Virus membrane-fusion proteins: more than one way to make a hairpin

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Abstract | Structure–function studies have defined two classes of viral membrane-fusion proteins that have radically different architectures but adopt a similar overall 'hairpin' conformation to induce fusion of the viral and cellular membranes and therefore initiate infection. In both classes, the hairpin conformation is achieved after a conformational change is triggered by interaction with the target cell. This review will focus in particular on the properties of the more recently described class II proteins.

Clathrin
A cellular protein that is composed of three heavy chains and three light chains. Clathrin is the main component of the coat that is associated with clathrin-coated vesicles, which are involved in membrane transport in both the endocytic and biosynthetic pathways.

Caveolae
Specialized regions that contain the protein caveolin and form flask-shaped, cholesterol-rich invaginations of the plasma membrane.

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Enveloped animal viruses are covered — or 'enveloped' — by a lipid bilayer that is derived from the host-cell membrane during virus budding. This membrane protects the genetic material of the virus until it is delivered into the cytoplasm of a new host cell to initiate infection. Delivery takes place by fusion between the virus membrane and the membrane of the host cell. This membrane-fusion reaction is a crucial step in virus infection and is mediated by transmembrane (TM) proteins that are anchored on the virus surface. Virus membranes are relatively simple compared to host-cell membranes, and the process of virus–host-cell fusion is devoid of the additional regulatory and recycling proteins that are present in many cellular fusion processes[1,2]. Therefore, understanding the molecular mechanism of membrane fusion catalysed by viral membrane-fusion proteins is important not only for understanding virus infection, but also as a paradigm for cellular membrane-fusion reactions.

Virus membrane fusion

Virus membrane fusion can take place either at the plasma membrane or at an intracellular location following virus uptake by endocytosis[3,4]. The fusion reactions of viruses that fuse directly at the plasma membrane are triggered by virus–receptor interactions at neutral pH, as discussed below. By contrast, the fusion of many other viruses is dependent on their internalization by receptor-mediated endocytic pathways such as clathrin-dependent, caveolae-dependent uptake or non-clathrin-dependent, non-caveolae-dependent uptake[4,5]. Viruses that use such routes frequently have fusion reactions that require exposure to mildly acidic pH within organelles of the endocytic pathway[6]. Although it is unclear why viruses use such a variety of entry pathways, it is possible that those viruses that have evolved to fuse intracellularly gain a selective advantage from releasing their genomes at specific intracellular sites[7].

Virus membrane-fusion proteins drive the fusion reaction by undergoing a major conformational change that is triggered by interactions with the target cell. The specific trigger depends on the virus. For example, influenza viruses, alphaviruses and flaviviruses are classic examples of viruses that fuse upon exposure to low pH in the endocytic pathway or in the test tube[3,6,8,9]. By contrast, fusion of HIV-1 occurs at neutral pH and is triggered by the sequential interaction of the virus fusion protein with the receptor CD4 and a co-receptor such as CCR5 or CXCR4, members of the 7-TM-domain chemokine receptor family[10,11]. Other variations include viruses with fusion reactions which seem to be triggered by interaction with a single receptor[3], by the binding of the receptor to a separate attachment protein[12], by a combination of receptor binding plus low pH[13,14], or by endosomal proteolysis[15]. Given the recently described variations on the pathways of fusion-protein activation, it is probable that there are additional interesting twists to be discovered.

To date, two classes of virus membrane-fusion proteins (FIG. 1) have been defined, based on key structural features, as detailed below. Viruses that are currently assigned to class I or class II are listed in TABLE 1. It is clear that for many viruses, the identity and/or functional features of the fusion protein have not yet been determined, making it difficult to classify them as class I or class II, or perhaps even as the inaugural members of a new fusion-protein class (BOX 1). This review will focus primarily on recent developments in the structure and function of the class II fusion proteins (FIG. 2), using the alphavirus E1 glycoprotein as a paradigm. Class I proteins will be introduced first

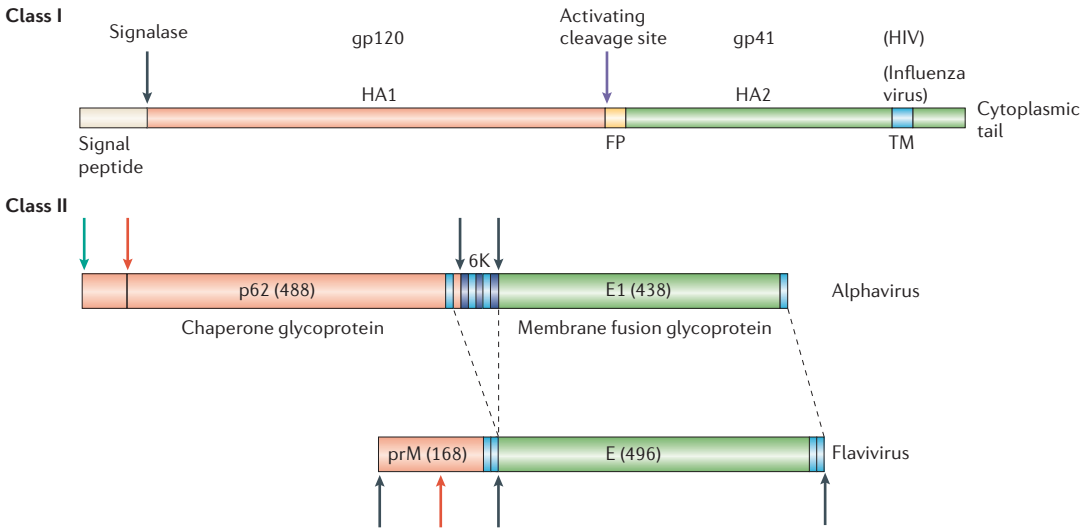

Figure 1 | **Linear diagrams of the class I and class II fusion proteins.** The nomenclature and organization of the class I proteins of influenza virus and HIV and the class II proteins of flaviviruses and alphaviruses are indicated. For class I, labels specify the positions of the fusion peptides (FP), processing sites (arrows) and transmembrane regions (TM). For class II, signalase cleavage sites are indicated by black arrows, furin-processing sites by red arrows, the capsid protease cleavage site by a green arrow and TM regions are light blue. The intervening 6K segment is unique to alphaviruses. Residue numbers are in parentheses.

to establish our current understanding of these fusion proteins, as they provide important context for the studies of the class II proteins.

Class I fusion proteins

Structure and function. The class I membrane-fusion reaction is mediated by the refolding of the fusion protein to a highly stable rod-like structure with a central trimeric α-helical coiled coil. Such coiled-coil structures are emblematic of class I proteins, and physical demonstration or computer prediction of such a structure is frequently used to help define a fusion protein as belonging to class I[16]. The class I proteins generally share several other important features, illustrated here by the example of the influenza-virus haemagglutinin (HA)[17] and summarized in TABLE 2 and in REFS 3,11,18.

HA is a trimeric type 1 TM protein that contains both the receptor-binding and fusion activities of influenza virus. It is synthesized as a fusion-inactive precursor, HA0, which is processed by host-cell proteases to produce two disulphide-bonded subunits — HA1 (328 amino acids), containing the receptor-binding site, and HA2 (221 amino acids), the TM subunit responsible for fusion (FIG. 1). HA2 is maintained in a 'metastable' state at the virus surface[19,20]. The N terminus of HA2 contains a conserved hydrophobic region known as the fusion peptide which inserts into the target membrane during fusion.

The structure of the HA0 ectodomain reveals a trimer with a large globular head region that is composed mainly of HA1, and a long α-helical coiled-coil stalk region[21]. After processing, the overall structure of HA is almost unchanged, but the N-terminal fusion peptide of HA2 becomes buried at the trimer interface within the α-helical stalk[22] (FIG. 3). Virus fusion is triggered by low pH, which destabilizes the HA1 trimer contacts at the head, causing the globular head domains to dissociate. This movement allows a loop-to-helix transition of a polypeptide segment of HA2 that was previously buried underneath the HA1 heads, projecting the fusion peptide ~100 Å towards the target membrane, where it inserts irreversibly[23]. This initial change is proposed to result in a 'pre-hairpin intermediate', an extended structure that is anchored both in the target membrane by the fusion peptide and in the virus membrane by the TM segment[11]. The C-terminal end of the long HA2 α-helix jackknifes back, reversing the direction of the viral-membrane-proximal segment of HA2, which then interacts in an anti-parallel fashion with the groove formed by the N-terminal trimeric coiled coil[23]. The final post-fusion conformation of HA2 is therefore a highly stable rod with the TM and fusion-peptide segments together at the same end of the molecule (FIG. 4), a structure termed a 'trimer of hairpins'[11].

Table 1 | **Class I and class II virus fusion proteins**

Virus family	Examples
Class I	
Orthomyxoviridae	Influenza virus HA2 protein
Paramyxoviridae	Simian virus 5 F1 protein
Filoviridae	Ebola virus GP2 protein
Retroviridae	Moloney murine leukemia virus TM protein, HIV-1 gp41 protein
Coronaviridae	Mouse hepatitis virus and SARS virus S2 proteins
Class II	
Flaviviridae	E proteins of flaviviruses tick-borne encephalitis and dengue viruses
Togaviridae	E1 protein of alphavirus Semliki Forest virus

HA, haemagglutinin; SARS, severe acute respiratory syndrome; TM, transmembrane; gp/GP, glycoprotein.

Co-receptor
A second receptor required for virus infection. In the case of HIV-1, fusion is triggered by sequential interaction of viral gp120 with the CD4 receptor, followed by a co-receptor.

Chemokines
Cytokines involved in specific inflammatory responses. They are differentiated into CC or CXC chemokines on the basis of their primary sequence.

Type 1 TM protein
A single-pass transmembrane protein that contains an N-terminal external domain and a C-terminal cytoplasmic domain.

Box 1 | **Other potential classes of virus membrane-fusion proteins**

Class I and class II fusion proteins share common features of target-membrane insertion of a hydrophobic fusion peptide and refolding/hairpin formation to drive the membrane-fusion reaction, whereas their structural characteristics are markedly different. What other classes of viral fusion proteins might there be?

One possibility would be proteins that mediate fusion using the same general mechanism but with a structural basis that belongs to neither class I nor class II. For example, rhabdoviruses such as vesicular stomatitis virus and rabies virus have a single homotrimeric membrane protein, G, which mediates fusion in a low-pH-dependent reaction that involves membrane insertion of a hydrophobic region of G[93] and biochemically detectable conformational changes (reviewed in REF. 94). However, unlike the class I and class II proteins discussed in this review, G is not proteolytically processed or synthesized with a companion protein, the G-protein conformational changes induced by the fusion trigger (low pH) are reversible and, as yet, no clear structural similarities to class I or class II proteins have been identified.

It is also probable that different overall mechanisms of fusion remain to be defined. For example, herpesvirus fusion involves several virus membrane proteins and might represent a different model from the single fusion proteins of class I and class II (reviewed in REF. 95). Likewise, recent data indicate that, in the case of the poxviruses, four proteins interact to induce fusion at low pH (see REF. 96 and references therein). The many open questions on the fusion of complex viruses such as herpesviruses and poxviruses will continue to be important and interesting topics in the future.

The non-enveloped viruses of the Orthoreovirus genus include some viruses that cause cell–cell fusion of infected cells. This fusion activity is due to non-structural membrane proteins termed FAST proteins (fusion-associated small transmembrane proteins), which range in size from ~10–15 kDa[97]. The ectodomains of FAST proteins are small, in some cases comprising only ~20 residues, and can contain short hydrophobic regions and/or *N*-myristic acid[97,98]. Although their small size argues against hairpin formation, FAST proteins are sufficient to mediate membrane fusion[99], and it will be important to determine how they fit into our growing knowledge of the mechanisms of protein-mediated membrane fusion.

The stability of the post-fusion conformation of HA is considerably higher than that of the metastable pre-fusion form, and class I membrane fusion is believed to be driven by the energy that is released during the conformational change[19,20,24,25]. Hairpin formation by HA2 is postulated to force the target and virus membranes into close apposition to trigger fusion, which occurs by the initial mixing of the outer lipid leaflets (termed 'hemifusion'), followed by mixing of the inner leaflets, opening of a small fusion pore, and widening of the pore to give complete fusion.

Membrane insertion. Viral fusion peptides generally tend to be apolar regions, conserved within a virus family, relatively rich in glycine and alanine residues, and containing several bulky hydrophobic residues[3]. For influenza HA and several other class I proteins, the fusion peptide is located at the N terminus of the TM subunit, but other class I fusion peptides are located internally in the amino-acid sequence, and presumably insert into the membrane as a loop. Structural studies of the HA fusion peptide indicate that it adopts a boomerang-shaped structure formed by a kinked amphipathic α-helix lying roughly in the plane of the outer leaflet of the membrane at the polar–aliphatic interface[26]. Membrane insertion seems to be primarily regulated by the initial release of the fusion peptide from its buried position to permit its amphipathic interaction with lipid bilayers.

Cooperativity. Fusion by HA is positively affected by protein density, suggesting cooperative HA interactions during fusion[27,28]. Intriguingly, studies of cell–cell fusion mediated by influenza HA suggest that 'bystander' HA molecules outside the zone of cell–cell contact have effects on expansion of the fusion pore[29]. The mechanism by which HA might interact during fusion and the role of bystander fusion proteins are not well understood.

Inhibitors of class I conformational changes and fusion. Structural information on class I fusion proteins has suggested several approaches for inhibiting fusion-protein refolding and membrane fusion[11,30]. For example, the HIV-1 inhibitor T20/enfuvirtide is a peptide that corresponds to part of the C-terminal helix of the envelope glycoprotein gp41 (REFS 31,32). T20 blocks the interaction of the C-terminal segment of the fusion protein with the groove of the central coiled coil (FIG. 4), and blocks virus fusion and infection. Importantly, small molecules that target crucial sites of interaction in the trimer of hairpins have also been shown to act as potent class I fusion inhibitors[33]. As small molecules can have higher bioavailability than peptides, they provide an important approach to develop more widely useful fusion inhibitors. Although, to date, the class I inhibitors have mainly targeted viruses such as HIV-1 that are triggered at the cell surface, small molecules can also target viruses that fuse within endocytic compartments[34,35].

Class II fusion proteins

The alphaviruses and flaviviruses are members of the *Togaviridae* and *Flaviviridae* families, respectively[36,37]. These small spherically shaped viruses are composed of a nucleocapsid core that contains the single-stranded positive-sense genomic RNA, surrounded by a membrane that contains the viral TM glycoproteins, which form an external icosahedral scaffold (FIG. 2). The X-ray structures of the fusion proteins of the alphaviruses and flaviviruses, described below, revealed that the ectodomains of these proteins have remarkably similar secondary and tertiary structures. This result strongly suggests that, despite the lack of any detectable amino-acid-sequence similarity, the corresponding genes were derived from a common ancestor. The three-dimensional structure of these fusion proteins is radically different from that of the influenza virus HA, a finding that introduced the concept of a separate class of fusion proteins, class II[38] (TABLE 2).

Hemifusion
Transient membrane-fusion intermediate in which only the two proximal leaflets of the two bilayers mix.

Fusion pore
Small opening that allows flux between two membrane-bound compartments. Fusion pores form at an early stage of membrane fusion and widen when they lead to full fusion.

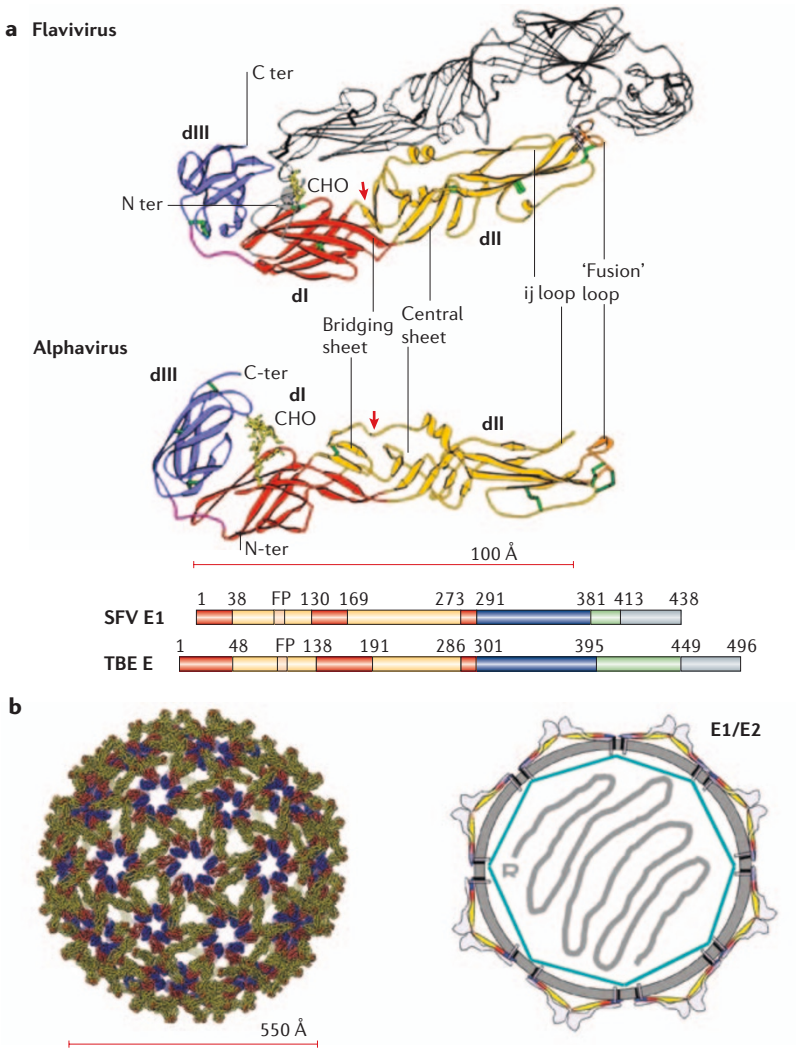

Figure 2 | **Class II virus membrane-fusion proteins. a** | Structure and sequence diagram of class II virus membrane-fusion proteins. The tick-borne encephalitis (TBE) flavivirus glycoprotein E and the Semliki Forest alphavirus (SFV) E1 protein are shown, with domains I, II and III represented in red, yellow and blue, respectively (dI, dII and dIII). Common elements and important loops in domain II are indicated. Red arrowheads point to the hinge region between domains I and II. The fusion loop is shown in orange, disulphide bonds as green tubes, and carbohydrates (CHO) in pale yellow. The flavivirus E protein dimer is indicated with one subunit in white. **b** | The icosahedral scaffold formed by E1 at the SFV surface. The left panel shows the lattice resulting from fitting the crystallographic model of E1 on the cryo-electron microscopy reconstruction of the SFV particle. The lattice is formed essentially by E1 interactions around the five-fold, quasi six-fold and quasi two-fold axes of the T = 4 icosahedral particle. All three-fold and quasi three-fold contacts are made exclusively by E2, within the trimeric spike depicted in FIG. 3b. The right panel shows a cartoon of the organization of the SFV particle, with E1 coloured as in FIG. 2a, E2 depicted in pale blue, and the transmembrane (TM) regions drawn as bars crossing the grey lipid bilayer. The internal icosahedral nucleocapsid is indicated by a blue polyhedron with the genomic RNA ('R') inside. Figure prepared using the RIBBONS program[100].

Biosynthesis and assembly. During biosynthesis, the alphavirus (E1) and flavivirus (E) fusion proteins fold co-translationally with a companion or regulatory protein, termed p62 (or PE2) for alphaviruses and prM for flaviviruses[36,37] (FIG. 1). This heterodimeric interaction is important for the correct folding and transport of the fusion protein. Both p62 and prM are cleaved by the cellular protease furin late in the secretory pathway, in a maturation reaction that is a crucial regulatory step for subsequent virus fusion[39–42].

One important difference between these two groups of viruses is the budding site[43,44]. In alphaviruses, the p62–E1 complex is transported to the plasma membrane, and the heterodimer interaction is maintained after p62 processing. New virions bud at the plasma membrane, in a process that is driven by lateral contacts between E2–E1 heterodimers (E2 being the mature companion protein) to induce the required curvature of the lipid bilayer, in combination with interactions of the cytosolic tail of E2 with the nucleocapsid. Budding results in formation of icosahedral enveloped particles of triangulation T = 4, containing 80 trimeric E2/E1 spikes[45,46].

By contrast, flavivirus particles bud into the endoplasmic reticulum as immature virions formed by 60 trimers of prM–E. The immature particles have an organization similar to mature alphaviruses, with each trimer forming a spike in which prM covers the fusion protein E[47]. The newly formed virions are then transported to the external milieu through the exocytic pathway. Processing of prM generates the mature M protein with a short (~40 residues) ectodomain. Presumably because of the removal of a large portion of the prM ectodomain, the flavivirus surface dramatically reorganizes after processing to give 90 E–E homodimers arranged with icosahedral symmetry[44,48–50]. The mature flavivirus particles display a smooth, spikeless surface, with E dimers ordered in a characteristic 'herringbone' pattern.

Structure. Crystal structures have been determined for the neutral pH ectodomains of the fusion proteins from the alphavirus Semliki Forest virus (SFV)[38,51] and the flaviviruses tick-borne encephalitis virus (TBE), dengue 2 virus, and dengue 3 virus[52–55]. The polypeptide chain of the class II proteins follows a complex path, resulting in three globular domains — essentially constituted by β-sheets — organized so that the C terminus and the fusion peptide are found at the two ends of a rod-like molecule of ~120 Å in length (FIG. 2). Domain I, which contains the N terminus and a conserved glycosylation site, is a β-barrel with an 'up-and-down' topology. Two of the connections between adjacent strands in this barrel are long and elaborated, and comprise the 'finger-like' domain II with the fusion loop at the tip of the molecule. Domain III, which lies at the opposite end of domain I, has an immunoglobulin-superfamily fold and is connected to the C terminus of domain I by an ~12 amino-acid polypeptide. The TM domain of SFV E1 and the two TM domains of TBE were proteolytically removed to generate the soluble ectodomains. The SFV E1 ectodomain, referred to here as E1*, retains part of the 'stem' region that connects domain III to the TM domain, although this flexible region is not visualized in the neutral pH structure. The stem regions of the flavivirus fusion proteins have been completely removed in the crystallized forms.

Table 2 | **Comparison of class I and class II virus membrane-fusion proteins**

Feature	Class I (influenza HA)	Class II (SFV E1)
Conformational change during fusion	Metastable fusion protein trimer to stable fusion protein trimer	Metastable dimer to stable fusion protein trimer
Predominant secondary structure of fusion protein	α-helix	β-sheet
Post-fusion structure	Trimer of hairpins with central α-helical coiled coil	Trimer of hairpins composed of β structure
Maturation to prefusion state through:	Proteolytic processing of fusion protein	Proteolytic processing of companion protein
Fusion peptide location in metastable structure	N-terminal peptide buried in trimer interface	Internal loop at fusion protein tip, capped by dimer interaction

HA, haemagglutinin; SFV, Semliki Forest virus.

Conformational changes during fusion. Unlike the class I fusion proteins, which are trimeric in both their pre-fusion and post-fusion conformations, class II fusion proteins undergo an oligomeric rearrangement during fusion[56–58], converting from the metastable pre-fusion dimer to a considerably more stable homotrimer conformation[59,60] (TABLE 2). Formation of the target-membrane-inserted homotrimer is required for class II virus fusion[61,62].

Binding of protons in the acidic endosomal environment triggers a complete rearrangement of the surface of the class II virus particles. The alphavirus E2–E1 heterodimers or the flavivirus E–E homodimers dissociate[58,63], resulting in disassembly of the icosahedral scaffold followed by a quaternary reorganization into E1 or E homotrimers inserted in the membrane by the fusion loops. Importantly, as discussed below, the homotrimers seem to display lateral contacts between each other, forming a different type of surface lattice. *In vitro* studies using the ectodomains of both the alphavirus and flavivirus proteins showed that trimerization requires insertion of the fusion peptide into target membranes[64,65]. Solubilization of the trimers from the liposomes using non-ionic detergent led to their crystallization[66,67] and to determination of their three-dimensional structure[68–70].

The crystal structures of the class II homotrimers showed a fold-back arrangement strikingly reminiscent of that of class I fusion proteins, despite the different architecture of the proteins (FIG. 4). The trimer is formed by a central parallel interaction of the subunits through roughly the N-terminal two-fifths of the molecule (including domains I and II, with the fusion peptide at one end). The remaining C-terminal portion of the protein folds back along the sides of the core trimer, with domain III moving ~30–40 Å towards the fusion loop. The portion of the stem region that is present in the SFV E1 protein interacts closely with the trimer core and extends towards the fusion loop. This hairpin organization is therefore the same as that observed for class I fusion proteins, resulting in a trimeric protein rod with the fusion peptide loops and the C-terminal membrane anchors together at the same end.

Membrane insertion. The class II fusion peptide forms a loop connecting β-strands c and d at the tip of domain II (FIG. 2). Monoclonal antibodies to this region have been used to show that the fusion loop inserts into membranes and that insertion is required for fusion[65,71,72]. The fusion loop is composed of relatively apolar and conserved residues, and fusion is blocked by substitution of negatively charged amino acids into this crucial region[61,62].

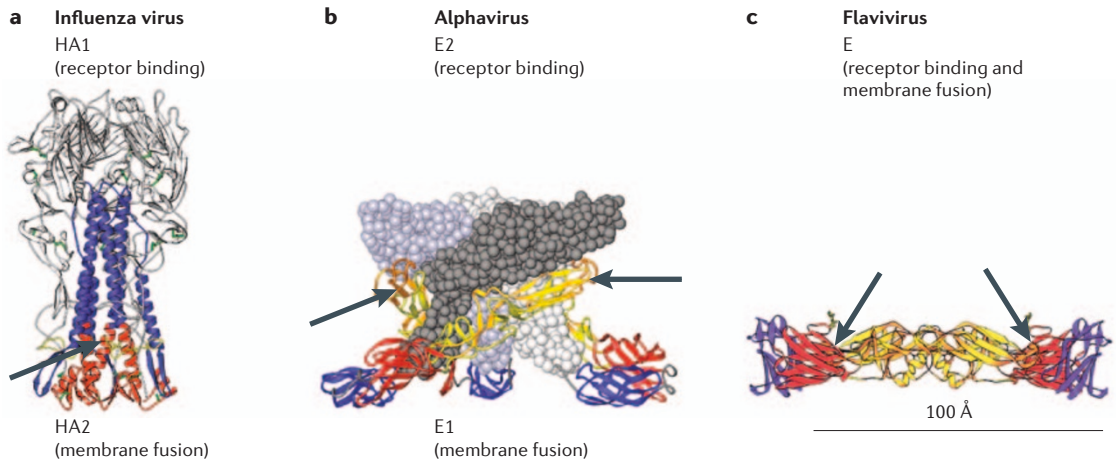

Figure 3 | **Pre-fusion complexes of class I and II viruses in the conformation present at the surface of infectious particles. a** | shows the influenza virus haemagglutinin $(HA1/HA2)_3$ complex with the HA1 receptor-binding subunits shown in white and the HA2 membrane-fusion subunits coloured in blue and red. The fusion peptide (yellow, residues 1 to 22) is buried at the trimer centre (arrow). **b** | shows the alphavirus Semliki Forest virus pre-fusion complex $(E1/E2)_3$. The E1 membrane-fusion subunits are drawn as tubes coloured by domains as in FIG. 2. The receptor-binding subunit, E2, is displayed as a sphere-filling model coloured grey, based on subtracting the E1 density from the cryo-electron microscopy reconstruction of the virus. The orange E1 fusion peptide (arrows) is buried at the E1–E2 interface. **c** | shows the pre-fusion complex $(E)_2$ of the flavivirus tick-borne encephalitis virus. The fusion peptide is buried at the E homodimer interface (arrows). All three panels are drawn at the same scale. The viral membrane would be tangential to a horizontal plane below the drawings. Figure prepared using the RIBBONS program[100].

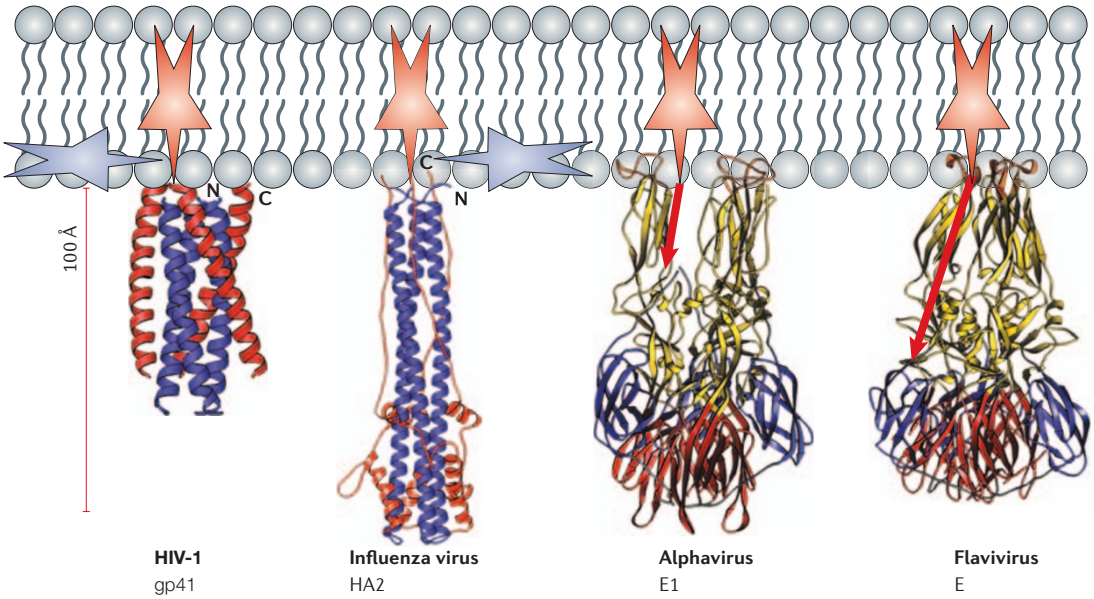

Figure 4 | **Post-fusion conformations of class I and class II fusion proteins.** The class I fusion proteins of HIV-1 and influenza virus (left), and the class II fusion proteins of Semliki Forest virus and tick-borne encephalitis virus (right) are shown. To demonstrate the suggested membrane interactions of the proteins, the final fused membrane is diagrammed in cartoon form. The class I proteins are drawn such that the N-terminal half of the hairpin is blue and the C-terminal part is red, with the missing fusion peptides and transmembrane domains indicated for one of the three trimer subunits as blue and red stars, respectively. For HA2, the residues are coloured as in the neutral pH form displayed in FIG. 3, and a membrane-proximal extended C-terminal 'leash' interacts with the central coiled coil of HA. In HIV glycoprotein 41 (gp41), the post-fusion structure is a six-helix bundle. The inhibitor T20 interacts at the site where the red helix is, effectively inhibiting the fusogenic conformational change. For the class II proteins, an elongated red star indicates the predicted location of the TM segment, with a red arrow pointing to the C terminus of one of the subunits in the trimer of the crystallized class II ectodomains, roughly showing the path of the missing 'stem' regions to complete the protein hairpin. Figure prepared using the RIBBONS program[100].

Interestingly, the process of insertion of the alphavirus fusion peptide into the target membrane seems to differ from that of the class I proteins. For example, although heterodimer dissociation exposes the SFV fusion peptide, this alone does not seem sufficient for membrane insertion. The fusion loop is solvent-accessible in the pH-7 form of the E1* ectodomain[38]. The crystal structure shows that in the monomeric form, the E1 fusion loop (which is longer than its flavivirus counterpart) folds back on itself, burying most of the bulky non-polar side chains. Its insertion into the target membrane apparently requires specific triggering by low pH and target-membrane cholesterol[64,71]. This agrees with the cholesterol dependence of alphavirus membrane fusion, as discussed below. Once E1* has stably inserted into the target membrane, it then requires detergent for solubilization, similar to the membrane-inserted class I proteins. The structure of the SFV detergent-solubilized E1* homotrimer shows that the fusion peptide unwinds, and the bulky non polar side chains are exposed, presumably within a detergent micelle in the crystal.

Role of cholesterol. Liposome experiments show that alphavirus fusion is promoted by the presence of cholesterol and sphingolipid in the target membrane[73–75]. Flavivirus–liposome fusion is also enhanced by cholesterol, although the stringency of the requirement seems considerably less than that of the alphaviruses[76,77]. For both viruses, the action of cholesterol is more specific than its bulk effects on membrane fluidity or 'raft' formation, with the sterol 3β-hydroxyl group being particularly important for promoting membrane insertion, trimerization and fusion. Interestingly, following membrane insertion, the SFV E1* ectodomain is strongly associated with cholesterol-rich membrane microdomains[72]. This could reflect a physical interaction of E1 with cholesterol, thereby coupling membrane insertion to the E1 conformational changes during fusion.

In vivo studies show that membrane fusion and infection of the alphaviruses SFV and Sindbis virus are decreased by 3–5 logs in cholesterol-depleted insect cells[78,79]. This system was used to select for SFV mutants that have increased cholesterol independence[80,81]. These *srf* (sterol requirement in function) mutants have single amino-acid changes in E1, located in the ij loop at the tip (the *srf-3* P226S mutation) or in the hinge region (the *srf-4* L44F and *srf-5* V178A mutations) of domain II (FIG. 2). The molecular mechanisms by which the *srf* mutations alter alphavirus cholesterol dependence are not yet known. For *srf-3*, the location in the ij loop suggests that the mutation could directly modulate the cholesterol dependence of the adjacent fusion loop. For *srf-4* and *srf-5*, the location in the hinge area suggests that these mutations might affect the inherent flexibility of this region, indirectly altering the angle of interaction of the fusion loop with the target membrane.

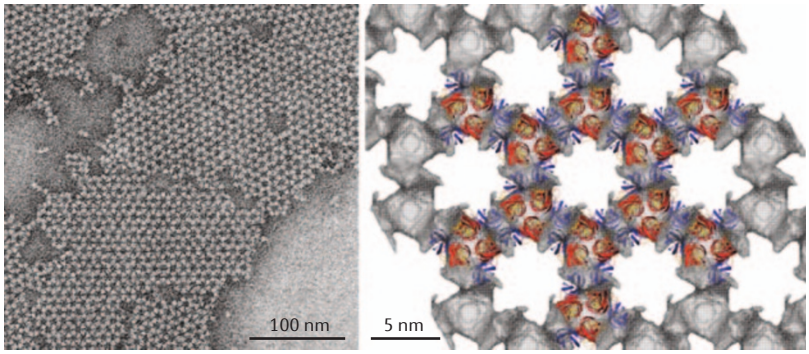

Figure 5 | **Lateral interactions between adjacent Semliki Forest virus E1 trimers.** The left panel shows a negatively stained sample of E1 ectodomain homotrimers (E1*HT) reconstituted at a low lipid-to-protein ratio to produce a planar hexagonal lattice. On liposomes, the E1*HT is proposed to display a fullerene-like architecture, in which insertion of rings of five instead of six trimers allows the required curvature to form a closed sphere (see text). Images of the planar lattice such as that shown on the left were used to generate a three-dimensional reconstruction into which the atomic model of the E1*HT was fitted (right panel). Direct lateral interactions between the trimer heads are observed from the fitting, as shown in this top view. Interactions between the fusion loops of adjacent trimers could also occur through adjustment of the hinge region (indicated in FIG. 2a). Figure prepared using the RIBBONS program[100].

Role of the ij loop. In addition to being the site of the *srf-3* mutation, several features of the alphavirus ij loop indicated its possible importance in the membrane-fusion reaction. Although the E1 homotrimer is highly resistant to proteolysis, under defined conditions protease cleavage sites were identified around the fusion loop[82]. Such protease cleavage releases the E1* homotrimer from the target membrane. Other than the cleavage in the fusion-loop region, the only other cleavage site was immediately N-terminal to histidine 230 (H230) in the ij loop. Sequence comparisons showed that, although the overall sequence of the ij loop is not highly conserved, a histidine residue is found in all of the reported alphavirus and flavivirus sequences[83].

The role of H230 in SFV fusion and infection was addressed by constructing an H230A mutation in the virus infectious clone[83]. The resulting mutant virus is not infectious and is completely blocked in membrane fusion, including the initial steps of lipid mixing. However, H230A virus undergoes apparently normal conformational changes upon exposure to low pH, including heterodimer dissociation and fusion-loop exposure, cholesterol-dependent target-membrane insertion, and formation of the E1 homotrimer. Therefore, the H230A mutation demonstrates a crucial role for this residue within the SFV ij loop, and identifies a novel late-stage intermediate in the class II fusion pathway.

Cooperative interactions of E1 trimers. Two important observations indicate that class II trimers interact cooperatively during membrane insertion and fusion. First, electron-microscopy studies of the membrane-inserted ectodomains of SFV and TBE show that insertion is highly cooperative and produces characteristic arrays of trimers organized in rings of five or six to form a lattice covering the liposome surface[67,84] (FIG. 5). Liposomes fully decorated with inserted SFV E1* display a fairly uniform diameter of ~120 nm, considerably smaller than the mean diameter of control liposomes without the protein. The surface-protein lattice therefore seems to control the curvature of the coated liposomes. Because hexagonal arrays of E1* trimers result in a flat lattice, as shown in FIG. 5, it is probable that rings of five E1* trimers are required to introduce the necessary overall curvature to make a fullerene-like[85,86], roughly spherical closed lattice at the liposome surface.

Second, the packing of SFV E1* trimers in the three-dimensional crystals largely occurs through two-fold interactions between the fusion loops of adjacent trimers[68]. The trimer axes and the two-fold axis relating them intersect in space, making an angle close to the angle between three-fold and two-fold axes in an icosahedron. As in an icosahedron, the combination of these two operations (three-fold and two-fold about the corresponding symmetry axes) generates a five-fold axis also intersecting the three-fold and two-fold axes at the same point in space (at the centre of the body). The trimers in the crystals therefore interact through the fusion peptide in such a way that the contacts can be propagated — by repeating the observed trimer–trimer contacts through the fusion loops — to generate a closed ring of five E1* trimers, all associated by their fusion loops, just like the rings observed by electron microscopy. The fusion peptides in the five-fold rings form a 'crater' ~100 Å in diameter and 40 Å deep, with a base composed of five fusion loops and a rim composed of the remaining ten fusion loops. As all three fusion loops in each trimer are believed to interact tightly with the lipid heads, the resulting arrangement of trimers is such that it creates a dome-like distortion in the target membrane, which is postulated to be important in fusion, as shown in the model in FIG. 6.

Therefore, electron-microscopy data and the crystal contacts provide support for two types of cooperative homotrimer interactions, producing rings of five and six trimers. We hypothesize that during fusion, five-fold interactions will occur between target-membrane-inserted trimers at the side of the particle closest to the cell membrane. Such five-fold interactions would act at the fusion site to induce formation of a 'dome' in the target lipid bilayer, whereas the refolding of E1 to a hairpin would pull the TM regions toward the centre of the five-fold ring, forming an opposing dome in the viral membrane and inducing hemifusion (FIG. 6). This 'lipid stalk' model for fusion is comparable to that proposed for the class I proteins[87], and in keeping with recent studies which show that alphavirus fusion progresses through a transient hemifusion intermediate[88]. The six-fold trimer interactions might occur between trimers that do not directly interact with the target membrane and insert instead into the virus membrane. The more planar interactions of such trimers could have a role in the enlargement of the initial fusion pore by providing energy to flatten the viral membrane[89]. Although these models are currently speculative, they provide a starting point for thinking about the geometry of the fusion reaction.

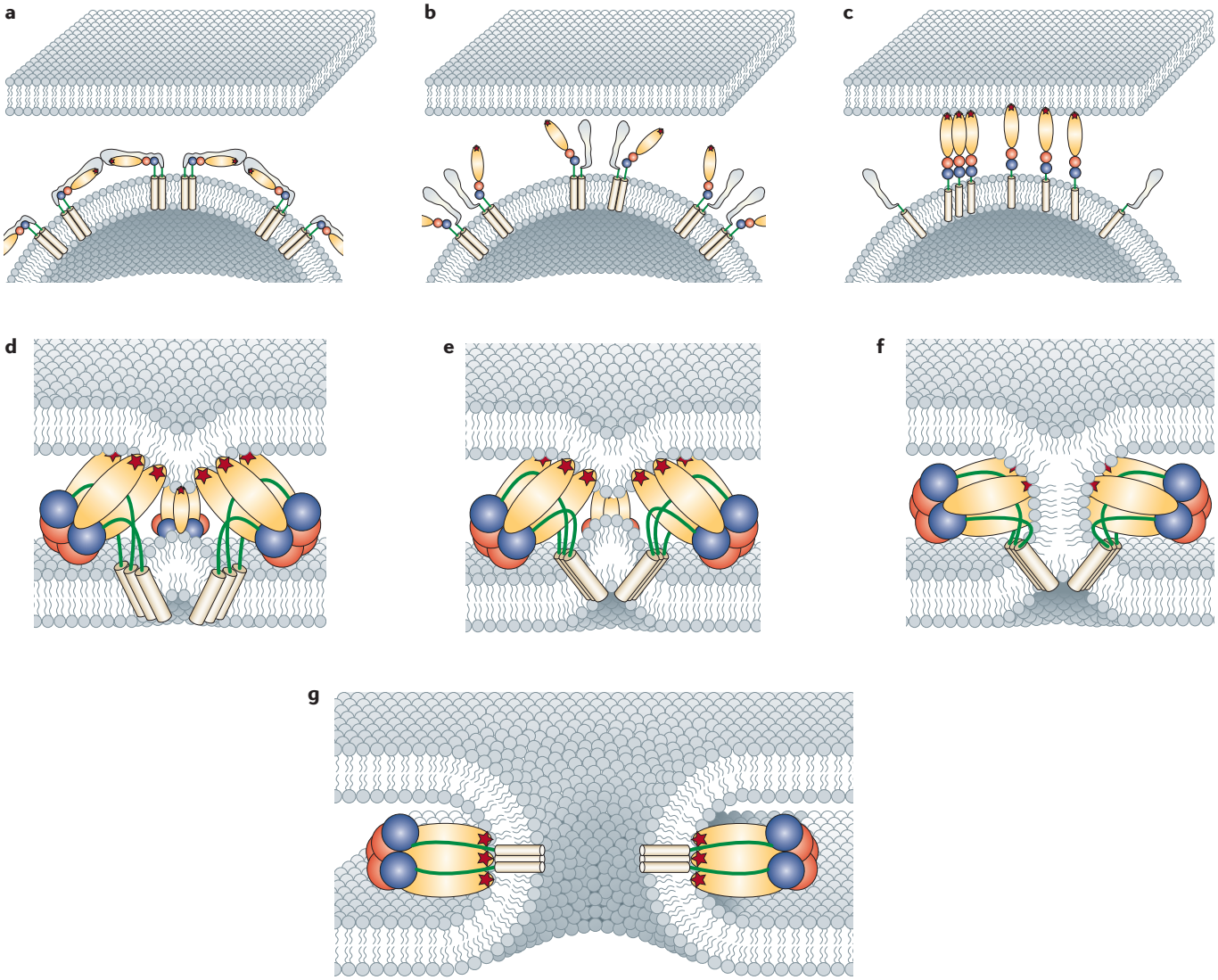

Figure 6 | **Model for class II membrane fusion, illustrated for Semliki Forest virus. a** | Native virions, showing E1 with domains coloured as in FIG. 2, and E2 in light grey interacting with E1 and covering the fusion loop. Only the target-cell-proximal side of the virion represented in FIG. 2b, right panel, is shown. **b** | Trigger 1: low pH triggers E1–E2 dissociation and exposure of the fusion loop. **c** | Trigger 2: low-pH- and cholesterol-dependent insertion of the fusion loop, leading to the alignment of E1 subunits parallel to each other, favouring trimerization. **d** | Fold-back process: domain III and the stem region move towards the fusion loop. Cooperative interactions between trimers through their fusion loops distort the target membrane. **e** | Folding of the domain III and stem segments against the body of the trimer pulls transmembrane (TM) segments against each other, distorting the viral membrane. **f** | Opposing dome-like deformations in the two membranes lead to mixing of the outer leaflets (hemifusion). **g** | To reach the final stable conformation, the TM segments have to be closely juxtaposed to the fusion loop. This is only possible by opening an initial fusion pore.

How general is cooperativity?

As discussed above, there is considerable evidence for fusion-protein cooperativity for members of the class I fusion proteins, particularly for the low-pH-induced influenza HA. Interestingly, however, recent data indicate that some class I virus fusion reactions might be mediated by as few as one or two trimers. Antibody-neutralization studies of HIV indicate that the target of neutralization is one trimer[90], and detailed studies using viruses containing mixtures of wild-type and dominant-negative envelope proteins showed entry of viruses containing a single active HIV Env trimer[91]. Other class I viruses where fusion is triggered by receptor binding (murine leukemia virus) or by receptor plus low pH (avian sarcoma/leukosis virus) also showed entry properties that are suggestive of fusion mediated by a single trimer. By contrast, parallel studies of influenza HA fusion supported a requirement for eight or nine trimers[91]. Further investigations will be required to determine if viruses with fusion directly triggered by low pH generally require multiple fusion proteins, the importance of concerted and cooperative interactions of those proteins, and whether fusion proteins with triggers that involve receptor binding typically activate fusion autonomously.

Implications and future directions

Studies of the class I and class II fusion proteins indicate that, although their structures are very different, both classes refold during fusion to give analogous hairpin conformations, with the fusion peptides or loops and the TM domains at the same end of a stable protein rod. These observations indicate that the overall mechanism of membrane fusion is probably the same for both class I and II, indicating that there might be a universal membrane-fusion mechanism, applicable to proteins that are clearly not homologous.

Although the structural and functional studies summarized here suggest an overall picture for class II fusion, many important questions remain and new questions can now be identified. For example, we do not understand the mechanism by which the class II fusion loop inserts into the target membrane, or the role of cholesterol in insertion. The current data indicate that insertion and fusion can be modulated by the ij loop and hinge region, but their mechanistic effects are unclear. We have a picture of the fusion-protein ectodomain before and after fusion, but we do not know how the fusion loop and TM domains are disposed in the fused membrane or how they might interact with each other. A series of conformational changes in class II fusion proteins can be proposed (FIG. 6), but their order, kinetics and role in each of the steps of membrane fusion remain to be elucidated. We also do not understand how the refolding of the fusion protein to the trimer conformation takes place in the context of the whole virus particle, the fate of the companion protein (E2 in the case of alphaviruses) during this particle reorganization, and the role(s) of the observed five-fold and six-fold trimer interactions in the overall membrane-fusion process.

An important question is whether the structure of the class II fusion proteins can allow the development of inhibitors of specific steps in fusion. Recent work indicates that exogenous domain III blocks class II membrane fusion and infection by binding to the fusion protein during the low-pH-induced conformational change[92]. Similar to the class I proteins, such inhibitors could prove important for dissecting the molecular mechanism of fusion and, ultimately, for developing novel antiviral strategies. Clearly, there is much exciting work to be done, and we look forward to future developments and discussions on the fusion of the class II viruses.

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Competing interests statement
The authors declare no competing financial interests.

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Entrez: http://www.ncbi.nlm.nih.gov/Entrez
dengue 2 virus | dengue 3 virus | HIV-1 | murine leukemia virus | rabies virus | SFV | Sindbis virus | TBE | vesicular stomatitis virus

UniProtKB: http://us.expasy.org/uniprot
CCR | CD4 | CXCR4 | gp41

FURTHER INFORMATION
Margaret Kielian's homepage:
http://www.aecom.yu.edu/cellbiology/kielian.htm
Felix A. Rey's homepage: http://www.pasteur.fr
Access to this interactive links box is free online.