Membrane fusion

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Fusion of biological membranes is governed by physical principles but it is unclear whether the transition states are primarily determined by lipid physics or by protein–lipid interactions. Recent advances in the field include the physical description of bilayer fusion, particularly new models beyond continuum models and the role of the SNARE proteins. Despite substantial progress, an integrated concept for protein-mediated membrane fusion is not yet available, and many open questions remain to be answered.

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Abbreviation
SNARE soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor

Introduction

Membrane fusion is essential for the life of eukaryotic cells. Intracellular fusion reactions are complex supramolecular events involving thousands of molecules. Research on fusion has been intense in recent years, but we are still far from a coherent molecular picture. New proteins are constantly being discovered, and their roles need to be defined. Less noticed by cell biologists, substantial progress has also been made in bilayer physics, in particular from coarse grained and atomistic simulations. In the following review, we will discuss recent progress in membrane fusion, with an attempt to integrate advances in the study of intracellular fusion events and of bilayer physics. We will also compare models for protein-mediated membrane fusion and discuss the role of SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) proteins in intracellular fusion reactions. The reader is referred elsewhere for recent discussions of docking and priming pathways and of viral fusion proteins [1–4].

Physics of bilayer fusion

Formation and fusion of bilayers is governed by the hydrophobic effect [5] that causes forces minimising solvent-exposed non-polar surfaces. Application of this concept to fusion intermediates suggests that fusion proceeds via formation of a stalk (Figure 1). According to the stalk hypothesis, fusion of pure lipid membranes requires at least five distinct steps: approach to small distances; local perturbation of the lipid structure and merger of proximal monolayers; stalk formation; stalk expansion, which in some variants of the stalk model is associated with a hemifusion diaphragm; and, finally, pore formation. For fusion to proceed, each of these steps needs to be driven by an energy gradient towards lower energies. Consecutive steps may be separated from each other by energy barriers. According to the Arrhenius law, the measured rates limit the height of these barriers to approximately 40 k_BT [6•]. The stalk hypothesis rests mainly on the observation that the merger of the proximal monolayers precedes the merger of the distal monolayers, which, in turn, precedes the exchange of intravesicular solvent, and on the effects of certain lipids on fusion rates. One of the challenges is to explain the low barrier height in light of the large membrane curvatures involved [6•,7•]. Another is to understand the energetics of the individual fusion steps and, in particular, the physical origin of the driving forces.

The physics of bilayer fusion is severely complicated by the involvement of a broad range of length and time scales. Whereas the stability of vesicles can be understood from continuum models, the creation of the fusion pore occurs within a volume of a few nanometers (i.e. at atomic scales) and is generally assumed to proceed much faster than microseconds. Thus, for the critical transition steps during fusion, the finite size and the thermal fluctuations of the lipid molecules may be relevant, in which case simple continuum models would be insufficient. Such atomistic effects have actually been observed for the formation of water nanodroplets [8].

Continuum models

Continuum models treat the lipid membrane as a homogeneous elastic surface. This approach has been successful in explaining the large variety of shapes that vesicles can adopt under varying conditions [9]. It has therefore been extended to describe bilayer fusion, in particular stalk formation. One problem, however, is that stalk formation requires the transient formation of large curvatures, and the saliently assumed linear and laterally homogeneous elastic constant leads to the prediction of unrealistically high activation energies for fusion. Furthermore, bending is supposed not to change the lipid composition.

The view that elastic models may be appropriate for describing the stalk is supported by the fact that they also provide a semi-quantitative description of the cubic phase, in which the bilayer is folded in a complicated way to form a three-dimensional, periodic lattice. However, in the cubic phase the average curvature is zero since the positive and negative contributions cancel. This is not the case for the fusion intermediates, where the average curvature for the distal monolayers is necessarily positive and large. Accordingly, continuum models would predict energy barriers of several hundred k_BT. Recent work has therefore

aimed at suitable extensions that reduced the activation energy considerably, for example bending or tilting of the lipid molecules [7•], rendering such effects relevant for fusion. Finally, continuum models depend on the *assumption* of fusion pathways rather than *predicting* them. Many fusion pathways studied so far within the framework of continuum models contain singularities (e.g. infinite curvatures) typically associated with membrane surface contact. These singularities are unphysical, and they create mathematical difficulties.

Coarse grained models

Coarse grained models aim at including the effects that arise from the finite size of the lipid molecules without spending unnecessary effort on the description of irrelevant atomic detail. Typically, the lipids are described as chains of beads, each of which represents a few atoms; for example, the polar lipid head. Subsequently, the collective motion of the beads under the influence of carefully tuned hydrophobic and hydrophilic interaction forces is computed numerically. Ideally, but not yet routinely, the parameterisation of interaction forces is derived from full atomistic detail [10,11], such that most studies cannot claim the predictive power of atomistic studies (see below). Despite this limitation, coarse grained simulations of spontaneous bilayer self-assembly [12], micelle self-assembly [13,14], membrane rupture [15] and fusion pore formation [16•,17] have significantly advanced our understanding of microscopic membrane dynamics. In particular, the latter studies are consistent with the stalk hypothesis insofar as the pore opening is induced by contact between the distal monolayers; but they cast some doubt on models involving pronounced hemifusion states.

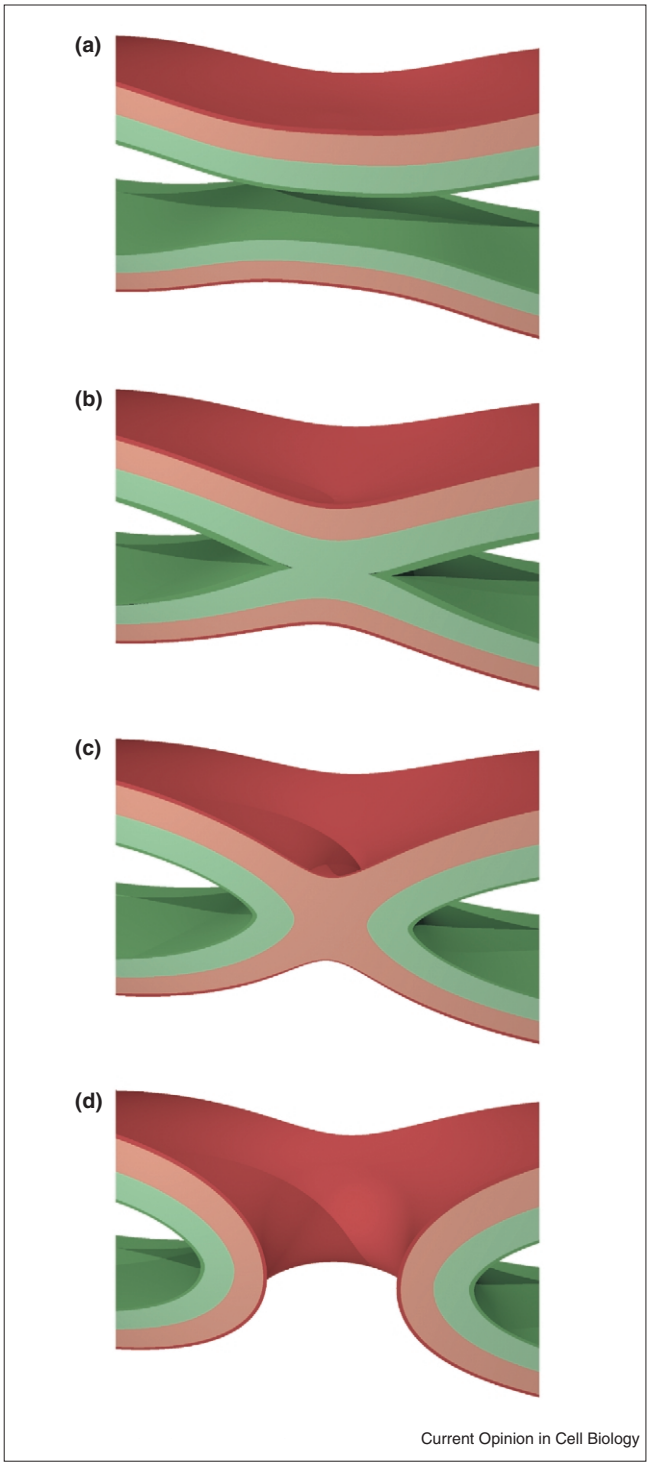
Inspection of snapshots from these fusion simulations (Figure 2) intuitively suggests that the fusion intermediate is more disordered and more complex than saliently assumed by contemporary continuum models.

Atomistic models

Atomistic models are derived from molecular dynamics simulations in which the forces and motions of *all* involved atoms are computed individually and in full detail. Therefore, these simulations generally rest on fewer assumptions compared with continuum or coarse grained models. Accordingly, they have recently been termed 'reality simulations' [18]. A sufficiently accurate description of the interatomic forces is essential, defined by the employed force field, which governs the dynamics of the lipid and water molecules. For a discussion of caveats related to these limitations, see Takaoka *et al.* (2000) [19].

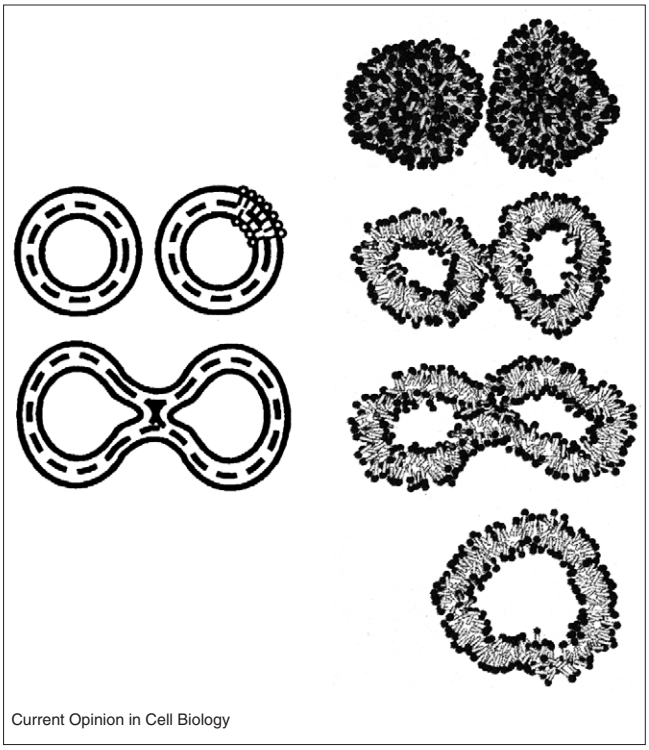
Lipid force fields have matured considerably in recent years, and they are now considered to be sufficiently accurate for the description of membrane dynamics. Insofar as these force fields are tested against experiments unrelated to membrane fusion, and are typically not changed in subsequent simulations, these atomistic simulations can be considered *first principles*. Thus, the predictive power of atomistic simulations is stronger than that of continuum models. Several reviews cover the period before 2000 [20–22]. More recent examples are the

Figure 1



Membrane fusion according to the stalk hypothesis. **(a)** Approach to small distances. **(b)** Local perturbation of the lipid structure and merger of proximal monolayers. **(c)** Stalk formation. **(d)** Stalk expansion and pore formation.

Figure 2



Recent simulations using coarse grained lipid models support the stalk hypothesis. However, rather than smoothly bent monolayers (left), a complex pattern of lipid orientations is seen for the fusion intermediate (right). Modified with permission from [16•].

successful calculation of the bulk modulus of a dimyristoyl phosphatidylcholine bilayer [23], the study of hydration effects [24] and the cubic phase [25], as well as the effects of sterols [26], of unsaturated fatty acids [27] and of peptides such as melittin [28].

The development of atomistic fusion models, derived from molecular dynamics simulations, is mainly hampered by the huge computational effort that these simulations imply, typically months of computer time. Recent technical and algorithmic advances have been considerable, however; and bilayer patches of up to 20 nanometers, requiring simulation systems of around 400,000 atoms, can now be simulated for several dozens of nanoseconds [29•,30]. These advances are, in fact, an essential step towards the atomistic simulation of fusion events. For example, at these length and time scales, membrane undulations become visible [29•]. These undulations probably contribute to overcoming the first fusion barrier, as do initial lipid distortions seen in a simulated approach of the proximal monolayers [31]. Most importantly, also at these time scales major conformational motions of membranes take place, as recently demonstrated in a landmark simulation of spontaneous bilayer formation from initially randomly distributed lipid molecules [32•]. In this simulation, a transmembrane water pore formed, the closure of which took about 15 ns, which turned out to be the rate-limiting step for the formation of an intact bilayer. Although the relationship of this pore to fusion intermediates is unclear, and although possibly relevant millisecond diffusive processes cannot be described by atomistic simulations at present, this result is encouraging and raises the hope that in the near future simulations of primary fusion dynamics steps will be achieved.

Protein-mediated membrane fusion

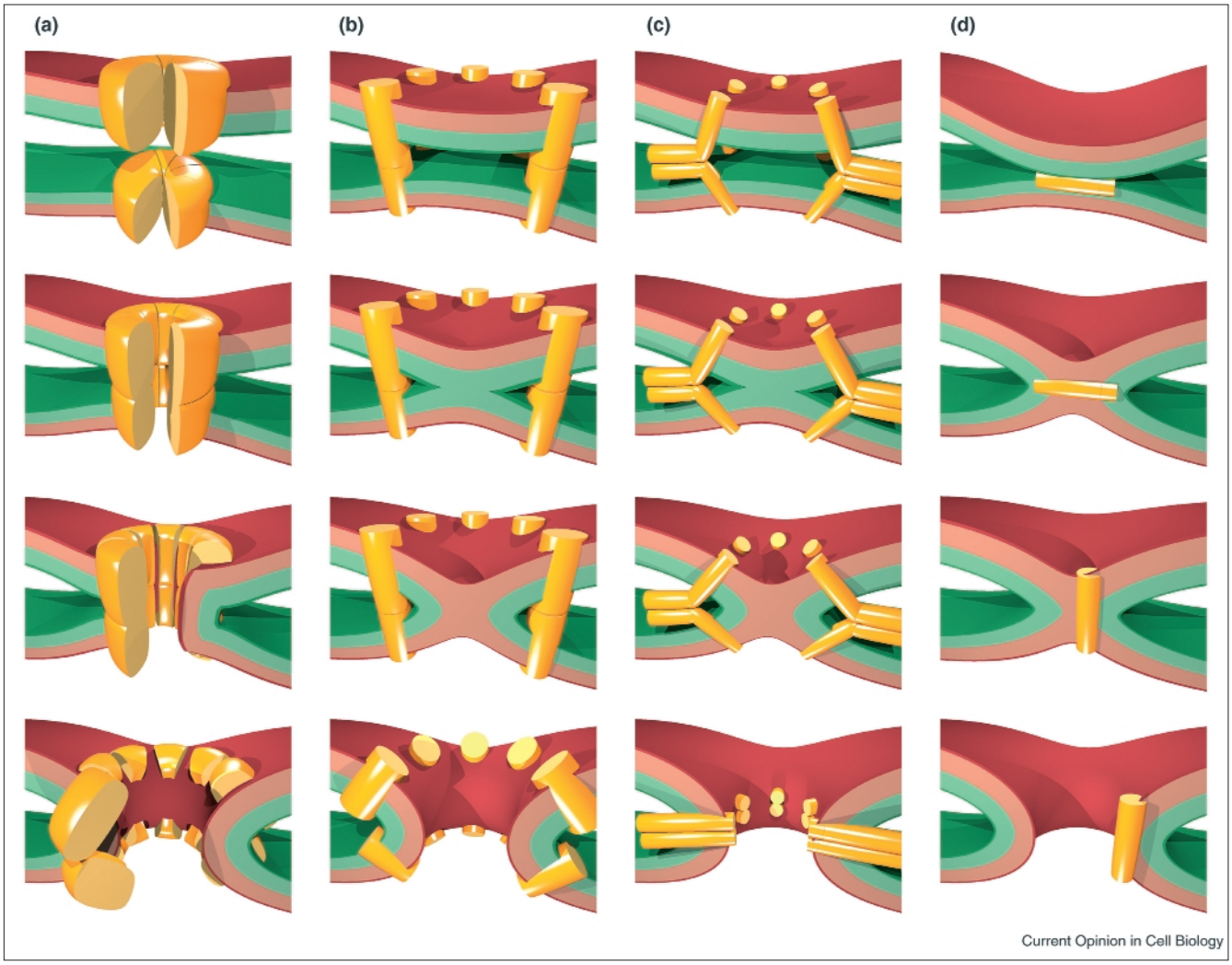
Fusion of biological membranes is more complex than that of lipidic bilayers, in that it requires specific proteins. As discussed above, the energy barrier for bilayer fusion in an aqueous environment is not very high and may be surpassed by local fluctuations or perturbations. Furthermore, it is not clear whether all fusion reactions involve similar intermediates (e.g. as suggested by the stalk hypothesis for protein-free membranes) or whether they exhibit greater diversity. Several models for protein-mediated fusion have been proposed that differ in the transition states, the interactions of the fusion proteins with membrane lipids, and the significance of conformational changes for the fusion pathway (Figure 3).

Proteinaceous fusion pore models

Proteinaceous fusion pores [33] are supposed to have gap junction (connexon)-like structures; that is, they are thought to consist of oligomeric transmembrane proteins with a hydrophilic channel in the middle that opens at the onset of fusion (Figure 3a). Proteinaceous fusion pores were originally proposed to explain the ion-channel-like flickering of fusion pores [34], and they have recently gained popularity among neuroscientists as an explanation for reversible fusion ('kiss-and-run' exocytosis) [35]. Formation of a proteinaceous fusion pore at the onset of fusion would also explain why in many fusion reactions an aqueous connection forms before lipids can diffuse from one membrane to the other [34].

How would fusion proceed according to this model (Figure 3a)? First, the subunits need to assemble into ring-like oligomers. Next, upon membrane contact '*trans*' complexes form via homophilic or heterophilic interactions involving the cytoplasmic faces of the proteins, followed by the opening of a central aqueous pore. For pore enlargement, the subunits (still attached in the *trans* configuration) need to dissociate radially, with phospholipids invading the space between them. This state requires polar lipid head groups to migrate alongside transmembrane regions. Alternatively, the ring may open at one side, allowing phospholipids to invade and a lipidic pore to 'grow' out of the central channel. Finally, the *trans* connections between the protein subunits need to break up to allow fusion to be completed. The last steps of this sequence are intuitively difficult to reconcile with basic biophysical principles, and a comprehensive physical description of proteinaceous fusion pore models is therefore urgently needed.

Figure 3



Schematic overview of four models for protein-mediated membrane fusion. **(a)** Proteinaceous fusion pores are assumed to form gap-junction-like oligomeric transmembrane complexes with a hydrophilic channel in the middle that opens at the onset of fusion. Subsequently, the subunits dissociate radially, with lipids invading the space in between. Fusion is completed by breaking the *trans* connections. **(b)** In fence models, fusion is essentially lipid-based, with 'fence posts' forming a ring restricting lipid flow. After formation of the fusion pore, the '*trans*' complexes dissociate. **(c)** In scaffold models, fusion is also essentially lipid-based but the role of the proteins is restricted in order to bring the membranes into contact, possibly by exerting mechanical forces and thereby helping to overcome the activation energy barrier. Note that this model does not require the proteins to have transmembrane domains. **(d)** Many amphiphilic peptides can induce membrane fusion, but the mechanism is still unclear. Possibly, such peptides cause local perturbation of the bilayer that lowers the activation energy barrier. Peptide-induced fusions are often leaky, and thus the intermediate states may be less ordered.

Recently, the V_o subunit of the vacuolar proton ATPase has been proposed to function as a proteinaceous fusion pore that is activated by calmodulin in a calcium-dependent manner [36]. The V_o subunit is composed of proteolipids that form transmembrane channels and that are structurally related to the F_o subunit of the mitochondrial ATP synthase. Peters *et al.* [36] observed that complexes form between V_o subunits derived from different sets of vacuoles at a late stage of the fusion reaction. While the data are intriguing, the conclusions are controversial. Conceptually it is difficult to envision how one and the same protein performs two radically different functions in the same organelle (i.e. a membrane channel for proton pumping, and fusion).

Fence models

Fence models (Figure 3b) also require the formation of oligomeric rings of transmembrane proteins at the onset of fusion, but they differ from proteinaceous pore models in that in the centre of the ring there is a patch of lipids. Fusion is essentially lipid-based, and thus there is no *a priori* need for corresponding protein rings in the two opposing membranes. The transmembrane segments surrounding the lipid patch may only loosely interact with

each other, representing individual 'fence posts' that are surrounded by bound phospholipid collars. Fences would restrict lipid flow between the membranes at the onset of fusion. Such fences have been discussed for several viral fusion proteins.

Scaffold models

The original version of the scaffold model (see Figure 3c) was proposed by Fernandez and co-workers (for an overview, see [37]) who found that flickering of fusion pores, until then considered as a hallmark of protein-mediated fusion, also occurs during fusion of protein-free bilayers. In this model, the role of the proteins is confined to bringing the membranes into close apposition, perhaps by exerting mechanical force, as suggested for the SNARE proteins ([38]; see also below), to overcome the activation energy barrier. In its extreme version, the scaffold proteins do not participate in the transition states that are considered to be exclusively lipidic. Furthermore, the model does not require (but also does not preclude) the scaffold proteins to span the membrane.

Local perturbation models

It is often overlooked by cell biologists that many amphiphilic peptides, once added to liposomes in micromolar concentrations, cause the spontaneous fusion of vesicles at ambient temperature, with a speed and efficiency that is orders of magnitude higher than, for instance, that of liposomes reconstituted with SNARE proteins [39,40]. Such amphiphilic peptides are part of many viral fusion proteins that insert them into the target membrane upon activation. The peptides may undergo structural changes upon membrane insertion [41]. Fusion is equally efficient if the fusion peptides are immobilized on the surface of only one of the fusion partners [42].

Peptide-induced membrane fusion (Figure 3d) highlights that the energy landscape of the fusion reaction is relatively flat [43]. Although fusion peptides have conformational constraints, they do not appear to be very rigorous. Furthermore, many amphiphilic proteins (including the chaperone ATPases NSF and p97 [44], and annexins [45]) are capable of fusing liposomes, and in some instances also biological membranes *in vitro*. While the role of such perturbations in biological fusion events remains to be established, these features show that fusion itself is not a very 'specific' reaction, as long as the phospholipid bilayers are sufficiently close to each other. Perhaps this explains why different classes of fusion proteins evolved, and it may also explain why most components of the complex machinery mediating intracellular vesicle fusion are involved in controlling the distance ('docking') between the membranes destined to fuse, rather than carrying out fusion itself.

Eukaryotic fusion proteins: SNARES as an example

Eukaryotic cells possess different classes of fusion proteins for specific fusion reactions. Extracellular fusions such as sperm–egg fusion appear to be carried out by proteins resembling viral fusion proteins [46] but so far only little is known about proteins involved in cell–cell fusion (syncytia formation). Within the cell, both mitochondria and peroxisomes appear to possess their own set of fusion proteins [47–49], although it is not known which of them carries out the fusion reaction. In the secretory pathway, a multitude of proteins are known to be required for docking and fusion, and several partial reactions are characterized. The SNAREs are presently the best candidates for universal fusion mediators in the secretory pathway, although it is still controversial whether they catalyse fusion or whether membrane merger is mediated by other factors. In the following paragraphs, we will focus on recent progress in our understanding of SNARE proteins.

SNAREs comprise a superfamily of small, mostly membrane-anchored proteins that share a motif of 60–70 amino acids, referred to as the 'SNARE' motif. SNARE proteins have been studied intensively during the past few years, and the reader is referred to recent reviews for a comprehensive overview [50–52]. SNAREs undergo an assembly/disassembly cycle that is crucial for their function. During assembly, four SNARE motifs form an extended, coiled-coil-like helical bundle, with the membrane anchor domains extending at one end of the complex [53,54•]. In the centre of the bundle, the helices are connected by layers of hydrophobic amino acids, with the exception of a hydrophilic central layer. This layer is formed by three conserved glutamines (Q) and one conserved arginine (R), leading to the classification into Q- and R-SNAREs, respectively [53,54•]. Assembly is spontaneous and irreversible [55]. Disassembly requires the concerted action of the evolutionarily conserved proteins αSNAP and the ATPase NSF [56].

Since fusion requires complementary sets of SNAREs to be present in both membranes, assembly into '*trans*' complexes would tie the membranes closely together and may cause fusion [1,38], with fusion proceeding largely as described by the scaffold model (Figure 3c). Indeed, proteoliposomes reconstituted with SNAREs fuse with each other, albeit very slowly [57], or with native vesicles containing corresponding SNAREs [58•]. Furthermore, as predicted, fusion is inhibited or reduced when the transmembrane domains are replaced with lipid anchors *in vivo* [59] or *in vitro* [60], or when additional flexible linkers are introduced [61]. Interestingly, even the transmembrane domains alone are capable of fusing liposomes when inserted into membranes [62•]. In cracked PC12 cells (i.e. that have been ripped open to gain access to the cytoplasm), soluble SNAREs compete with or substitute for endogenous SNAREs, with the effects of mutations and substitutions precisely in agreement with the model [51].

While most scientists agree that formation of *trans*-SNARE complexes is essential for fusion, it is debated whether SNAREs are indeed required for membrane merger or whether their role is confined to setting up the fusion site, with fusion being executed by other proteins. Support for

the latter view stems mainly from studies on the fusion of yeast vacuoles [63]. Several proteins have been proposed to operate downstream of the SNAREs, including protein phosphatase 1 [64], calmodulin [65], the V_o subunit of the vacuolar ATPase (see above) [36], and Vac8p, an armadillo-repeat protein [66,67]. In all cases, the evidence for a role downstream of SNAREs critically depends on the ability to reliably measure *trans*-SNARE complexes, and to arrest the reaction sequence leading up to fusion with stage-specific inhibitors. Unfortunately, so far the only assay for measuring SNARE complexes is by immunoprecipitation from detergent extracts, which principally cannot distinguish between *cis* and *trans* configuration.

In yeast, this problem is circumvented by using vacuoles from yeast strains lacking one each of the complementary SNAREs. Thus, the precipitated complexes can only be derived from an interaction between different vacuole populations [68,69]. Recent evidence suggests, however, that additional SNARE proteins might be involved [70] that were not measured in these studies. Similarly, formation of fully assembled core complexes has been postulated to precede exocytosis in isolated nerve terminals [71]. However, studies on exocytosis in chromaffin cells have suggested that the formation of *trans* complexes is rapidly reversible until fusion occurs [72]. Low stability of *trans* complexes is also suggested by competition experiments with soluble SNAREs (reviewed in [51]), questioning whether fusion-relevant *trans* complexes can be reliably quantitated after detergent treatment.

The view that SNAREs execute fusion is also challenged by observations showing that some SNAREs (mostly R-SNAREs) appear to be expendable for fusion. For instance, in neurons deletion of the R-SNARE synaptobrevin/VAMP or of the Q-SNARE SNAP-25 abolishes calcium-dependent transmitter release but does not block spontaneous exocytosis [73,74•,75•]. Similarly, several yeast R-SNAREs (including Sec22p and Snc1/2p) are not essential for the respective fusion steps (for a review, see [52]). In none of these studies, however, can it be ruled out that another R-SNARE substitutes for the damaged or missing one, particularly when considering that R-SNAREs can be more easily replaced by other R-SNAREs than corresponding sets of Q-SNAREs [51].

In summary, the SNAREs remain the best candidates for carrying out the fusion reaction, but it would be premature to count out alternative proposals.

Concluding remarks

Despite progress in both the physics of bilayer fusion as well as the characterization of proteins involved in the fusion of biological membranes, we are still only beginning to understand the fusion event. For protein-free fusion, simulations have recently provided support for the stalk hypothesis. Intracellular fusion reactions are apparently carried out by supramolecular machines that temporarily recruit additional proteins for specific tasks. However, fusing bilayers in the test tube requires much less specificity than, for example, the import of a protein into the ER, or the splicing of a precursor mRNA, complicating the validation of *in vitro* fusion assays. Furthermore, linear reaction sequences, worked out under defined *in vitro* conditions, may not accurately represent the intracellular process that involves interdependent networks of protein–protein and protein–lipid interactions operating in parallel.

What is needed are more refined assays in which partial reactions can be studied with high resolution, and simpler model systems that are accessible to modern physical techniques and that ultimately may be accessible to atomistic simulations.

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