Mechanisms of Virus Membrane Fusion Proteins

Margaret Kielian

Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461; email: margaret.kielian@einstein.yu.edu

Abstract

Enveloped viruses infect host cells by a membrane fusion reaction that takes place at the cell surface or in intracellular compartments following virus uptake. Fusion is mediated by the membrane interactions and conformational changes of specialized virus envelope proteins termed membrane fusion proteins. This article discusses the structures and refolding reactions of specific fusion proteins and the methods for their study and highlights outstanding questions in the field.

Keywords

virus entry, influenza virus, influenza hemagglutinin, alphavirus, flavivirus, rhabdovirus, pestivirus
INTRODUCTION

The genomes of enveloped animal viruses are enclosed within lipid bilayers (envelopes) that protect these viruses during extracellular transport to a new host cell. Infection is initiated when the viral genome is delivered into the host cell cytoplasm via fusion of the virus membrane with that of the cell. This critical membrane fusion event can occur either at the cell surface or, following virus internalization, within cellular endocytic compartments. Fusion is mediated by specialized transmembrane proteins on the virus envelope, termed fusion proteins. The activity of these proteins is highly regulated to ensure that they are deployed at the correct time and cellular location during infection but are silenced during virus biogenesis and transport. Inhibition or inactivation of the viral fusion protein blocks virus infection.

Given the central role of membrane fusion in enveloped virus infection, viral fusion proteins have been the subject of many structural and functional studies (for general virus fusion reviews, see 1–6). This article highlights examples of the key features of fusion proteins, describes some of the methods used for their study, draws analogies and contrasts among known fusion proteins, and discusses some important and intriguing unsolved problems in the field. The reader is referred to additional reviews and primary literature for detailed coverage of specific fusion proteins and mechanisms, with apologies for those topics not fully covered.

Location of the Membrane Fusion Reaction

What determines whether a virus fuses at the plasma membrane versus in an endocytic compartment? Viruses initially interact with cells through attachment factors and/or specific receptors at the cell surface (7). In some cases, these interactions are sufficient to trigger the fusion reaction. In other cases, triggering requires the low pH of endocytic compartments or additional events that occur after internalization. Although the location of some viral fusion reactions is optimized for virus infection (7, 8), fusion can often be successfully relocalized by supplying an intracellular trigger such as low pH to virus bound at the cell surface (e.g., 9).

The determination of the cellular location of virus fusion and infection involves careful evaluation of the functional effects of inhibitors of endocytosis and endosomal acidification, combined with morphological and biochemical studies to test for virus internalization during infection (7, 10). A number of issues are important to note in this context. Endocytosis is generally very rapid, and many of the pathways continue at a reduced but significant rate even at lower temperatures such as 10°C (7, 11). Inhibitors can have secondary effects and require careful controls for the steps in the life cycle that are affected and the specificity of the effect (7). Inhibition also needs to be assessed using amounts of virus in the linear range for infection and sensitive assays for fusion or early steps in primary infection. When possible, comparative studies with a virus or fusion protein that is independent of the inhibitor or pathway can provide important controls. For some viruses, the inhibition of a specific cellular internalization pathway, such as that mediated by clathrin, can upregulate infection via other pathways that are not normally as important (12). Live cell imaging studies that track single virus particles versus cellular endocytic markers can help in differentiating the most physiologically relevant entry pathways (12–17). Lastly, some viruses can spread from cell to cell rather than requiring production and release of free extracellular virus particles (18–20) and thus may not be amenable to classical methods to assay virus-cell binding, uptake, or fusion.

OVERVIEW OF THE FUSION PROTEIN MECHANISM

General Features

Membrane fusion involves the close approach of two lipid bilayers, followed by local deformations of the individual bilayers and finally by their merger into a single membrane (Figure 1) (reviewed...
Figure 1
General model for virus membrane fusion. (a) A metastable viral fusion protein in the prefusion conformation has its fusion peptide (or loop) hidden or inactive. (b) The fusion protein is triggered, and the fusion peptide or loop inserts into the target cell membrane to form a monomeric or trimeric extended intermediate that bridges the two membranes. (c) Protein insertion and refolding cause initial distortions in the viral and/or target membranes. (d) Fold-back of the proteins in a hairpin-like fashion promotes the formation of a hemifusion intermediate in which the contacting membrane leaflets mix. (e) The final packing of the outer layer onto the core trimer transitions the protein to the postfusion hairpin and drives the opening of the fusion pore. Steps c–e may involve multiple fusion protein trimers (two are shown). The virus membrane is shown in purple and the target cell membrane in blue.

The approach of two bilayers to distances of less than $\sim 30$ Å is strongly disfavored due to repulsive forces including that of the hydration barrier, a layer of water associated with the polar lipid head groups (23). The deformations that occur during fusion are also disfavored because lipid bilayers have intrinsic preferred curvatures conferred by their lipid compositions (22). Thus, even when fusion is energetically favored overall, there are high kinetic barriers (1, 21, 22). The energy to overcome these kinetic barriers is provided by the virus membrane fusion protein, which refolds from a metastable, relatively high-energy prefusion conformation to a more stable, lower-energy postfusion conformation (Figure 1). The transmembrane fusion protein inserts into the target membrane via a hydrophobic fusion peptide (or loop), and its dual-membrane interaction then couples the energy of protein refolding to the deformation and fusion of the two membranes. The final state of the fusion protein is a stable hairpin conformation in which the transmembrane (TM) domain and the fusion peptide or loop are at the same end of the molecule. Fusion proceeds by a pathway that involves the initial mixing of the outer membrane leaflets (a step termed hemifusion) followed by the opening of a small fusion pore that then widens to complete the fusion reaction (Figure 1). The key steps in fusion are discussed in detail below.

Production of the Fusion Protein
Generation of the metastable form of the virus fusion protein occurs during virus biogenesis or subsequent maturation. In some cases, the fusion protein is synthesized in an inactive precursor form that is proteolytically matured to generate the metastable, fusion-competent form (as is the case for the fusion proteins of influenza virus and HIV-1) (24, 25). In other cases, the fusion protein is synthesized together with a viral chaperone or companion protein that promotes its folding into the metastable form (as is the case for the fusion proteins of alphaviruses and flaviviruses) (26–30). For some viruses, additional priming occurs during entry. For example, the Ebola virus fusion protein GP is primed through processing by cathepsin proteases in endocytic compartments, a process that can be recapitulated by in vitro cleavage (31, 32). Together, such prefusion events promote the generation of a fusion-competent protein without triggering target membrane insertion and the refolding steps of fusion.
A Generic Model for the Virus Membrane Fusion Process

The first step in the fusion pathway is the triggering of the metastable virus fusion protein to initiate the fusion cascade (Figure 1a,b). The specific trigger for fusion depends on the virus (reviewed in 2). The currently described triggers (and examples of viruses that use them) include receptor binding to the fusion protein (retroviruses), receptor binding to a separate attachment protein (paramyxoviruses), receptor plus coreceptor binding (HIV), low pH (alphaviruses, flaviviruses, and influenza virus), receptor plus low pH (avian sarcoma/leukosis virus), and receptor binding plus additional unknown triggers (Ebola virus). Though diverse, these triggering mechanisms all initiate the thermodynamically favored process of refolding the fusion protein into the stable final postfusion form. Indeed, for certain fusion proteins such as the influenza hemagglutinin, simply heating the virus can trigger refolding and fusion (33, 34).

An initial effect of triggering for many fusion proteins involves changes in their oligomeric associations. This may mean dissociation and rearrangement of interactions between fusion proteins, as in the case of dissociation of the flavivirus E protein homodimer (35, 36) or the rhabdovirus fusion protein G trimer (4). Such intersubunit rearrangements and/or conformational changes within the fusion protein promote the release and reorientation of the hydrophobic fusion peptide toward the target membrane. The fusion peptide then inserts into the outer leaflet of the target membrane (Figure 1b). For some viral fusion proteins, insertion initially occurs via trimers (37). Others insert as monomers, and subsequent trimerization is promoted by the orientation and local concentration provided by the membrane interaction (38–40). An important extended trimeric intermediate (sometimes termed a prehairpin) is thus formed, bridging the target and viral membranes. Note that the definition of the extended intermediate is still incomplete, and the step at which trimerization occurs is unclear for some fusion proteins (reviewed in 4). The intermediate then folds back to a hairpin conformation in which the target membrane-inserted central trimer is packed by an outer layer that connects to the virus membrane. This dramatic conformational change brings the target membrane and fusion peptides into close proximity with the virus membrane and transmembrane domains. Initial nipple-like distortions deform the two membranes toward each other, a process that appears to play an important mechanistic role in fusion (Figure 1c) (41). Such bending of the target membrane may occur as a result of the inserted fusion peptide or fusion loops acting to asymmetrically expand the outer leaflet, as has been observed in vitro (e.g., 42, 43). Viral membrane distortions may occur due to stresses produced during hairpin formation as well as via interactions of the membrane-proximal region of the fusion protein (1, 2, 44). These distortions are followed by formation of a lipidic stalk or hemifusion intermediate (22), which is driven by the fold-back process (Figure 1d). The stalk opens into a small and transient fusion pore. This initial pore then expands into an irreversible open state that joins the virus interior with the cytoplasm, allowing genome delivery and infection (Figure 1e). Pore expansion is thought to be the most energy-intensive part of the fusion process (45, 46) and may involve several fusion proteins (1, 6). In spite of their varied architectures in the prefusion form, to date all postfusion structures of viral fusion proteins show a trimeric hairpin conformation.

Assays for Fusion Steps

A wide variety of assays has been developed for the individual fusion protein stages diagrammed in Figure 1. The characteristics of the fusion protein (and virus) in question affect the utility and specificity of such assays.

Overall changes in fusion protein conformation or oligomeric arrangement can be detected by following changes in epitope exposure or in sensitivity to protease or reducing agents; by using
classical methods to follow oligomers such as cross-linking, coimmunoprecipitation, or gradient sedimentation; and by employing electron microscopy and circular dichroism (e.g., 24). Careful analysis can often develop a general protein test such as protease sensitivity into a more targeted assay for a specific conformational change.

Exposure of the hydrophobic fusion peptide is reflected by its binding to detergents or to site-specific antibodies (47, 48). Membrane insertion of the fusion peptide may result in cofloatation of the virus or fusion protein with liposomes (49) or may be reflected in the receptor-independent interaction of the virus with target cells prior to fusion (50). More specifically, the membrane-inserted fusion peptide can be labeled with photoactivatable lipid probes (51, 52) or protected from recognition by a site-directed antibody (53).

Formation of an extended intermediate that bridges the virus and target membranes has been observed by electron microscopy (e.g., 40, 54, 55). The prehairpin trimer has been assayed by its specific interaction with an exogenous peptide derived from the outer layer. Such peptides can inhibit fusion and also act to capture this intermediate (54, 56–58). Detailed stages of hairpin formation can be dissected by such inhibitors and by biochemical and immunological analysis. Virus mutants that are blocked in specific steps of fusion have also been informative in defining fusion intermediates (e.g., 24).

The stages in lipid bilayer fusion can be followed independently. Either extracellular virus particles or expressed viral proteins can cause cell-cell fusion, which can be quantified by following the intercellular transfer of a content or membrane marker (59). Morphological detection of syncytia formation can also be a useful assay, but care is required because morphological changes can be inhibited by treatments that do not affect membrane fusion per se. Virus-cell fusion can be assayed by following the delivery of viral membrane or content markers to the host cell or the virus genome into the cytoplasm. Similarly, such markers can be used to follow virus fusion with artificial membranes such as liposomes or planar lipid bilayers. Formation of a hemifusion intermediate is detected as lipid mixing in the absence of content or inner leaflet mixing (60). Electrophysiological measurements of fusion can be performed in a number of contexts, often following the fusion of cells expressing virus fusion proteins with target cells or planar lipid bilayers (59). These methods can detect the formation of even very small fusion pores and allow assessment of their dynamics. In addition, content markers of varying size can be used to follow the enlargement of the fusion pore (59). Imaging of single virus particles can be used to monitor virus fusion during cell entry (12) or with membranes in vitro (61). These approaches allow localization of the fusion reaction within the host cell (15, 16) and dissection of the detailed kinetics of fusion steps (62, 63). Such imaging techniques hold considerable promise for the future.

EXAMPLES OF THE PRE- AND POSTFUSION CONFORMATIONS OF VIRUS FUSION PROTEINS

Virus fusion proteins are frequently grouped into classes (I, II, and III) based on structural and functional similarities (2, 4, 6, 64). These classifications highlight important features, although many differences exist within each class (1). This section briefly describes a fusion protein from each group, each of which forms a structurally distinct postfusion hairpin. Recent information on novel fusion protein structures is also considered.

Influenza Virus Hemagglutinin

The influenza virus hemagglutinin (HA) (Figure 2a) is a key example of the class I fusion proteins, all of which are initially synthesized as inactive precursors and rearrange into postfusion trimers.
Figure 2
Examples of the structures of virus membrane fusion proteins in the pre- and postfusion conformations. In each example the fusion protein is shown in color, with the fusion peptide or loop (labeled FP and FL, respectively) in green and the domains that will form the core trimer in yellow and red. Blue indicates both the residues that jackknife to allow movement of the outer layer and those that form the outer layer of the postfusion hairpin. The yellow domain carries the fusion peptide or loop in each case. The prefusion forms are oriented with the virus membrane at the bottom. The postfusion forms are oriented with the fused membrane at the top. For the postfusion forms, each postfusion trimer is shown on the left side, and on the right side the chain at the front of the trimer is pulled out to illustrate the hairpin. The C terminus that connects to the transmembrane domain is indicated as C'. Panels a, b, and c represent examples of class I, II, and III fusion proteins, respectively. (a) The influenza virus fusion protein hemagglutinin (HA), with HA1 indicated in gray and HA2 in colors in the prefusion structure (PDB ID 1RUZ). The postfusion structure (PDB ID 1QU1) shows HA2 alone, with the position of the fusion peptide (not present in the structure) indicated by an arrowhead. The yellow domain translocates to the top of the red α-helix in the postfusion form. (b) The alphavirus fusion protein E1. The prefusion structure (PDB ID 3N42) shows mature E2 in gray and E3 in pink. For clarity, only one E2-E1 heterodimer of the trimeric spike structure is shown. The postfusion structure shows only E1 (PDB ID 1RER). A flexible hinge connects the yellow and red domains and rotates during the transition to the postfusion form. (c) The rhabdovirus vesicular stomatitis virus fusion protein G. The prefusion trimer (PDB ID 26[6]) is also shown with the chain at the front pulled out (right) to enable comparison with postfusion G (PDB ID 2CMZ). The yellow domain rotates toward the target membrane during the transition to the postfusion form.

containing a central α-helical coiled coil. This group includes proteins from the orthomyxoviruses, paramyxoviruses, retroviruses, filoviruses, arenaviruses, and coronaviruses (reviewed in 2).

The influenza HA is synthesized as a fusion–inactive precursor HA0. Cellular proteases cleave HA0 to produce N-terminal HA1 and C-terminal membrane-anchored HA2, which remain linked by a disulfide bond (24). Structures have been determined for the HA0 (65), prefusion HA1/HA2
(66), and postfusion HA2 (67) forms, making HA one of the best-understood fusion proteins. There are also pre- or postfusion structures for a number of the other class I proteins, in particular those of the parainfluenza virus F protein in its uncleaved precursor, prefusion, and postfusion forms (68–70).

Influenza HA is a trimer and forms a vertically oriented structure on the virus surface with the HA1 globular head region at the top and a stalk region composed of HA2 connecting to the TM domain (Figure 2a). HA1 contains the receptor-binding site, which interacts with sialic acid residues on glycoproteins and glycolipids (37). The HA2 stalk is composed of a three-stranded α-helical coiled coil. In the HA0 form, the protease cleavage site is in an accessible loop near the base of the stalk. Cleavage causes little change in the overall structure but acts to free the ∼20–25-residue fusion peptide at the N terminus of HA2. Following cleavage, the fusion peptide becomes buried in a pocket within the trimer interface of the stalk.

Upon exposure to low pH in endosomes, the HA1 head regions move apart, although HA1 and HA2 remain linked by the disulfide bond (71). The HA2 fusion peptide is released from the trimer interface, in part by protonation of charged residues within the pocket (72). A loop region just C-terminal to the fusion peptide becomes an extension of the α-helical stalk, moving the fusion peptide about 100 Å toward the top of the molecule, where it interacts with the target membrane. A region of the original α-helix at the base then jackknifes to allow the packing of the C-terminal region of HA2 in the grooves of the coiled coil, forming the outer layer of the postfusion hairpin and bringing the fusion peptide and TM domains to the same end of the trimer. Evidence suggests that refolding of several target membrane–inserted HA trimers is required to mediate membrane fusion (62, 73), whereas trimers outside of the contact region may play a role in pore expansion (74).

**Alphavirus E1 Protein**

The alphavirus E1 protein is discussed as an example of the class II fusion proteins, which also include the flavivirus E (75–78), rubivirus (rubella virus) E1 (79), and phlebovirus Gc (80) proteins. These proteins are all initially synthesized as part of a polyprotein that also contains an N-terminally located companion or chaperone protein. All are triggered by low pH during endocytic entry.

The structure of alphavirus E1 has been determined in association with the companion protein (81, 82), as a prefusion monomer (64, 83), and as a postfusion trimer (84). The prefusion form of alphavirus E1 is an elongated molecule with three domains (DI–III) composed primarily of β-sheets. The central DI connects via a flexible hinge to DII with the fusion loop at its tip, and on the other side via a linker region to DIII, which has an immunoglobulin (Ig)-like fold. The C terminus of DIII connects to a stem region followed by the TM anchor. During biosynthesis, E1 associates with the chaperone/companion protein p62 (also termed PE2) within the endoplasmic reticulum (85). p62 is processed to the mature TM E2 and the peripheral E3 peptide in the late secretory pathway, with minimal change in protein conformation other than disordering of the cleaved E3-E2 linker (82). The bound E3 protects the virus from low pH during exit. Maturation is completed when E3 is released at extracellular neutral pH, thus priming the virus for fusion during endocytic entry (86). On the mature virus surface, the proteins are organized as spikes composed of trimers of E2-E1 heterodimers. E2 is an elongated molecule with three Ig-like domains. The E2-E1 heterodimers are tightly but noncovalently associated, with E1 oriented tangential to the virus membrane and the fusion loop clamped between domains A and B of E2. A required first step in fusion is the release and rearrangement of E2-E1 interactions at low pH (87). E1 then inserts into the target membrane via the fusion loop at the tip of DII. Three DI-DII regions associate to form the core trimer, and DIII and the stem pack against it to generate a postfusion
conformation with the fusion loops and TM domains at the same end of the molecule (84). Thus, in spite of its very different structure, during fusion alphavirus E1 refolds to form a trimeric hairpin arrangement similar to that of postfusion HA.

The available information for the flavivirus, rubella virus, and phlebovirus fusion proteins suggests that their structures in the pre- and postfusion forms are quite similar to those of alphavirus E1. Among the interesting differences, the rubella virus fusion protein E1 contains two fusion loops at the tip of DII (79). This extensive membrane-interacting surface also contains a metal-binding site that can interact with calcium or sodium, a feature that is unique among fusion proteins to date. Although very different from p62 in structure, the flavivirus prM protein acts similarly to protect the fusion protein from low pH during exit and is proteolytically processed to prime the virus for fusion and infection (88–90). In contrast, the rubivirus E2 and phlebovirus Gn companion proteins are not proteolytically processed (reviewed in 91), and pH protection and priming of these viruses thus appear to use mechanisms that differ from those of alphaviruses and flaviviruses.

Vesicular Stomatitis Virus G Protein

The third class of fusion proteins includes the G protein of vesicular stomatitis virus (VSV), the gB proteins of herpes simplex virus and Epstein–Barr virus, and the gp64 protein of baculovirus (reviewed in 4, 92). The postfusion structures of all of these proteins have been determined; both the pre- and postfusion structures of VSV G are known (93, 94) and are discussed here.

VSV G is a trimer in the prefusion form and contains two fusion loops (Figure 2c) (94). Unlike the examples of influenza HA and alphavirus E1, in the prefusion G protein the fusion loops are not buried and are pointing down toward the virus membrane. As with the class II proteins, the G fusion loops are located at the tip of an elongated β-sheet-rich domain, but its topology differs from that of the class II proteins. As with the class I proteins, the G trimer also contains a central α-helical coiled coil. At low pH, G undergoes dramatic rearrangements involving domain rotations that reorient the fusion loops toward the target membrane, extension of the coiled coil, and fold-back of a C-terminal region (Figure 2c) (93). The net result is the generation of a trimeric hairpin, as in the case of the class I and class II fusion proteins. G does not undergo proteolytic maturation and is not synthesized with a companion protein. The low-pH-triggered conformational changes in G are reversible in the absence of a target membrane (4). Why G does not progress to irreversible inactivation during transit through the acidic exocytic pathway is intriguing but still unclear (1, 4). However, given this reversibility, G may not require a pH protection or priming mechanism.

Novel Structures and Puzzles

Although the fusion proteins described thus far all fit into a trimer of hairpins pattern, not every fusion protein appears to do so. The hepacivirus hepatitis C virus (HCV) and the pestivirus bovine viral diarrhea virus (BVDV) are both members of the Flaviviridae family. Both encode a polyprotein containing an N-terminally located E1 glycoprotein followed by an E2 glycoprotein, both of which are required for fusion. This arrangement and modeling studies suggested that the C-terminal E2 proteins could be class II fusion proteins, with the E1 proteins serving as chaperones (91, 95–98). However, recent structural studies demonstrate that the E2 protein from BVDV has an elongated structure that, despite containing Ig-like domains, does not contain an apparent fusion peptide or resemble class II or other structurally characterized fusion proteins (99, 100). These findings leave both the identity of the pestivirus fusion protein (E1, E2, or both) and its mechanism as open questions. Several characteristics suggested that the HCV E2 protein has a
similar structure to that of pestivirus E2 (reviewed in 99, 100). However, the structure of the HCV protein (101, 102), while containing an Ig-like domain, overall shows a very different, globular structure compared to pestivirus E2 and does not contain an obvious fusion peptide. Again, this leaves the identity and mechanism of the HCV fusion protein undefined.

Some members of the nonenveloped reoviruses encode transmembrane proteins termed FAST proteins that cause syncytia formation of infected cells (103). Given the small size of these proteins—some of which have ectodomains of only \( \sim 20 \) residues—definition of the mechanism by which they cause membrane fusion and determination of the potential involvement of cellular proteins will be important to our understanding of membrane fusion. At the other extreme, poxviruses carry out membrane fusion via a complex of 11–12 nonglycosylated transmembrane proteins (104). The roles of these proteins during fusion have not yet been determined.

THE ALPHAVIRUS FUSION PATHWAY

The alphavirus E1 protein is used here to illustrate events that take place during virus membrane fusion (also reviewed in 105). Although information about all of these stages is incomplete, a fairly detailed model can be proposed from the results of structural, biochemical, and mutational analyses (Figure 3). As for all virus fusion proteins studied to date, alphavirus fusion occurs through the formation of a hemifusion intermediate and initial fusion pore (106). The alphavirus envelope proteins form a highly organized lattice on the virus surface, and cryo–electron microscopy reconstruction and fitting studies provide important information about the arrangement of the individual proteins in the prefusion virus particle (e.g., 83, 107). Importantly, fusion involves not only the refolding of the E1 protein as shown in the pre- and postfusion E1 structures (Figure 2b) but also reorganization of the E2-E1 heterodimer and the virus particle.

**Priming and pH Protection**

The cellular enzyme furin cleaves p62 in the late secretory compartment (108, 109). The p62 form of the virus will fuse at very low pH (\( \leq 5.0 \)), but maturation to E2 allows fusion in the pH range of early endosomes (\( \sim 6.0 \)) (87). The secretory pathway has a pH range that would trigger the mature virus, but E3 remains bound to E2 during transit of the envelope proteins to the cell surface, stabilizing the dimer against low pH (86). Mutation of key residues within the E3-E2 interface inhibits E1 transport and virus production, and both are rescued by neutralization of the exocytic pathway (110). Together, these data indicate that bound E3 inhibits premature fusion, whereas its release at neutral pH allows the virus to fuse during subsequent virus entry.

**Rearrangements of the E2-E1 Heterodimer**

The mature E2 protein regulates the fusion activity of E1 through stepwise rearrangements of the heterodimer interaction. The initial rearrangement of the heterodimer involves the movement of the E2 B domain, which flips up to uncap the E1 fusion loop (81, 82). This movement occurs by the destabilization of the interactions between the connector (a flexible ribbon that links E2 domain B to domains A and C) and the underlying E1 protein (111). This interaction is sensitive to low pH (111), and it may also be influenced by receptor binding to the E2 protein (82, 112, 113).

Further rearrangements of the heterodimer must occur in order for the E1 trimer to form, but the driving force for these rearrangements and their exact nature are unclear and are revisited with the discussion of the virus particle to follow.
Figure 3

A model for the steps in alphavirus membrane fusion. (a) The immature p62-E1 heterodimer (for clarity, only one of the three heterodimers of the trimeric spike is shown). E2, including domains A, B, and C, is shown in gray; E3 in pink; and E1 domains I, II, and III in red, yellow, and blue, respectively, with the fusion loop indicated by a star. (b) p62 processing by the host protease furin. E3 remains bound during transit through the low pH of the secretory pathway, protecting E1 from premature triggering. (c) E3 is released at neutral extracellular pH, priming the virus for fusion at the pH of the endosome compartment. (d) The mature trimeric spike containing E2-E1 heterodimers. (e) At low pH, domain B of E2 rearranges, exposing the E1 fusion loop. E2-receptor binding may also play a role in this conformational change. (f) Low pH triggers further rearrangements on the particle surface that release the heterodimer interaction. E1 inserts into the target membrane outer leaflet as a monomer. Three E1 proteins then interact to form the core trimer (indicated by a bracket), the target for binding and inhibition by exogenous DIII proteins. (g) Distortion and bending of the virus membrane and target membrane, mediated by fusion loop insertion, strain on the virus membrane, and initial movements of DIII to interact with the core trimer. (h) DIII-stem interaction with the core trimer, mediating hemifusion of the virus and target membranes. (i) Completion of DIII-stem packing drives fusion pore formation. This process may require several trimers at the fusion site, cooperative interactions between trimers, and interactions between the fusion loops and TM domains. The virus membrane is shown in purple and the target cell membrane in blue. See Figure 2b for the structures schematized in panels b and i.
The E1 Refolding Pathway

Although the pH dependence of the heterodimer interactions clearly affects the pH dependence of fusion, there are also independent requirements for low pH during E1 refolding. Following the uncapping of the fusion loop, E1 inserts into the target membrane as a monomer (38, 39, 114). Fusion loop insertion (and fusion) is promoted by the presence of cholesterol in the target membrane (reviewed in 105), and photolabeling studies demonstrate direct and specific binding of cholesterol to the inserted E1 protein (115). It is not yet clear whether the insertion of the fusion loop itself requires low pH or how it is promoted by cholesterol. The E1 monomers then associate reversibly to form the core trimer, stabilized in part by a circle of salt bridges within the trimer interface (116) but also by interactions between the fusion loops (117). The fold-back of the outer layer is promoted by a network of interactions between a conserved histidine residue on DI at the top of the core trimer and the flexible linker that joins DI and DIII (118). In this low-pH-dependent step, the linker acts to clamp and stabilize the core trimer. DIII and the stem region then sequentially bind to the core trimer, driving final homotrimer formation. In vitro studies indicate that once the core trimer is formed and clamped by the linker, the packing of the outer layer does not require low pH (42). The binding of DIII-stem to the core trimer is a high-affinity interaction ($K_d \sim 85$ nM) that is conserved across various alphaviruses (119).

Definitive measurements of the number of alphavirus E1 trimers required for fusion have not yet been performed. E1 membrane insertion is highly cooperative, and E1 trimers associate to form rings of 5–6 trimers (42, 84, 120). Even truncated core trimers composed of DI-DII alone have such interactions, suggesting that they are mediated by intramembrane contacts between the fusion loops of adjacent trimers (42). While indirect, these results support a role for cooperativity between multiple trimers in fusion.

Virus Particle Rearrangements

Cryo–electron microscopy studies have been performed on alphaviruses treated at acidic pH for varying times. In the absence of target membranes, relatively modest conformational changes involving swiveling movements within the spike complex are initially observed (121–124), with a possible preferential occurrence of these changes at the fivefold axis of icosahedral symmetry (123). Longer incubation times and/or more acidic pH levels lead to loss of symmetry or virus aggregation. When virus is mixed with liposomes, a study at neutral pH suggested preferential virus approach to the target membrane along the fivefold axis (125). Recently the virus–liposome interaction was compared at neutral pH versus a pH just below the virus fusion threshold (55). Virus–target membrane interaction is observed only under low-pH conditions. Although overall the icosahedral symmetry of the particle is maintained, rearrangement occurs specifically in the zone of membrane contact. The central E2 protein in the trimer does not significantly change position, but the target membrane promotes or captures a monomeric form of E1, which forms a bridge connecting the virus and target membranes. The observed distance between the bridged membranes compared with the length of prefusion E1 suggests the formation of an extended conformation through changes in the E1 DI-DIII linker, the DI-DII hinge, or the stem region, or through a combination of these events. These results are in keeping with an initial decapping of the E1 fusion loop and its membrane interaction, but the more extensive subsequent rearrangements of the E2 protein remain to be visualized.

INHIBITION OF VIRUS FUSION

Virus fusion reactions can serve as targets for antiviral therapy (reviewed in 1, 126, 127) and for inhibition by host immune responses including interferon-inducible transmembrane proteins.
A number of steps in fusion protein rearrangement can act as antiviral targets. For example, formation of the core trimer may be altered by small molecules that insert into conserved hydrophobic pockets (e.g., 129–132). Peptides derived from the outer layer can inhibit final hairpin formation by binding to the core trimer (e.g., 9, 56, 57, 133, 134). Such peptide design is the basis of enfuvirtide, a licensed antiretroviral therapeutic (126). Monoclonal antibodies can block fusion via inhibition of the required particle rearrangements (e.g., 135). More generally, the membrane-bending steps involved in fusion can be inhibited by intercalation of lipids or other amphiphiles into the virus membrane (reviewed in 128, 136). Many of these amphiphiles act through the photoactivated oxidation of virus envelope lipids, thereby making the virus membrane physically resistant to fusion (137, 138). While such molecules act as broad-spectrum inhibitors of enveloped viruses, host cells are relatively resistant due to their capacity to synthesize lipids and repair membranes (139, 140). Fusion inhibitors such as those summarized here have been important research tools and will hopefully yield additional useful therapeutics in the future.

**CELLULAR HOMOLOGS OF VIRAL FUSION PROTEINS**

The class I fusion proteins have cellular counterparts in the SNARE proteins (141). SNARES, with the participation of additional proteins, mediate the specific and regulated fusion reactions of intracellular membrane vesicles (142, 143). SNARE proteins on the vesicle and target membranes interact in trans to form a coiled coil composed of four \( \alpha \)-helices, analogous to the postfusion structure of the class I proteins.

A recent report defined the postfusion structure of EFF-1, a cell-cell fusion protein in *Caenorhabditis elegans* (144). The structure is a trimer analogous to the postfusion trimers of viral class II fusion proteins but with an acidic patch in the fusion loop that precludes its membrane insertion. Fusion is driven by the trans-trimerization of EFF-1 proteins on the surface of two cellular partners. As with class II viral fusion proteins, EFF-1 fusion is blocked by the addition of exogenous domain III, supporting a mechanism involving the fold-back of domain III and the stem against the core trimer. Thus, in each of these examples, cellular fusion proteins present on the two membranes interact in trans to drive the fusion reaction.

**SUMMARY POINTS**

1. Enveloped viruses use the energy stored in metastable transmembrane fusion proteins to drive the fusion of the virus membrane with that of the host cell. To mediate membrane fusion, fusion proteins insert a fusion peptide or loop into the target membrane and refold to a trimeric hairpin conformation.

2. These fusion protein conformational changes are induced by specific triggers that can include receptor binding or exposure to the low pH of endocytic compartments.

3. Although structures of a number of viral fusion proteins provide key insights into their functions, the transition between the pre- and postfusion conformations remains unclear. Several viruses appear to have fusion mechanisms that do not fit the current paradigms.

4. Virus fusion can be targeted by the immune response, and fusion inhibitors can act as antiviral therapies.
FUTURE ISSUES

1. The insertion of the fusion peptide or loop into the target membrane is not well understood. How does insertion occur, and what are the interactions of the protein with membrane lipids? Are there specific and required interactions of the fusion peptide with the transmembrane domain or between the fusion peptides of adjacent trimers?

2. The pre- and postfusion conformations of fusion proteins were defined as static structures using crystallography. By contrast, the intermediates that link them are dynamic and remain relatively uncharacterized. These intermediates may also be fertile ground for the development of fusion inhibitors.

3. All of the currently characterized postfusion structures are trimers, including those of fusion proteins that are dimers in their prefusion forms. Why is the trimeric conformation favored, and will exceptions to this paradigm be found?

4. Do viruses preferentially use fusion proteins at specific locations on the virus particle to carry out fusion? What are the importance and role of bystander proteins—fusion proteins outside of the membrane fusion site—in driving fusion?

5. How do the fusion proteins of hepatitis C virus, pestiviruses, poxviruses, and reoviruses (FAST proteins) mediate fusion?

6. What are the evolutionary relationships between viral and cellular fusion proteins? Are there cellular homologs of each of the fusion protein classes? What other types of cellular fusion proteins remain to be discovered?

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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LITERATURE CITED


111. Fields W, Kielian M. 2013. A key interaction between the alphavirus envelope proteins responsible for initial dimer dissociation during fusion. *J. Virol.* 87:3774–81


RELATED RESOURCES

History of structural studies of the influenza virus fusion protein: http://virologyhistory.wustl.edu/influenza.htm
Oral histories by the influenza researchers Don Wiley, John Skehel, Ian Wilson, and Judy White: http://virologyhistory.wustl.edu/contributors.htm
Online lectures on virus entry by Ari Helenius: http://www.ibiology.org/ibioseminars/microbiology/ari-helenius-part-1.html
Online lectures on virus structure and membrane fusion proteins by Stephen Harrison: http://www.ibiology.org/ibioseminars/microbiology/stephen-c-harrison-part-1.html
“Snapshot” comparing the structures of viral and cellular fusion proteins by Sebastien Igonet and Felix A. Rey: http://dx.doi.org/10.1016/j.cell.2012.11.041
Animation summarizing the changes in structure of VSV G protein during fusion: http://www.sciencemag.org/content/315/5813/843/suppl/DC1 (movie 1)
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