CHAPTER 8

CLASS II FUSION PROTEINS

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Abstract: Enveloped viruses rely on fusion proteins in their envelope to fuse the viral membrane to the host-cell membrane. This key step in viral entry delivers the viral genome into the cytoplasm for replication. Although class II fusion proteins are genetically and structurally unrelated to class I fusion proteins, they use the same physical principles and topology as other fusion proteins to drive membrane fusion. Exposure of a fusion loop first allows it to insert into the host-cell membrane. Conserved hydrophobic residues in the fusion loop act as an anchor, which penetrates only partway into the outer bilayer leaflet of the host-cell membrane. Subsequent folding back of the fusion protein on itself directs the C-terminal viral transmembrane anchor towards 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 each other, resulting in membrane fusion. In class II fusion proteins, the fold-back is triggered by the reduced pH of an endosome, and is accompanied by the assembly of fusion protein monomers into trimers. The fold-back occurs by domain rearrangement rather than by an extensive refolding of secondary structure, but this domain rearrangement and the assembly of monomers into trimers together bury a large surface area. The energy that is thus released exerts a bending force on the apposed viral and cellular membranes, causing them to bend towards each other and, eventually, to fuse.

INTRODUCTION

Enveloped viruses acquire a lipid bilayer membrane when they bud across the plasma membrane or the membrane of the endoplasmic reticulum (ER) during assembly of the virion.1,2 During infection, the viral membrane must be fused 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 the central molecular event during the entry of
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enveloped viruses into cells. Adjacent membranes do not fuse spontaneously; membrane fusion requires considerable energy (on the order of 100 kJ mol⁻¹ or 40 kT).³,⁴ Envelope proteins anchored in the viral membrane provide this energy in the form of a conformational rearrangement that bends the apposed membranes towards each other, inducing them to fuse.⁵⁻⁷ Most ‘fusion proteins’ (or their cleavage products) also effect cellular attachment of the virus prior to the membrane fusion event by binding to a receptor on the cell surface, except the paramyxo- and alphaviruses, in which a second envelope protein binds the receptor.

Fusion proteins of enveloped viruses fall into two structural classes. The influenza virus haemagglutinin (HA) is the prototype of class I fusion proteins,⁸ which encompass those of other orthomyxo- and paramyxoviruses such as measles virus, retroviruses such as human immunodeficiency virus (HIV), filoviruses such as Ebola virus, and coronaviruses such as SARS (see Chapters 4-6). Class II fusion proteins are a structurally and evolutionarily distinct class of proteins found in Flaviviridae, such as dengue, yellow fever, and West Nile viruses, and on alphaviruses, such as Semliki Forest and Sindbis viruses. Hepatitis C has a 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 before⁹⁻¹⁵ and after⁵⁻¹⁶⁻²⁹ their fusogenic conformational rearrangements have provided us with a detailed molecular understanding of the fusion mechanism (Table 1). The structures show that, despite the absence of similarities in the protein folds of the two classes, fusion proteins from both classes 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, thirty as in influenza and HIV,³¹ or an internal loop, as in SARS coronavirus,³² avian sarcoma leucosis virus³³ and all class II enveloped viruses.³⁴ Second, the fusion protein folds back on itself, directing the (C-terminal) viral transmembrane anchor towards 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

Figure 1. Cell entry of class II enveloped viruses. 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 acidic pH of these compartments causes conformational rearrangements in the viral envelope proteins that catalyze the fusion of the viral and host-cell membranes. Upon membrane fusion, the viral genome enters the cytoplasm.
anchor) against each other, resulting in fusion of the two membranes. In this chapter, I describe our current picture of how class II fusion proteins drive viral membrane fusion, based on the structural and biochemical data available to date.

OVERALL ARCHITECTURE

Three-dimensional structures of eight class II fusion proteins in their native, or prefusion states, that is, the conformation that they adopt on the surface of a mature virus particle, have been determined at near atomic resolution. Figure 2 shows the three-domain structures of E13 and E12, the fusion proteins of dengue virus (a representative flavivirus) and of Semliki Forest Virus (an alphavirus), respectively. The two proteins share a common molecular architecture, despite a lack of significant sequence similarity. 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, a fusion loop in class II proteins, at its tip (Figs. 2, 4). Domain II contains twelve β-strands and two α-helices. Domain III is an IgC-like module, with ten β-strands. Domain III contains most of the antigenic sites on E, as well as most of the structural determinants of virulence and tropism. This observation, and the widespread occurrence of immunoglobulin modules in cell-adhesion proteins, suggest that domain III participates in attachment to a cellular receptor. 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. Both E1 and E have one or more glycosylation sites. These glycans can aid viral attachment to the cell surface, in

Table 1. Class II fusion protein crystal structures and corresponding electron cryomicroscopy structures

| Virus                                | Fusion Protein | Fusion State | Quaternary Structure in Solution | References |
|--------------------------------------|----------------|--------------|----------------------------------|------------|
| Tick-borne encephalitis E1           | Prefusion      | Dimer        | 10                               |
| Semliki Forest virus E1              | Prefusion      | Monomer     | 12, 46, 50                       |
| Dengue virus type 2 E1              | Prefusion      | Dimer        | 13, 35, 45, 48                   |
| Dengue virus type 2 E1              | Postfusion     | Trimer      | 29                               |
| Semliki Forest virus E1              | Postfusion     | Trimer      | 55                               |
| Dengue virus type 3 E1              | Prefusion      | Dimer        | 14                               |
| Tick-borne encephalitis E            | Postfusion     | Trimer      | 56                               |
| West Nile virus E                    | Prefusion      | Monomer     | 93                               |
| Dengue virus type 1 E                | Postfusion     | Trimer      | 94                               |
| Chikungunya virus E1                | Prefusion      | Monomer     | 95                               |
| Sindbis virus E1                    | Intermediate   | Trimer      | 96                               |
| Dengue virus type 4 E                | Prefusion      | Dimer        | 97                               |
| Japanese encephalitis virus E        | Prefusion      | Monomer     | 98                               |

Table 1. Class II fusion protein crystal structures and corresponding electron cryomicroscopy structures
Figure 2. Representative class II fusion protein structures. A) The three domains of the flaviviral fusion protein of dengue virus (DEN), E: Domain I (residues 1-52, 133-193, 281-296), domain II (residues 53-132, 194-280), domain III (residues 297-394). A 53-residue ‘stem’ links the ectodomains to a two-helix C-terminal transmembrane anchor. B) The domains of an alphaviral fusion protein, Semliki Forest virus (SFV) E1: Domain I (residues 1-38, 131-169, 274-291), domain II (residues 39-130, 170-273), domain III (residues 292-381). A 32-residue ‘stem’ links the ectodomains to a single-helix C-terminal transmembrane anchor. C) Crystal structure of DEN E in the prefusion conformation, as found in the mature virus particle. The fusion loop in A-C is marked with an asterisk. A second subunit of E, forming the dimer found on the viral surface and in solution, is shown in light gray. D) View rotated 90° relative to C, with the second subunit omitted for clarity. E) Crystal structure of SFV E1 in the prefusion conformation, as found in the mature virus particle. The fusion loop is marked with an asterisk. F) View rotated 90° relative to E.
the case of dengue virus by binding to the lectin DC-SIGN.\textsuperscript{37,38} As expected from their sequence identities (≥ 37%), flaviviral E proteins have very similar overall structures, and differ only in the length and structure of surface-exposed loops, some of which have been implicated in receptor binding.\textsuperscript{10,39,40} Despite these hints on the basis of cellular attachment, however, a cellular receptor that specifically recognizes an envelope protein on a class II enveloped virus has yet to be conclusively identified, although candidate receptors for dengue virus type \textsuperscript{41} and West Nile virus\textsuperscript{42} were recently suggested.

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, and has been intentionally omitted in constructs targeted for crystallization to facilitate expression and handling, and to promote crystallization. The crystallized species are therefore referred to as soluble fragments of the ectodomains of the full-length fusion protein. Furthermore, all available crystal structures of class II fusion proteins lack the ‘stem’ region,\textsuperscript{43} a 30-55 amino acid linker between Domain III and the C-terminal transmembrane anchor (Figs. 2A-B, 3). As I will discuss 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.\textsuperscript{8}

**MATURATION AND PRIMING**

Both class I and class II fusion proteins rely on a proteolytic cleavage event to become primed to respond to the environmental conditions appropriate for fusion. These conditions are usually the acidic pH of an endosome (Fig. 1), but for some class I enveloped viruses, such as HIV, coreceptor 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 M (for membrane protein) in flaviviruses or E2 in alphaviruses. The protector protein is cleaved by furin when immature virus particles assembled in the ER reach the trans-Golgi network.\textsuperscript{44} The cleavage produces mature virus particles, which are then released from the host cell by exocytosis. The cleavage of the protector protein releases a conformational constraint on the fusion protein, which allows it to adopt its mature conformation (described above) in a large rearrangement on the viral surface. In the mature conformation, the fusion protein is primed to respond to acidic pH and induce membrane fusion with a further conformational rearrangement (described below).

Structures from electron cryomicroscopy of both immature\textsuperscript{45,46} and mature\textsuperscript{47-50} flavivirus and alphavirus particles, provide a detailed picture of the rearrangement that accompanies maturation in these viruses. Alphaviruses retain the $T = 4$ icosahedral packing of their envelope proteins, but domains that form spikes on the immature virion swing in towards the threefold symmetry axis, during maturation.\textsuperscript{46,50} The rearrangement is more dramatic in flaviviruses, in which the fusion protein E breaks the $T = 3$ icosahedral symmetry of the immature virion\textsuperscript{45} to adopt an unusual icosahedral herringbone pattern in the mature virion.\textsuperscript{47,51} In both alphaviruses and flaviviruses, the fusion proteins form dimers in the mature virion albeit in different configurations.\textsuperscript{10,12} The key feature of the maturation process in both genera, however, is that cleavage of the protector protein allows the fusion loop to reposition itself so that it is poised to insert into the host-cell membrane in response to acidification of the solute in the endosome. Mature virus particles are
therefore infectious,\textsuperscript{44,52} unlike immature virions,\textsuperscript{53,54} which are insensitive to pH. The fusion loop is shielded from the viral surface in mature virions by E-E dimer contacts in flaviviruses, or by protein E2 in alphaviruses (Figs. 3A,B, 5A).

\textbf{THE FUSOGENIC CONFORMATIONAL REARRANGEMENT}

The three-dimensional structures of four class II fusion proteins in their postfusion states\textsuperscript{29,55,56,94} reveal striking differences from the prefusion forms (Fig. 3), and suggest a

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Pre- and postfusion structures of class II fusion proteins, and proposed intermediates. A) A dimer of DEN E,\textsuperscript{13} and B) two SFV E1\textsuperscript{12} molecules in the prefusion conformation as found on the viral surface, viewed perpendicular to the viral membrane. The fusion loop is buried, either in the dimer interface (A), or under E2 (B). The outer (proximal) bilayer leaflets of the cellular and viral membranes are shown to scale as solid rectangles. 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. C,D) Upon acidification of the solute in the endosome, the domain II rotates 15-30\degree about a hinge in the domain II-domain I interface. This exposes the fusion loop, which then inserts into the host cell membrane. The postfusion, trimeric structures of DEN E\textsuperscript{29} (E) and SFV E1 (F).\textsuperscript{55} 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.}
\end{figure}
molecular mechanism for membrane fusion (see below and Fig. 5). Like class I fusion proteins, flaviviral E proteins and alphaviral E1 proteins are both homotrimers in their postfusion conformations. Class II proteins form trimers from monomers on the viral surface, while class I proteins are trimeric in their prefusion state.\(^8\) However, 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 about 70°, and its center of mass shifts by 30-40 Å towards domain II. This folding-over brings the C-terminus of domain III about 40 Å closer to the fusion loop, at tip of domain II (Fig. 3). Domain II rotates 15-30° with respect to domain I about a hinge region\(^13\) in which mutations affect the pH threshold of fusion in various flaviviruses.\(^57-62\) These conformational rearrangements position the end of domain III—and the beginning of the stem region that links domain III to the C-terminal viral transmembrane anchor—towards the fusion loop (Fig. 3E-F).\(^29,55\) A deep channel extends from the C-terminus of the crystallized fragment along the intersubunit contact between domains II to the fusion loops, in both the dengue and Semliki Forest virus postfusion trimer 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.\(^29,48\) This proposed stem conformation 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 class II viral proteins is irreversible. The refoldings just described may impart irreversibility by contributing a high barrier to initiation of trimerization and an even higher barrier to dissociation of postfusion trimers once they have formed. Moreover, many new polar and nonpolar contacts are formed during the fusion transition, in several different areas along the threefold axis of the trimer. The total surface buried is 13,000-15,000 Å\(^2\); nearly four times more than is buried in the prefusion dimer. The stem, which is missing from currently available crystal structures, most likely forms additional contacts with the core trimer structure. The stem does indeed promote trimer assembly even in the absence of liposomes.\(^43\)

THE FUSION LOOP

The process of viral membrane fusion in both class I and class II enveloped viruses 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 Trp or Phe. Sequence conservation is poor between fusion proteins of both classes. 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 envelope 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.\(^15\) 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.63,64 None of the currently available postfusion class I protein crystal structures contain information on the fusion peptide.

The recently determined crystal structures of class II fusion proteins in pre10,12-14 and postfusion29,55,56 conformations offer the first direct views of fusion anchors—in this case, the fusion loops—as they insert into a target membrane (Fig. 4). Like the class I fusion

Figure 4. Close-up of the aromatic anchor formed by the fusion loop in: A) dengue virus E, and B,C) SFV E1. In flaviviruses (A), three clustered fusion loops form a nonpolar, bowl-shaped apex, with three 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 fusion loop has a rigid structure. In alphaviruses, the fusion loop is flexible and can adopt very different conformations. Two conformations of the fusion loop from different subunits of the SFV E1 postfusion crystal structure are shown in B and C. The alphaviral fusion loops do not appear to cluster around the threefold axis of the trimer.
peptide, the class II fusion loop penetrates only partway into the hydrocarbon layer of
the target membrane. Exposed carboxyls and charged residues prevent the fusion loop
from penetrating further than 6 Å. In flaviviruses, the fusion loop adopts a tightly
folded conformation, which is stabilized by a disulfide bond (Fig. 4A). The structure of
the fusion 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 hydrophobic residues in the fusion loop (Trp,
Leu, and Phe) become exposed on the molecular surface. Three fusion loops end up in
close proximity at the tip of the trimer in the postfusion conformation, where they form
a crater-like surface with a hydrophobic rim (Fig. 3E). Electron cryomicroscopy and
mutagenesis studies 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, which are required for fusion.
In alphaviruses, the fusion loop is also rich in aromatic and other hydrophobic residues.
Unlike flaviviral fusion loops, however, alphavirviral fusion loops do not form trimer
contacts (Fig. 3F). Indeed, in the postfusion structure of the Semliki Forest virus E1 trimer,
the fusion loops have high temperature factors and exhibit a high degree of flexibility
despite the presence of two disulfide bonds. Thus, the structures of the fusion loops are
poorly defined, but each fusion loop seems to adopt a very different conformation (Fig.
4B,C). The fusion loops in the postfusion Semliki Forest virus E1 structure form quite
polar surfaces, with many mainchain carboxyls and some polar or charged sidechains
exposed on the surface. This suggests that, in contrast to flaviviral fusion loops, alphaviral
fusion loops either change their conformation upon membrane insertion to shield polar
groups from the membrane, or the fusion loops only interact with the polar headgroups
of the lipids, and do not penetrate into the hydrocarbon layer.
Semliki Forest virus E1 trimers form irregular clusters, or ‘rosettes’ of about 40-60
trimers through contacts between fusion loops in adjacent trimers. This is reminiscent
of influenza virus HA, which aggregates into rosettes through interactions between the
fusion peptide, at low pH and after proteolytic activation. 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 (see below).

MECHANISM OF MEMBRANE FUSION

Combined with previous knowledge, the structures of the fusion proteins from class
II viruses in their postfusion states have led to a much better understanding of how
conformational changes in the 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.
In the current model, viral membrane fusion proceeds as follows (Fig. 5). First,
receptor binding by an envelope protein, which in flaviviruses is also the fusion protein,
leads to clathrin-mediated endocytosis of the virus (Figs. 1, 5A). When the virus reaches
endosomal compartments the low pH of the lumen (pH 6) causes an initial conformational
rearrangement that leads to the exposure of the previously buried fusion loop at the
tip of domain II. In flaviviruses, domains I and II flex relative to each other by 30°. This hinge motion causes domain II, and therefore the fusion loop, to swing away from the viral surface and towards the host-cell membrane (Fig. 5B). Indeed, mutations at the domain I-domain II interface in various flaviviruses alter the pH threshold of fusion. 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 monomers to rearrange laterally in the plane of the membrane. The stem may also be able to extend away from the membrane at this stage. In alphaviruses, constraints are released in response to low pH by the dissociation of the protector (and receptor-binding) protein E2. This exposes the fusion loop and allows domain II of E1 to swing towards the nearest threefold symmetry axis in the virus particle in a 15° hinge motion relative to domain I, leading to the formation of trimer contacts with adjacent E1 molecules.

The second key step in the fusion process is insertion of the exposed fusion loop into the host-cell membrane (Fig. 5C). Alphaviral E1 has already formed some trimer contacts at this stage, but flaviviral E proteins probably insert their fusion loops as monomers. Membrane insertion probably catalyzes trimerization of the fusion loops, by lateral rearrangement of E monomers. This trimeric prefusion intermediate (Fig. 5C) bridges 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.\textsuperscript{68}

Upon insertion of the fusion loops into the host-cell membrane, formation of trimer contacts spreads from the fusion loops at the trimer tip to domain I at the trimer base. Domain II shifts and rotates, folding the stem and C-terminal anchor back towards the fusion loop (Fig. 5D), and burying additional protein surfaces. Free energy released by this refolding drives the two membranes to bend towards each other,\textsuperscript{5-7} as the C-terminal anchor is forced closer to the fusion loop, forming apposing nipples in the membranes (Fig. 5D).\textsuperscript{3} Fusion-loop insertion may induce positive bilayer curvature, which 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 have a destabilizing effect on the membrane, as has been postulated for fusion peptides in class I fusion proteins.\textsuperscript{65} Based on the energy required to deform lipid bilayers, it seems likely that a ring of trimers refolding in concert is needed to provide sufficient energy to form nipples in the membranes.\textsuperscript{3,4} It is unclear exactly how many trimers are needed to drive membrane fusion in class II viruses, nor how their conformational changes are coupled. In the case of influenza, fusion requires the concerted action of at least three HA trimers,\textsuperscript{69} and is more likely driven by rings of 6-8 trimers.\textsuperscript{70} 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 been observed in flaviviruses. It is possible that coupling occurs via the membrane: only when several trimers fold back in concert can they overcome the resistance of the membrane to deformation and reach their final, most stable postfusion conformation (Figs. 5D-F).

As the fusion transition proceeds, the stem zippers up onto the core of the trimer, along a channel that spans domain II, at the intersubunit contact regions (Figs. 3, 5D-F). The zipperring up 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 thought to be an essential intermediate of membrane fusion.\textsuperscript{3,4,71} (Fig. 5E) illustrates the need for shallow penetration of the viral fusion anchor into the host-cell membrane: assuming several trimers do in fact act in concert around a single fusion site, fusion anchors from different trimers would collide if they inserted beyond the outer (proximal) lipid bilayer leaflet. 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 ‘flicker’ open into narrow fusion pores.\textsuperscript{71} In order to prevent the transient fusion pores from closing, the stem must complete its zipperring up 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. Thus, the replacement of the C-terminal transmembrane anchor of influenza HA with a glycosylphatidylinositol (GPI) lipid anchor,\textsuperscript{72-74} or with a half-length protein α-helical anchor,\textsuperscript{75} stalls the fusion reaction at the stage of hemifusion. Other viral fusion proteins and cellular SNARE fusion proteins also require at least one transmembrane anchor.\textsuperscript{76-83} Upon completion of fusion, the trimer has reached the conformation seen in the postfusion crystal structures.\textsuperscript{29,55,56} The stems (not present in the structures) are docked along the surface of domains II, and the fusion loops and transmembrane anchors lie next to each other in the fused membrane (Fig. 5F).

Some class II fusion proteins, including those of alphaviruses, can only fuse membranes containing cholesterol and sphingolipids.\textsuperscript{84} The structural basis for this requirement is still
not well understood. Several mutations in different regions of the Semliki Forest virus fusion protein E1 lower its dependence on cholesterol and/or sphingolipids for membrane fusion. It is unclear, however, whether the lower dependence on cholesterol of these mutants is due to an apparent destabilization of the E1 homotrimer, or to the different physical properties of membranes lacking cholesterol and sphingolipids. In flaviviruses, cholesterol facilitates fusion, but neither cholesterol nor sphingolipids are essential for fusion.

STRATEGIES FOR FUSION INHIBITION

Many class II viruses, especially the flaviviruses, represent important human pathogens such as dengue, hepatitis C, yellow fever, West Nile, Japanese encephalitis and tick-borne encephalitis viruses. 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. Recently determined three-dimensional structures of class II fusion proteins suggest new strategies for inhibiting viral entry by blocking membrane fusion. One such strategy stems from the discovery in dengue virus E of a long, tapering channel lined with hydrophobic side chains. In the crystal structure, the channel is occupied by a molecule of the detergent n-octyl-β-D-glucoside. In the absence of detergent, a β-hairpin covering the channel swings towards the protein, and closes up the channel. 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. Most of these mutations involve side chains lining the ligand-binding pocket. The postfusion structure of dengue virus E shows that this region acts as a hinge between domains I and II during the fusogenic conformational rearrangement (see above). The opening up of a ligand-binding pocket just at the locus of a hinge suggests that compounds tightly inserted at this position might hinder the conformational changes required for membrane fusion (Fig. 6A). The mechanism of action of such compounds might resemble that of some of the well-studied antipicornaviral compounds, which block a concerted structural transition in the icosahedral assembly. Alternatively, small molecules that pry open the β-hairpin on binding in the pocket may inhibit infection by facilitating the low-pH conformational change, causing premature triggering. Knowledge of the structure of the binding pocket with a bound ligand will guide efforts to design derivative ligands with higher affinities for use as inhibitors of flaviviral membrane fusion.

The postfusion structures of dengue and Semliki Forest viruses suggest a second possible strategy for fusion inhibition, related to an approach successful in developing an HIV antiviral compound. 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 way in which the stem is likely to fold back in class II viral fusion proteins (Figs. 3, 5D-F) suggests that an analogous strategy may be successful with class II viruses. Peptides derived from stem sequences could block completion of the fusogenic conformational change, by competing with the 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 class II enveloped viruses by preventing the final folding back of the fusion protein that is required to drive the viral and host-cell membranes together to fuse.
All viral membrane fusion proteins use the same physical principles and topology to drive membrane fusion. Class II fusion proteins are structurally and evolutionarily distinct class of proteins found in Flaviviridae, such as dengue, yellow fever, and West Nile viruses, and on alphaviruses, such as Semliki Forest and Sindbis viruses. Unlike class I fusion proteins such as influenza HA, which undergoes extensive refolding during membrane fusion, the three domains of class II fusion proteins retain most of their folded structures. Instead, the domains undergo major rearrangements in their relative orientations, through flexion of the interdomain linkers. Class II fusion proteins rely on a hydrophobic fusion loop to anchor themselves in the target cellular membrane. Like the class I fusion peptide, the class II fusion loop penetrates only partway into the hydrocarbon layer of the target membrane. Class II fusion proteins drive membrane fusion in a foldback rearrangement of a trimeric protein assembly. Crystal structures of class II envelope proteins have suggested two specific strategies for fusion inhibition, with hydrophobic small molecules and “stem”-like peptides or peptidomimetics, respectively.
NOTE ADDED AFTER PROOFS

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