

12. Molecular Mechanisms of Flaviviral Membrane Fusion

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Abstract

Enveloped viruses rely on transmembrane fusion proteins to fuse the viral membrane to the host-cell membrane and deliver the viral genome into the cytoplasm for replication. Although the structures and evolutionary origins of viral fusion proteins vary widely, all fusion proteins use the same physical principles and topology to drive membrane fusion. First, exposure of a hydrophobic fusion anchor allows them to insert into the host-cell membrane. Conserved hydrophobic residues in the fusion anchor penetrate part way into the outer bilayer leaflet of the host-cell membrane. The fusion protein then folds back on itself, directing the C-terminal viral transmembrane anchor toward the fusion loop. This fold-back forces the host-cell membrane (held by the fusion loop) and the viral membrane (held by the C-terminal transmembrane anchor) against one another until they fuse. In West Nile virus and other flaviviruses this fold-back in the fusion protein, E, is triggered by the reduced pH of an endosome, is accompanied by the assembly of E monomers into trimers, and occurs by domain rearrangement rather than by an extensive refolding of secondary structure. The rearrangement releases a large amount of energy, which is used to exert a bending force on the apposed viral and cellular membranes, propelling them toward each other and, eventually, causing them to fuse. The conserved regions of E that are responsible for driving membrane fusion are attractive targets for antiviral therapies.

Keywords

Virus cell entry, envelope protein, membrane fusion, fusion loop, conformational change, fusion inhibitor

1 Introduction

Flaviviruses and other enveloped viruses acquire a lipid bilayer membrane when they bud across the plasma membrane or the membrane of the endoplasmic reticulum (ER) during virion assembly (Lindenbach and Rice, 2001; Schlesinger and Schlesinger, 2001). During infection, the viral membrane must fuse to the host-cell membrane to deliver the viral genome into the cytoplasm for replication (Fig. 1). The fusion of the viral and host-cell membranes is therefore one of the key molecular events during viral entry. Adjacent membranes do not fuse spontaneously. Membrane fusion requires considerable energy – on the order of 100 kJ mol^{-1} (or 40 kT). Most of this energy is used to generate a force that is strong enough to bend the two membranes toward each other until they are separated by only a few Ångströms (Kozlov and Chernomordik, 1998; Kuzmin et al., 2001). In flaviviruses, the envelope (E) protein anchored in the viral membrane exerts this force during a pH-induced conformational rearrangement (Modis et al., 2004). In addition to their role as fusion proteins, flavivirus E proteins are also responsible for cellular attachment of the virus by binding to a receptor on the cell surface and are targets for antibody neutralization.

Fusion proteins of enveloped viruses fall into two structural classes. The influenza virus hemagglutinin (HA) is the prototype of class I fusion proteins (Skehel and Wiley, 2000), which encompass those of other orthomyxoviruses and paramyxoviruses, retroviruses, filoviruses, coronaviruses, and herpesviruses. Class II fusion proteins are a structurally and evolutionarily a distinct class of proteins found in the flaviviruses,

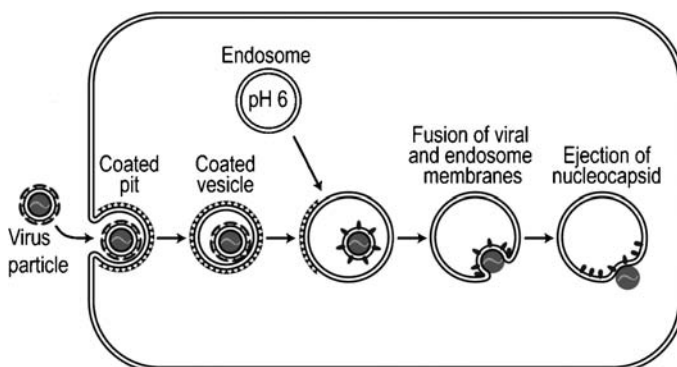


Figure 1. Cell entry of flaviviruses. Virus particles bind target cells through a surface receptor, which is linked to the clathrin-dependent endocytic pathway. Internalized vesicles fuse with endosomal compartments. The reduced pH of these compartments promotes conformational rearrangements in the viral envelope proteins that catalyze the fusion of the host-cell and viral membranes. Upon membrane fusion, the viral genome is delivered into cytoplasm. (See Color Plates)

including West Nile, dengue and yellow fever viruses, and alphaviruses, such as Semliki Forest, Sindbis, and the equine encephalitis viruses. Hepatitis C has a relatively similar genomic organization to the flaviviruses, and therefore most likely relies on a class II fusion protein as well. Crystal structures of several class I and class II fusion proteins (including West Nile E) before and after their fusogenic conformational rearrangements have provided a detailed molecular understanding of the fusion mechanism (Baker et al., 1999; Bullough et al., 1994; Caffrey et al., 1998; Chan et al., 1997; Chen et al., 1999; Fass et al., 1996; Kanai et al., 2006; Kobe et al., 1999; Lescar et al., 2001; Malashkevich et al., 1998; Modis et al., 2003, 2004, 2005; Nybakken et al., 2006; Rey et al., 1995; Rosenthal et al., 1998; Tan et al., 1997; Weissenhorn et al., 1997, 1998; Wilson et al., 1981; Xu et al., 2004a, b; Yin et al., 2005, 2006; Zhao et al., 2000). The structures show that the two classes of fusion proteins have completely different structural folds, and that fusion proteins from both classes nevertheless remarkably use the same physical principles and general topology to drive membrane fusion. First, the fusion protein inserts a hydrophobic fusion anchor partway into the outer bilayer leaflet of the host-cell membrane. The fusion anchor is either an N-terminal peptide (Gething et al., 1978), as in influenza and HIV (Gallagher, 1987), or an internal loop, as in SARS coronavirus (Supekar et al., 2004), avian sarcoma leucosis virus (Cheng et al., 2004) and all class II enveloped viruses including flaviviruses (Allison et al., 2001). Second, the fusion protein folds back on itself, directing the (C-terminal) viral transmembrane anchor toward the fusion anchor. This fold-back forces the host-cell membrane (held by the fusion anchor) and the viral membrane (held by the C-terminal transmembrane anchor) against each other, resulting in fusion of the two membranes. In this chapter, we describe our current paradigm of how West Nile E and other flaviviral fusion proteins drive viral membrane fusion, based on the existing structural and biochemical data. We also review how this knowledge may be translated into ant flaviviral therapies.

2 Overall Architecture of Flaviviral Membrane Fusion Proteins

In flaviviruses, membrane fusion is catalyzed by the envelope protein, E. Three-dimensional structures of four flaviviral E proteins in their native, or prefusion state have been determined at near atomic resolution (Table 1) (Kanai et al., 2006; Modis et al., 2003, 2005; Nybakken et al., 2006; Rey et al., 1995; Zhang et al., 2004). Figure 2 compares the three-domain structures of the E proteins of West Nile

Table 1. Flavivirus E fusion protein crystal structures and corresponding electron cryomicroscopy structures (listed in chronological order of publication)

Virus	Fusion state	Oligomeric state in solution	References
Tick-borne encephalitis	Prefusion	Dimer	Rey et al. (1995)
Dengue type 2	Prefusion	Dimer	Modis et al. (2003) and Zhang et al. (2003a, b, 2004)
Dengue type 2	Postfusion	Trimer	Modis et al. (2004)
Dengue type 3	Prefusion	Dimer	Modis et al. (2005)
Tick-borne encephalitis	Postfusion	Trimer	Bressanelli et al. (2004)
West Nile	Prefusion	Monomer	Kanai et al. (2006) and Nybakken et al. (2006)

virus (Kanai et al., 2006) and dengue virus (Modis et al., 2003). As expected from sequence identities of over 37% across the flavivirus genus, and of up to 49% between dengue and West Nile viruses, flaviviral E proteins share a common molecular architecture and the structures of the individual domains of West Nile and dengue E are very similar. Domain I, an eight-stranded β -barrel, organizes the structure. Two long insertions between pairs of consecutive β -strands in domain I form the elongated domain II, which bears the fusion anchor, or fusion loop, at its tip (Figs. 2 and 4). Domain II contains 12 β -strands and two α -helices. Domain III is an IgC-like module, with ten β -strands. However, unlike dengue and tick-borne encephalitis E proteins, West Nile E does not form head-to-tail dimers and is a monomer in solution (and in the crystalline form). The monomeric state of West Nile E in solution is mainly due to the different relative orientation of domain II with respect to domains I and III. Indeed, domain II participates in all of the dimer contacts in the dimeric dengue and TBE E structures, and the orientation of domain II in West Nile E is not compatible with dimer formation (Fig. 2).

Many of the antigenic sites on E map to domain III, as do most of the structural determinants of virulence and tropism (Rey et al., 1995; Sanchez et al., 2005). This observation, and the widespread occurrence of immunoglobulin modules in cell-adhesion proteins, suggests that domain III participates in attachment to a cellular receptor (Rey et al., 1995). Indeed, positively charged patches on the surface of domain III in dengue virus have been suggested to promote attachment by binding heparan sulfate on the cell surface (Chen et al., 1997). Also, while E proteins have very similar overall structures, they differ in the length and structure of surface-exposed loops, which may affect the specificity of receptor binding (Crill and Roehrig 2001; Hung et al., 2004; Rey et al., 1995). However, despite these hints on the basis of cellular attachment, a cellular

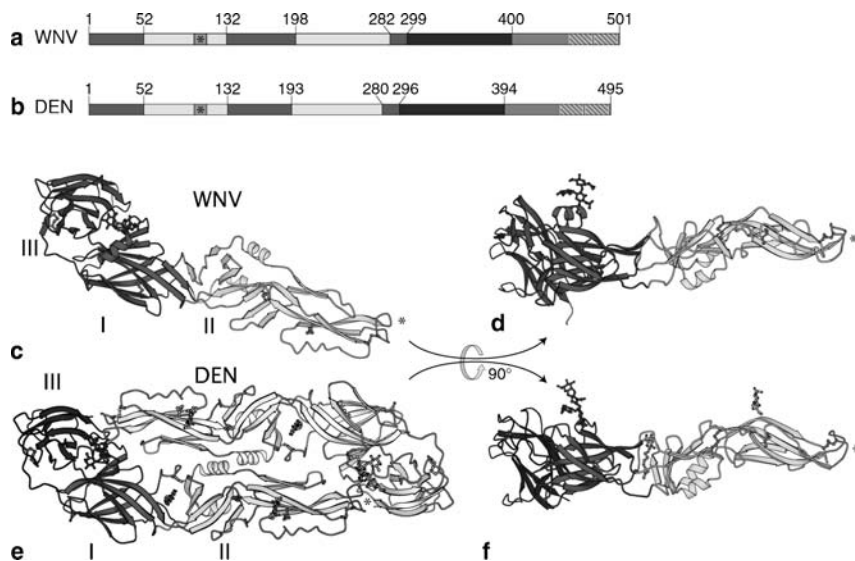


Figure 2. Representative flavivirus fusion protein structures. **(a)** The three domains of the fusion protein of West Nile virus, E: domain I (residues 1–51, 132–197, 282–298), domain II (residues 52–131, 198–281), domain III (residues 299–501). A 53-residue “stem” (cyan) links the ectodomains to a two-helix C-terminal transmembrane anchor (hatched areas). **(b)** The three domains of dengue type 2 E: domain I (residues 1–51, 132–192, 280–295), domain II (residues 52–131, 193–279), domain III (residues 296–495). A 53-residue stem links the ectodomains to the transmembrane anchor. **(c)** Crystal structure of West Nile E in the prefusion conformation (Kanai et al., 2006). The relative orientations of domains I and II are slightly different from those observed in the mature virus particle (Kanai et al., 2006; Mukhopadhyay et al., 2003). The fusion loops in **(a)**–**(f)** are marked with an asterisk. **(d)** View rotated 90° relative to **(c)**. **(e)** Crystal structure of dengue type 2 E in the prefusion conformation (Modis et al., 2003), as observed in the mature virus particle (Zhang et al., 2003a). A second subunit of E, forming the dimer found on the viral surface and in solution, is shown in light gray. **(f)** View rotated 90° relative to **(e)**, with the second subunit omitted for clarity. (See Color Plates)

receptor that specifically recognizes a protein epitope on an envelope protein of a class II enveloped virus has yet to be conclusively identified.

Flavivirus E proteins have either one or two N-linked glycosylation sites. West Nile virus and dengue virus use these glycans to attach to the surface of dendritic cells. In West Nile virus, the single glycan on E (at residue 154) is recognized by the C-type lectin DC-SIGNR (Davis et al., 2006). In the case of dengue virus, a different glycan (at residue 67) is recognized by a related lectin, DC-SIGN (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003). The basis for the differential recognition of the glycans on West Nile and dengue virions by different dendritic cell receptors may be due to a match in the spacing of the respective glycans on the viral surface with the spacing of the carbohydrate recognition

domains of the receptors (Kanai et al., 2006; Mitchell et al., 2001). Indeed, DC-SIGN appears to require two adjacent glycans approximately 18 Å apart for optimal binding, as illustrated by the binding pattern of DC-SIGN on dengue virus particles (Pokidysheva et al., 2006). In contrast, the spacing between glycans on the surface of West Nile virus is 50 Å. This is close to the 54-Å separation between carbohydrate recognition domains in the crystal structure of the DC-SIGNR tetramer (Feinberg et al., 2005), suggesting that DC-SIGNR could bind multiple (up to four) viral glycans simultaneously. In support of this notion, the specificity of carbohydrate recognition of DC-SIGN and DC-SIGNR depends largely on whether glycans can bind all four carbohydrate recognition domains simultaneously (Feinberg et al., 2005; Mitchell et al., 2001).

It is important to note that all the crystal structures of fusion proteins determined so far, from both classes and regardless of their conformational state, lack the C-terminal viral membrane anchor. This anchor consists of one or two transmembrane helices. The crystallized species should therefore be referred to strictly as soluble fragments of the ectodomains of the full-length fusion protein. Furthermore, all available crystal structures of class II fusion proteins also lack the “stem” region (Allison et al., 1999), a 30–55 amino acid linker between domain III and the C-terminal transmembrane anchor (Figs. 2a, b and 3). As will be discussed below, the stem region plays a key role in the final stages of membrane fusion. Its function is analogous to that of the “outer helix” in class I fusion proteins (Skehel and Wiley, 2000).

3 Maturation and Priming of Fusion-Competent Virions

All viral membrane fusion proteins rely on a proteolytic cleavage event to become primed to respond to the environmental conditions appropriate for fusion. These conditions are usually induced by the acidic pH of an endosome (Fig. 1), but for some class I enveloped viruses, such as HIV, co-receptor binding is required instead. In contrast to class I fusion proteins, however, class II fusion proteins rely on a priming proteolytic cleavage that does not cleave the fusion protein itself. Instead, class II proteins associate with a second, “protector” protein, called prM in flaviviruses (or E2 in alphaviruses). PrM is cleaved by furin-like proteases when immature virus particles assembled in the ER reach the trans-Golgi network (Stadler et al., 1997). The cleavage of prM to its mature product, M, releases a conformational constraint on E (the fusion

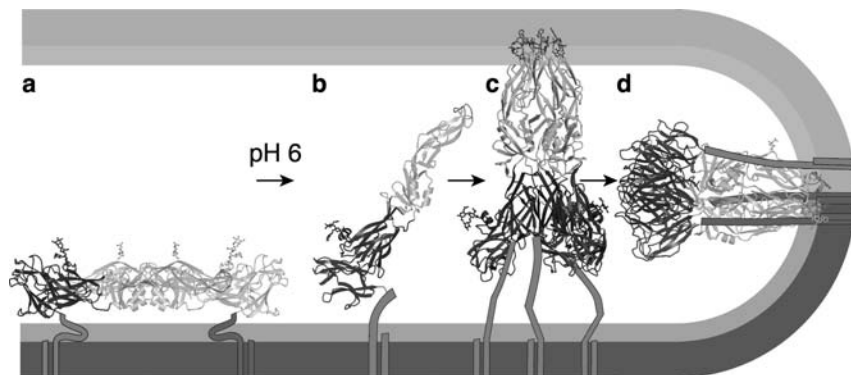


Figure 3. Pre- and postfusion structures of dengue E, and proposed intermediates. (a) A dimer of dengue E (Modis et al., 2003) molecules in the prefusion conformation as found on the viral surface, viewed perpendicular to the viral membrane. The fusion loop is buried in the dimer interface. The outer (proximal) bilayer leaflets of the cellular and viral membranes are shown to scale as blue and red bands, respectively. The thin outer layer within each leaflet represents the polar headgroup layer, and the thicker inner layer represents the hydrocarbon layer. The stem-anchor segments are absent from the crystal structure, but are represented here schematically as rods in the viral membrane. (b) Upon acidification of the solute in the endosome, domain II rotates 15–30° about a hinge in the domain II–domain I interface. This exposes the fusion loop, which then inserts into the host-cell membrane. (c) Insertion of the fusion loop into the target membrane leads to domain II self association to form trimers. The panel shows a hypothetical West Nile E trimer constructed by superimposing domain II of the West Nile E monomeric prefusion structure onto the dengue E trimeric postfusion structure (Modis et al., 2004). This intermediate bridges the viral and cellular membranes. (d) The postfusion, trimeric structure of dengue E. After insertion of their fusion loops into the target membrane, the fusion proteins form trimers and fold back on themselves, bringing the fusion loops close to the C-terminal transmembrane anchors. (See Color Plates)

protein), which allows E to reach its mature, prefusion conformation in a large rearrangement on the viral surface. The mature virus particles are then released from the host cell by exocytosis. In the mature conformation, the fusion protein is primed to respond to acidic pH and induce membrane fusion with a further conformational rearrangement.

Structures from electron cryomicroscopy of both immature and mature flavivirus particles provide a detailed picture of the rearrangement that accompanies maturation in these viruses (Kuhn et al., 2002; Mukhopadhyay et al., 2003; Zhang et al., 2003a, b). Unlike alphaviruses, which retain the $T = 4$ icosahedral packing of their envelope proteins during maturation (Ferlenghi et al., 1998; Mancini et al., 2000), flaviviruses undergo a dramatic rearrangement in the organization of their E proteins on the viral surface during maturation. Indeed, when the protector protein prM is cleaved in flaviviruses, the fusion protein

E breaks the $T = 3$ icosahedral symmetry of the immature virion (Zhang et al., 2003b) to adopt an unusual icosahedral herringbone pattern in the mature virion (Kuhn et al., 2002; Zhang et al., 2002). The E fusion proteins form dimers in the mature virion, including in West Nile virus, despite the preference for West Nile E to adopt a monomeric conformation in the absence of icosahedral constraints (see Fig. 2). This has led to the suggestion that icosahedral assembly might impose some physical strain on West Nile E, and that this strain could serve to “spring-load” E, allowing some of the energy required for membrane fusion to be stored in the metastable mature virus particle (Kanai et al., 2006). The key feature of the maturation process, however, is that cleavage of prM allows the fusion loop of E to reposition itself so that it is poised to insert into the host-cell membrane in response to acidification in the endosome. Mature virus particles are therefore infectious (Elshuber et al., 2003; Stadler et al., 1997), unlike immature virions (Guirakhoo et al., 1991, 1992), which are insensitive to pH. The flavivirus fusion loop is shielded from the viral surface in mature virions by E-E dimer contacts (Figs. 3a and 5a).

4 The Fusogenic Conformational Rearrangement

The three-dimensional structures of three class II fusion proteins (dengue, tick-borne encephalitis, and Semliki Forest viruses) in their postfusion states reveal striking differences from the prefusion forms (Fig. 3), and suggest a molecular mechanism for membrane fusion (see Fig. 5) (Bressanelli et al., 2004; Gibbons et al., 2004; Modis et al., 2004). Like class I fusion proteins, flaviviral E proteins and other class II fusion proteins are homotrimers in their postfusion conformations. E proteins form trimers from monomers on the viral surface, however, whereas class I proteins are trimeric in their prefusion state (Skehel and Wiley, 2000). A comparison of the pre- and postfusion states of influenza HA – the only example in its class where both structures are known for the same protein – shows that, as in class II fusion proteins, nearly all of the trimer contacts in the postfusion state are formed during the fusogenic conformational rearrangement.

Unlike influenza HA, which undergoes extensive refolding during membrane fusion, the three domains of class II fusion proteins retain most of their folded structures (Fig. 3). Instead, the domains undergo major rearrangements in their relative orientations, through flexion of the interdomain linkers. Domain III undergoes the most significant displacement in the fusion transition. It rotates by approximately 70° ,

and its center of mass shifts by 30–40 Å toward domain II. This shift brings the C terminus of domain III about 40 Å closer to the fusion loop, located at the tip of domain II. Domain II rotates 15–30° with respect to domain I about a hinge region near the interface with domain I (Modis et al., 2003). Mutations in this region affect the pH threshold of fusion in various flaviviruses (Beasley and Aaskov, 2001; Cecilia and Gould, 1991; Hasegawa et al., 1992; Hurrelbrink and McMinn, 2001; Lee et al., 1997; Monath et al., 2002). These conformational rearrangements place the end of domain III – and the beginning of the stem region that links domain III to the C-terminal viral transmembrane anchor – pointing toward the fusion loop (Fig. 3d) (Gibbons et al., 2004; Modis et al., 2004). A deep channel between domains II of adjacent subunits in the trimer extends from the C terminus of the crystallized fragment to the three clustered fusion loops at the tip of the trimer, in the flaviviral postfusion structures. In the full-length fusion proteins, it is thought that the stem binds in this channel in an extended, but mainly α -helical conformation (Modis et al., 2004; Zhang et al., 2003a). This proposed conformation for the stem places the viral transmembrane anchor in the immediate vicinity of the fusion loop, just as in the postfusion conformation of class I viral fusion proteins.

The fusion transition in flaviviruses is irreversible. The structural rearrangements just described may impart irreversibility by contributing a high barrier to the initiation of trimerization and an even higher barrier to the dissociation of postfusion trimers once formed. Moreover, many new polar and nonpolar contacts are formed during the fusion transition, in several different areas, mostly near the threefold axis of the trimer. The total surface buried in the dengue E trimer is 15,000 Å² (Modis et al., 2004), nearly four times more than is buried in the dengue prefusion E dimer. The stem, which is missing from currently available crystal structures, most likely forms additional contacts with the core trimer structure (Figs. 3 and 5). The stem does indeed promote trimer assembly *in vitro*, even in the absence of liposomes (Allison et al., 1999).

5 The Flaviviral Fusion Loop

In both class I and class II enveloped viruses, the process of viral membrane fusion begins with the exposure of a fusion anchor, and its subsequent insertion into the host-cell membrane. Fusion anchors from both viral classes vary in length but are in general rich in glycines and hydrophobic residues, particularly aromatic residues such as tryptophan

or phenylalanine. Viruses from different genera rarely have significant levels of sequence identity in their fusion proteins. The fusion anchor in class I fusion proteins – the “fusion peptide” – is a region of approximately 20 residues at or near the N terminus of the fusion protein. The crystal structure of the parainfluenza virus 5 fusion (F) protein in its prefusion form reveals the fusion peptide wedged between two subunits of the protein, in a partly extended, partly β -sheet and partly α -helical conformation (Yin et al., 2006). Structural studies on influenza HA in its postfusion conformation using NMR and other spectroscopic techniques show that the fusion peptide is mostly α -helical in character and that its structure changes only subtly as it inserts partway into the outer leaflet of the host-cell lipid bilayer (Dubovskii et al., 2000; Han et al., 2001). The fusion peptide is disordered or absent in all of the currently available postfusion class I protein crystal structures.

In contrast, the crystal structures of class II fusion proteins in postfusion conformations offer direct views of fusion anchors – in this case, fusion loops – as they insert into a target membrane (Fig. 4) (Bressanelli et al., 2004; Gibbons et al., 2004; Modis et al., 2004). Like the class I fusion peptide, the class II fusion loop penetrates only partway into the hydrocarbon layer of the target membrane. Exposed carbonyls and charged residues prevent the fusion loop from penetrating further than 6 Å (Bressanelli et al., 2004; Modis et al., 2004). In flaviviruses, the fusion loop adopts a conserved and tightly folded conformation, which is stabilized by a disulfide bond (Fig. 4a). The structure of the fusion

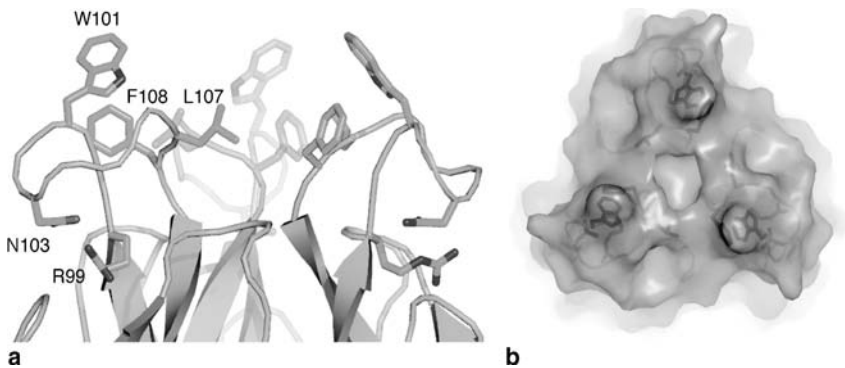


Figure 4. Close-up of the aromatic anchor formed by the fusion loop in dengue virus E. (a) In flaviviruses, the fusion loop has a rigid structure, with three conserved hydrophobic residues (Trp, Phe, and Leu) protruding at the tip of domain II. These three residues insert into the hydrocarbon layer of the target cell membrane. The three fusion loops in the dengue E postfusion trimer (Modis et al., 2004) are shown, with the residues of the one of the protomers labeled. (b) The clustered fusion loops form a nonpolar, bowl-shaped apex, with the three conserved hydrophobic residues at the rim of the bowl, shown here in surface representation. (See Color Plates)

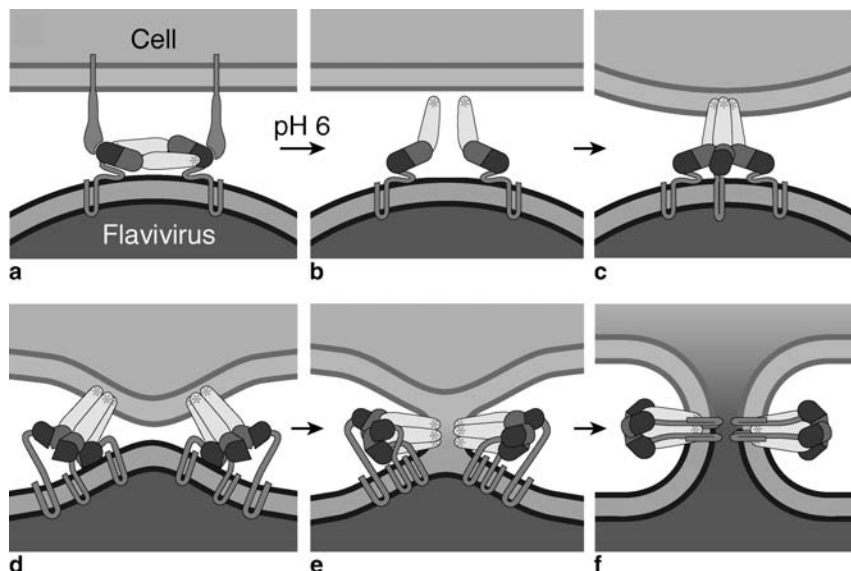


Figure 5. Proposed fusion mechanism for fusion mediated by class II fusion proteins. (a) The virus binds to a receptor on the cell surface. In flaviviruses, the fusion protein E binds the receptor. Following attachment, the virus is internalized to an endosome. (b) Acidic pH in the endosome causes domain II to hinge outward from the virion surface, exposing the fusion loop, and allowing E monomers to rearrange laterally in the plane of the membrane. (c) The fusion loop inserts into the hydrocarbon layer of the host-cell membrane, promoting trimer formation. (d) Formation of trimer contacts spreads from the fusion loop at the tip of the trimer, to the base of the trimer. The protein folds back on itself, directing the fusion loop toward the C-terminal transmembrane anchor. Energy release by this refolding bends the apposed membranes. (e) Creation of additional trimer contacts between the stem anchor and domain II leads first to hemifusion and then (f) to formation of a lipidic fusion pore. (See Color Plates)

loop is essentially identical in the pre- and postfusion conformations of the protein, suggesting that membrane insertion has no effect on the structure of the fusion loop. During the fusion transition, three conserved hydrophobic residues in the fusion loop (a tryptophan, leucine, and phenylalanine) become exposed on the molecular surface. Three fusion loops end up tightly clustered at the tip of the trimer in the postfusion conformation, where they form a crater-like surface with a hydrophobic rim (Fig. 4). Electron cryomicroscopy (Modis et al., 2004) and mutagenesis studies (Allison et al., 2001) confirm that these hydrophobic, mostly aromatic residues on the crater rim insert into the host-cell membrane, acting as an “aromatic anchor” for the fusion protein. The concave shape of the crater is thought to be important in generating distortions or perturbations in the host-cell membrane (Modis et al., 2004), which are required for fusion with the viral membrane (Tamm et al., 2002).

Semliki Forest virus E1 (a class II fusion protein) forms irregular clusters, or “rosettes,” consisting of 40–60 postfusion trimers through contacts between fusion loops in adjacent trimers (Gibbons et al., 2004). This is reminiscent of influenza virus HA, which aggregates into rosettes through interactions between the fusion peptide, at low pH and after proteolytic activation (Ruigrok et al., 1988). This fusion loop/peptide clustering may provide a mechanism for the direct coupling of several E1/HA trimers to work in concert around a single fusion site. However, flavivirus E protein trimers have never been observed to form clusters or rosettes, and current evidence suggests that although the energetics of membrane fusion requires more than one E trimer to deliver enough energy for fusion, the trimers work in concert without any direct protein-protein interactions between trimers (see below).

6 Mechanism of Flaviviral Membrane Fusion

Combined with previous knowledge, the structures of flavivirus fusion proteins in their postfusion states (Bressanelli et al., 2004; Modis et al., 2004) have elucidated how conformational changes in these proteins drive membrane fusion. The structures confirm two major principles of membrane fusion machineries: (1) the fusion protein must insert an anchor into each of the two membranes to be fused and (2) the protein folds back on itself in a thermodynamically favorable conformational rearrangement that drives membrane fusion by forcing the two anchors into close proximity of one another.

In the current model, viral membrane fusion proceeds as follows (Fig. 5). First, receptor binding by the envelope protein (which in flaviviruses is also the fusion protein) leads to clathrin-mediated endocytosis of the virus (Figs. 1 and 5a). When the virus reaches an endosomal compartment, the reduced pH of the lumen (~pH 6) causes an initial conformational rearrangement that exposes the previously buried fusion loop (Zhang et al., 2003a), at the tip of domain II. In flaviviruses, domains I and II flex relative to each other by 30° (Modis et al., 2004). This hinge motion causes domain II, and therefore the fusion loop, to swing away from the viral surface and toward the host-cell membrane (Fig. 5b). The notion that the domain I-domain II interface acts as a hinge early in the fusion transition is supported by the observation that mutations at this interface alter the pH threshold of fusion in various flaviviruses (Beasley and Aaskov, 2001; Cecilia and Gould, 1991; Hasegawa et al., 1992;

Hurrelbrink and McMinn 2001; Lee et al., 1997; Modis et al., 2003; Monath et al., 2002). As domain II swings away from the viral surface, constraints imposed by the tight packing of E on the viral surface are released, allowing E to diffuse freely in the plane of the viral membrane. The stem may provide enough flexibility at this stage to allow the E ectodomains to extend away from the membrane (Fig. 4b, c).

The second key step in the fusion process is insertion of the exposed fusion loop into the host-cell membrane (Fig. 5c). Flavivirus E proteins probably insert their fusion loops as monomers, but membrane insertion quickly promotes trimerization of the fusion loops (Stiasny et al., 2002), by lateral rearrangement of E monomers in the plane of the viral membrane. The resulting trimeric prefusion intermediate (Figs. 4c and 5c) bridges the host-cell and viral membranes, anchored by its fusion loops in the former and by the viral transmembrane anchors in the latter. This proposed intermediate is analogous to the “prehairpin” intermediate postulated for class I viral fusion mechanisms (Chan and Kim, 1998).

After the fusion loops have inserted into the host-cell membrane, formation of trimer contacts spreads from the fusion loops at the tip of the trimer to domain I at the base of the trimer. Domain II rotates and shifts, folding the stem and C-terminal anchor back toward the fusion loop (Fig. 5d), and burying additional protein surfaces. Energy released by this refolding drives the two membranes to bend toward one another (Baker et al., 1999; Melikyan et al., 2000b; Russell et al., 2001), as the C-terminal anchor is forced closer to the fusion loop, forming apposing nipples in the membranes (Fig. 5d) (Kuzmin et al., 2001). Fusion-loop insertion is expected to induce bilayer curvature as lipid molecules are laterally displaced in the leaflet. Such a curvature would stabilize the lateral surfaces of the nipples. The concave shape of the crater-like surface formed by the fusion loops at the trimer tip may also destabilize membranes, as has been postulated for fusion peptides in class I fusion proteins (Tamm et al., 2002). Based on the amount of energy required to deform lipid bilayers, the concerted action of at least three trimers is needed around the fusion site of enveloped viruses to provide sufficient energy to form nipples in the membranes. In the case of influenza, fusion requires at least three HA trimers (Danieli et al., 1996), and is more likely to be driven by rings, or “rosettes,” of 6–8 trimers (Blumenthal et al., 1996). The concerted refolding of each trimer in the ring allows the energy released by each refolding to be combined to generate a total force that is great enough to create the necessary distortions in the host-cell and viral membranes (Kozlov and Chernomordik, 1998;

Kuzmin et al., 2001). It is unclear exactly how many trimers are needed to drive membrane fusion in flaviviruses, or how their conformational changes are coupled. The clustering of fusion loops may provide a mechanism for the direct coupling of several E1 trimers to work in concert around a single fusion site in alphaviruses, but such clustering has not yet been observed in flaviviruses. It is possible that coupling occurs through the membrane: only when several trimers fold back in concert can they overcome the resistance of the membrane to deformation and reach their final, stable postfusion conformation (Fig. 5d–f).

As the fusion transition proceeds, the stem anneals onto the core of the trimer, along a channel that spans the length of domain II, at the interface between adjacent subunits (Figs. 3d and 5d–f). The annealing of the stem onto the domain II forces the fusion loop and the viral transmembrane anchor closer and closer, until the proximal leaflets of the two membranes fuse to form a “hemifusion stalk” (Fig. 5e). Hemifusion is an obligatory intermediate of membrane fusion in general (Kozlov and Chernomordik, 1998; Kuzmin et al., 2001; Razinkov et al., 1999). Figure 5e illustrates the need for shallow penetration of the viral fusion anchor into the host-cell membrane: assuming several trimers act in concert around a single fusion site, fusion anchors from different trimers cannot insert beyond the outer (proximal) leaflet of the lipid bilayer without colliding with each other. This constraint on the length of the fusion anchor holds true for both class I fusion peptides and class II fusion loops.

Hemifusion stalks can briefly “flicker” open into narrow fusion pores and then return to the hemifused state (Razinkov et al., 1999). To prevent transient fusion pores from closing, the stem must complete its annealing onto the core of the trimer, and the C-terminal transmembrane anchor must migrate into the pore (Fig. 5f). Indeed, the transition from hemifusion stalk to full fusion pore appears to require that the viral transmembrane anchor span the membrane completely, in all biological membrane fusion systems. This requirement was clearly demonstrated in an experiment in which the C-terminal transmembrane anchor of influenza HA was replaced with a glycosylphosphatidylinositol (GPI) lipid anchor (Kemble et al., 1994; Melikyan et al., 1995; Nussler et al., 1997), or with a half-length α -helical anchor (Armstrong et al., 2000). In both of these truncated HA mutants, the fusion reaction stalls at the stage of hemifusion. Other viral fusion proteins and cellular SNARE fusion proteins also require at least one transmembrane anchor (Bagai and Lamb, 1996; Dutch and Lamb, 2001; Januszski et al., 1997; McNew et al., 1997, 2000, 2000a; Saifee et al., 1998; West et al., 2001). In flaviviruses, the C-terminal anchor is also essential to resolve fusion

intermediates into a fully fused pore. Upon completion of fusion, the E trimer reaches the conformation seen in the postfusion crystal structures of dengue and tick-borne encephalitis E (Bressanelli et al., 2004; Modis et al., 2004). The stems (not present in the structures) are thought to dock along the surface of domains II, with the fusion loops and transmembrane anchors lying next to each other in the fused membrane (Fig. 5f).

Some viral fusion proteins require a specific lipid composition to catalyze membrane fusion. Alphavirus E1, for example, can only fuse membranes that contain cholesterol and sphingolipids (Nieva et al., 1994). Mutations in different regions of the Semliki Forest virus E1 lower its dependence on cholesterol and/or sphingolipids for membrane fusion (Chanel-Vos and Kielian 2004; Vashishtha et al., 1998); however, the molecular basis for this requirement is still not well understood (Chatterjee et al., 2002). In flaviviruses, cholesterol facilitates fusion, but neither cholesterol nor sphingolipids are essential for fusion (Stiasny et al., 2003).

7 Strategies for Fusion Inhibition

Many flaviviruses are important human pathogens including dengue, hepatitis C, yellow fever, Japanese encephalitis, and tick-borne encephalitis viruses in addition to West Nile virus (Burke and Monath, 2001). For most of these viruses, there are no specific treatments for infection, their control by vaccination has proved elusive, and the number of infections is on the rise. The three-dimensional structures of flavivirus fusion proteins described above suggest novel strategies for inhibiting viral entry by blocking membrane fusion. One such strategy stems from the discovery in dengue E of a long, tapering channel lined with hydrophobic side chains (Modis et al., 2003). In the crystal structure, the channel is occupied by a molecule of *n*-octyl- β -*d*-glucoside, a nonionic detergent. In the absence of detergent, a β -hairpin covering the channel swings shut, closing up the channel (Modis et al., 2003). The location of this ligand-binding pocket at the domain I–domain II interface coincides with that of mutations affecting the pH threshold of fusion in various flaviviruses (Beasley and Aaskov, 2001; Cecilia and Gould, 1991; Hasegawa et al., 1992; Hurrelbrink and McMinn, 2001; Lee et al., 1997; Monath et al., 2002). Most of these mutations affect side chains lining the ligand-binding pocket. The structure of dengue virus E in the postfusion conformation shows that this region acts as a hinge between domains I and II during the fusogenic conformational rearrangement

(see above) (Modis et al., 2004). The existence of a ligand-binding pocket just at the locus of a hinge suggests that compounds, which bind tightly to this position, may hinder the conformational changes required for membrane fusion (Fig. 6a). Such small molecules may have a similar mechanism of action as some of the well-studied anti-picornavirus compounds (e.g., disoxaril, pleconaril), which block a concerted structural transition in the icosahedral assembly by binding in a hydrophobic canyon on the viral surface (Smith et al., 1986). Alternatively, small molecules that pry open the β -hairpin in the pocket may inhibit infection by facilitating the fusogenic conformational change, causing premature triggering. Knowing the structure of the binding pocket with a bound ligand should guide efforts to design derivative ligands with higher affinities for use as inhibitors of flavivirus membrane fusion.

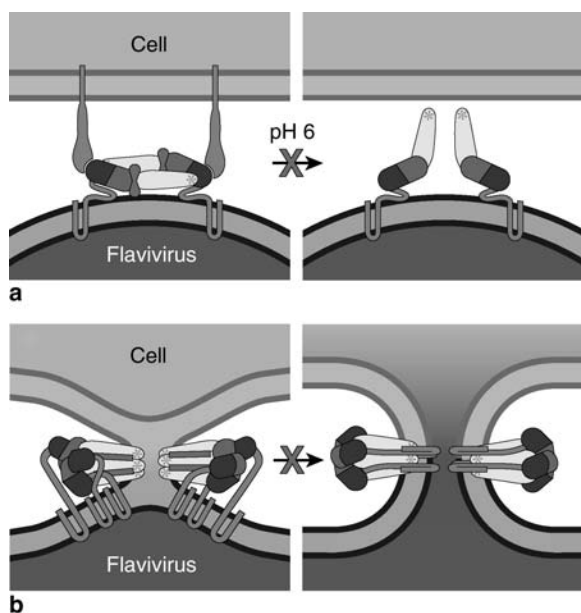


Figure 6. Fusion inhibition strategies. **(a)** The discovery of a ligand-binding pocket at the interface between domains I and II in dengue virus E (Modis et al., 2003), just at the locus of a hinge motion required for fusion, suggests that compounds inserted in the pocket might hinder the hinge motion and hence inhibit the fusion transition. This approach would block the first step in the fusion mechanism (Fig. 5a, b). **(b)** Peptides corresponding to the stem region of the fusion protein may inhibit viral entry by binding to the trimeric core of the protein in its postfusion conformation, and interfering with the folding back against it of the fusion protein's own stem (Modis et al., 2004). An analogous strategy has been successful with HIV gp41 (Baldwin et al., 2003; Kilby et al., 1998). This approach would block the last step in the fusion mechanism (Fig. 5e, f). Antibody and vaccine-based strategies also offer promise in the treatment of flaviviral infection by inhibiting membrane fusion (see text). (See Color Plates)

The postfusion structures of dengue (Modis et al., 2004) and Semliki Forest (Gibbons et al., 2004) viruses suggest a second possible strategy for fusion inhibition, related to an approach successful in developing the HIV fusion inhibitor T-20, or enfuvirtide (Baldwin et al., 2003; Kilby et al., 1998). Peptides corresponding to the stem region of the gp41 fusion protein inhibit HIV entry by binding to the trimeric, N-terminal “inner core” of the protein and interfering with the folding back against it of the stem and C-terminal viral transmembrane anchor. The expected annealing of the stem into a deep channel in flavivirus E proteins during the fusion transition (Figs. 3c, d and 5d–f) suggests that an analogous strategy may be successful with flaviviruses. Peptides derived from stem sequences could block completion of the fusogenic conformational change, by competing with the intramolecular stem for interaction with surfaces on domain II, at the trimer interface (Fig. 6b). Stem-like peptides or peptidomimetic compounds could thus inhibit viral membrane fusion in flaviviruses, and other class II enveloped viruses, by preventing the final folding back of the fusion protein, which is required to drive the viral and host-cell membranes together to the point of fusion. Indeed, a 33-residue peptide from the dengue E stem inhibits infection of cells in culture by both dengue and West Nile viruses at $<25 \mu\text{M}$ concentrations (Hrobowski et al., 2005).

A third strategy for fusion inhibition in flaviviruses is to use an antibody to prevent the fusogenic conformational change from proceeding to completion. Indeed the conventional view that neutralizing antibodies against flavivirus E proteins inhibit virus infection by blocking cellular attachment (Rey et al., 1995; Sanchez et al., 2005) was recently challenged when it was found that the domain III-specific antibody E16 protected mice from a lethal dose of West Nile virus even when the antibody was administered several days postinfection (Oliphant et al., 2005). E16 is a poor inhibitor of attachment and was therefore proposed to sterically interfere with a postattachment step – most likely one of the fusogenic domain rearrangements described above (Nybakken et al., 2005). This mechanism of antibody neutralization presents the advantage that it is independent of the receptor used by the virus to attach and enter the cell, which is particularly important given the apparent ability of flaviviruses to use multiple attachment receptors (Davis et al., 2006; Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003). Hence, a promising avenue in vaccine design is to raise antibodies against E protein antigens that either directly participate in fusion, such as the highly conserved fusion loop (Goncalvez et al., 2004; Oliphant et al., 2006; Stiasny et al., 2006), or that preclude the fusogenic domain rearrangement from proceeding to completion, such as the E16 epitope on domain III.

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