



Membrane fusion: a structural perspective on the interplay of lipids and proteins

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The fusion of biological membranes is governed by the carefully orchestrated interplay of membrane proteins and lipids. Recently determined structures of fusion proteins, individual domains of fusion proteins and their complexes with regulatory proteins and membrane lipids have yielded much suggestive insight into how viral and intracellular membrane fusion might proceed. These structures may be combined with new knowledge on the fusion of pure lipid bilayer membranes in an attempt to begin to piece together the complex puzzle of how biological membrane fusion machines operate on membranes.

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Abbreviations

| | |
|---------------------------------|-------------------------------------------|
| CHOL | cholesterol |
| DOPC | dioleoyl-phosphatidylcholine |
| DOPE | dioleoyl-phosphatidylethanolamine |
| EM | electron microscopy |
| EPR | electron paramagnetic resonance |
| HA | hemagglutinin |
| NSF | <i>N</i> -ethylmaleimide-sensitive factor |
| PC | phosphatidylcholine |
| PE | phosphatidylethanolamine |
| PEG | polyethylene glycol |
| PS | phosphatidylserine |
| PtdIns(4,5)P₂ | phosphatidylinositol-4,5-bisphosphate |
| SM | Sec1/Munc-18 |
| SM | sphingomyelin |
| SNAP-25 | synaptosome-associated protein of 25 kDa |
| SNARE | soluble NSF-attachment protein receptor |
| t-SNARE | target membrane SNARE |
| VAMP | vesicle-associated membrane protein |
| v-SNARE | vesicle membrane SNARE |

Introduction

Membrane fusion is a ubiquitous cell biological process. It occurs intracellularly in membrane trafficking and exocytosis, including neurotransmitter release in synaptic transmission, and extracellularly in virus infection, gamete formation in sexual reproduction and myotube formation in organ development. In all these cases, two

distinct membranes that separate different cellular compartments have to merge and thereby connect the two compartments, whose contents are then free to mix and react. Lipid bilayer membranes do not spontaneously fuse. Energy must be invested to overcome hydration repulsion between membranes that approach each other and to disrupt the normal bilayer structure of the fusing membranes. This energy is expended on removing water molecules from the cleft between the two membranes, on bending the membranes that are to be fused and on creating nonbilayer lipid structures that function as fusion intermediates. The energy to drive biological membrane fusion is provided by highly specialized fusion proteins. The structures of several membrane fusion proteins and fragments of such proteins have been solved by X-ray crystallography and NMR. Structural work on membrane fusion proteins up to about 2001 has been covered in several excellent reviews [1,2,3].

Among the many structures of fusion proteins that have been solved, influenza hemagglutinin (HA) is unique because it is the only membrane fusion protein for which structures of the core fragment before and after fusion are known. It has been known for several years that this core fragment undergoes a dramatic conformational change upon fusion [4]. This advanced knowledge of the pertinent structural transitions of HA has inspired many experiments on HA-mediated and other fusion systems. Therefore, influenza HA-mediated fusion has often served as the prototype fusion system and has also greatly influenced the interpretation of experimental results obtained with many other fusion systems. Having solved the structures of several soluble membrane fusion protein fragments, the central question in the field now is to determine how these proteins work on their substrates, that is, how they reshape the membranes that they are designed to fuse. In the following, we summarize recent progress in the structural biology of membrane fusion, drawing on influenza HA-mediated fusion as a paradigm.

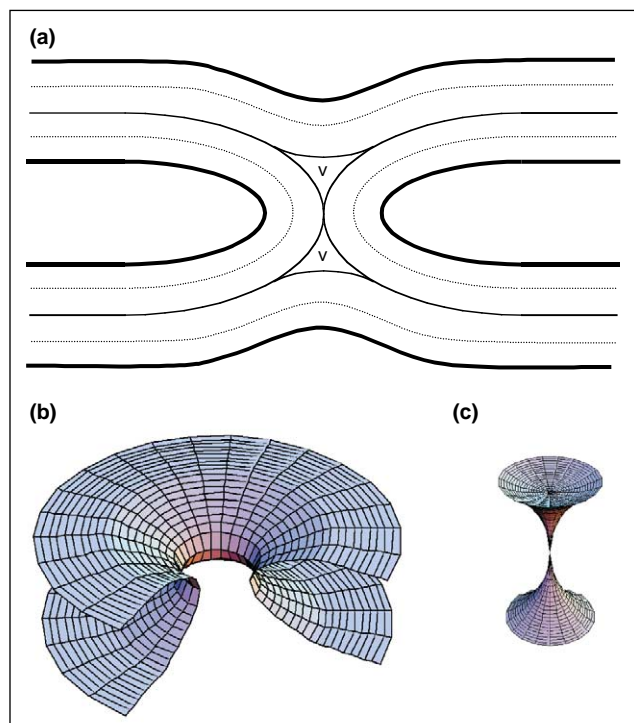
Pathways and possible intermediate lipid structures in membrane fusion

Experimental and theoretical studies have suggested the existence of several intermediates on the pathway to membrane fusion. To put biological membrane fusion into perspective, we first discuss the fusion of pure lipid bilayer systems. As fusion of pure lipid bilayers does not occur spontaneously, external forces have to be applied to make them fuse. A conceptually simple and straightforward experiment is to mechanically push two mica-supported lipid bilayers together in the surface forces

apparatus. Force versus distance measurements show that bilayers of zwitterionic lipids attract each other by van der Waals attraction at fluid separation distances of $\sim 5\text{--}20$ Å. Hydration repulsion becomes dominant for fluid separation distances below ~ 5 Å for PEs and ~ 15 Å for PCs. When bilayers are partially depleted of lipids and then forced into close contact, they fuse into a single bilayer by radial expansion of a hemifusion defect [5,6]. ‘Hemifusion’ in these experiments is structurally defined as a state in which the *trans*-monolayers of the two bilayers are closely apposed to each other over an extended area. Extended hemifused bilayers have been previously observed in optical and electrophysiological experiments with black lipid membranes [7]. The hemifused state is thought to arise from a hydrophobic point defect between the two apposed bilayers and is then thought to progress to an hourglass-shaped stalk intermediate (Figure 1). Although they have not been observed experimentally, ideas about the possible involvement of point defects and lipid stalks in membrane fusion have evolved since their first description in the late 1970s and early 1980s [8,9]. The energy of the postulated lipid stalks between two

membranes has been calculated using known elastic moduli and intrinsic curvatures of the lipids from which they are formed [10] and, additionally, energy penalties for creating ‘voids’ in the membrane due to the nonideal packing of the lipid sidechains [11,12]. These original calculations produced unrealistically large energies for the proposed stalk intermediates, so that lipid stalks would be unlikely to be formed in any real fusion process. However, three teams of theoreticians recently solved this problem either by adjusting the geometry of the stalk itself or by changing (tilting) the orientation of the lipids within the stalk. Markin and Albanesi [13[•]] let the shape of the stalk relax to its optimum shape to create a so-called ‘stress-free’ stalk. Kuzmin *et al.* [14[•]] introduced lipid tilt in the two *trans*-monolayers to create a ‘modified’ stalk that can transform easily into a fusion pore without the creation of an extended hemifusion diaphragm. Kozlovsky and Kozlov [15[•],16] present a stalk model with tilted lipids in the *trans*- and *cis*-monolayers. The appealing feature of this last model is that the troubling hydrophobic ‘voids’ are eliminated. However, these calculations create a new ‘problem’, namely a negative total energy of the stalk, which would make the stalk a thermodynamically stable rather than a transient structure.

Figure 1



Stress-free stalk. (a) Cross-section through a lipid stalk formed between two lipid bilayers. Thin lines represent hydrophobic monolayer contact surfaces. Dotted lines represent ‘neutral’ surfaces between the polar and monolayer contact surfaces. Hydrophobic ‘voids’ are marked with ‘v’s. (b) Three-dimensional rendition of the neutral surface of the *cis* leaflet of a stress-free stalk with a spontaneous curvature of -0.1 nm^{-1} . (c) Three-dimensional rendition of hydrophobic voids of a stress-free stalk with a spontaneous curvature of -0.1 nm^{-1} . (Adapted from [13[•]] with permission.)

Do stalks really occur or are they just convenient models to explain certain experimental behavior of membrane fusion? The first direct proof for the existence of membrane stalks comes from recent X-ray diffraction data on oriented lipid systems at low hydration [17^{••}]. Oriented bilayers of diphytanoyl phosphatidylcholine (i.e. a lipid with negative intrinsic spontaneous curvature) undergo two phase transitions — from lamellar to rhombohedral and from rhombohedral to hexagonal — as a function of increasing hydration and temperature. The rhombohedral phase observed at relative humidities of 70–80% consists of periodically spaced stalks that are similar in shape to the ‘stress-free’ stalks predicted by Markin and Albanesi [13[•]]. Similar rhombohedral stalk phases have also been detected for certain DOPC/DOPE mixtures at low hydration [18[•]]. DOPE is a fusion-promoting lipid with a high negative intrinsic curvature. The fact that fusion-promoting lipids actually do form stalks under certain conditions provides good support for the notion that stalks might exist as intermediates in membrane fusion. However, rhombohedral stalk phases have not yet been demonstrated in fully hydrated systems. Also, stalks have not been directly experimentally observed in any (lipid- or protein-mediated) fusion system. Therefore, the occurrence of the lipid stalk as an intermediate in membrane fusion is still contentious, although it has been frequently invoked to explain experimental results.

Lentz and co-workers [19] developed fluorescence techniques to follow the kinetics of several aspects of lipid bilayer fusion. In this work, small unilamellar vesicles are induced to fuse by the addition of, typically, 15–20%

PEG of molecular weight 8000. PEG reduces the activity of water and thereby aggregates the vesicles. The lipid bilayers are compressed by the osmotic pressure imposed by the addition of PEG and the net result is similar to what is achieved by the surface forces apparatus. A major advantage of osmotic compression is that it can be applied to lipid vesicles rather than supported planar bilayers, which cannot bend. The older work has been refined more recently [20]. The kinetics of outer leaflet lipid mixing (~0.5 min), transbilayer lipid movement (~3.5 min), inner leaflet mixing (~4.5 min) and contents mixing (~6 min) have been resolved (approximate half-times for 20 nm mixed PC vesicles at 23°C). Increasing temperature increases these rates and increasing vesicle size decreases them. These data clearly show that outer leaflet lipid mixing (~0.5 min) and pore formation (4–6 min) are two distinct kinetic processes, even in pure lipid bilayer systems. Whether the first process corresponds topologically to the postulated stalk, the delay between the first and second processes to the maturation of the stalk into a hemifusion diaphragm (sometimes also called *trans*-monolayer contact [11,12]), and the second process to the ‘popping’ of the hemifusion diaphragm is not known with certainty, although it has been frequently interpreted in this manner. The kinetic data do not address the structures of these intermediates in membrane fusion. Therefore, the ‘hemifusion’ intermediate must be operationally defined in these and all other kinetic experiments as a state that promotes lipid but not contents mixing.

A large variety of lipid compositions were tested with regard to their ability to promote hemifusion, fusion and vesicle rupture [21•]. The lipid composition that produced the most efficient fusion without contents leakage to the environment was a mixture of PC:PE:SM:CHOL (35:30:15:20). Interestingly, this composition is close to the lipid composition of synaptic vesicles, except for the lack of PS. The negatively charged lipid PS had a negative effect on the fusion of pure lipid vesicles. In presynaptic membrane fusion, the inhibitory effect of PS is presumably eliminated by the binding of basic membrane proteins. The presence of PE in lipid bilayers promotes fusion, whereas the SM:CHOL components at appropriate ratios reduce vesicle rupture upon osmotic compression. Vesicles under hypotonic osmotic stress fuse more readily than unstressed vesicles [22]. Reasons for this may include more extensive contact areas in flattened vesicles, a decreased bending modulus of hypotonic vesicles (softer bilayers) or compression of hydrophobic mismatch (‘void’) volumes in fusion intermediates. Excimer fluorescence of pyrene cholesterol increases and then decreases during fusion [23]. This clearly demonstrates that this compound transiently accumulates in fusion sites. The authors interpret this as evidence for the hydrophobic voids postulated in the stalk and *trans*-monolayer contact models of fusion intermediates. How-

ever, other (dynamic) intermediate structures may also accumulate pyrene cholesterol.

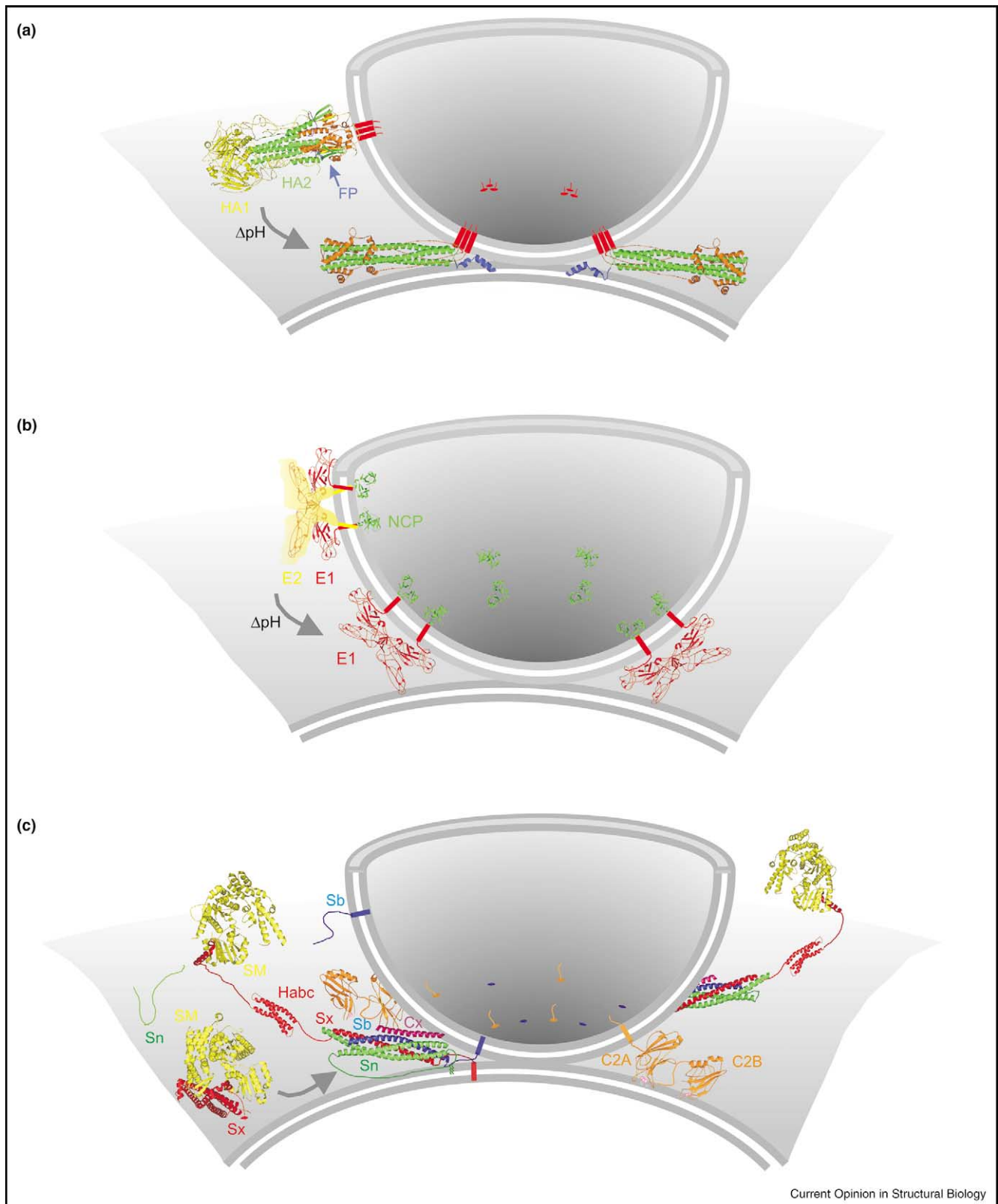
Fusion of pure lipid bilayers and biological membranes is strongly modulated by lipid additives that alter membrane curvature [24]. Lyso-PC added to the *cis* monolayers and oleic acid added to the *trans* monolayers promote fusion, whereas lyso-PC added to the *trans* monolayers and oleic acid added to the *cis* monolayers inhibit fusion. Lyso-PC induces positive membrane curvature, but oleic acid induces negative membrane curvature. As lipid stalks formed from the *cis* monolayers have negative overall curvature and as the fusion pore is characterized by positive curvature of the *trans* monolayers, these results have been taken as evidence for the ‘stalk-pore’ model of membrane fusion. In this model, fusion progresses from a stalk to a pore either directly or through a hemifusion (*trans*-monolayer contact) second intermediate. Although the correlation between experiment and expectation based on the stalk-pore model is extremely compelling, there are alternative or additional explanations for the observed effects of the lipid additives on membrane fusion (see below).

Multiple strategies have been developed to capture intermediates of biological (i.e. mostly viral fusion protein-mediated) membrane fusion. One strategy examines the effect of mutations of the fusion proteins and their ability to arrest fusion at intermediate states. For example, a hemifused state (characterized by lipid mixing, but not contents mixing) was first detected with an influenza HA that had its transmembrane domain replaced with a glycolipid anchor [25]. The same phenotype was later produced under conditions of suboptimal concentrations or cold-arrested wild-type HA. The arrested state(s) could be pushed to full fusion by raising the temperature or by adding fusion-promoting lipid curvature agents [26–29]. These experiments strongly supported the notion not only that hemifusion is an intermediate that is observable in pure lipid model systems, but also that intermediates exhibiting a hemifusion phenotype occur in biological membrane fusion.

Structures of membrane fusion proteins

The first membrane fusion protein whose structure was solved by X-ray crystallography was HA from influenza virus [30] (Figure 2a). The ectodomain of HA, from which the transmembrane domains have been proteolytically removed for crystallization reasons, consists of a trimer whose most prominent feature is a long central coiled coil formed from three long α helices, each contributed by a different subunit. HA is synthesized as a precursor, HA0, whose trimeric structure has also been solved [31]. HA0 is activated by proteolytic cleavage at Arg329, resulting in HA1 and HA2 subunits. The first ~20 residues of the newly formed N terminus of HA2 are quite hydrophobic, are very critical for membrane fusion and, therefore, have

Figure 2



Structures and probable sites of action of proteins involved in viral and intracellular vesicle membrane fusion. **(a)** Class I viral membrane fusion — influenza HA. Upon lowering the pH, HA1 (yellow) partially dissociates, the trimeric helical bundle of HA2 (green) undergoes the 'spring-loaded' conformational change, and an outer layer of helices and a long unstructured 'leash' (orange) pack against the inner core of coiled coils,

been termed the ‘fusion peptide’. Upon proteolytic activation, the fusion peptide moves into a cavity in the trimer, which protects it from hydrophobic self-association. The structures of a proteolytic product of low-pH-treated HA [4] and an equivalent expressed fragment of HA2 [32] also reveal very long trimeric coiled coils, although these consist of different portions of the polypeptide chains (Figure 2a). Major refolding of HA takes place when the protein is exposed to pH 5, that is, the endosomal pH that triggers fusion of the viral and endosomal membranes after receptor-mediated endocytosis of the virus. The refolding can be seen as the product of two major molecular motions: an extension of the central coiled-coil helices towards their N terminus, relocating the fusion peptides (which are also removed in these constructs for crystallization reasons) to the top; and the formation of reverse turns in the middle of the original coiled helices, which results in a 180° reorientation of the C-terminal halves of the helices and adjacent newly formed extended structures (‘leashes’) that fit into the groove between adjacent helices. It is not known whether these two refolding steps occur simultaneously or sequentially, but many fusion models assume that the former occurs before the latter. The net result of the complete refolding is that the fusion peptides and the transmembrane domains are delivered to the same end of the rod-shaped molecule. The HA1 receptor-binding domains probably dissociate from the HA2 core domain at low pH. The structures of the receptor-binding domains are not altered at pH 6 in solution [33], but may be altered when bound to membranes at pH 5 [34]. Some early components of the pH-induced conformational change of HA are reversible [35,36], but the final trimeric helical hairpin structures are irreversible products of the energy-releasing ‘spring-loaded’ conformational change [37–39]. Firmly locking the last leash residues into the grooves of the coiled coil is required for fusion (JM White, personal communication).

Influenza HA is a class I viral fusion protein. The core structures of many other class I viral fusion proteins have been solved. They include those of HIV [40–42], SIV [43,44], MoMLV [45], HTLV [46], SV5 [47], HRSV [48] and Ebola [49,50]. The common theme of all these class I

structures is a trimeric bundle of helical hairpins with the N and C termini pointing in the same direction, that is, they have a very similar general architecture (but not in detail) to the described pH 5 structure of HA2. It should be noted that all of these structures are only fragments of the entire fusion protein and that they are believed to represent activated fusion proteins (i.e. the structures that form during fusion or after fusion is complete). For no fusion protein other than influenza HA is the prefusion structure known. Peptides that competitively inhibit helical hairpin formation also inhibit the growth of fusion pores in HIV gp120/gp41-mediated membrane fusion, indicating that the energy from sustained six-helix bundle formation is required to maintain and enlarge these fusion pores [51].

Class II viral membrane fusion proteins consist of three domains and form dimers on the membrane surface of flaviviruses and alphaviruses. Like influenza, these viruses also fuse at low pH in the endosome. Tick-borne encephalitis virus E protein consists of three mostly β -sheet domains (Ig-like, central and dimerization domains) [52]. A recent structure of the whole Semliki Forest virus was solved by a combination of cryo-EM and X-ray crystallography [53**] (Figure 2b). The internal fusion peptides protrude as short loops from the tips of the dimerization domains of the E1 fusion protein, which has the same domain structure as the tick borne encephalitis virus E protein. In the virion, the fusion protein dimers are assembled in a regular T4 lattice on the surface of the membrane, but their long axis is slanted so that the tips with the fusion peptides project farthest from the membrane surface. To prevent fusion, the fusion peptides are protected at neutral pH by the receptor-binding E2 protein, which moves away at pH 5. The transmembrane domains of E1 and E2 are in close proximity to each other, and project from the end opposite to the fusion peptide and to the other (viral membrane) side of the slanted dimer. The fusion peptides exposed at low pH may insert into the cellular membrane as a β barrel. A similar mechanism has been proposed for the pH-induced rearrangement of the E protein of dengue virus, whose structure was also solved by a combination of X-ray crystallography and cryo-EM [54**]. In this case, 90 E

(Figure 2 Legend Continued) bringing the fusion peptides (FP, blue) and transmembrane domains (red) into close proximity (structures taken from [30,32,92**]). **(b)** Class II viral membrane fusion — Semliki Forest virus. Upon lowering the pH, the receptor-binding protein E2 (yellow) moves and deprotects the fusion peptide loop at one end of the elongated E1 fusion protein (red), which forms dimers arranged in a regular lattice on the virus surface. NCP, nucleocapsid protein from the related Sindbis virus (structures taken from [53**,122,123]). **(c)** Intracellular vesicle membrane fusion. Available structures from synaptic and Golgi vesicle fusion are combined in this figure. The t-SNAREs syntaxin (Sx, red) and SNAP-25 (Sn, green) combine with the v-SNARE synaptobrevin (Sb, blue) to form the SNARE complex, which is further stabilized by complexin (Cx, purple) at the site of fusion. The SNARE motif of Sx is preceded by an N-terminal peptide, which binds to an SM protein (Sly1p, upper yellow structure), and the Habc domain in the open form. Sx can also bind as a fusion precursor in the closed form to another SM protein (Munc-18, lower yellow structure). In Ca^{2+} -regulated exocytosis, synaptotagmin (orange), consisting of C2A and C2B domains, is resident in the vesicle membrane and binds in the presence of Ca^{2+} (pink spheres) in *trans* to the target cell membrane via its Ca^{2+} -binding loops. It may also bind to syntaxin. Rab and associated proteins that are involved in the tethering of the vesicles and the disassembly complex NSF/ α -SNAP are not shown. (structures taken from [62,70,71**,75**,76]). In viral and intracellular membrane fusion, transmembrane domains and/or fusion peptides or Ca^{2+} -binding loops are proposed to cooperate to create hemifusion and full fusion intermediates (see Figure 3 for an example).

protein dimers form a relatively flat icosahedral lattice on the surface of the viral lipid bilayer and there is no separate receptor-binding protein. In this case, the internal fusion peptides at the tip of the dimerization domains are protected by the dimer interface. It is proposed that low pH induces a rotation of the dimers so that a bare area of viral membrane is created. A proposed conformational change may project the tips with the fusion peptides towards the cellular membrane, where they may insert in the form of a β barrel [54^{••}].

Intracellular membrane fusion is promoted by a complex assembly of proteins, in which the SNARE (soluble NSF-attachment protein receptor) complex plays a central role (see [55[•],56[•]] for recent reviews, but see also [57] for an alternative view) (Figure 2c). Vesicular and target membranes that are to be fused contain v- and t-SNAREs, respectively. The v-SNARE consists of a single polypeptide chain with a single transmembrane span (synaptobrevin or VAMP [vesicle-associated membrane protein]). The t-SNARE is much larger. It consists of membrane-spanning syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa), which is membrane anchored by multiple palmitylations. Together, the t- and v-SNAREs form the SNARE complex and, according to a widely held view, the pairing of t- and v-SNAREs drives membrane fusion [58]. The core structures of two SNARE complexes have been solved [59,60[•],61[•]]. Interestingly, the SNARE complex forms a helical rod, as do class I viral fusion proteins, but the rod consists of four parallel helices without antiparallel outer layer helices or 'leashes'. Syntaxin and synaptobrevin contribute one helix each to this complex and SNAP-25 contributes two α helices. In the uncomplexed form, the groove of the t-SNARE, into which the v-SNARE fits, is occupied by the N-terminal regulatory domain of syntaxin, which by itself forms a small antiparallel three-helix bundle (Habc domain) [62] (Figure 2c). Alternatively, a second syntaxin polypeptide chain can fill the groove of an open t-SNARE and thereby complete the four-helix bundle [63[•]]. Association of the helices in the SNARE complex is guided by the SNARE motif, which consists of a regular heptad repeat of apolar residues required for coiled-coil formation and one polar residue (glutamine or arginine) in the center of the sequence. These residues interact and thereby put the four helices into the correct register. Apparently, all functional SNARE complexes consist of three glutamine-containing Q-SNAREs (one from syntaxin and two from SNAP-25) and one arginine-containing R-SNARE (synaptobrevin) [64,65]. The cross-sectional plane where these polar residues interact has been termed the 'Q-layer'. In analogy to viral fusion proteins, it is thought that the assembly (zippering of the coiled coils) of functional SNARE complexes provides the energy to drive membrane fusion. However, and in contrast to viral fusion proteins, SNARE complexes can be recycled after one round of fusion. This is accomplished by NSF

(*N*-ethylmaleimide-sensitive factor) and other accessory proteins, which use ATP to dissociate SNARE complexes and thereby reset their high-energy uncomplexed forms. SNARE complex assembly is under kinetic control [66] and is coupled to the rate of dissociation of the N-terminal Habc domain from the SNARE motif of syntaxin [66–68]. The rate of complex assembly and thus the rate of fusion is enhanced by a synaptobrevin-derived peptide that binds to the C-terminal groove of the t-SNARE and thus displaces the Habc domain, while still providing a binding site for the N-terminal portion of synaptobrevin [69[•]]. These studies also establish that folding proceeds in the N→C-terminal direction of the complex (i.e. towards the two fusing membranes).

SM (Sec1/Munc-18) proteins are soluble proteins of 60–70 kDa that interact with SNAREs via several different mechanisms. Although their precise role in membrane fusion is still debated and an area of extensive current research, they are proposed to regulate or perhaps even control SNARE assembly, and thereby add another layer of specificity to intracellular membrane fusion. The problem with SNAREs providing the sole specificity filter for controlling which intracellular membranes fuse is that the many different SNAREs (>20 syntaxins and analogs in mammals) are apparently recycled through the different vesicle compartments and therefore would be available as potential fusion partners at several stages of vesicle trafficking. There are fewer SM proteins, four in yeast and seven in mammals. Therefore, SM proteins may serve as cellular compartment tags. They may also serve to prevent premature promiscuous SNARE assembly. Consistent with this idea is that SM proteins interact with SNAREs in different compartments and perhaps at different stages of fusion via different modes. Two crystal structures of SM protein–SNARE complexes highlight two modes of interaction. In both complexes, the structures of the SM proteins, although only distantly related, are quite similar. They consist of three domains that form an arch with a central, about 15 Å wide cavity. Neuronal Sec1 (n-Sec1), which operates in exocytosis, binds syntaxin 1A in the closed conformation in this cavity [70]. The four-helix bundle of the closed conformation of syntaxin 1A, consisting of the SNARE motif and the Habc domain, fits about half way into the cavity of n-Sec1 (Figure 2c). Most contacts to n-Sec1 are formed with helix Hc and the SNARE motif of syntaxin 1A. Sly1p is an SM protein that operates in the Golgi, where it interacts with the extreme N terminus of the syntaxin Sed5p. The structure of the Sly1p–Sed5p complex (the N-terminal peptide of Sed5p was used for co-crystallization) reveals a different binding site on the SM protein [71^{••}] (Figure 2c). The N terminus of the syntaxin forms two helices that wrap around the outside of domain 1 (i.e. the thicker leg of the arch). It is possible that this represents the most frequent mode of interaction between SNAREs and SM proteins [72[•]]. As this mode involves neither the SNARE

motif nor the Habc domain of syntaxins, binding may occur in the open or closed conformation, and independently of SNARE complex assembly. Yeast Sec1p, which operates at the plasma membrane, appears to bind only assembled SNARE complexes [73]. The precise mode of interaction in this case is not known. Finally, in endosome and vacuole fusion, SM proteins may also interact with SNAREs indirectly through multiprotein complexes that may include Rab effector proteins. Therefore, SM proteins probably link the tethering of vesicles, which is accomplished by Rab and Rab effector proteins (reviewed in [74]), to SNARE assembly.

Complexins are small (15–16 kDa) soluble proteins that bind to assembled SNARE complexes. They are essential for efficient Ca^{2+} -evoked neurotransmitter release. The structure of the complex between complexin and the SNARE complex shows that complexin forms a fifth antiparallel helix on the four-helix parallel bundle of the SNARE complex [75••] (Figure 2c). It covers about two-thirds of the C-terminal (membrane-proximal) end of the groove between the synaptobrevin and syntaxin helices of the rod-shaped SNARE complex. Therefore, it may stabilize the fully assembled *trans*-SNARE complex, whose structure it does not otherwise alter.

It is possible that SNARE assembly and the binding of complexin in exocytosis proceed to just to the verge but not to the completion of fusion. The final step may await the action of Ca^{2+} and its interaction with synaptotagmin. Synaptotagmin is an integral membrane protein of the vesicular membrane that consists of two slightly different C2 domains, C2A and C2B, preceded by a small intravesicular domain (~60 residues in synaptotagmin 1) and a single-helix transmembrane anchor. The structures of both C2 domains have been solved [76–79] (Figure 2c). They each bind two to three Ca^{2+} ions. Both C2 domains bind to acidic membranes in the presence of Ca^{2+} . C2B binding to membranes may require prebinding of C2A or the presence of phosphatidylinositol-4,5-bisphosphate [$\text{PtdIns}(4,5)\text{P}_2$] in the membrane, but these findings are not uncontroversial. The orientation and penetration of C2A docking onto PS-containing membranes in the presence of Ca^{2+} has been determined by site-directed spin labeling [80•]. Two Ca^{2+} -binding loops are found to penetrate the bilayer to about 5 Å below the lipid phosphates. Although monomeric in solution, linked C2A and C2B domains of synaptotagmin form heptamers on membranes [81•]. These heptamers, as visualized by EM, form barrels consisting of two rings, presumably comprising seven C2A and seven C2B domains. Assembly of the rings is driven by the C2B domains, but only in the presence of Ca^{2+} and PS-containing lipid monolayers. Whether the observed oligomeric structures with dimensions of 11×11 nm have time and space to form at the fusion site in the <1 ms required to trigger exocytosis with Ca^{2+} remains an open question. Regardless, the hypothesis

that the C2B and C2A domains bind upon Ca^{2+} release in a concerted fashion to the *trans* membrane opposite to the *cis* membrane, in which they are anchored via their transmembrane domain, is appealing as a decisive contributing mechanism to complete fusion that has been preset by SNARE assembly.

Synaptotagmin also binds to assembled SNARE complexes. Binding, which mostly occurs *trans* to the t-SNARE polypeptides syntaxin and SNAP-25, appears to be Ca^{2+} independent. Co-reconstitution of synaptotagmin in the v-SNARE membrane but not in the t-SNARE membrane accelerated SNARE complex assembly and fusion in a Ca^{2+} -independent manner in an *in vitro* fusion assay [82•]. Addition of soluble linked C2A and C2B domains also increased the rate of SNARE-mediated fusion. The relative importance of synaptotagmin binding to SNAREs versus phospholipid binding is unclear at present. Although the reconstitution results appear to indicate a clear role in *trans*-SNARE activation, the insensitivity of this effect to Ca^{2+} is puzzling. Moreover, recent results using Sr^{2+} as a Ca^{2+} agonist in cultured hippocampal neurons indicate that SNARE binding to synaptotagmin is dispensable, but phospholipid binding is not for fast Ca^{2+} -triggered neurotransmitter release [83]. It is conceivable that SNARE and phospholipid interactions are important in synaptotagmin control of synaptic membrane fusion. A possible missing link could be the involvement of $\text{PtdIns}(4,5)\text{P}_2$, which has been shown to weakly interact with t-SNARE syntaxin 1A–SNAP-25 [84].

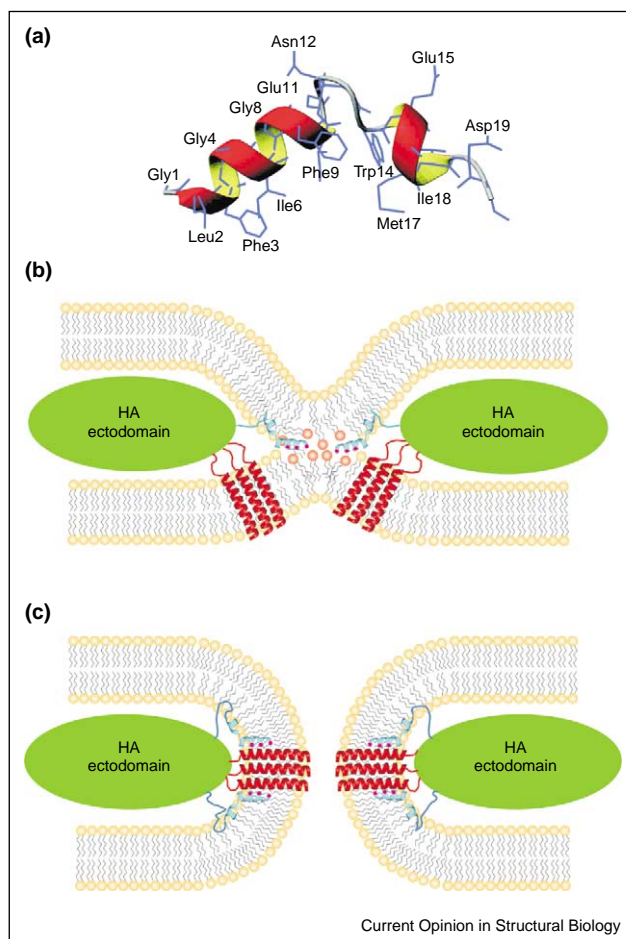
Fusion peptides and transmembrane domains of fusion proteins

There is a fundamental difference between viral and intracellular fusion proteins. Because viruses are parasites, they need to package the entire fusion machinery into their own membrane and, usually, into a single viral fusion protein. By contrast, intracellular fusion machines consist of several proteins that are resident in both membranes that are to be fused. Although SNARE pairing provides a mechanism to link the two fusing membranes via the transmembrane domains of the respective SNAREs, viral fusion proteins carry a cryptic potential membrane anchor, namely the hydrophobic fusion peptide, in a hydrophobic cavity of the resting structure (Figure 2a). Only upon activation (low pH in the case of influenza HA) is the fusion peptide exposed and available for insertion into the target membrane. Sequences of fusion peptides are highly conserved between different strains of individual viruses, but not between different virus families [85,86]. They typically comprise about 20–30 residues, potentially fold into amphipathic helices, and are rich in glycines and alanines. This last property endows them with a high degree of conformational flexibility. Fusion peptide structures appear to be polymorphic and strongly dependent on

the environment. Indeed, the fusion peptide of influenza HA has been observed in random coil, α -helical and β -sheet conformations in different environments [87]. It is possible that all three forms have some physiological relevance. For example, the peptide may be unstructured in solution on its way to the target membrane. The helical form prevails at low concentrations in membranes, but self-associated β sheets are induced at higher concentrations in the membrane interface. This self-assembly step may help recruit several HA trimers to individual fusion sites. Because, at least in influenza HA, fusion peptides are connected to the ectodomain via a flexible linker [32], they probably form independently folded domains in membranes [88]. Conformational transitions in membranes have also been observed with the fusion peptide of HIV gp41, which adopts a helical or β -sheet structure depending on concentration, lipids and ionic conditions [89]. A structural characterization of the β form has been carried out by solid-state NMR [90].

There is quite strong evidence that it is the helical form of the influenza HA fusion peptide that promotes fusion; fusion peptide mutants that inhibit membrane fusion at various stages are less helical and have a higher tendency to self-associate into β sheets [91]. The structure of the fusion peptide of influenza HA in membranes has recently been solved by a combined NMR and spin-label EPR approach [92**] (Figure 3a). Interestingly, the pH 5 structure exhibits a kink defined by Asn12 and Gly13 that separates an N-terminal and a short C-terminal helix, which together form a boomerang-shaped structure. Both helical arms are amphipathic, with bulky hydrophobic residues facing the membrane interior, a ridge of glycines (some of which are highly conserved) facing the membrane surface on the N-terminal arm and two acidic residues facing the membrane surface on the C-terminal arm. Because of their location in a membrane interface, Glu11 and Glu15 in a fusion peptide analog have unusually high pK_a s of 5.6 [93]. Protonation of these side-chains may explain the deeper membrane penetration and enhanced fusogenicity of the fusion peptide at pH 5 [92**]. The totally conserved N-terminal glycine residue, which is very critical for fusion, is also critical to the structure of the fusion peptide in membranes, presumably because of its unique interaction with the membrane; when changed to a valine, the N-terminal helix is partially unwound, and the fusion peptide becomes linear [94] and fusion inactive in the context of full-length HA [95]. The hypothetical 'spring-loaded boomerang mechanism' of membrane fusion proposes that, at a late stage in fusion, the fusion peptide may associate with the transmembrane domains of the fusion protein, become transmembrane itself, and thereby form an early fusion pore [96*] (Figure 3c). However, although appealing from a structural perspective, this mechanism has not yet been verified experimentally.

Figure 3



Membrane-inserted structure of the influenza HA fusion peptide and possible intermediates of influenza HA-mediated fusion. **(a)** Structure of the influenza HA fusion peptide determined by NMR in detergent micelles and confirmed with spin-label EPR in membranes at pH 5 [92**]. **(b)** Dynamic 'lipid mixer' model of the hemifusion intermediate. Water is withdrawn from the cleft between the two fusing membranes by the presence of the boomerang-shaped fusion peptides (light blue). The fusion peptides, which are lined with bulky hydrophobic residues on the inside of the boomerang and with glycines (pink dots) on the outside of the N-terminal arm, facilitate easy lipid mixing between the two apposed membranes owing to their amphipathic properties. **(c)** A fusion pore is formed by latching the fusion peptides onto the transmembrane helices (red), possibly via interactions of the conserved glycine ridge on the fusion peptide. The fusion peptides may at this stage open their angle and extend more deeply into the membrane. For clarity, only one or two fusion peptides per trimer (instead of three) are shown in (b) and (c), respectively.

The transmembrane anchor domains of influenza HA and vesicular stomatitis virus (VSV) G fusion proteins have been demonstrated to form regular transmembrane helical structures as expected [97,98]. The transmembrane domain of HA forms oligomers in SDS micelles and contains a large number of exchangeable amide hydrogens, indicating that the helices may form water-accessible pores in membranes [97]. The sequence

requirements for transmembrane domains are not as stringent as for fusion peptides [99,100], although some glycines seem to be important in both proteins [101,102]. Glycine motifs are well known to support helix interactions in membranes [103] and may also be of importance in fusion proteins to support interactions between transmembrane helices, and/or between transmembrane helices and fusion peptides.

Conserved amino acid motifs in the transmembrane domains of syntaxin and synaptobrevin have been reported to drive homodimer and heterodimer helix association of these SNAREs in detergent micelles and membranes [104,105]. The transmembrane helix interactions of the synaptobrevin homodimer have been modeled [106]. However, recent results using refined methods found only very weak interactions between synaptobrevin transmembrane helices and therefore questioned their biological significance [107]. The juxtamembrane peptide of syntaxin 1A, which comprises short sequences of basic residues intervening between the SNARE motif and the TM domain, interacts with acidic lipid bilayers by a surprisingly deep insertion into the membrane interface [108]. Peptide models of the v- and t-SNARE transmembrane domains, and some derived mutant peptides promote fusion of liposomes in a fashion that is anticorrelated to their propensity to form helices in membrane-mimetic environments and lipid bilayers [109]. Although probably still helical in membranes, this unexpected result indicates that marginal stability of the SNARE transmembrane helices may be functionally important. Reconstituted synaptobrevin, but not syntaxin, exchanges relatively easily between model membranes, indicating that its transmembrane domain is more weakly bound to membranes than those of most single-span membrane proteins [110]. The conformational flexibility of both SNAREs and the reversible membrane insertion of the v-SNARE are reminiscent of the properties of viral fusion peptides described above. Perhaps the transmembrane domain of the t-SNARE serves the function of the transmembrane domain of the viral fusion protein, whereas the transmembrane domain of the v-SNARE serves the role of the viral fusion peptide.

Putting it together

The structures of many components of viral and intracellular fusion machines have provided highly suggestive insights into how these machines might work. Despite the huge impact that structural biology has had on the study of membrane fusion, we still need to assemble the components in the correct spatial and temporal order. Refined and new structural and biophysical methods will be needed to develop a coherent mechanistic picture of how biological membrane fusion works. From the structural perspective, key questions that still await future investigation are: how do fusion peptides and transmembrane domains perturb and reshape lipid bilayers, and

how do they accomplish the merger of two lipid bilayers in an essentially nonleaky fashion? Also, what exactly is the role, if any, of synaptotagmin in actual membrane merging during intercellular membrane fusion? It has long been conjectured that bending of bilayers is important in fusion (reviewed in [111,112]). Some fusion peptides change membrane curvature, as shown by their ability to induce nonbilayer lipid phases under certain conditions [113,114]. However, curvature effects of transmembrane domains of SNAREs on lipid bilayers have not been described. Fusion and transmembrane peptides also alter the water structure on lipid bilayers and probably partially dehydrate their surfaces [97,115]. Finally, it has been argued that fusion peptides accelerate liposome fusion by filling hydrophobic packing defects in stalk intermediates [116,117]. A problem with the curvature models is that the structure of the influenza HA fusion peptide in membranes is not compatible with bending bilayers towards more negative curvature, as required to stabilize a stalk intermediate. It also does not fit the 'hydrophobic voids' (Figure 1) proposed in some stalk models because this would require translocation of a major portion of the fusion peptide towards the mid-plane of the membrane. Given the many polar residues in the C-terminal half of the fusion peptide, this seems unlikely. We therefore favor a different mechanism by which fusion peptides promote fusion of biological membranes. This model, which we call the 'lipid mixer' model, is easily incorporated into the spring-loaded boomerang mechanism of membrane fusion [96]. We think that fusion peptides and the membrane-proximal residues of the transmembrane domains replace water molecules in the thinning cleft between the two membranes that are forced to approach each other by the zippering of the coiled coils of the ectodomains. Membrane dehydration coupled with the asymmetric insertion of bulky hydrophobic and hydrophilic sidechains into the bilayer probably disrupts the lamellar order of the lipids because the thermodynamic stability of the bilayer structure is defined only in the presence of water and/or adequate polar headgroup interactions [118]. If water and headgroup interactions are removed by the presence of inserted peptides, the bilayer ordering principles are removed from the lipids. The lipids will be 'confused' and therefore will have a high probability of flipping (mixing) between two closely apposed bilayers (Figure 3b). This is exactly what is experimentally observed in the 'hemifusion' intermediate of biological membrane fusion without a need for an hourglass-shaped lipid stalk. The dehydration/lipid mixing model is compatible with most previous experimental data that were used to support the stalk model. For example, the positive curvature agent lyso-lecithin with its bulky polar headgroup may block fusion by preventing dehydration and deep insertion of the fusion peptide. Conversely, negative curvature agents such as oleic acid may promote fusion peptide insertion and thus dehydration. Future experimental studies of these interactions, as

well as Brownian and molecular dynamics simulations [119–121], will probably shed more light on the nature of the controlled lipid mixing process in membrane fusion.

Update

The position in the membrane and accessibility for SNARE complex formation of reconstituted synaptobrevin (VAMP-2) was recently determined in PC/PS lipid model membranes by site-directed spin labeling [124*]. The juxtamembrane domain (membrane-proximal residues 85–95) of VAMP-2 was found to be immersed into the membrane interface, possibly forming a one- to two-turn interfacial helix. The t-SNARE syntaxin 1A–SNAP-25 did not bind and therefore did not assemble into a SNARE complex with VAMP-2 that was reconstituted into membranes at close to physiological concentration (1:300 protein:lipid). The authors conclude that these residues form a regulatory domain, which can only assemble into a SNARE complex upon release from the membrane surface. To reconcile the results from Kweon *et al.* [124*] with the fusion experiments of Weber *et al.* [58], which have been conducted at much higher protein:lipid ratios, one would have to postulate an equilibrium between the surface-bound and released forms of the juxtamembrane domain of VAMP-2. The slow fusion observed by Weber *et al.* may be explained if the membrane-surface-bound form dominates the equilibrium and if the exchange between the two forms is slow. However, another recent study demonstrates that cognate SNAREs expressed on the surfaces of two populations of cells at concentrations close to those found on synaptic vesicles are sufficient to cause fusion of t- and v-SNARE expressing cell pairs [125*], supporting the notion that SNAREs may indeed constitute a minimal fusion machinery [58]. Fusion is quite efficient (up to 40% of the cells that are in contact fuse) in this system and occurs over a time course of one to two minutes when monitored at the level of individual cells.

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