In Vivo Analysis of Membrane Fusion

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Membranes provide a barrier that allows chemical reactions to be isolated from the environment. The plasma membrane, for example, delineates self from nonself, and thus must have played an essential role in the evolution of life. Yet under numerous circumstances it is equally important that membranes be breached. Numerous forces oppose the spontaneous fusion of membranes; thus, specialized proteins have evolved to fuse membranes. The most well-understood fusion proteins are the viral fusion proteins and the SNARE proteins used in the secretory pathway. In addition, recent discoveries have lead to models for the fusion of organelles such as mitochondria and peroxisomes, as well as for cell–cell fusion. Despite the diverse structures of fusion proteins, it is possible that they function to drive membranes through a series of common lipid intermediates. Here we review the mechanisms of fusion for biological membranes, and highlight the similarities and differences in these processes.

Introduction

The cells of living organisms are defined by membranes. This flexible, yet impenetrable, barrier isolates chemical reactions from the external environment. It is important that these membranes exist, yet under numerous circumstances it is equally important that membranes be breached. Controlling membrane fusion is an essential process for everything from a virus entering a cell to the functions of a synapse in the brain. Several forces contribute to prevent membranes from spontaneously fusing. These forces include: hydrophobic effects that seek to minimize solvent-exposed surfaces, elastic forces that serve to resist strong monolayer deformation and the high repulsive forces generated by negatively charged phospholipids 1–2 nm apart (Chernomordik and Kozlov, 2003; Cohen and Melikyan, 2004). Overcoming these forces requires specialized proteins that drive membrane fusion. This article covers the known proteins involved in this process with a focus on *in vivo* analyses of their functions. We highlight similarities and differences among the various fusion reactions. **See also**: Cell Membrane Features; Lipid Bilayers

By far the two best-understood membrane fusion reactions are those of viruses and those of the secretory pathway. For each of these reactions the specialized proteins mediating membrane fusion, the fusogens, are known. The specialized proteins used by viruses as well those in the secretory pathway meet the criteria of 'necessity' and 'sufficiency': In their absence fusion is eliminated and they can induce fusion in reconstituted systems. We present the current models for how proteins mediate the lipid rearrangements required to merge membranes during viral entry and secretory vesicle exocytosis. **See also**: Flaviviruses; Influenza Viruses; Synaptic Vesicle Fusion

Other fusion reactions are less understood. Interestingly, it appears that cell–cell fusion might be catalysed by a variety of unrelated proteins rather than the related proteins that are used throughout the secretory pathway. But the identities of the molecules mediating cell–cell fusion are in considerable dispute and a description of their mechanism of action is currently lacking. Finally, we discuss the fusion of organelles such as mitochondria and peroxisomes which are separate from the secretory pathway.

Common Lipid Intermediates?

Fusing membranes require dramatic rearrangements of lipids. However, this process must also be carefully controlled. Two lipid bilayers must merge into one while

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maintaining a degree of integrity such that the membrane-enclosed contents are not lost.

It is likely that all membrane fusion reactions pass through common lipid intermediates. The intermediates in membrane fusion were initially proposed based on mathematical modelling of pure lipids (Kozlov and Markin, 1983). According to the model, the initial step is the formation of a lipid stalk between the proximal leaves of the lipid bilayers (**Figure 1**). This structure may contain as few as a dozen lipid molecules assembled in an hourglass-like structure (Yang and Huang, 2002). The lipid stalk then expands to form a hemifusion diaphragm where the previously distal leafs of the lipid bilayer are now in contact. The rupture of this hemifusion diaphragm opens the initial fusion pore. Expansion of the pore completes the fusion of the membranes and the contents of the compartments mix. However, the opening of the fusion pore is reversible and does not always lead to full fusion. Perhaps surprisingly, mathematical modelling suggests that each of these steps requires an increasing demand for energy (Cohen and Melikyan, 2004). The requirement for energy at all steps suggests that proteins must operate from initial membrane contact through full membrane merger. In later sections, we shall see that this has been shown for the fusogens of viruses and the fusogens of the secretory pathway.

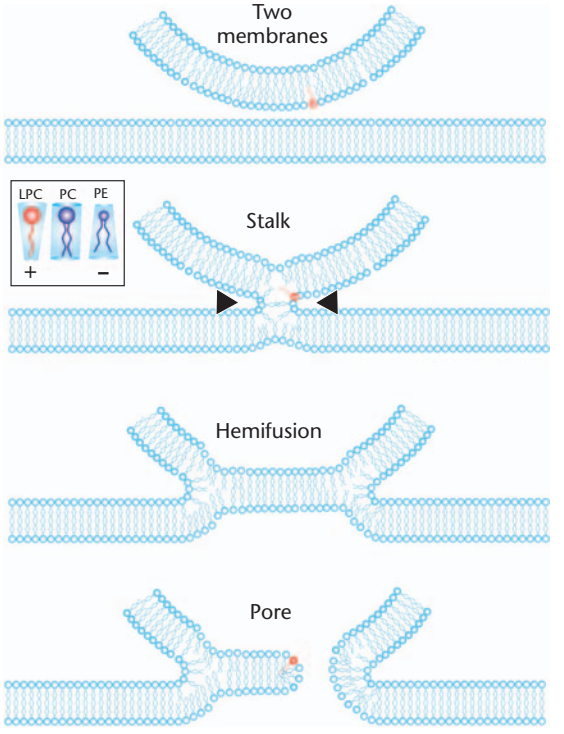

Figure 1 Common lipid intermediates during fusion. The proximal leaflets of the plasma bilayers are brought into close proximity forming the hourglass-like structure known as a lipid stalk. This stalk is then expanded forming a hemifusion diaphragm. In the hemifusion diaphragm the distal leaflets of the bilayer are now in direct contact. A rupture in the hemifusion diaphragm leads to the initial opening of a fusion pore that is then expanded leading to full fusion. The insert shows the shape of lysophosphatidylcholine (LPC), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). LPC induces positive curvature whereas PE induces negative curvature. Adding lipids with positive curvature to the proximal leaflets inhibits stalk formation (red lipid – 'stalk' and 'two membranes' steps). Adding lipids with positive curvature to the distal leaflets promotes fusion (red lipid – 'pore step'). Arrowheads point to areas where the lipid must adopt a net negative curvature.

The hemifusion model for fusion has been experimentally validated in viral fusion (Chernomordik *et al.*, 1998; Kemble *et al.*, 1994; Razinkov *et al.*, 1998). Considerable evidence points to hemifusion as an intermediate in the fusion reactions in the secretory pathway (Grote *et al.*, 2000; Lu *et al.*, 2005; Reese *et al.*, 2005; Wong *et al.*, 2007; Xu *et al.*, 2005; Yoon *et al.*, 2006). Recently, experiments have hinted that the same lipid intermediates might also be used in cell–cell fusion (Podbilewicz *et al.*, 2006). The details of these experiments will be described in the respective sections later.

Viral Fusion

To be propagated, enveloped viruses must fuse their membranes with the membrane of the host. Fusion releases the virus genome into the cytoplasm and initiates a new cycle of viral replication. Unlike all other known membrane fusion reactions, viral fusion is unique in that the process is not cooperative – the fusion proteins are located only on the virus itself rather than on both membranes destined to fuse. The fusion proteins are transmembrane glycoproteins (**Figure 2**). In this section we explain the common structural rearrangements that all viral proteins undertake during fusion – this despite three unique classes of fusion proteins. We then present the evidence that all viral fusion proteins are likely to mediate fusion through the common lipid intermediates described in the previous section. Finally, we will see that viral fusion involves multimerization of the fusion proteins – a trait in common in the fusion proteins of the secretory pathway.

Although the sequences of viral fusion proteins can vary considerably, they share several common characteristics. The fusion proteins are first activated. Depending on the virus the trigger for activation can be either receptor binding for viruses fusing at the cell surface, or low pH for viruses fusing along the endosomal pathway (**Figure 2a**). Upon activation all viral fusion proteins form extended structures that expose a hydrophobic loop or patch, the so-called fusion peptide (**Figure 2c** and **d**). This peptide is inserted into the target membrane. Once inserted, another structural rearrangement takes place which brings the transmembrane domain attached to the virus into close contact with the fusion peptide located in the target membrane. This rearrangement involves the folding back of the *C*-terminal region onto the trimeric *N*-terminal region, illustrated in yellow and blue, respectively, for type I fusion and black and yellow for type II (**Figure 2c** and **d**). The zippering together of these two domains brings the membranes into close proximity. Importantly, full zippering is not complete at the time of fusion pore opening but is necessary for full pore opening (Markosyan *et al.*, 2003). Full

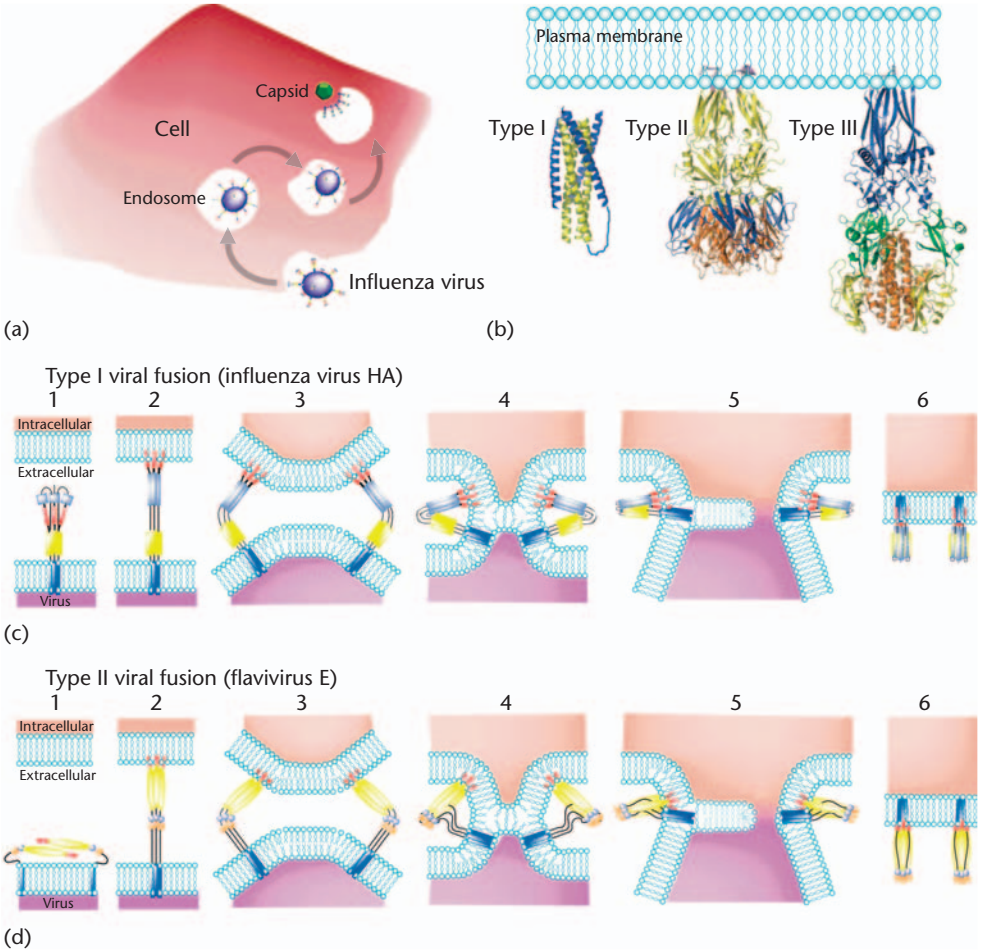

Figure 2 Viral fusion using the influenza HA fusion protein as the primary example. Structural rearrangements of the E fusion protein from flavivirus are shown to illustrate commonalities and differences between type I and type II fusion proteins. (a) The influenza virus enters the host by being taken up through the endocytic pathway. Upon endocytosis the acidic environment in the endosome activates the HA fusion proteins and the viral capsid is released into the cytoplasm. Structural rearrangements are described in detail in (c), then lead to the fusion of viral and host membranes releasing the contents of the influenza virus into the cytoplasm. (b) The structures of the three classes of viral fusion proteins vary widely, yet all undergo common rearrangements that catalyse fusion (compare (c) and (d)). The post-fusion structures of all three classes of viral fusion proteins are shown. The transmembrane domains and fusion peptides are not shown. The examples of each class are: HIV gp41 (type I), Flavivirus fusion protein E (type II) and VSV glycoprotein G (type III). HA is a type I fusion protein. (c) All viral fusion reactions are catalysed by common rearrangements in the viral fusion proteins. Here HA and E are taken as the examples for comparison. Initially, HA and E are present only on the viral membrane (1). Upon activation, in this case by low pH, the fusion peptide (in red) is exposed and inserted into the host membrane (2). In the case of the type II E fusion protein, E monomers come together to form a trimer after low pH activation. By contrast, the type I HA fusion proteins are thought to exist as trimers before activation. After activation, the viral and host membranes are then brought into close proximity (3). The zippering of the HA and E proteins induces lipid stalk formation in which the proximal leaflets of the membranes are fused (4). Full zippering induces pore formation, in which the distal leaflets have now become one (5). The post-fusion HA and E proteins are left on the membranes of the host having accomplished their function – catalysing the release of the viral genome into the host cell (6). Structures illustrated in (b) are adapted from Weissenhorn *et al.* (2007) Virus membrane fusion. *FEBS Letters* **581**: 2150–2155.

zippering is therefore directly coupled to membrane fusion. Viral fusogens act from initial membrane contact through the final steps fusion pore expansion (Markosyan *et al.*, 2003; Melikyan *et al.*, 2000; Russell *et al.*, 2001). These structural rearrangements drive the membranes through a series of defined steps that lead to full fusion (Melikyan *et al.*, 2005). Specifically, the viral fusion proteins catalyse the transition from a lipid stalk, though a hemifusion intermediate, to fusion pore expansion, and finally full fusion (**Figure 2c** and **d**). As we will see in subsequent sections, these lipid intermediates might be common in other fusion reactions (**Figure 1**, **Figure 2** and **Figure 3**).

Based on structural characteristics, the fusion proteins come in three classes (**Figure 2b**). The most well understood are the class I fusion proteins. The class I fusion proteins include haemagglutinin (HA) from the influenza A virus and gp41 from the human immunodeficiency virus 1 (HIV) (**Figure 2b** and **c**). The class I fusion proteins are characterized by a trimer of hairpins composed of a central α-helical coiled-coil structure. The class II fusion proteins include the dengue 2 and 3 virus E and the Semliki forest virus E1 (**Figure 2b** and **d**). Class II fusion proteins also form a trimer of hairpins but assemble via a β sheet rather than α-helical motifs. Finally, the recently discovered class III fusion

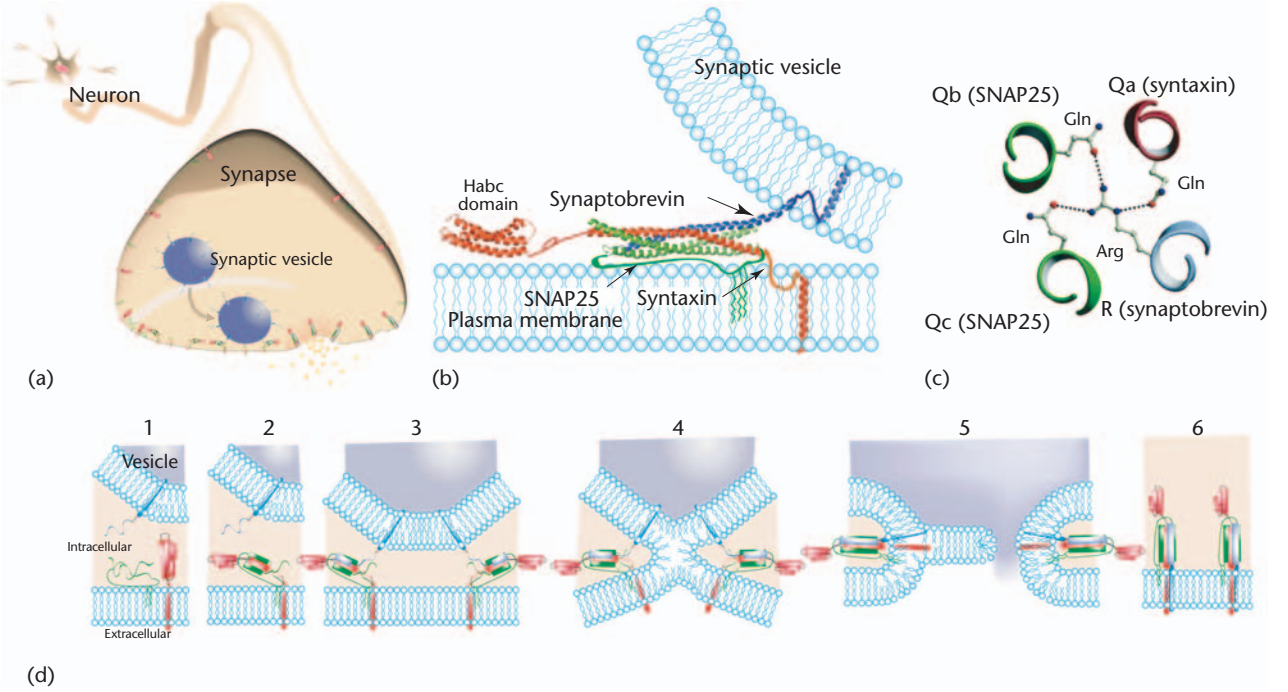

Figure 3 SNARE-based fusion using neurotransmission as the example. (a) Synaptic vesicles fuse at synapses and release neurotransmitters into the synaptic cleft. SNARE proteins are present on synaptic vesicles (synaptobrevin – blue) and on the plasma membrane (syntaxin – red and SNAP25 – green). Structural rearrangements in the SNARE proteins are described in detail in (d). (b) The structure of the fully assembled SNARE complex. The example shown is of the SNAREs used in neurotransmission; however, fusion reactions in the secretory pathway all use related SNARE proteins. These proteins form a four-helix bundle during fusion – the so-called core complex. Syntaxin contains an additional inhibitory domain known as the Habc domain. (c) The four SNARE family members are characterized by conserved residues that face into the centre of the four-helix bundle. Illustrated is a crosswise slice through the core complex showing the interactions between arginine on the R-SNARE (synaptobrevin) and the three glutamines of the Qa-SNARE (syntaxin) and the Qbc-SNARE (SNAP25). In many fusion complexes the Qb and Qc helices are located on separate proteins rather than on a single molecule as is the case with SNAP25. (d) Unlike viruses, SNAREs are initially located on both membranes destined to fuse (1). Before assembly into the core complex, the SNARE domains are unstructured except for syntaxin which adopts a closed confirmation where the inhibitory Habc domain folds over the SNARE motif. The plasma membrane SNAREs are thought to form an acceptor complex in which syntaxin and SNAP25 partially assemble (2). Synaptobrevin then joins the complex, by making initial contact at the *N*-terminal membrane-distal portion (3). The SNAREs become progressively more structured in an *N*- to *C*-terminal direction as they zipper up. The zippering of the SNAREs pulls the proximal leaflets of the membranes together so that they fuse and form a lipid stalk (4). The continued zippering opens the initial fusion pore (5) – the distal leaflets of the membranes have now become one. Post-fusion, all of the SNARE proteins are located in the plasma membrane (6).

proteins, like those found in Herpes simplex virus gB, appear to contain elements of both class I and class II fusion proteins (**Figure 2b**). The trimer of class III fusion proteins is composed of a core α-helical coiled-coil structure; however, the fusion peptide is presented at the tip of an elongated β sheet which shows striking convergence with class II fusion proteins. We will see that these core motifs of interacting coiled-coils and β sheets have parallels in the structural rearrangements of the fusion proteins used in the secretory pathway (**Figure 2** and **Figure 3**).

Despite these structural differences, all three classes of fusion proteins mediate a common series of steps leading to fusion. **Figure 2c** illustrates the common protein transitions using the type I HA fusion protein from the influenza virus as the example whereas **Figure 2d** illustrates the transitions using the type II E protein from flavivirus. These protein rearrangements lead the lipids through a common set of intermediates (Chernomordik *et al*., 1998; Chernomordik *et al*., 2006; Kozlov and Markin, 1983) (**Figure 1**). The initial evidence for a hemifusion intermediate came from studies of the HA fusion protein. The replacement of the transmembrane domain of the fusion protein with a glycosyl-phosphatidylinositol (GPI) anchor trapped fusion at a hemifusion intermediate (Kemble *et al*., 1994). Experimentally the primary evidence for a hemifusion intermediate comes from two observations: lipid exchange without virus content mixing and the sensitivity of the fusion reaction to lipids of different intrinsic curvature. Lipid exchange is often determined by use of the fluorescent tags targeted to the outer membrane of the virus. Intrinsic curvature of lipids is determined by the ratio of the size of their head group to their acyl tails. For example, a lipid with a single acyl tail (big head, little body) would promote positive intrinsic curvature (convex); whereas, a lipid with multiple tails (little head, big body) will introduce negative curvature (convex) (**Figure 1**). Lysophosphatidylcholine (LPC), for example, has only a single acyl tail and would therefore induce positive curvature. At stalk structures and hemifusion diaphragms the outer, fused monolayer, must adopt a negative curvature (concave) (arrowheads in **Figure 1**). When added to the proximal monolayer, lipids with negative curvature stimulate fusion whereas those with

positive curvature inhibit the reaction (Melikyan *et al*., 1997; Razinkov *et al*., 1998). LPC (illustrated in red) inhibits formation of the lipid stalk structure (**Figure 1**, 'stalk') because it stabilizes positive curvature (**Figure 1**, 'two membranes'). Addition of these different lipids to the inner monolayer of the vesicle has the opposite effect, negative curvature inhibits whereas positive curvature stimulates fusion (**Figure 1**, 'pore'). These observations are consistent with a lipid-based hemifusion state as a transition during viral fusion. Studies of haemagglutinin provided the first evidence for a hemifusion intermediate (Kemble *et al*., 1994; Melikyan *et al*., 1997; Razinkov *et al*., 1998). However, other viral fusion reactions also show lipid exchange without content mixing. These include fusion reactions mediated by additional class I proteins such as the HIV env fusion protein (Muñoz-Barroso *et al*., 1998) as well as fusion reactions using class II proteins including aphavirus E1 (Zaitseva *et al*., 2005) and paramyxovirus F fusions (Russell *et al*., 2001). Thus, the hemifusion transition state is likely to be a common step in all viral fusion reactions.

Another commonality in viral fusion reactions is the requirement for multimerization of the viral fusion proteins. Only in one example, the HIV env fusion protein, has it been proposed that single viral fusion proteins are sufficient for fusion (Yang *et al*., 2005). However, even in this case, the presence of multiple env proteins increases fusion rates. Importantly, the presence of multiple env proteins speeds up the kinetics of single fusion events rather than simply increasing the number of viruses fusing (Reeves *et al*., 2002). For all other fusion reactions it is estimated that fusion requires from 3 to 8 fusion proteins (Danieli *et al*., 1996; Gibbons *et al*., 2003).

SNARE-based Fusion

Fusion in the secretory system is mediated by the SNARE proteins (soluble *N*-ethylamine sensitive factor attachment receptor proteins). Unlike viral fusion, the SNAREs are initially present on both membranes destined to fuse. Genetic removal of individual SNAREs in mice, yeast, *Drosophila* and *Caenorhabditis elegans* results in the elimination of fusion in most cases (Hammarlund *et al*., 2007; Hardwick and Pelham, 1992; Schoch *et al*., 2001; Schulze *et al*., 1995). The remaining fusion seen in SNARE null animals can be attributed to redundant SNARE proteins (Bhattacharya *et al*., 2002; Borisovska *et al*., 2005; Liu and Barlowe, 2002). Thus, the SNARE proteins are necessary for fusion. The SNAREs have also been reconstituted into artificial liposomes and shown to be sufficient to induce fusion (Weber *et al*., 1998). These experiments have been extended to native membranes by 'flipping' the SNAREs to face outside the cell. In such a configuration, the SNAREs can induce cell–cell fusion rather than fusing compartments within the cell – a topologically inverse fusion reaction (Hu *et al*., 2003). Thus, in both artificial lipid bilayers as well as in endogenous lipid bilayers, the SNAREs are sufficient to fuse membranes.

The SNAREs are characterized by a conserved 60–70 amino acid SNARE motif (**Figure 3b**). They can be classified into four families (Bock *et al*., 2001; Fasshauer *et al*., 1998; Kloepper *et al*., 2007). In solution they are largely unstructured, but when the four family members come together they form a four-helix parallel coiled-coil bundle (Fasshauer *et al*., 1997; Sutton *et al*., 1998). This four-helix bundle is known as the core complex. The core complex is remarkably stable and can only be disassembled by boiling in the presence of sodium dedocyl sulfate (SDS) (Fasshauer *et al*., 2002; Hayashi *et al*., 1994). Like leucine zippers, the centre of the coiled-coil bundle is lined with hydrophobic residues. Unlike leucine zippers the SNARE complex possesses a single charged residue halfway through the intertwined α-helices. This residue is located at the so-called zero layer in the middle of the core complex. The four family members are named based on these invariant residues. Three of the family members contain a glutamine at this location and are known as the Qa, Qb and Qc SNAREs (Fasshauer *et al*., 1998). These glutamines are coordinated via hydrogen bonding to an arginine residue in the R SNARE (**Figure 3c**) (Sutton *et al*., 1998). The majority of SNARE complexes contain these conserved residues, yet it is not known what function the zero layer plays. Proposed models for zero layer function include: keeping the SNARE in register during fusion; and, SNARE disassembly after fusion. However, neither of these two models has been fully supported by *in vivo* tests (Fasshauer *et al*., 1998; Hanson *et al*., 1997; Lauer *et al*., 2006; Scales *et al*., 2001). Although other complexes can be assembled *in vitro*, only SNAREs containing a QabcR 'QQQR' core complex can efficiently support fusion *in vivo* (Dilcher *et al*., 2001; Fratti *et al*., 2007; Gil *et al*., 2002; Ossig *et al*., 2000; Wang *et al*., 2001; Wei *et al*., 2000). At each trafficking reaction along the secretory pathway a unique core complex is assembled, leading to the model that the SNAREs provide the specificity of fusion (McNew *et al*., 2000a; Parlati *et al*., 2002; Scales *et al*., 2000; Söllner *et al*., 1993b).

Three characteristics of the SNAREs are believed to be vital for their role in membrane fusion. First, the stability of the core complex provides the energy source that overcomes the barrier to fusion (Fasshauer *et al*., 1997; Hayashi *et al*., 1994). *In vitro*, the assembled core complex can only be disassembled by boiling in the presence of SDS. The assembly of the core complex is thus essentially irreversible, making it perfectly suited to overcome the high repulsive forces of membranes in close proximity. Second, the SNARE complex must contain at least two SNAREs with transmembrane domains. The transmembrane domains must be inserted into both membranes destined to fuse (Parlati *et al*., 2000). Third, the SNAREs assemble in a parallel orientation (Hanson *et al*., 1997; Lin and Scheller, 1997; Poirier *et al*., 1998; Sutton *et al*., 1998). As a result of this parallel orientation, SNARE assembly brings the transmembrane domains into close proximity and thus also the membranes themselves (**Figure 3b**) (Hanson *et al*., 1997). These three characteristics are responsible for leading the lipids through the sequential intermediates of a lipid stalk,

a hemifusion diaphragm, a fusion pore and finally full fusion (**Figure 3**).

Like viral fusion proteins, the SNAREs undergo structural rearrangements that enable them to catalyse membrane fusion. The SNAREs initially contact each other at the membrane distal *N*-terminal (**Figure 3d**). This conformation of the SNARE proteins is termed a 'loose' SNARE complex. The SNAREs are held in this 'loose' conformation until triggered to zipper into a 'tight' conformation where the SNARE complex is fully assembled (**Figure 3d**) (Chen *et al*., 2001; Fasshauer and Margittai, 2004; Fiebig *et al*., 1999; Hanson *et al*., 1997; Hua and Charlton, 1999; Lin and Scheller, 1997; Melia *et al*., 2002; Pobbati *et al*., 2006; Sorensen *et al*., 2006; Xu *et al*., 1999). The synaptic SNARE proteins are triggered to zipper by calcium (Bai and Chapman, 2004; Brose *et al*., 1992; Chapman, 2008; Davis *et al*., 1999; Fernandez-Chacon *et al*., 2001; Sabatini and Regehr, 1996). During assembly into a 'tight' conformation torque is transferred via a short linker to the transmembrane domains (McNew *et al*., 2000b). Thus, the SNAREs both bring the membranes into close proximity and actively disrupt lipids via the transmembrane domains (McNew *et al*., 2000b). Membrane proximity alone is therefore not sufficient to catalyse fusion.

Close proximity may, however, be sufficient to achieve a hemifusion state. The early steps of hemifusion require less energy than the later steps of fusion pore formation and expansion (Chernomordik and Kozlov, 2005; Cohen and Melikyan, 2004; Kuzmin *et al*., 2001). When the SNARE transmembrane is replaced by an artificial lipid anchor or when it is truncated, fusion is markedly reduced and in many cases no longer proceeds (Giraudo *et al*., 2005; Grote *et al*., 2000; McNew *et al*., 2000b; Nonet *et al*., 1998; Saifee *et al*., 1998; Xu *et al*., 2005). However, these perturbations do lead to a state in which lipids can exchange – a hallmark of hemifusion (Chernomordik and Kozlov, 2005; Giraudo *et al*., 2005; Grote *et al*., 2000; Xu *et al*., 2005). This observation parallels the replacement of the transmembrane domain of the HA fusion proteins from viruses described in the preceding section (Kemble *et al*., 1994). Importantly, the fusion arrest resulting from transmembrane domain replacement or removal can be bypassed by addition of lipids with positive curvature to the inner membrane or those with negative curvature to the outer membrane (Grote *et al*., 2000; Xu *et al*., 2005). The hemifusion state in the secretory pathway is an 'on pathway' reaction and in numerous instances can be quite long lasting (Jun and Wickner, 2007; Lu *et al*., 2005; Reese *et al*., 2005; Reese and Mayer, 2006; Wong *et al*., 2007; Xu *et al*., 2005). Like viral fusion reactions, LPC inhibits SNARE-mediated fusion with a similar dose response and acyl tail length dependence (Chernomordik *et al*., 1997; Reese *et al*., 2005). Thus, proximity resulting from SNARE pairing may be sufficient to achieve a hemifusion state, whereas full fusion requires the transmembrane domains of the respective fusion proteins.

The dependence of the transmembrane domains for fusion suggests that force is transduced from SNARE zippering to the transmembrane domain during fusion. This model has been tested by adding flexible linkers between the SNARE motif and the transmembrane domains. In each of the tested cases the addition of flexible linkers results in incremental decreases in fusion to full elimination (Deak *et al*., 2006; Kesavan *et al*., 2007; McNew *et al*., 1999, 2000b; Siddiqui *et al*., 2007; Wang *et al*., 2001). Of particular interest, are studies of synaptobrevin in chromaffin cells lacking the synaptobrevin homologues used in dense-core vesicle secretion (Borisovska *et al*., 2005; Kesavan *et al*., 2007). Synaptobrevin with extended linkers was introduced into these cells and fusion studied using capacitance and amperometric measurements. Importantly, these experiments demonstrated that the SNAREs operate throughout the fusion process, from the initial priming of vesicles at sites of fusion through the final opening and expansion of the fusion pore (Kesavan *et al*., 2007).

The stage of initial fusion pore opening can be analysed for both SNARE-based fusion and viral fusion using capacitance measurements. Capacitance is proportional to membrane area. During fusion, the membrane of a secretory vesicle or viral membrane becomes continuous with the plasma membrane. The fusion of a secretory vesicle, for example, can add between 30 aF and 1 fF of membrane capacitance depending on vesicle size (He *et al*., 2006). A comparison of capacitance recordings between synaptic vesicles and viral particles provides further evidence for a common lipid-based fusion pore. Two characteristics are found in common. First, in both instances fusion pores 'flicker' indicating that the step of fusion pore formation is reversible. Second, the sizes of the initial fusion pores were found to have similar conductances, although they do have considerable variability. In secretory vesicle fusion, pores range from around 19 pS to expanded fusion pores that are still reversible and have conductances of 8 nS (He *et al*., 2006; Klyachko and Jackson, 2002; Lollike *et al*., 1995; Spruce *et al*., 1990). Capacitance studies in viral fusion have indicated initial pore sizes ranging from 1 to 600 pS (Lanzrein *et al*., 1993; Spruce *et al*., 1989). At the lower end, these pores are equivalent to those of ion channels; at 8 nS the pore would be around 20 nm and readily visible in electron microscopic studies (Lollike *et al*., 1995; Spruce *et al*., 1990).

Like viral fusion, SNARE-mediated fusion requires higher order multimerization. Electron microscopy studies indicate that SNAREs form star-shaped structures with the transmembrane domains at the vertex (Rickman *et al*., 2005). By assembling into these higher-order multimers the SNAREs might thus locally disrupt lipids – perhaps by delineating a hemifusion diaphragm (Montecucco *et al*., 2005). The *in vivo* experimental evidence for multimerization comes from the cooperative action of the SNAREs as well as the dose dependence of inhibition by peptide blocker and the botulinum neurotoxins. Together the evidence has suggested multimers containing between 3 and 15 SNARE complexes (Han *et al*., 2004; Hua and Scheller, 2001; Montecucco *et al*., 2005; Raciborska *et al*., 1998; Rickman *et al*., 2005; Stewart *et al*., 2000). Nonetheless, at

present working models for multimerization are quite preliminary and how they might aid in fusion is unknown.

Like viral fusion the fusion reaction ends with all membrane anchors in the same membrane (**Figure 2** and **Figure 3a**). However unlike viral fusion proteins, the SNAREs are subsequently recycled. The disassembly of the SNARE proteins is accomplished by the triple A+ ATPase (adenosine triphosphotase) *N*-ethylmaleimide sensitive factor (NSF) (Söllner *et al*., 1993a). The action of NSF allows the SNAREs to be pulled apart to undergo further rounds of fusion (Littleton *et al*., 1998, 2001; Mayer *et al*., 1996; Nichols *et al*., 1997).

One final difference of note between viral fusion and SNARE-based fusion is that they are topologically inverse reactions. Viral fusion proteins face out of the cell, whereas SNARE proteins are oriented towards the cytoplasm. SNAREs flipped to face outside of cells can accomplish induce fusion of whole cells, demonstrating that topological differences do not necessitate different fusion proteins (Hu *et al*., 2003). However, because the SNAREs face the cytoplasm they can be extensively regulated by the controlled environment of the interior of the cell in a fashion that is not possible with viral fusion proteins. The list of SNARE regulatory proteins is extensive and includes the regulatory proteins conserved from yeast to man, such as the SM superfamily, as well as regulatory proteins unique to specific fusion events (Lang and Jahn, 2008; McNew, 2008; Rizo and Rosenmund, 2008). For example, synaptotagmin and complexin are part of the key evolutionary modifications that allowed to SNAREs to be adapted to the unique requirements of neurotransmission (Melia, 2007). Though calcium is needed for fusion in other membrane trafficking steps, it usually functions as a facilitator of fusion rather than directly functioning as the trigger for fusion (Flanagan and Barlowe, 2006; Starai *et al*., 2005). At synapses the time delay between elevation of calcium of calcium and a postsynaptic response can be as little as 60–200 μs (Sabatini and Regehr, 1996). The addition of complexin and synaptotagmin is likely to impart the calcium trigger needed to satisfy the rapid and tightly regulated fusion unique to the release of neurotransmitters from neurons.

Cell–Cell Fusion

By far the best understood fusion reactions are those involving viral entry and those of the secretory system. Yet, these two well-studied cases represent only a small subset of the fusion reactions that occur in living organisms. Other fusion reactions include: cell–cell fusion, mitochondrial fusion, peroxisome fusion, chloroplast fusion and nuclear fusion. Among these reactions is the fundamental fusion reaction that underlies all sexually reproducing organisms, namely the fusion of egg and sperm in animals or pollen and stigma in plants. Surprisingly little is known about the molecules governing these fusion reactions. We briefly cover those reactions where a molecular foothold is apparent.

Cell–cell fusion is required for numerous biological processes including hypodermal cell fusion in *C. elegans*, sperm–egg fusion, yeast mating pair fusion, placenta formation in mammals and muscle and bone formation. The molecular players in these reactions have only been recently uncovered. In the next section, we will cover those reactions for which a fusogen has been proposed with a focus on how it was discovered and along with evidence for its role as a fusogen.

At present the best understood cell–cell fusogen is the epithelial fusion failure (EFF)-1 protein used in *C. elegans* hypodermal fusion. It is the only putative fusogen that has been shown to be both necessary and sufficient for cell fusion. Nonetheless, it is not understood how EFF-1 drives membrane fusion.

Many cells in organisms are multinucleate. They often become so by cell–cell fusion. For example, skin cells in the nematode *C. elegans* undergo fusion to form a large multinucleate syncytium. The epidermis of this species is formed via repeated cell–cell fusions to eventually form a single large cell containing 138 nuclei. Genetic screens for epidermal fusion failure identified a recessive mutant known as *eff-1* (Mohler *et al*., 2002). *eff-1* encodes a type I transmembrane protein with no apparent homology outside of nematodes. Misexpression of EFF-1 was sufficient to cause ectopic fusion in *C. elegans*. Interestingly, EFF-1 could also fuse insect cells when expressed on both cells (Podbilewicz *et al*., 2006). Thus, like the SNAREs and viral proteins, sufficiency has been demonstrated for EFF-1 (**Figure 4**). Unlike SNARE and viral fusion, EFF-1 has a homotypic fusion machinery. In other words, both membranes must have EFF-1 for fusion to occur. A protein related to EFF-1, called AFF-1, is involved in the fusion of vulval cells in *C. elegans* (Sapir *et al*., 2007). EFF-1 and anchor cell fusion failure (AFF)-1 do not mediate all cell–cell fusions in *C. elegans* – in a double mutant sperm and egg still fuse. The mechanism for EFF-1 and AFF-1 cell fusion is not known but because LPC (an 'inverted cone' lipid, see **Figure 1**) in the proximal membranes blocks EFF-1-mediated fusion it is likely that it requires a hemifusion intermediate (Podbilewicz *et al*., 2006). Thus, despite a complete lack of sequence or structural homology, SNAREs, viral fusion proteins and EFF-1 proteins are all likely to catalyse a common intermediate during fusion.

Among the most fundamental cell–cell fusion events is that of egg and sperm. Currently it remains unknown what the fusogen is that mediates this process. Early experiments implicated a family of proteins known as the fertilins (Primakoff *et al*., 1987). The fertilins α and β are the founding members of the ADAM (A Disintegrin and Metalloprotease) family. Intriguingly, fertilin α possesses motifs related to viral fusion proteins. However, knockouts of the fertilins had only a minor effect on sperm and egg fusion. Two additional proteins have been implicated for which stronger cases can be made. Izumo, a protein related to the immunoglobulin superfamily and CD9, a tetraspanin, have been suggested to mediate sperm–egg fusion (**Figure 4**). Unlike the fertilins, knockouts of Izumo or CD9

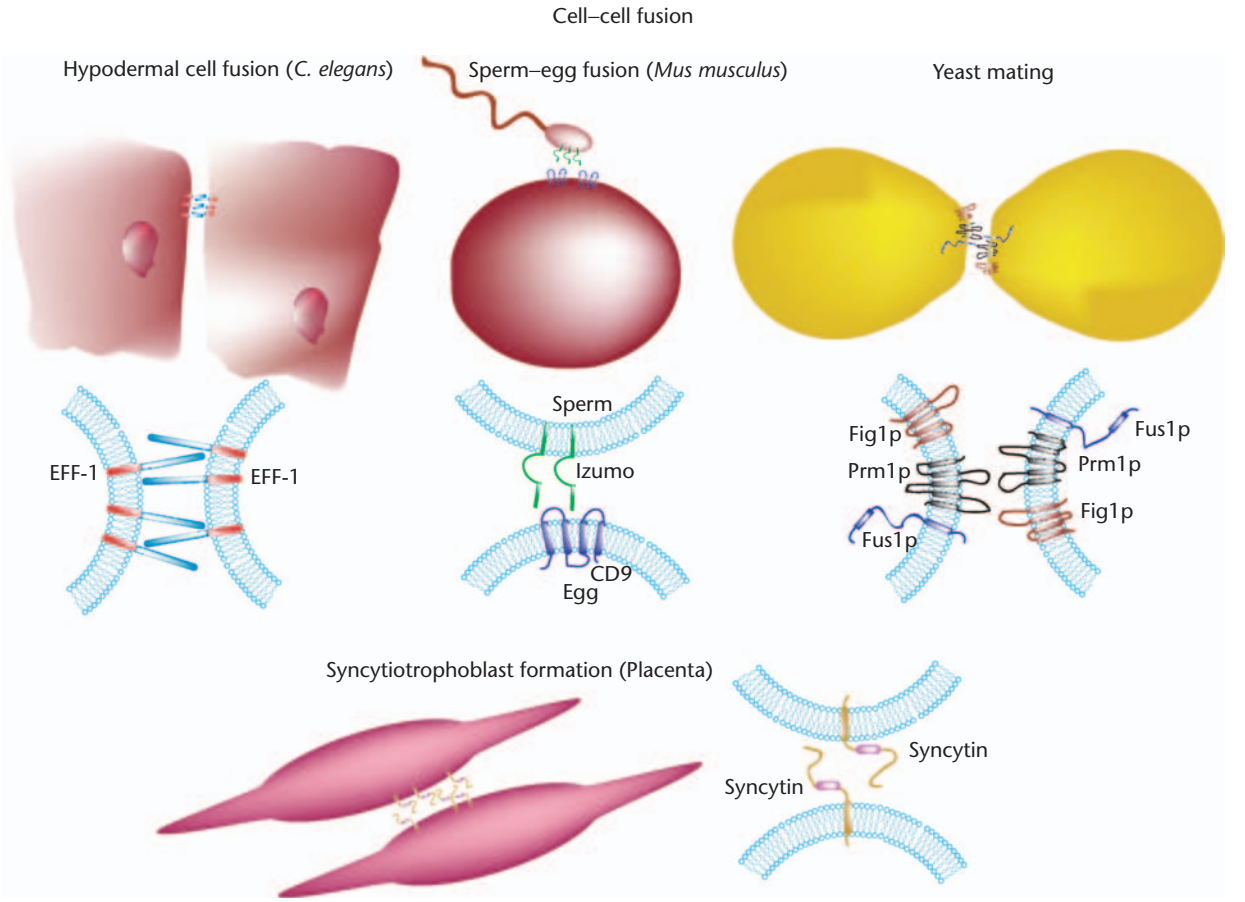

Figure 4 Cell–cell fusion reactions are catalysed by an assortment of unique fusion proteins. EFF-1 is used in *C. elegans* during the formation of the hypodermis. EFF-1 mediates homotypic fusion – it must be present on both membranes destined to fuse. It has been demonstrated to be necessary and sufficient for fusion. The best candidates for sperm–egg fusion are Izumo localized on sperm and CD9 localized on egg. Izumo encodes a member of the immunoglobulin superfamily. CD9 is a member of the tetraspanin superfamily. It is not known how Izumo and CD9 might catalyse fusion. Yeast haploid cells fuse during mating. Molecules suggested to mediate yeast mating cell fusion are: Prm1p, Fig1p and Fus1p. Prm1p is multipass membrane protein whose expression is induced by the mating pheromone. Fus1p is a single pass membrane protein in whose absence the fusion pore opening is delayed and inefficient. Fig1p is a multipass membrane protein. Fig1p fusion defects can be rescued by increasing calcium concentration. Placenta development requires cell–cell fusion in the syncytiotrophoblast layer. This layer forms the interface between fetus and mother. Fusion of the syncytiotrophoblast is reduced by antibodies against syncytins. Syncytins are fusogens from an endogenous retrovirus expressed specifically in the syncytiotrophoblast layer.

do eliminate fusion (Inoue *et al*., 2005; Kaji *et al*., 2000; Le Naour *et al*., 2000; Miyado *et al*., 2000). Nonetheless sufficiency for these proteins has not yet been demonstrated. At present, at least two alternative interpretations for the lack of fusion in the knockouts are equally probable. The proteins could function to localize an as yet undiscovered fusogen or they could function in an initial tethering step between sperm and egg. Myoblast fusion in *Drosophila* for instance also involves at least four immunoglobulin domain proteins; however, all of them function in myoblast attachment and not directly in the fusion step (Artero *et al*., 2001; Bour *et al*., 2000; Ruiz-Gómez *et al*., 2000; Strünkelnberg *et al*., 2001). **See also**: Sperm–Egg Interactions: Sperm–Egg Binding in Mammals

In yeast cell–cell fusion, haploids of different mating types fuse during the mating cycle (**Figure 4**). Prm1p was discovered in a bioinformatic screen as a membrane protein induced by mating pheromone (Heiman and Walter, 2000). The defining characteristic of *prm1* mutants is that mating pairs are found with their membranes in contact but unfused. Nonetheless, *prm1* mutants only have a phenotype when both mating pairs lack the protein and even then they only exhibit a 75% reduction in fused cells. Thus, if Prm1p is a fusogen it cannot represent the sole fusogen. This role may be played in part by Fus1p. In *fus1* mutants the size and expansion of the fusion pore between mating pairs is smaller and its expansion slower (Nolan *et al*., 2006). An additional protein involved in the final steps of fusion is Fig1p (Muller *et al*., 2003). Double mutants between *prm1* and *fig1* reduce fusion to 10% of wild type (Aguilar *et al*., 2007). Fig1p, however, may be involved in a calcium-sensing step rather than in the fusion step itself since *fig1* mutants can be fully bypassed by increasing calcium concentration (Muller *et al*., 2003).

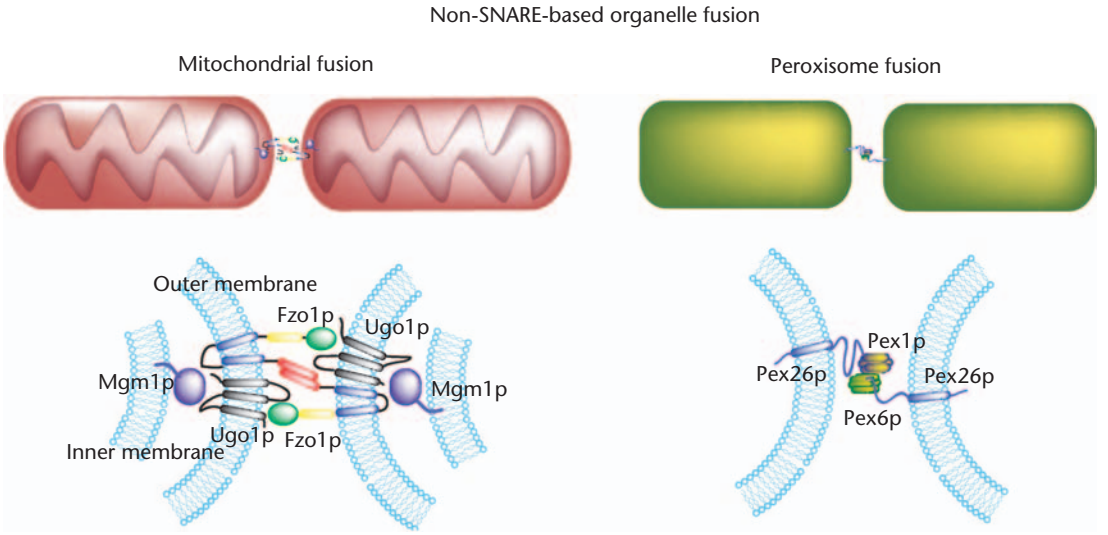

Figure 5 Outside of the secretory pathway, organelle fusion is not mediated by the SNARE proteins. These non-SNARE fusion reactions include those that maintain mitochondria and peroxisomes. Mitochondria are unique in that they contain an inner and outer membrane whose fusion must be coordinated. Fusion of the outer membrane is catalysed by fuzzy onions (Fzo1p), known as mitofusins in mammals. Fzo1p contains two transmembrane domains anchoring it in the outer membrane. The transmembrane domains are separated by a loop that extends into the space between outer and inner membranes and may play a role in coordinating the fusion of these two membranes. Facing into the cytoplasm, Fzo1p contains a GTPase domain (green oval) and α-helices (red rods) that form coiled-coils between two mitochondria destined to fuse. Inner membrane fusion is catalysed by Mgm1p, known as Opa1 in mammals. The dynamin-related Mgm1p contains a GTPase domain (purple oval) and is peripherally localized to the inner membrane. In yeast the coordination of outer and inner membrane fusion might be accomplished by Ugo1p. Peroxisomes also do not appear to use SNAREs for membrane fusion. Two triple A+ ATPases, related to NSF, have phenotypes consistent with a membrane fusion defect. The triple A+ ATPases, Pex1p and Pex6p are localized to peroxisomes via Pex26p. Currently there are no specific models for their role in fusion.

One final molecule used in cell–cell fusion was perhaps acquired by outright theft from viruses. During mammalian development the placenta provides nutrients to the developing fetus. At the interface of mother and fetus lies the syncytiotrophoblast layer that is generated by cell–cell fusion (**Figure 4**). In a screen for secreted proteins Mi *et al.* found the envelope protein from the human endogenous retrovirus and named it syncytin (Mi *et al.*, 2000). Syncytin is specifically expressed in the syncytiotrophoblast layer and its inhibition, by RNAi (ribonucleic acid interference) or antibody, reduces syncytiotrophoblast fusion (Frendo *et al.*, 2003; Mi *et al.*, 2000). Finally, ectopic expression of syncytin in numerous cell lines can induce their fusion (Dupressoir *et al.*, 2005; Mi *et al.*, 2000). The human genome is littered with endogenous retroviruses. It seems that rather than using an endogenous fusogen, the genome has commandeered a fusion protein from a virus to use for cell fusion during placental development.

Non-SNARE-based Organelle Fusion

The SNAREs mediate fusion throughout the secretory pathway. Thus, organelles such as the Golgi, the endoplasmic reticulum and the lysosome are all maintained by SNARE-based fusion reactions. However, numerous organelles undergo fusion without SNARE proteins. These include mitochondria, peroxisomes and the cell nucleus. Although no fusogen has been proposed for the nucleus, candidates have been proposed for mitochondria and peroxisomes.

Mitochondria must undergo rounds of fission and fusion to maintain optimal respiratory function and, in some cases, to be passed to daughter cells. Unlike other fusion reactions, mitochondria have an unusual challenge; they have both an inner and an outer membrane whose fusion and fission must be coordinated (**Figure 5**). For fusion, the key molecular foothold came from the cloning of a gene know as *fuzzy onions* (*fzo*) from *Drosophila*. *fzo* encodes a large GTPase (guanosine triphosphotase) (Hales and Fuller, 1997), which is conserved from yeast to man (Hermann *et al.*, 1998; Rapaport *et al.*, 1998). The mammalian homologues are known as the mitofusins 1 and 2 (mfn1 and mfn2) (Santel and Fuller, 2001). Models for the role of the fuzzy onions family in fusion have been proposed based on our understanding of SNARE and viral fusion mechanisms (Mozdy and Shaw, 2003). The fuzzy onions family all contain two transmembrane domains as well as α-helices that are predicted to form coiled-coils. The antiparallel assembly of the coiled-coils between fuzzy onions on opposing mitochondria bring the membranes destined to fuse together (Koshiba *et al.*, 2004) (red domains in **Figure 5**). Like SNARE and viral-based fusions, the composition of lipids is also critical for fusion in mitochondria. Mutations in a mitochondrial-specific phospholipase D fail to generate phosphatidic acid. Phospholipase D is also necessary for SNARE-based fusion reactions (Nakanishi *et al.*, 2006; Vicogne *et al.*, 2006). Importantly, the phospholipase acts

downstream of mitofusin 1, thus a lipid-based fusion pore might also initiate fusion in mitochondria (Choi *et al.*, 2006). Sufficiency has not yet been demonstrated for FZO and at least two additional proteins might be directly involved in the fusion process, Ugo1p and Mgm1p/OPA1. Ugo1p does not appear to have a mammalian orthologue and so may not be part of the conserved fusion machinery (Sesaki and Jensen, 2001). *MGM1* encodes a GTPase related to dynamin (Jones and Fangman, 1992) and unlike Ugo1p has a clear mammalian homologue in OPA1 (Alexander *et al.*, 2000). Mgm1p/OPA1 is localized on the inner mitochondrial membrane facing the inner membrane space and appears to fuse the inner membrane (Wong *et al.*, 2000; Meeusen *et al.*, 2006). It is still unknown how inner and outer membrane fusion is coordinated. Direct interactions between Mgm1p and Fzo1p have not yet been demonstrated. In yeast, it is possible that the coordination is accomplished in part by Ugo1p which binds both Mgm1p and Fzo1p (Coonrod *et al.*, 2007; Sesaki and Jensen, 2004).

Peroxisomes participate in the metabolism of fatty acids and other metabolites. Like mitochondria, rounds of fission and fusion are needed to maintain peroxisomes. Unlike mitochondria, they are enclosed in a single membrane bilayer and are functionally linked to the secretory pathway. Currently, very little is known about their mechanism of fusion. Nonetheless, two triple A+ ATPases have been implicated in their fusion (Erdmann *et al.*, 1991; Spong and Subramani, 1993) (**Figure 5**). These two ATPases, Pex1p and Pex6p, were identified in genetic screens for lack of peroxisome inheritance and share the unique phenotype of accumulating numerous small peroxisome fragments, a phenotype highly reminiscent of the fragmented mitochondria seen in *fzo1*, *ugo1* and *mgm1* mutants (Heyman *et al.*, 1994; Spong and Subramani, 1993). They are localized to peroxisomes through the action of the Pex26p (Matsumoto *et al.*, 2003). However, as of yet they have not been demonstrated to be sufficient for fusion. It is equally possible that pex1 and pex6 function in an analogous manner to the triple A+ ATPase, NSF, which disassemble the SNARE proteins for further rounds of fusion (Mayer *et al.*, 1996; Nichols *et al.*, 1997; Söllner *et al.*, 1993a). Thus, instead of being the fusogens themselves they may instead be activating an as yet unidentified fusion protein.

Conclusions

The evolution of membranes was a key step for the origin of life. Membranes allowed chemical reactions to be isolated from the environment – delineating the self from the nonself. Yet, at numerous points these membranes must also be breached: viruses must enter cells to replicate, organelles must empty their content during trafficking, sperm must fuse with egg. These are but a few of the important biological functions that membrane fusion accomplishes. The merger of membranes involves dramatic lipid rearrangements but must also be carefully controlled such that the encapsulated material is not lost. The fusion of membranes is likely to involve common lipid-based intermediates whose transition is catalysed by specialized proteins. By far the best understood fusogens are the viral fusion proteins and the SNAREs used in the secretory pathway. Although the viral proteins and SNAREs are unrelated by sequence, they have structural steps that are conceptually conserved. Both involve bringing together the plasma membranes followed by 'zippering up' of the fusion proteins which drive the membranes to complete merger. In both cases energy released in zippering must be transferred to transmembrane domains that are likely to actively disrupt the stability of the lipid bilayer. The membrane fusion reactions in viral entry and in the secretory pathway are relatively well understood, yet these represent only a small subset of the fusion reactions happening in living organisms. The molecules that mediate cell–cell fusion and the molecules that fuse organelles outside the secretory pathway are only now coming to light and at present mechanistic insight into their action is virtually nonexistent.

Several outstanding questions remain. We have presented a model where the specialized proteins act to drive lipids through conserved steps. Experimental evidence for the viral fusion proteins and the SNAREs supports this model, but does it hold true for cell–cell fusion and non-SNARE organelle fusion? In other words, is the hemifusion step a common intermediate in all fusion reactions? In cell–cell fusion the putative fusogens do not seem to be conserved. Does this imply that hemifusion is not a common intermediate or does it mean that there are numerous ways to push the lipids through this transition state? The interrelation of the structural rearrangements in the fusion proteins and the lipid transitions is still mostly a mystery. How does the energy released by these transitions catalyse membrane fusion? Multimerization of the fusion proteins also appears to be a common trait. Do the multimerized fusion proteins act to delineate a hemifusion diaphragm? Do multimerized fusion proteins act cooperatively to deliver the energy from the fusogen to lipid rearrangements?

Undoubtedly, uncovering additional fusogens and gaining mechanistic insight into their functions will help us understand how fusion proteins catalyse the lipid rearrangements that lead two membranes to merge into one.

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