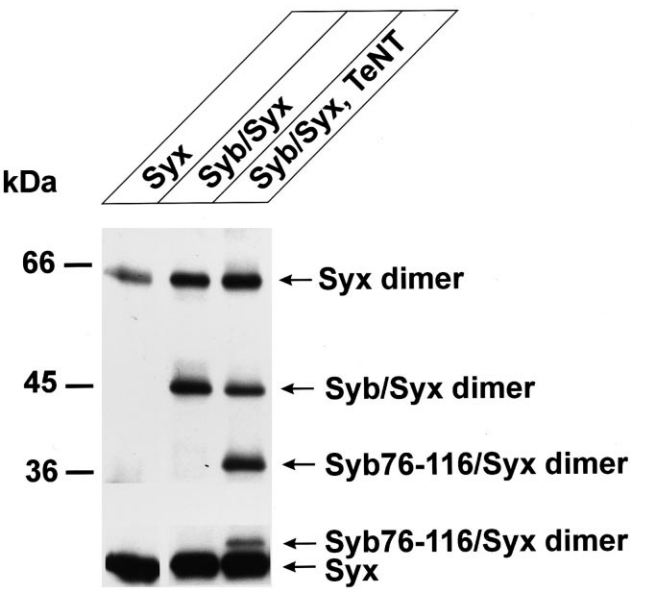

Fig. 1. Co-reconstitution of synaptobrevin 2 and syntaxin 1a into proteoliposomes leads to the formation of a SDS-resistant heterodimer (Syx/Syb dimer) that is partially cleaved by TeNT light chain. All samples were analyzed by SDS-PAGE and immunoblotting, using a mixture of monoclonal antibodies 69.1 (synaptobrevin 2) and HPC-1 (syntaxin 1) for detection. Left lane, proteoliposomes reconstituted with syntaxin 1a; middle lane, co-reconstitution of synaptobrevin 2 and syntaxin 1a; right lane, co-reconstitution of synaptobrevin 2 and syntaxin 1a, followed by digestion with TeNT light chain. Top panel: 8% separation gel; lower panel: 14% separation gel (to show equal loading of syntaxin). Note that a syntaxin homodimer (Syx dimer) is also observable.

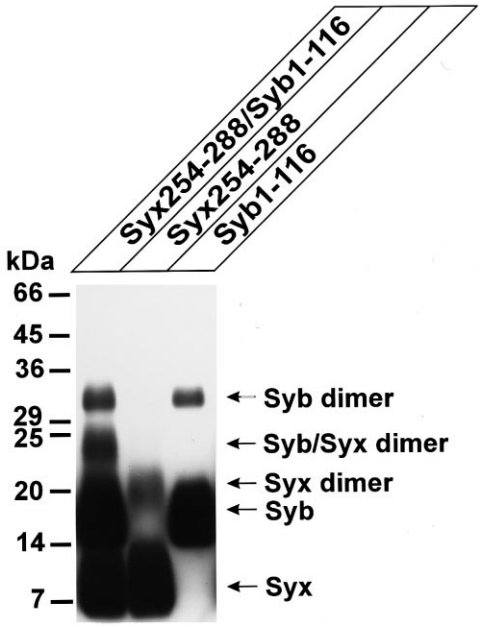

Fig. 2. Heterodimer formation after reconstitution of synaptobrevin with an N-terminally truncated version of syntaxin 1a (left lane). Analysis was performed as in Fig. 1 except that an anti-myc antibody was used instead of HPC-1. Both, truncated syntaxin 1a and synaptobrevin form homodimers (middle and right lane) that are separated from the heterodimer.

myc antibodies. The complex migrated with an apparent M_r of 23 000, i.e. an M_r expected for a 1:1 complex between synaptobrevin and the syntaxin fragment.

Together, these data demonstrate that syntaxin and synaptobrevin form a stable binary complex upon co-reconstitution in liposomes. Complex formation was dependent on the two proteins being present in the same membrane. When synaptobrevin and syntaxin 1 were reconstituted into separate liposome populations and mixed subsequently, no complex formation was observed (not shown). This also demonstrated that the complex could not have formed after addition of SDS. We then investigated whether the complex needs to be preformed in detergent micelles or whether it can form while one of the proteins is residing in a membrane. To address this question, we reconstituted synaptobrevin into proteoliposomes and then added syntaxin in the presence of increasing concentrations of the detergent octylglucoside (Fig. 3). At low detergent concentrations, no complex formation was observed. When the octylglucoside concentration was increased to 0.6%, the binary syntaxin-synaptobrevin complex was detectable. At this concentration, which is still below the critical micelle concentration, octylglucoside is known to facilitate insertion of proteins into preformed vesicles without disrupting the membrane [34]. When the detergent concentration was increased well above the critical micelle concentration, the amount of complex was reduced. This was expected because the proteins are dissolved in detergent micelles instead of being concentrated in the membrane. Similar results were obtained when synaptobrevin-containing proteoliposomes were digested with TeNT light chain, followed by centrifugation and resuspension, before syntaxin was added. Again, a complex was observed at 0.6% (w/v) octylglucoside which was reduced upon solubilizing the liposomes (Fig. 3, lower panel).

In the last series of experiments, we investigated whether the binary syntaxin-synaptobrevin interaction is detectable after NSF-driven disassembly. A preformed ternary complex, consisting of full-length syntaxin, synaptobrevin, and SNAP-25, was reconstituted into proteoliposomes. In the first experiment, these liposomes were incubated in the presence of NSF and α-SNAP under conditions that either block or favor disassembly. Analysis of the protein complexes by SDS-PAGE revealed that partial disassembly of the ternary complex (visible as a major band migrating at an M_r of 60 000) induced the formation of the synaptobrevin-syntaxin complex (Fig. 4, left). As control, an identical experiment was carried out with ternary complex formed from recombinant proteins lacking their transmembrane domains. As expected, no syntaxin-synaptobrevin complex was observed (Fig. 4, right).

4. Discussion

Syntaxin 1a and synaptobrevin II are essential for neuronal exocytosis and are thought to operate via regulated protein-protein interactions. Our results demonstrate that both inte-

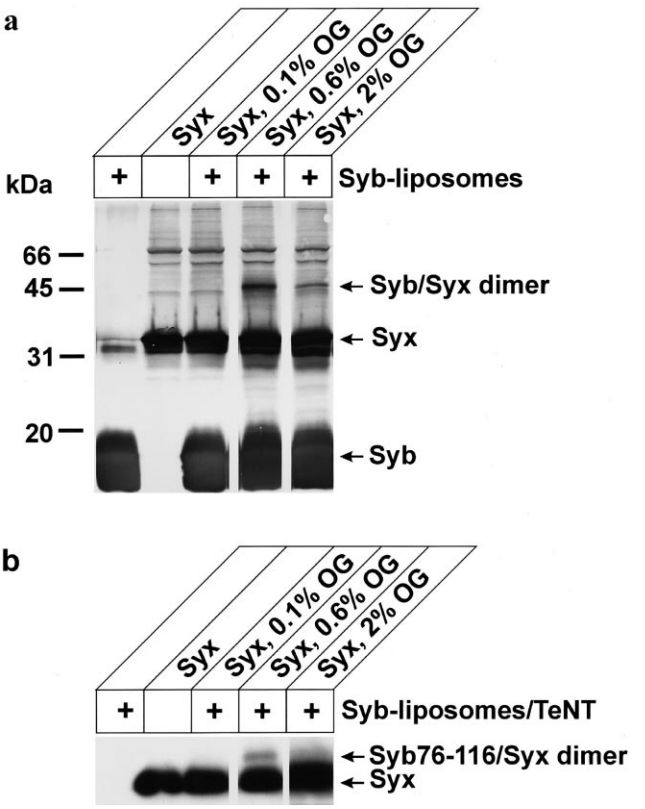

Fig. 3. Detergent assisted insertion of syntaxin 1a into preformed proteoliposomes containing synaptobrevin 2. a: Synaptobrevin was reconstituted into proteoliposomes. Syntaxin (molar ratio of synaptobrevin:syntaxin ≈ 2:1) was added in the presence of increasing concentrations of the detergent octylglucoside (OG, final concentrations (in % (w/v)). Left lane: no addition (control). Syx: recombinant syntaxin without liposomes. For detection an alkaline phosphatase coupled secondary antibody was used. Note that the dimer of synaptobrevin (left lane) migrates at a position similar to that of syntaxin. b: As a but the synaptobrevin liposomes were first treated by TeNT light chain (1.3 times molar access of light chain over synaptobrevin). Before addition of recombinant syntaxin, the liposomes were sedimented by ultracentrifugation and resuspended in order to remove the cytoplasmic fragment of synaptobrevin. Note that dimer formation is maximal at intermediate (sub-lytic) detergent concentrations.

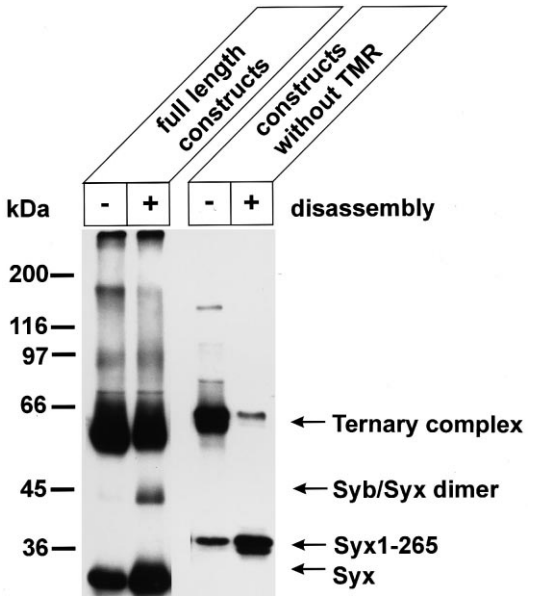

Fig. 4. Disassembly of the ternary SNARE complex in proteoliposomes by α-SNAP and NSF leads to the formation of a syntaxin/synaptobrevin heterodimer. Synaptobrevin and syntaxin were expressed either as full-length proteins (left lanes) or as truncated proteins lacking their transmembrane domains (right lanes) and combined with recombinant SNAP-25 to form ternary SNARE complexes. The complex containing full-length proteins was reconstituted into proteoliposomes. Note that no dimer is formed upon disassembly of the truncated complex. Syntaxin without transmembrane domain migrates slower than its full-length counterpart. This is due to the presence of a larger tag.

gral membrane proteins are capable of forming a hitherto undetected binary complex. Formation of this complex is mediated by the respective transmembrane domains and is at least partially resistant to denaturation by SDS.

It should be noted that in all of our experiments protein constructs were used that contain additional amino acids N-terminal of the transmembrane region. Theoretically, these cytoplasmic portions might contribute to the interaction described here. However, we think that the interaction is confined to the transmembrane domains for the following reasons. Firstly, the syntaxin fragment used in the experiment shown in Fig. 2 contains only 12 cytosolic amino acids, which is probably too short to fold into a defined domain. Secondly, this region is unstructured in monomeric syntaxins lacking their transmembrane domains [17,35]. More importantly, no binary complexes are observed when proteins were used that contained only the cytoplasmic portion.

A limitation of the assay used here for detecting complex formation should be highlighted. SDS is known to denature proteins even though hydrophobic interactions are usually more resistant against SDS-denaturation. For these reasons, we cannot exclude that binary complexes form oligomers of a higher stoichiometry than the 1:1 ratio observed in our experiments. Similarly, it is difficult to estimate the efficiency of complex formation. In our hands, only a relatively small proportion of the proteins present in the assay formed binary complexes. Possibly, the proteins are unevenly distributed in the liposome population (we estimate an average concentration of about 15 copies of each protein/liposome, assuming 100% efficiency in reconstitution). Also, the formation of homodimers, observable both for synaptobrevin (see also [25]) and syntaxin, may compete with the binary complex. Finally, the binary complex may only be partially stable during sample preparation and SDS-electrophoresis, causing a further reduction of the detectable complex.

The significance of the interaction between the transmembrane domains of syntaxin and synaptobrevin remains to be established. According to our current hypothesis, assembly of the ternary complex begins at the N-terminal tips of the binding domains (i.e. distal from the membranes) and progresses towards the membrane anchors, forcing the membranes close together. The helix bundle is very densely packed in the region close to the membranes, and even a small truncation of SNAP-25 as induced by BoNT/A poisoning [36] blocks exocytosis. Thus it is conceivable that an extension of the helix bundle into the transmembrane domain is promoted by the binary interaction described here. It may contribute to the

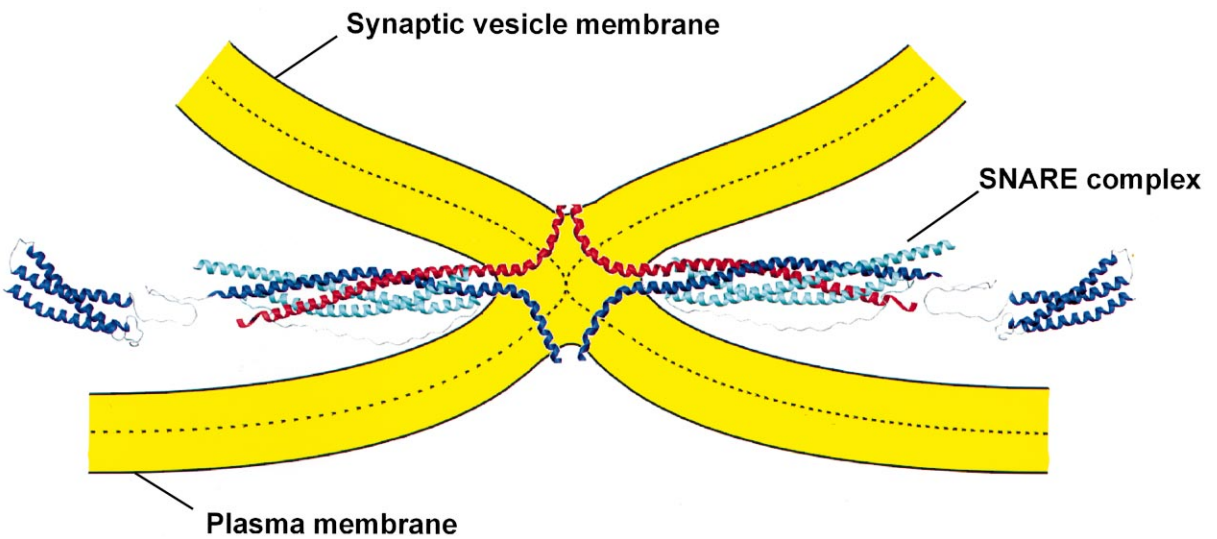

Fig. 5. Schematic drawing illustrating the potential role of the binding between the transmembrane domains during the formation of fusion intermediates. According to the model, the interaction between the transmembrane domains may be associated with the formation of a fusion stalk, i.e. the creation of continuity between the proximal membrane leaflets.

formation of membrane continuity between the proximal leaflets of the fusing membranes, thus promoting the formation of a prefusion stalk (Fig. 5), a structure believed to be an intermediate in all fusion reactions [37].

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