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Review

The role of transmembrane domains in membrane fusion

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Abstract. Biological membrane fusion is driven by different types of molecular fusion machines. Most of these proteins are membrane-anchored by single transmembrane domains. SNARE proteins are essential for intracellular membrane fusion along the secretory and endocytic pathway, while various viral fusogens mediate infection of eukaryotic cells by enveloped viruses. Although both types of fusion proteins are evolutionarily quite distant from each other, they do share a number of structural and functional features. Their transmembrane domains are now known to be critical for the fusion reaction. We discuss at which stages they might contribute to bilayer mixing.

Keywords. Membrane fusion, hemifusion, transmembrane domain, SNARE, fusion pore.

Introduction

Biological membrane fusion is central to cellular secretion and endocytosis, infection of eukaryotic host cells by enveloped viruses, cell-cell fusion, *etc*. Several excellent recent reviews cover the mechanism of fusion [1–17]. Depending on the perspective of the different authors, these reviews emphasize different aspects of fusion, like the specificity of membrane-membrane recognition prior to fusion, regulation of fusogenic proteins by accessory factors and the biophysics of bilayer mixing, to name a few. Here, we review the mechanism of the fusion reaction and focus on the role of the transmembrane domains (TMDs).

Amphipathic phospholipid molecules self-assemble in aqueous solution into bilayers that normally do not fuse spontaneously. How do the lipids then rearrange along the pathway from the unfused to the fused membrane and how do fusogenic proteins facilitate bilayer restructuring? Functional dissection of the fusion reaction is primarily based on two major model systems. One system comprises envelope proteins from different viruses, of which the trimeric class I fusion protein influenza hemagglutinin (HA) represents the most thoroughly investigated case [2, 12]. Class I viral fusogens are synthesized as precursor molecules that are proteolytically cleaved to yield the mature forms. The membrane-spanning subunit contains an N-terminal amphipathic fusion peptide, an ectodomain that folds into a coiled-coil structure and a single TMD. In the case of class II proteins, internal fusion sequences are embedded within the ectodomains [1, 4, 13]. SNAREs [soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptors], on the other hand, form a family of orthologs that are essential for most types of intracellular membrane fusion, ranging from constitutive vacuole-vacuole fusion in yeast cells to regulated exocytosis of neuro-

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transmitters from mammalian presynaptic terminals [4, 10, 14, 17]. According to their preferential subcellular localization, SNARE proteins were initially classified as vesicle (v) or target membrane (t)-SNAREs [18]. This nomenclature is somewhat ambiguous, since t-SNAREs are also found on vesicles and v-SNAREs can be found on target membranes. A systematic sequence analysis revealed that most v-SNAREs have an arginine residue in the center of the SNARE domain (R-SNAREs), whereas a glutamine (or aspartate) residue is found in syntaxins and SNAP-25-like t-SNARE proteins (Q-SNAREs) [19, 20]. The N-terminal domains of SNAREs show considerable variability. The N terminus of Q-SNAREs consists of a three-helix bundle [10] that can interact with the central SNARE domain. R-SNAREs have either a complex *longin*-fold (longins) or short, presumably unstructured, sequences (brevins) [14]. Most SNAREs are membrane anchored via a single C-terminal proteinaceous TMD. In some cases, anchoring is mediated by palmitate (the Q-SNAREs SNAP-25 and SNAP-23 [21, 22]), palmitate and farnesyl chains (Ykt6, [23, 24]) or by an N-terminal PX domain (the Q-SNARE Vam7, [25, 26]). However, SNARE-mediated fusion can occur only if at least one SNARE on each bilayer is anchored by a TMD [27, 28].

SNAREs do not share sequence homology with viral envelope proteins and thus both types of fusogens must be regarded as distinct classes of molecules. Nevertheless, they do share a number of basic architectural features: both are integral membrane proteins with a single-span transmembrane topology, both assemble to quaternary complexes via formation of membrane-extrinsic coiled-coil domains, and in both cases the quaternary complexes form supramolecular multimers [4, 8, 29, 30]. The TMDs of SNAREs [31–33] and of hemagglutinin [34] insert as α-helices that span the bilayer and assume oblique angles relative to the normal bilayer.

To assess the potential function of fusion protein TMDs in fusion, they have been replaced with lipid anchors, mutated or truncated. Table 1 lists a number of examples where alteration of viral fusion protein TMDs affects the efficiency of fusion; in some other reports, TMDs were not functionally relevant. Table 2 compiles the evidence for a role of TMDs in SNARE function.

In addition to the structural analogies between viral fusogens and SNAREs, fusion reactions that are driven by these proteins share essential mechanistic features. In both cases can bilayer mixing be divided into subreactions: (i) membrane apposition, (ii) formation of stalk and hemifusion intermediates, (iii) fusion pore formation, and (iv) the hemifusion-to-fusion transition. By outlining the various stages of the fusion reaction, we discuss the stage(s) at which the TMDs may contribute to membrane fusion.

Membrane apposition

Depending on their content in charged lipid species and integral membrane proteins, hydrated membranes are held at an equilibrium distance of 2–20 nm [13]. Accordingly, the first step leading to fusion is the establishment of an intermembrane contact by proteins that bridge both cognate bilayers. To our knowledge, no influence of TMDs at this stage is currently known. Therefore, this stage is only reviewed briefly here.

Virus docking to cellular membranes involves exposition of the amphipathic fusion peptides by a conformational change of ectodomains; this is followed by fusion peptide insertion into the target bilayer. Subsequent structural rearrangements of the ectodomains are probably required to closely juxtapose both bilayers [1, 12, 35] (Fig. 1a). In addition, there is direct evidence that the heptad repeats within the ectodomains of HIV and Sendai virus are directly involved in membrane fusion [36–40].

The situation is more complex with intracellular fusion since membrane-membrane recognition along the secretory pathway needs to be specific. Different subcellular membranes contain distinct sets of SNAREs, which form defined *trans* complexes upon initial membrane contact (Fig. 1b, c) [41]. Thereby, the cytoplasmic SNARE domains assemble to intermolecular coiled coils [42, 43]. SNAREs appear to zipper up from the N to the C terminus [44–46], although another recent study contradicts this view [47]. This folding reaction leads to the close apposition of membranes prior to lipid mixing. Contrary to the original expectation [18], it is likely that the specificity of organelle-organelle recognition in the cell does not exclusively rest on *trans* SNARE pairing [48, 49], although evidence for specificity has subsequently been reported [50, 51]. Rather, initial membrane contact is at least in part mediated by Rab proteins and tethering factors that act upstream of SNARE complex formation. Proteins discussed as tethers include Uso1p, TRAPP, the exocyst, the GARP/VFT complex, the HOPS complex and EEA1 [52–55]. Evolutionarily distant organisms such as yeast, invertebrates and mammals appear to present different variations of the common theme of *trans* SNARE pairing. In addition, different types of fusion – such as organelle-organelle fusion or neurotransmitter release – may have specific requirements. In symmetric vacuole-vacuole fusion, *cis* complexes of R- and Q-SNAREs exist prior to fusion [56]. They have to be

Table 1. Functional defects displayed by viral fusion proteins after replacing or altering their TMDs.

Protein	Type of TMD alteration	Functional defect	Reference
Influenza hemagglutinin	Replacement by GPI anchor	Abolished contents mixing but retained outer leaflet mixing	[63, 64]
Influenza hemagglutinin	Replacement by GPI anchor	Abolished contents mixing that is partially rescued by chlorpromazine	[67]
Influenza hemagglutinin	Replacement by GPI anchor	Inefficient fusion pore formation and growth	[105]
Influenza hemagglutinin	Replacement by unrelated TMDs	Fusion retained	[148]
Influenza hemagglutinin	TMD G520L mutation (Japan strain)	Abolished contents and reduced inner leaflet mixing, absence of fusion pores, fusion rescued by chlorpromazine	[149]. [65]
Influenza hemagglutinin	Shortening of TMD by 12 residues	Abolished contents mixing but retained outer leaflet mixing, partially rescued by chlorpromazine	[66]
VSV G-protein	Replacement by GPI anchor	Abolished fusion	[77]
VSV G-protein	Deletion of TMD residues or mutation of a GxxxG motif	Abolished contents mixing but retained outer leaflet mixing	[68]
VSV G-protein	Replacement by unrelated TMDss	Fusion retained	[77]
HIV gp120	Replacement by GPI anchor	Reduced syncytia formation	[150]. [151]
HIV gp120	Different truncations and mutations	Reduced syncytia formation	[152]
HIV gp120	Replacement by CD22 TMD or R696I mutation	Viral particle release maintained	[153]
HIV gp120	Replacement by glycophorin A or VSV G-protein TMD	Reduced outer and inner leaflet mixing	[154]
HIV gp120	Mutation of GGxxG motif	Reduced cell-cell fusion	[155]
Measles virus F protein	Cysteine residues mutated	Reduced palmitoylation and cell-cell fusion	[156]
HN protein of Newcastle disease virus	Mutation of leucine zipper repeat	Reduced fusion-promoting activity	[157]
Moloney murine leukemia virus envelope protein	Mutation of Pro617	Reduced fusion and infectious particle formation	[158]
Reovirus fusion associated small transmembrane protein p10	Mutation of tri-glycine motif	Reduced syncytia formation	[159]
Herpes simplex virus type 1 glycoprotein gD	Replacement by GPI anchor	Reduced cell-cell fusion	[160]
Herpes simplex virus type 1 glycoprotein gH	Various point mutations	Reduced cell-cell fusion	[161]
VSV TMD peptide	Mutating GxxxG motif and other point mutations	Reduced liposome-liposome fusion	[85]
VSV TMD peptide	Mutating GxxxG motif	Reduced liposome-liposome fusion	[86]
Semliki forest virus E1 protein	Mutation of conserved Gly residues	Reduced cell-cell fusion and increased dependence of liposome fusion on cholesterol	[162, 163]

Table 2. Functional defects displayed by SNARE proteins after replacing or altering their TMDs.

Protein	Type of TMD alteration	Functional defect	Reference
Caenorhabditis elegans Snb-1	Frameshift within TMD of naturally occurring mutant	Reduced neurotransmission	[164]
Caenorhabditis elegans Unc-64	Truncated TMD	Reduced neurotransmission	[165]
Synaptobrevin II and syntaxin 1A	Replacement by phosphatidyl-ethanolamine anchor	Reduced liposome-liposome fusion	[166]
Yeast exocytotic Snc1p and Sso2p	Replacement by isoprenoid anchor	Reduced exocytosis, rescue by lysolipid addition to distal leaflet	[27]
Synaptobrevin II TMD peptide	Multiple point mutations	Reduced liposome-liposome fusion	[84]
Yeast vacuolar Vam3p	Replacement by isoprenoid anchor	Reduced vacuole-vacuole fusion	[28]
Yeast exocytotic Snc1p	Truncation of TMD to half of its original length	Reduced inner leaflet mixing in liposome-liposome fusion	[31]
Synaptobrevin II and syntaxin 1A	Replacement by GPI anchor	Abolished inner leaflet mixing in cell-cell fusion ("flipped" SNAREs)	[75]
Yeast vacuolar Vam3p (full-length protein and TMD peptide)	Multiple point mutations	Reduced vacuole-vacuole fusion reduced inner leaflet mixing in liposome-liposome fusion	[87]

dissociated by the AAA-ATPase NSF and α-SNAPs prior to assembly of *trans* complexes that bridge the membranes [57] (Fig. 1c). In contrast, fusion of secretory vesicles to the plasma membrane is asymmetric. It requires SNAP-25 orthologues, which combine two SNARE domains in one protein (Fig. 1b). Syntaxin and SNAP-25 assemble in *cis* at the plasma membrane prior to *trans* complex formation with the R-SNARE of the secretory vesicle [4, 10, 14, 29, 46]. Although most researchers agree that SNARE pairing sets the stage for fusion, controversy still exists. For example, quantitative removal of SNAREs did not inhibit the ability of sea urchin cortical vesicles to fuse [58]. In agreement with this cautious note, a recent study reports that synaptic SNAREs that are reconstituted in liposomal membranes at very low protein/lipid ratios accelerate lipid mixing even without detectable *trans* complex formation, provided that the membranes were dehydrated by poly(ethylene glycol) [59].

TMDs in formation of stalk and hemifusion intermediates

Lipid mixing is thought to involve intermediate membrane structures, and this has been reviewed extensively [6, 8, 13, 60]. Here, we briefly summarize the current concepts and discuss the potential involvement of the TMDs.

Mixing of outer membrane leaflets requires that lipid molecules leave their bilayer orientation. This step is thought to result in a 'stalk' structure that provides a local link between both membranes (Fig. 2). The stalk corresponds to an hourglass-shaped structure that may contain only a few dozen lipid molecules. Expansion of the stalk is then believed to result in a hemifusion diaphragm. In hemifusion, the contacting outer monolayers mix over a more extended area, while the distal monolayers stay intact, thus preserving the separation of both aqueous compartments before a fusion pore forms. The concept of stalk formation and hemifusion was originally inspired by studies on viral fusion protein function [13, 61, 62]. This issue is not uncontroversial since experimentally detectable lipid mixing is preceded by fusion pore formation under conditions that allow for complete fusion. One line of evidence supporting the existence of stalk and hemifusion intermediates rests on the sensitivity of the fusion reaction to lipids of different intrinsic curvature. The intrinsic curvature is defined by the ratio of the cross-sectional area of the hydrated lipid head group to that of the acyl chain moiety. It dictates the curvature of a monolayer composed of the respective lipid species. Accordingly, lysophosphatidylcholine with its single acyl chain has a highly positive intrinsic curvature. It thus stabilizes positive curvature of the outer monolayer of a spherical, lipid-enclosed particle, such as a liposome or a small secretory vesicle. Conversely, the negative curvature of the inner monolayer of the same particle is stabilized by lipids with negative intrinsic curvature, such as phosphatidylethanolamine, diacylglycerol and phosphatidic acid or even by free fatty acids. At the stalk structure and at the rim where a hemifusion diaphragm meets the adjoining bilayers, the outer, *i.e.*,

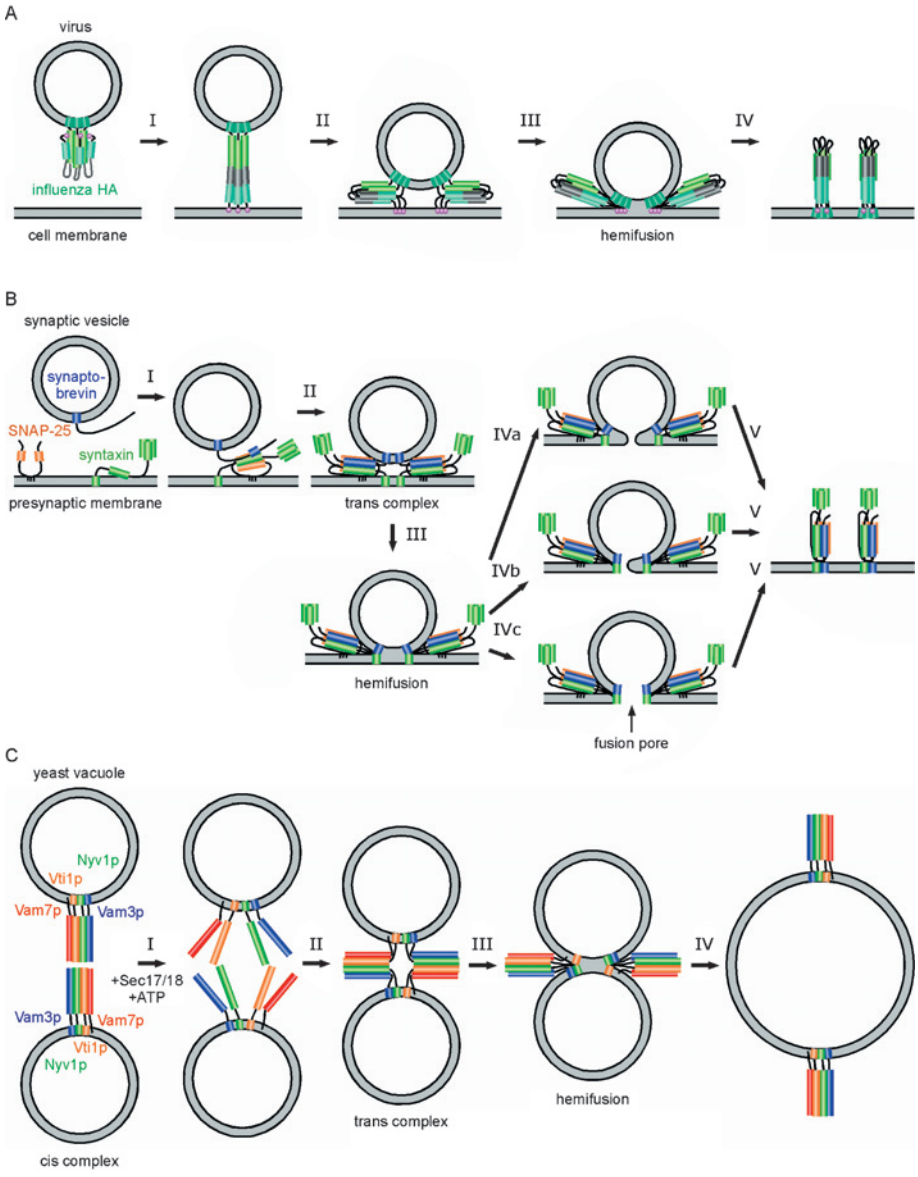

Figure 1. Models of protein-mediated membrane fusion. (*a*) Influenza hemagglutinin HA2 subunit as a representative of class I viral fusion proteins. In the resting state, the coiled-coil domain of the trimeric molecule constitutes a six-helical bundle. Upon a pH-triggered extension of the coils the N-terminal fusion peptides (red dots) insert into the target membrane (I). Chain reversal is probably required to mediate membrane apposition (II), hemifusion (III) and fusion pore formation (not shown) prior to complete fusion (IV) where TMDs and fusion peptides might be close. Two trimers are shown per fusion site to visualize the known cooperative nature of hemagglutinin function. (*b*) SNAREs and synaptic vesicle fusion in neurotransmitter release. Synaptobrevin associates with a preformed syntaxin/SNAP-25 heterodimer by sequential N- to C-terminal complex assembly (I) to yield the complete *trans* complex (II). *Trans* complex formation is thought to result in hemifusion (III). A fusion pore develops that is either primarily composed of lipid (IVa), lipid plus protein (IVb) or protein (IVc). The process is terminated by full fusion (V). (*c*) SNAREs and yeast vacuole fusion. *Cis* SNARE complexes in resting vacuoles are dissociated by Sec17/Sec18/ATP (I) prior to *trans* complex formation (II), hemifusion (III) and contents mixing (IV). A fusion pore is not depicted here. In (*b*) and (*c*) two *trans* complexes per fusion site are shown to visualize the known cooperative nature of SNARE function.

proximal, monolayer of a fusing membrane has to adopt a more net negative curvature compared to the unfused bilayer. Thus, the fusing membrane is probably stressed at these sites. Indeed, fusion is stimulated by molecules with negative intrinsic curvature and inhibited by molecules with positive intrinsic curvature within the proximal monolayer. The concept of stalk formation and hemifusion rationalizes these observations since local bilayer stress is decreased by the presence of lipids with the appropriate shape.

Hemifusion can be observed directly with certain mutant fusion proteins that favor outer leaflet mixing over fusion pore formation and full membrane fusion. For example, influenza hemagglutinin loses its ability

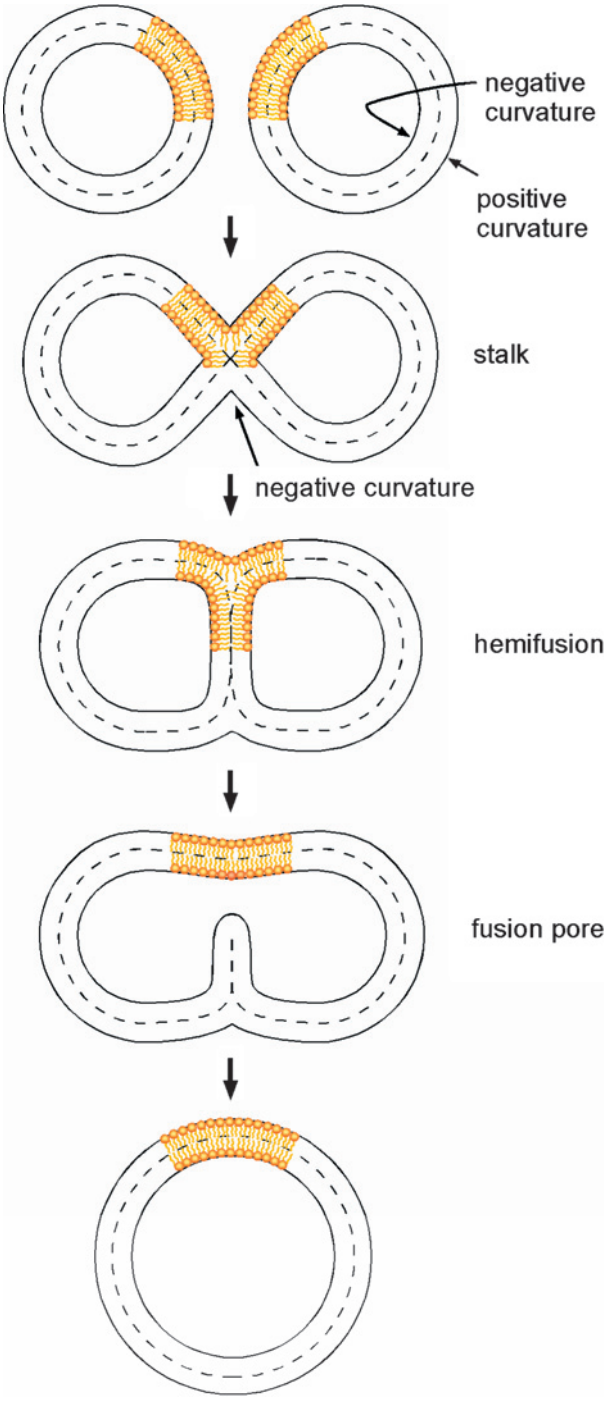

Figure 2. Lipid topology in fusion intermediates. The schematic depicts hypothetical lipid arrangements in stalk and hemifusion diaphragm that are thought to be on-pathway intermediates in the reaction pathway from unfused liposomes to complete lipid mixing. The fusion pore is putatively placed at the boundary between bilayer phase and hemifusion diaphragm. The stalk structure was modeled after [147].

to induce enlarging fusion pores and full membrane fusion when a glycosylphosphatidylinositol (GPI) membrane anchor replaces its TMD (GPI-HA). Nevertheless, GPI-HA induces the redistribution of fluorescent lipids between the outer monolayers of fusing cells, the hallmark of hemifusion [63, 64]. A hemifusion phenotype is also seen upon introducing a point mutation into the hemagglutinin TMD [65] or after truncating it by more than 12 residues [66]. Notably, chlorpromazine, an agent that is thought to specifically destabilize the inner monolayer – of which the hemifusion diaphragm is composed – rescues full fusion with GPI-HA [67] and the point mutant [65]. Hemifusion is also seen after truncating or mutating the TMD of the vesicular stomatitis virus (VSV) G-protein [68]. Collectively, the implications of these results are twofold: First, they suggest that hemifusion is a true intermediate of the fusion reaction. Under normal conditions, hemifusion is thought to be 'restricted' and to rapidly give way to fusion pore formation and inner leaflet mixing. In those cases where outer leaflet mixing can be experimentally observed, the hemifusion diaphragm may have expanded into an 'unrestricted' structure that may even represent a kinetically stable dead-end. Formation of stalk and hemifusion intermediates is supported by molecular dynamics simulations of fusion. In one recent study, fusion between tethered, protein-free liposomes was suggested to follow a branched pathway, in which a common stalk-like intermediate can either directly form a fusion pore or enter a metastable hemifused state on the way to fully fused vesicles [69]. The need for fusogenic proteins may be obliterated in these calculations due to the unrealistically small diameter (14 nm) of the liposome model, and to its exclusive composition of phosphatidylethanolamine. The negative intrinsic curvature of this lipid does not permit formation of experimental liposomes that are composed of only this lipid.

Apart from revealing the existence of hemifusion, characterization of the TMD mutants also uncovered the apparent involvement of fusion protein TMDs in the hemifusion-to-fusion transition, which is discussed in detail in the corresponding section below.

Recent studies indicate that hemifusion is also an authentic intermediate of SNARE-driven membrane fusion. The first hint in this respect was provided upon replacement of the TMDs of yeast exocytotic SNAREs (Snc1p and Sso2p) by C16 geranyl-geranyl moieties. While this abolished exocytosis, the fusion defect could be rescued by addition of lysolipid to the distal membrane leaflets, *i.e.*, from the cells' exterior [27]. This suggested that increasing the positive curvature of the distal monolayer overcame arrest at a hemifusion intermediate. At the same time, this study demonstrated that SNARE pairing by itself is insufficient for complete fusion but requires TMDs; replacement of the TMD of the vacuolar Q-SNARE Vam3p corroborated this notion [28]. A more direct

indication for hemifusion was obtained when yeast vacuoles were shown to exchange fluorescent lipid labels of the outer monolayer under conditions where SNARE pairing occurs, but where content mixing is apparently blocked [70]. In line with the curvature concept, addition of lysolipid to proximal vacuolar membrane leaflets reversibly prevented hemifusion [71], while diacylglycerol accelerated fusion [72]. Evidence for hemifusion also comes from an *in vitro* assay that examines fusion between artificial liposomes containing recombinant SNAREs [73]. A low surface density of yeast exocytotic SNARE proteins in the membrane or shortening the R-SNARE TMD to about half of its original length partially arrests liposomes at hemifusion. Hemifusion appears to be an on-pathway intermediate in this reaction, as suggested by a progressive decrease of the percentage of hemifused membranes in the course of the reaction [31]. Finally, "flipped" synaptic SNAREs can be expressed with an inverted transmembrane topology on the surface of eukaryotic cells if they are equipped with an N-terminal cleavable signal sequence. In this configuration, they induce cell-cell fusion [74]. One third of the individual fusion events correspond to hemifusion as determined by the redistribution of aqueous *versus* lipidic markers. In analogy to previous experiments done with viral fusogens, flipped SNAREs whose TMDs had been replaced by GPI-anchors exclusively induce hemifusion. Hemifusion is partially reversible and considered by the authors as an off-pathway reaction since a progressive decrease of the number of hemifusion events in favor of complete fusion was not seen [75].

These results convincingly demonstrate that hemifusion is relevant in SNARE-mediated fusion. Moreover, they suggest that hemifusion can easily be reached upon membrane apposition. Theoretical estimates have indeed indicated that the repulsive force separating hydrated bilayers as well as the energy required for local lipid rearrangement in stalk formation would not present major barriers on the fusion pathway [6, 13]. It is thus possible that *trans* complex formation plus thermal energy suffice to induce outer leaflet mixing. Hemifusion of model membranes can indeed be triggered by physical means. It was shown that stacked pure dehydrated bilayers that contained lipids with negative spontaneous curvature formed a stalk-like structure that could be observed by X-ray scattering [76].

Is outer leaflet mixing influenced by SNARE TMDs? While this issue is currently unresolved, some observations suggest that this might indeed be so. For example, the VSV G-protein appears to require a proteinaceous TMD for fusion initiation since its replacement by a GPI-anchor reduced fusion without inducing hemifusion [77]. Further, GPI-anchored HA is only ~40 % as efficient as the wild-type protein in outer leaflet mixing [66]. Similary, flipped presynaptic SNAREs whose TMDs had been replaced by GPI-anchors induced only ~50–70 % of outer leaflet mixing (the sum of complete fusion plus hemifusion) compared to the wild-type SNAREs. Full-length and GPI-linked proteins exhibited comparable surface densities in the plasma membrane [75]. These results are thus compatible with the view that the proteinaceous TMDs stimulate outer leaflet mixing.

TMDs may contribute to fusion initiation by bilayer deformation. It has been argued that fusogenic proteins might locally deform the bilayer by pulling on the lipids that surround their TMDs. This would result in 'nipples' or 'dimples', whose local curvature would increase their energy and therefore favor stalk formation [6, 13]. Thus, one function of the TMD could simply be to translate *trans* complex formation or conformational changes of ectodomains into local bilayer stress. In addition, it has recently been suggested that SNAREs may destabilize the bilayer via the TMDs since membranes holding the reconstituted proteins were leaky even without complex formation [59]. Protein/lipid interactions are notoriously difficult to study. Which forces could mediate the interaction of fusion protein TMDs and lipids?

First, most SNAREs contain a cluster of lysine and arginine residues within the flanking region close to their TMD N-termini [19, 78]. In case of synaptobrevin, the positively charged residues have been proposed to interact with the negatively charged phosphatidylserine in *cis* or in *trans*, depending on the experimental conditions. The *trans* interaction may play a role in membrane docking and/or fusion [79]. On the other hand, omission of phosphatidylserine had no effect on SNARE-mediated liposome fusion [80]. Alternatively, therefore, positively charged residues may interact with the phosphate moiety of phospholipid headgroups in *cis*, and thereby stabilize a TMD/lipid interaction as seen with other membrane proteins [81]. The issue of whether these positively charged residues are important for fusion or not is confounded by the observation that their mutation within the yeast Q-SNARE Ssolp affects fusion *in vivo* but not *in vitro* [82]. Second, a conserved pair of tryptophan residues is located between the positively charged cluster and the hydrophobic core of R-SNARE TMDs. This tryptophan motif inserts into the acyl chain region of the membrane in *cis*, and has been proposed to bend the SNARE domain towards the bilayer [83]. Third, the TMDs may mechanically interact with the surrounding lipids by virtue of their intrinsic structural dynamics. This is suggested by studies with synthetic peptides that harbor the hydro-

phobic cores of fusion protein TMDs, but dispense with the soluble domains [84–87]. In this approach, the reductionist philosophy that motivated reconstitution and functional characterization of isolated full-length recombinant SNAREs [73, 88–90] was extended to correlate functional and structural properties of the TMDs. Indeed, reconstituted peptides that mimic the TMDs of synaptic [84] or yeast vacuolar SNAREs [87] or of the VSV G-protein [85, 86] drive liposome fusion, while pure liposomes only show negligible background fusion. Fusion is detected at peptide/lipid-ratios that correspond to 20–100 TMD copies per liposome (30 nm diameter), which is close to the surface density of SNAREs in synaptic vesicles [91]. Like natural fusion reactions, fusion by TMD-peptides is sensitive to lysophosphatidylcholine and enhanced by the negative-curvature lipid phosphatidylethanolamine [84, 85]. Although the TMD peptides cannot bridge membranes prior to fusion, the macroscopic kinetics of peptide-induced liposome fusion is comparable to that seen with full-length SNAREs. It has been estimated previously that the macroscopic fusion kinetics of the latter is dominated by the low probability by which randomly colliding liposomes fuse [88]. It is thus likely that the frequency by which liposomes collide randomly dominates the probability by which they enter fusion, regardless of whether they hold full-length proteins or TMD peptides. More recently, however, it has been shown that stabilizing the syntaxin/SNAP25 complex by a peptide representing part of the synaptobrevin SNARE domain allowed for fast liposome fusion which saturated within seconds [46]. By comparison, individual SNARE-driven liposome fusion events occur within milliseconds [92–94]. TMD peptide-driven fusion is sequence specific [84]. For example, the same point mutations reduce the fusogenicity of full-length VSV G-protein in a cell-based fusion assay [68] and the fusogenicity of the corresponding synthetic TMD in the liposome assay, while an oligo-leucine peptide is non-fusogenic [85]. Thus, hydrophobicity of a peptide does not cause fusogenicity. Rather, the function of the TMD-peptides may be related to the stability of their α-helical conformations. In solution, peptides corresponding to wild-type SNARE TMDs exist as concentration-dependent mixtures of α-helical and β-sheet structures and mutations that increase helicity decrease fusogenicity [84]. Similarly, mutations that stabilize the VSV G-protein TMD helix diminish fusion [86]. Although other transmembrane helices have been observed to refold into β-sheets [95, 96], reversible helix/sheet transitions are unlikely to take place in the hydrophobic environment of a membrane. Rather, it is conceivable that local and transient opening of backbone hydrogen bonds renders a membrane-embedded helix conformationally flexible. Recent electron spin resonance spectroscopy data support this idea in demonstrating increased motional dynamics of the oligo-valine stretch that constitutes the C-terminal half of the yeast SNARE Sso1p helix in a bilayer [33]. A flexible helix may induce local membrane defects and thereby promote outer leaflet mixing upon collision with a partner liposome. The hypothesis of fusion-promoting TMD flexibility is consistent with the overrepresentation of β-sheet-promoting β-branched residues (isoleucine, valine) in SNARE TMDs [84] and of isoleucine and glycine in viral fusion protein TMDs [68]. Although β-branched residues support formation of hydrophobic model helices in micelles [97], they are known to destabilize soluble helices [98, 99]. In a hydrophobic environment, these residue types may promote local and transient helix unfolding that is too subtle to be detected by CD spectroscopy. Helix destabilization by isoleucine and valine may be related to an increased entropy loss upon helix formation since β-branched side-chains exhibit a smaller number of side-chain conformations in the helical than in the unfolded state [100] and/or to steric clashes between side-chain and local backbone [99]. Glycine is known to destabilize helices since it cannot promote folding by side-chain/side-chain interaction [101]. That structurally flexible TMDs may contribute to lipid mixing is in line by the successful *de novo* design of fusogenic TMD peptides. Here, the hydrophobic cores of a series of peptides consist of helix-promoting leucine, sheet-promoting valine and/or helix-destabilizing proline and glycine residues. Depending on the relative ratio of these residues, the peptides display a range of different fusogenicities. Interestingly, the more fusogenic peptides can be readily refolded from the α-helical conformation to β-sheet or *vice versa* by changing solution polarity [102].

TMDs in fusion pore formation

The existence of fusion pores has originally been indicated by studying viral fusion proteins. These pores are likely to form within the hemifusion diaphragm and their dilation is thought to complete bilayer mixing. The rim of a hemifused membrane is particularly suited for pore formation since local bilayer stress is expected to promote rupture at this site [103]. This rim may correspond to the vertex of apposed bilayers. Indeed, video microscopy has revealed intravacuolar disc-shaped membrane fragments that pinch off from the vertices of fusing yeast vacuoles. The vertex is enriched in the Q-SNARE

Vam3p and a number of known regulatory proteins [104].

The walls of fusion pores may primarily be composed of lipids or of protein domains, like the TMDs. Lipids are clearly involved since pore formation induced by viral fusogens depends on the intrinsic curvature of lipids in a way that is inverse to the lipid dependence of hemifusion [61]. Thus, the cone-shaped lysophosphatidylcholine stabilizes a fusion pore presumably by forming a positively curved cover over the exposed acyl chains of a bilayer crack. Further, even GPI-HA is capable of forming small fusion pores [105]. Since these pores fail to enlarge, the TMD of hemagglutinin may be dispensable for pore formation but be required for its expansion.

Lipids also play a role in SNARE-mediated fusion. The fusion-active vertices in vacuoles accumulate several regulatory lipids, including ergosterol, diacylglycerol and 3- and 4-phosphatidylinositides in a mutually interdependent manner with SNAREs and other fusion factors [106]. While ergosterol and diacylglycerol might affect bilayer tension by virtue of their intrinsic negative curvature, the phosphatidylinositides appear to form acceptor sites for the soluble SNARE Vam7p and actin; ergosterol regulates Sec17p release. Palmitoylated proteins such as Vac8p may facilitate fusion by intercalating into the lipids at the fusion site [104, 107–109]. In vertebrates, fusion may be modulated by cholesterol. Its selective removal from membranes, selective sequestering within membranes or enzymatic modification causes a significant inhibition of the extent, Ca^{2+} sensitivity and kinetics of fusion of cortical vesicles isolated from sea urchin eggs [110]. Cholesterol may stimulate fusion by way of is negative intrinsic curvature; alternatively, it may also be related to raft formation at fusion sites since depletion of sphingomyelin has a similar effect [111]. In SNARE-dependent insulin exocytosis, opening of a fusion pore was actually preceded by unrestricted lateral diffusion of lipids. This is a rare example where lipid mixing can be observed prior to formation of an aqueous continuity [112]. It is no surprise to see that neurotransmitter release is also lipid dependent. Snake venom phospholipase A2 hydrolyses phospholipids to lysolipids and fatty acids; addition of these products stimulates exocytosis at the neuromuscular junction and thus mimics the effect of the toxin. Again, the action of these agents is explained by their effects on membrane curvature [113].

A natural fusion pore may also require TMDs. For example, a set of residues along the same face of the syntaxin TMD helix appears to determine neurotransmitter flux and pore conductance [114]. Although these results suggest that the syntaxin TMD lines the fusion pore, the sensitive side chains may not be polar enough to allow passage of solutes, and their volumes are not conserved in evolution [115]. In any case, one may wonder whether the R-SNARE TMD would also contribute to pore formation and, if so, whether this TMD would form a half-channel within the vesicular bilayer that connects to a Q-SNARE TMD half-channel (Fig. 1b). There is indeed indirect evidence for a contribution of the R-SNARE TMD to the pore as the kinetics of pore expansion has been found to depend on whether exocytosis is mediated by synaptobrevin or its orthologue cellubrevin whose TMDs are not conserved [116]. It has been suggested that dilation of a predominantly proteinaceous fusion pore would require dissociation of the TMD lining with concomitant lipid intercalation. This does not necessarily need to be so. Rather, a fusion pore may be partially composed of TMDs and of lipid (Fig. 1b). Even a purely proteinaceous pore may require lateral opening at only one site for lipid mixing to proceed. Fusion pores in neurotransmitter release do not irreversibly lead to complete fusion. Rather, the 'kiss-and-run" mode may be favored [117] depending on the intracellular Ca^{2+} concentration [118], the subtype of the Ca^{2+} sensor synaptotagmin [119], or other factors that determine the release probability of the nerve terminal [120]. In the kiss-and-run mode, the vesicles may remain tightly docked or even hemifused to the plasma membrane as suggested by recent *in vitro* data [121].

The formation of fusion pores during intracellular fusion has also been ascribed to non-SNARE membrane proteins such as the V0 subunit of the ATPase [122] and the associated Vtc proteins [123]. It is likely that these proteins support the organization of lipids and SNAREs at the fusion site. In favor of this idea, lipid and content mixing is deficient for vacuoles lacking the V0 subunit Vph1p or Vac8p [70].

Transmembrane domains in the hemifusion-to-fusion transition

Fusion pore dilation is thought to initiate the transition from the hemifused to the fully fused state of the membrane. As outlined above, replacing the proteinaceous TMDs of various viral fusion proteins or of SNAREs by lipid anchors or altering their primary structures leads to partial or full arrest of the fusion reaction at hemifusion [31, 63–67, 75, 105, 124]. In addition to revealing the existence of a hemifused state, these results also demonstrate a crucial role of the TMDs in the transition from hemifusion to complete bilayer mixing.

To date it is not clear how a TMD may accomplish this

transition. Nevertheless, a recent study might shed light on the issue. In this study, the fusogenic function of a synthetic peptide mimicking the vacuolar Q-SNARE Vam3p TMD was examined in the liposome system. Interestingly, the extent to which the TMD elicited complete bilayer mixing *versus* hemifusion non-linearly increased with the surface density of the helical peptide. Thus, TMD helices appear to cooperate in the hemifusion-to-fusion transition. Further, the fraction of hemifused membranes decreased with reaction time, which qualifies hemifusion as an on-pathway intermediate. Interestingly, introducing multiple mutations on one, but not on the other, face of the helix significantly favored hemifusion, while covalent dimerization of the mutant TMD restored complete fusion to the level seen with the wild type. Therefore, TMD-TMD interaction via the mutated residues appears to support the hemifusion-to-fusion transition. When tested in the context of full-length Vam3p, the crucial TMD mutation also reduced mixing of contents of yeast vacuoles [87]. Moreover, the same mutation or TMD deletion abolished non-covalent homodimerization of recombinant Vam3p but not quaternary complex formation with partner SNAREs [125]. In sum, these findings suggest a role of homotypic lateral TMD-TMD interactions in inner leaflet mixing.

Indeed, TMDs have previously been demonstrated to mediate SNARE/SNARE interactions. The synaptic R-SNARE synaptobrevin II and the Q-SNARE syntaxin 1A homo- and heterodimerize in SDS micelles and in a natural membrane through a conserved motif within their respective TMDs [126–129]. The synaptobrevin motif forms a tightly packed interface as indicated by molecular modeling. Efficient packing of these residues requires that the helices cross each other at a negative angle [126, 130]. Although the synaptobrevin TMD-TMD interactions have been proposed to be of very low affinity [131], they are significant under carefully controlled experimental conditions [129]. A fusion protein of *Staphylococcus aureus* nuclease A and the syntaxin 1A TMD homodimerizes with a free energy of –3.5 kcal mol^{-1} by analytical ultracentrifugation. In contrast, homodimerization of an analogous synaptobrevin TMD construct could not be detected under these conditions unless its interaction motif [128] was rendered fully identical to the one of syntaxin 1A by a single mutation [132]. The low synaptobrevin affinity may be due to steric hindrance of the TMD by the nuclease A domain as indicated by a previous study [126]. Evidence for SNARE TMD-TMD interaction has also been obtained by electron paramagnetic resonance spectroscopy of membrane-reconstituted spin-labeled proteins. Accordingly, alternate surfaces of the yeast exocytotic R-SNARE Snc2p TMD helix may interact with partner TMDs [31], while residues that mediate tertiary contacts of the Q-SNARE Sso1p TMD are confined to one face within its N-terminal half. Sso1p exists at a monomer/oligomer equilibrium of approximately 6:4 at a nominal protein-to-lipid ratio of 1:300 [33].

How can protein-protein interaction in the membrane facilitate the hemifusion-to-fusion transition? It has been argued that a restricted hemifusion diaphragm is delimited by a ring of fusion protein TMDs at its perimeter [13]. This ring could form if individual oligomeric proteins assemble to supramolecular multimers. Evidence for SNARE multimers containing from 3 to 15 complexes has indeed been obtained [30] based on cooperative SNARE action in exocytosis [133], their inhibition by botulinum neurotoxins [134] and electron microscopy of purified proteins [135]. Likewise, viral fusion proteins multimerize as indicated by the cooperative function of influenza hemagglutinin [136] and by aggregation of baculovirus glycoprotein 64 [137] or Semliki Forest virus E1 fusion trimers [138]. Multimerization may be supported by lateral interactions between the TMDs [30]. Currently, any mechanistic model that tries to connect TMD-TMD interaction to bilayer mixing is bound to be speculative. In one scenario, homo- and/or heterotypic TMD-TMD interactions may support multimerization of quaternary SNARE complexes to the proposed ring-like structures. Alternatively, the quaternary complexes may be laterally connected by SNARE homodimers. Homodimeric forms of synaptobrevin have indeed been detected in neuronal membranes [129, 139–142] and a Vam3p homodimer is present in detergent extracts of yeast vacuoles [125]. Failure of mutant proteins to assemble a stable ring may cause arrest at an apparently non-restricted, readily detectable hemifused state. In yet another scenario, the TMDs of R- and Q-SNAREs from apposed membranes may enter heterotypic interactions [127, 128] subsequent to the zippering up of the SNARE domains [45, 46, 143]. A similar situation may pertain to class I viral fusion proteins. It has been postulated that the amphipathic fusion peptide of influenza hemagglutinin interacts with its TMD late in fusion since certain mutations within the fusion peptide produce a hemifusion phenotype [144].

Recent results have added yet another layer of complexity to the hemifusion-to-fusion-transition. SNARE-mediated fusion is clamped in the presence of complexin [121, 145, 146]. Complexin is a small protein that stabilizes the synaptic SNARE complex by binding into a groove of the cytoplasmic coiled coil [9]. The fusion block is released in the presence of Ca^{2+}/synaptotagmin. This explains the strict Ca^{2+}

dependence of fast neurotransmitter release since the SNAREs can be tightly controlled by complexin and synaptotagmin. It is presently not clear whether similar clamps exist for other SNARE-mediated fusion reactions since complexins are restricted to neurons. Possibly, SNAREs that are not clamped by complexin drive fusion in a constitutive fashion [146]. Interestingly, complexin clamps the fusion reaction at the hemifusion intermediate [121]. The transition to complete fusion can therefore be regulated by manipulating the cytoplasmic SNARE domains. It will be interesting to unravel how complexin and TMDs collaborate in regulating lateral SNARE-SNARE interactions at the fusion site.

Conclusion and perspective

Understanding the heart of membrane fusion is intrinsically related to the question how proteins and lipids communicate in space and time. We have outlined that fusion protein ectodomains set the stage for fusion. To what extent these soluble domains suffice to initiate lipid mixing may depend on the efficiency by which they juxtapose or disturb the membrane. TMDs actively participate in outer and inner leaflet mixing. This function may be related to TMD/lipid interactions via conserved amino acid motifs and/or via TMD conformational flexibility. Further, TMD-TMD interactions and multimerization of oligomeric complexes may fence off a hemifusion diaphragm and affect bilayer tension. The efficiency of pore formation and inner leaflet mixing may depend on the stability of this fence. If membrane defects leading to fusion pores develop at the TMD-lipid interface, pore function would depend on protein as well as on lipids. The future will tell whether the fusion reaction moves along a well-defined pathway or, rather, whether it comprises a variety of structurally different intermediates.

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