Flavivirus membrane fusion

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Flavivirus membrane fusion is mediated by a class II viral fusion protein, the major envelope protein E, and the fusion process is extremely fast and efficient. Understanding of the underlying mechanisms has been advanced significantly by the determination of E protein structures in their pre- and post-fusion conformations and by the elucidation of the quaternary organization of E proteins in the viral envelope. In this review, these structural data are discussed in the context of functional and biochemical analyses of the flavivirus fusion mechanism and its characteristics are compared with those of other class II- and class I-driven fusion processes.

Introduction

Infection of cells by enveloped viruses requires a step in which the viral membrane fuses with a cellular membrane. This process has a dual function, i.e. the release of the viral nucleocapsid from its protective envelope and the transfer of the viral genetic information into an intracellular compartment where early replication events can be initiated. The merger of the two membranes does not occur spontaneously, but requires the action of specific viral fusion proteins or fusion-protein complexes. These exist in metastable conformations at the surface of mature virions primed to undergo extensive structural changes that drive the fusion process (Schibli & Weissenhorn, 2004; Harrison, 2005). In order for fusion to occur at the right time and at the right place, specific triggers are required (reviewed by Earp et al., 2005). Two types of trigger have been identified: interactions with cellular receptors and/or co-receptors – resulting in fusion at the plasma membrane [e.g. Human immunodeficiency virus (HIV) and paramyxoviruses] – or the acidic pH in endosomes after the virus is taken up by receptor-mediated endocytosis – resulting in fusion from within endosomes (e.g. influenza viruses, alphaviruses and flaviviruses). Some retroviruses, such as avian sarcoma and leukemia viruses (ASLV), apparently require a combination of both triggers (Earp et al., 2005).

In many cases, viral fusion proteins are synthesized as inactive precursors or precursor complexes that are unable to mediate membrane fusion unless a proteolytic cleavage event activates their fusion potential. Such maturation cleavages are carried out intracellularly in the course of virus maturation, e.g. by furin in the case of alpha-, flav-, retro-, paramyxo- and certain influenza viruses and, in other instances, by extracellular proteases after virus release, e.g. with certain strains of influenza virus (Wenger & Wenger, 1989; Salminen et al., 1992; Klenk & Garten, 1994; Stadler et al., 1997; Moulard & Decroly, 2000; Skehel & Wiley, 2000; Zhang et al., 2003b; Zhang & Kielian, 2004). With some enveloped viruses (Ebola virus, Severe acute respiratory syndrome coronavirus), the cleavage activation of fusion proteins is delayed until the stage of virus entry and is mediated by cathepsins after virus uptake into endosomes (Chandran et al., 2005; Matsuyama et al., 2005; Simmons et al., 2005; Qiu et al., 2006; Schornberg et al., 2006).

In the best-studied models, the viral membrane-fusion process involves only a single fusion protein, and detailed structures of both the pre- and post-fusion conformations of such proteins or parts thereof have been determined for representatives of several virus families (Wilson et al., 1981; Bullough et al., 1994; Rey et al., 1995; Modis et al., 2003, 2004, 2005; Bressanelli et al., 2004; Gibbons et al., 2004b; Zhang et al., 2004; Yin et al., 2005, 2006; Roussell et al., 2006).

It must be emphasized, however, that there are more complex viruses, such as herpesviruses (Spear et al., 2006) and poxviruses (Moss, 2006), that require the concerted action of several envelope proteins for membrane fusion. In the case of poxviruses – which have two lipid envelopes – the non-fusogenic dissolution of a viral membrane has also been described (Law et al., 2006). Even with the simplest viral systems, however, there are striking structural and mechanistic similarities to the fusion machineries controlling cellular vesicle fusion (Söllner, 2004; Chen & Olson, 2005), making them excellent models for studying the biological principles and concepts of membrane fusion in general.

Among the viruses with single fusion proteins, two completely unrelated structural classes have been identified and these are designated class I and class II fusion proteins (Lescar et al., 2001). The class I proteins exhibit structural analogies to the cellular SNARE fusion proteins (Skehel & Wiley, 1998; Söllner, 2004) and have been identified in orthomyxo-, paramyxo-, retro-, filo- and coronaviruses.
Flaviviruses

Several features make flaviviruses interesting and important models for studying viral membrane fusion: they exhibit the fastest and most efficient fusion machinery of all enveloped viruses analysed to date, which is probably linked to their peculiar, smooth-surfaced envelope structure (described in detail below) – in contrast to the spiky surface of most other enveloped viruses. Furthermore, essential portions of the flavivirus fusion protein are known in atomic detail in both their pre- and post-fusion conformations, making it feasible to understand the mechanistic details of the flavivirus fusion process at a structural level. Such knowledge can lead to the specific design of antiviral agents, which are not yet available for the treatment of flavivirus-induced diseases.

Flaviviruses comprise about 70 different viruses that form a genus in the family Flaviviridae and, in the majority of cases, they are transmitted to their vertebrate hosts by mosquitoes or ticks (Heinz et al., 2000). In terms of disease incidence, the most important human-pathogenic flaviviruses are Dengue virus (DenV), Yellow fever virus (YFV), Japanese encephalitis virus (JEV), Tick-borne encephalitis virus (TBEV) and West Nile virus (WNV) (Burke & Monath, 2001). The expansion of flaviviruses beyond their native conformation, but becomes exposed through these conformational changes, interacts with the target membrane and thus initiates the fusion process, and (iii) the conversion of the fusion protein into a hairpin-like post-fusion structure in which the FP and the membrane anchor are juxtaposed (Jardetzky & Lamb, 2004; Kielian & Rey, 2006). In this review, we will discuss the currently known details of flavivirus membrane fusion and compare its characteristic features with those of other class II and class I viral-fusion systems.

Structure of the E protein

The basic structural organization of flaviviruses and a schematic depiction of their life cycle are displayed in Fig. 1. An isometric nucleocapsid (Fig. 1a) (composed of a single protein designated C) contains the positive-stranded RNA genome of about 11 000 nt, which has a single open reading frame (ORF) flanked by non-coding regions at its 5’ and 3’ ends (Fig. 1b). The structural proteins are encoded in the 5’ one-third of the ORF in the order C, prM/M, E, followed by a series of non-structural proteins (Lindenbach & Rice, 2001). Virus assembly takes place in the endoplasmic reticulum (ER) (Mackenzie & Westaway, 2001) (Fig. 1c) and first leads to the formation of non-infectious immature virions (Fig. 1a, left), which are studded with spikes containing a tight complex of prM and E proteins (Zhang et al., 2003c). Upon transport of the immature virion through the cellular exocytotic pathway (Fig. 1c), the prM–E protein complex undergoes a conformational change, induced by the low pH in the trans-Golgi network (TGN), that allows prM to be cleaved by furin or a furin-like protease before the virus particles are released from the cell (Stadler et al., 1997; Elshuber et al., 2003). This cleavage causes a major rearrangement of E proteins at the particle surface, leading to the formation of mature infectious virions (Kuhn et al., 2002; Mukhopadhyay et al., 2003) (Fig. 1a, right) that carry the dimeric E protein in a metastable conformation (Stiasny et al., 2001), which is now primed to mediate low pH-triggered membrane fusion in the endosome upon fusing with the receptor (Fig. 1c). It is believed that the function of the prM protein in immature virions is to prevent the induction of low pH-induced conformational changes in E that would already lead to fusion in the TGN during exocytosis (Heinz et al., 1994; Stadler et al., 1997).

An interesting aspect of the flavivirus life cycle is the production of capsidless, membrane-containing particles, which have been observed as natural by-products in the supernatants of flavivirus-infected cells (Stollar, 1969; Smith et al., 1970). The fact that such capsidless particles can be generated in recombinant form (recombinant subviral particles) by the coexpression of only the prM and E proteins suggests that particle formation in the ER is driven by lateral interactions of the prM and E proteins and does not require any interactions with the capsid protein (reviewed by Heinz & Allison, 2000, 2003).

Structural details of the flavivirus fusion machinery

The atomic structures of the E proteins from TBEV, DenV2 and DenV3 have been determined by X-ray crystallography using dimeric complexes isolated either from purified virions (TBEV) or produced as recombinant proteins in Drosophila cells (DenV) (Rey et al., 1995; Modis et al., 2003, 2005; Zhang et al., 2004). These forms of E represent the metastable, neutral-pH conformation of the protein and are soluble (therefore termed sE) because they are truncated and lack the double transmembrane anchor, as well as a ‘stem’ structure of about 50 aa that is located between the viral membrane and the crystallizable ectodomain (Fig. 1a, Fig. 2d). Although the DenV and TBEV E proteins have <40% amino acid identity, the basic structural features of both proteins are virtually identical and can be assumed to apply to all flavivirus E proteins. Corresponding to its orientation parallel to the viral membrane, the E dimer is gently curved (Fig. 2c) and the two monomeric subunits are
oriented antiparallel to each other (Fig. 2a, b). Each monomer is made up of three distinct domains – DI, DII and DIII (Fig. 2a, b). DI, the central β-sandwich domain, contains the amino terminus and – in many of the flaviviruses – carries an N-linked carbohydrate side chain (Fig. 2a, c). Two long, disulfide-stabilized loops emanate out of this central domain and interact to form domain II, which has an elongated structure and provides most of the intersubunit contacts (‘dimerization domain’). Some flaviviruses, including DenV2 and -3, have a second carbohydrate side chain attached to this domain (Modis et al., 2003, 2005). On the opposite side, DI is flanked by DIII, which has an Ig-like fold and constitutes the carboxy terminus of the crystallized fragment. The junctions between the three domains are flexible and capable of hinge-like motions that are important both in the conversion of immature to mature

Fig. 1. Schematic representations of flavivirus particles (a), their genome organization (b) and the viral life cycle (c). (a) Immature virions contain two membrane-associated proteins (prM and E) that form a tight, heterodimeric complex. In the course of virus maturation, the prM protein is cleaved, resulting in the rearrangement of E into homodimers (mature virions). The carboxy-terminal cleavage product of prM (M) remains associated with the viral membrane. In contrast to this schematic representation, the surface proteins are packed tightly in virus particles, as shown in Fig. 3. (b) Genome organization. The positive-stranded RNA genome consists of approximately 11 000 nt and contains a single, long ORF that encodes the structural proteins C, prM/M and E and a set of seven non-structural proteins. The ORF is flanked by non-coding regions (NCRs) at both ends. (c) Flavivirus life cycle: virus entry occurs by receptor-mediated endocytosis, and the acidic pH in the endosome induces structural alterations in E that lead to membrane fusion and the release of the nucleocapsid into the cytoplasm. After uncoating, the plus-stranded RNA genome is translated to initiate virus replication. Virus assembly occurs in the endoplasmic reticulum (ER) and leads to the formation of immature (prM-containing) particles that are transported through the exocytic pathway. There is evidence that the acidic pH in the trans-Golgi network (TGN) causes an irreversible conformational change in the prM–E complex that is required for the maturation cleavage by cellular furin or a related protease (Stadler et al., 1997). Mature infectious particles are released by exocytosis.
virions (Zhang et al., 2004) and in the process of membrane fusion (see below). Of prime importance with respect to fusion is a loop structure at the tip of DII, which is almost completely conserved among flaviviruses and functions as an internal FP during membrane fusion (Allison et al., 2001). In the native dimeric structure of E, this sequence element is buried in a hydrophobic pocket provided by DI and DIII of the second subunit (Fig. 2a–d) and thus shielded from interactions with membranes in its neutral-pH configuration (Rey et al., 1995; Modis et al., 2003, 2005; Zhang et al., 2004).

In addition to its function in fusion, the E protein also mediates receptor binding. Although our understanding of these virus–cell interactions is still far from complete, the currently available information suggests the involvement of both ancillary attachment receptors (such as glycosaminoglycans), which can interact with different parts of the exposed surface of E, as well as more specific receptors that interact with DIII (Chen et al., 1997; Sankaran et al., 1997; Ni & Barrett, 1998; Gril & Roehrig, 2001; Lee & Lobigs, 2002; Kroschewski et al., 2003; Suksanpaisan & Smith, 2003; Hung et al., 2004). The specific interaction of the carbohydrate side chains of E with DC-SIGN or DC-SIGNR has been shown to be involved in the attachment of DenV (DC-SIGN) and WNV (preferentially DC-SIGNR) to immature dendritic cells (Navarro-Sanchez et al., 2003; Tassaneenrithep et al., 2003; Davis et al., 2006). For DenV2, the structural details of this interaction were recently investigated by cryo-electron microscopy (cryo-EM) and were shown to involve an interaction of DC-SIGN with a specific subset of E protein molecules at the particle surface and their carbohydrate side chain, attached to Asn67 (Pokidysheva et al., 2006).

**Structural organization of the viral envelope**

cryo-EM and image reconstructions of mature DenV, YFV and WNV have revealed a very unusual icosahedral envelope organization (Kuhn et al., 2002; Mukhopadhyay et al., 2003) consisting of 30 ‘rafts’, each of which is composed of three E dimers oriented in parallel (Fig. 3b, c). The centre of each raft is coincident with the icosahedral twofold axis and each icosahedral asymmetric unit consists of three E monomers (Fig. 3c). This specific configuration generates different chemical environments for individual E monomers, which can explain the observed non-equivalent binding of DC-SIGN to carbohydrates of different E molecules on the same virion (Pokidysheva et al., 2006). Because of the densely packed arrangement of E proteins, the lipid membrane is largely inaccessible from the exterior and – consistent with its putative role in receptor binding – DIII makes the highest protrusion from the otherwise smooth particle surface (Fig. 3b, c) (Kuhn et al., 2002; Mukhopadhyay et al., 2003).

cryo-EM has also revealed structural details of the arrangement of the parts of E that are absent in the crystallographic structures of truncated E proteins, but play an important role in the fusion process (see below), i.e. the so-called ‘stem’ region and the membrane anchor (Fig. 2d) (Zhang et al., 2003a). As predicted from the mechanism of polyprotein processing (Lindenbach & Rice, 2001), flavivirus E proteins possess a double membrane anchor, with their carboxy termini being exposed at the external surface of the membrane (Fig. 2d). The two amphipathic α-helices of the stem are oriented parallel to the viral membrane and – through their hydrophobic sides – are half-buried in the outer leaflet of the lipid bilayer (Zhang et al., 2003a).

In contrast to mature virions, the surface of immature virions is studded with 60 prominent spikes, each of which is
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Fusion characteristics

Consistent with their use of receptor-mediated endocytosis for entry, the studies that have been carried out so far on flavivirus membrane fusion have demonstrated a dependence on acidic pH, with a relatively high pH threshold around 6-6, suggesting that fusion can already occur in early endosomes. Such studies have been conducted with DenV, JEV, St. Louis encephalitis virus (SLEV), WNV, Murray Valley encephalitis virus (MVEV) and TBEV by using cell–cell fusion or virus–liposome fusion assays (Ueba & Kimura, 1977; Gollins & Porterfield, 1986; Summers et al., 1989; Randolph & Stollar, 1990; Guirakhoo et al., 1991, 1993; Després et al., 1993; McMinn et al., 1996; Corver et al., 2000; Stiasny et al., 2003), but detailed kinetic data have so far only been published for TBEV (Corver et al., 2000). These were obtained by the analysis of the interaction of pyrene-labelled virus with model liposomes and revealed that fusion under these conditions (i) has a broad optimum range below pH 6.2 (threshold above pH 6.6), (ii) does not exhibit any lag phase in the temperature range 15–37 °C (even at 4 °C, the lag phase was found to last for only a few seconds), (iii) is extremely fast and efficient, with a fusion rate of 40 % s⁻¹ under optimal conditions, and (iv) apparently does not require an interaction with specific receptors. This extraordinary efficiency of fusion may be related to specific features of the organization of flavivirus particles, including their smooth surface structure and the orientation of the fusion protein parallel to the membrane – allowing close apposition of the two membranes at the initial stage of fusion – as well as the arrangement of E in an icosahedral lattice that probably favours the coordinated action of fusion-protein clusters to generate a fusion pore. Additional aspects are discussed below in the sections on ‘Structural transitions driving fusion’ and ‘Comparative aspects of viral membrane fusion’.

Role of lipids in fusion

An important role of the lipid composition of target membranes on flavivirus-mediated fusion was revealed in studies with WNV (Gollins & Porterfield, 1986) and TBEV (Stiasny et al., 2003). In both cases, the presence of cholesterol facilitated fusion, but – in contrast to studies with alphaviruses – an absolute requirement could not be demonstrated (see ‘Comparative aspects of viral membrane fusion’). In the TBEV system, it was shown that cholesterol already promoted the early steps of fusion, i.e. membrane attachment and concomitant structural rearrangements in E, and that these effects were dependent on interactions with the 3β-hydroxyl group of cholesterol (Stiasny et al., 2003). The precise nature of these interactions, however, has not yet been elucidated.

Structural transitions driving fusion

Experiments conducted with TBEV have shown that exposure to acidic pH leads to a complete oligomeric rearrangement of the E proteins in the viral membrane from dimers to trimers (Allison et al., 1995), which are significantly more thermostable than the E dimers.

Fig. 3. cryo-EM reconstructions of DenV particles. (a) Immature virion with one of the spikes highlighted (E in green, prM in grey). Reproduced from Zhang et al. (2003c) with permission from EMBO. (b) Mature virion. Reproduced from Kuhn et al. (2002) with permission from Elsevier. (c) Packing of E protein dimers in the mature virus in a herringbone-like arrangement. One of the so-called ‘rafts’ consisting of three parallel dimers is highlighted. The stars label the three Dlls in one of the icosahedral asymmetric units. Reproduced from Zhang et al. (2004) with permission from Elsevier. Twofold, threefold and fivefold symmetry axes are labelled 2, 3 and 5, respectively.

composed of a trimer of prM–E heterodimers (Figs 1a, 3a) (Zhang et al., 2003c). In this configuration, the hinge angle between DI and DII in E is significantly different from that in mature virions (Zhang et al., 2004) and the FP is covered by prM, which extends along the side of E and makes most of its contact with E via the ‘pr’ part of the molecule (Zhang et al., 2003c). Considering the strikingly different organization of E molecules in immature virions compared with mature virions, it is clear that prM cleavage induces massive structural rearrangements at the virion surface, which are poorly understood, but are likely to involve the flexible hinge regions between the individual domains of E, as well as the structural elements of the ‘stem’ (Mukhopadhyay et al., 2005).
As virions exposed to low pH lose infectivity and are no longer fusion-competent (Heinz et al., 1994; Corver et al., 2000), it is assumed that the trimeric form of E represents its final post-fusion structure. The conversion of dimers to trimers requires the disintegration of the cage-like structure of the viral envelope (Fig. 3c), as well as the dissociation of the E dimers into monomers (Fig. 2a–d). This dissociation also leads to the exposure of the FP loop at the tip of DII (which is buried in the native dimer structure), allowing it to interact with a target membrane for initiating the fusion process (Allison et al., 1995; Stiasny et al., 2002).

Structural data on the full-length E trimer have not yet been obtained for any of the flaviviruses. It has been shown, however, that the sE dimers of TBEV and DenV (which dissociate into monomers at acidic pH, but do not trimerize in solution) can be converted into their trimeric post-fusion conformation in the presence of liposomes, and this technology formed the basis for their crystallization and structure determination (Stiasny et al., 2002, 2004; Modis et al., 2004). The structure of a single monomer of the TBEV sE protein in its trimeric post-fusion conformation is shown in Fig. 2(f) in comparison with its native conformation (Fig. 2e) (Bressanelli et al., 2004). The conformational switch involves a reorganization of the molecule from an antiparallel dimer that is oriented horizontally with respect to the viral membrane (Fig. 2c) into a perpendicularly oriented trimer in which the monomeric subunits are arranged in parallel (Fig. 2g). Most importantly, these transitions do not involve a significant refolding of the polypeptide chain and the structural integrity of the three domains (except for some minor secondary-structure changes in DI) remains unchanged. Instead, the major changes involve the reorientation of the domains relative to each other, which is made possible by the hinge-like structures and built-in flexibility at the junctions between DI and DII and between DI and DIII (Fig. 2e, f). The position of DII relative to DI is not dramatically different in both forms, except for a rotation of about 20° at the DI–DII junction. Mutations of residues at the DI–DII interface can alter the pH threshold of fusion, consistent with an important role of this structure for the fusion mechanism (Modis et al., 2003).

DIII, on the other hand, adopts a completely new position and relocates from the end of the native E protein rod to the side of DI. In order to allow this domain movement, the tight interactions at the DI–DIII interface of the native E monomer – involving a number of van der Waals contacts, salt bridges and hydrogen bonds – must be broken. It has been proposed that the protonation of conserved histidines at this interface is an essential driving force for its dissociation and thus a prerequisite for fusion (Bressanelli et al., 2004), but experimental evidence for this hypothesis is still lacking.

Through the relocation of DIII, the structure acquires a hairpin-like orientation and the position of the carboxy terminus of DIII supports the ‘stem’ would follow the grooves generated at the interfaces between the DIIIs, leading to the juxtaposition of the membrane anchor and the membrane-inserted FP loops (Fig. 2h). Modelling studies have indeed shown that the best fit is obtained when conserved residues of helix 1 of the ‘stem’ are directed toward DII from the same polypeptide chain (Bressanelli et al., 2004). The FP loops have the same conformation in both the pre- and post-fusion structures, and it has been proposed that during fusion – because of the presence of exposed carbonyl groups and charged residues on the outside rim of the trimer – they can dip only about 6 Å (0.6 nm) into the outer leaflet of the target membrane (Modis et al., 2004).

The formation of a hairpin-like structure of E in the course of fusion (Fig. 4) is strikingly reminiscent of the hairpin-like post-fusion structures of class I fusion proteins and suggestive of a mechanistically related fusion process, despite the completely different conformations of the proteins involved (Jardetzky & Lamb, 2004). Most of the intermediate stages and structures in this process, however, are still only hypothetical and need further experimental confirmation. In the case of flaviviruses, it is clear that the E dimers must dissociate (Fig. 4b) in order to generate a post-fusion trimer, but it has not yet been resolved whether the initial contact with the target membrane is mediated by

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**Fig. 4.** Schematic of the proposed stages of the flavivirus membrane-fusion process. Colour codes: viral membrane: outer leaflet, yellow; inner leaflet, blue; target membrane: outer leaflet, grey; inner leaflet, red. The colour code of E is the same as in Fig. 2. (a) Metastable E dimer at the surface of a mature virion. (b) Low pH-induced dissociation of E dimer and interaction of E monomers with the target membrane via the FP loop. Dotted lines indicate flexibility at the junction between DI and DII. (c) Initiation of hairpin formation and E trimerization through the relocation of DIII and zipperping of the stem along DII. (d) Hemifusion intermediate in which only the leaflets of the two membranes that face each other (outer leaflets) have mixed. (e) Generation of the final post-fusion structure (E trimer) and opening of the fusion pore.
monomeric or trimeric forms of E. Recent data obtained with TBEV, however, provide evidence for the involvement of membrane-associated, monomeric, pre-hairpin intermediates (K. Stiasny, C. Koessl, J. Lepault, F. A. Rey & F. X. Heinz, unpublished results) and that trimerization does not occur before, but rather as a consequence of, DIII relocation. It has therefore been hypothesized that such a sequence of events could also allow the formation of ‘mixed trimers’, with individual subunits having their FPs inserted either into the target membrane or the viral membrane, an arrangement that could exert strong destabilizing forces on the lipid bilayers and thus facilitate their fusion.

The fusion mechanisms proposed for both the class I and class II systems involve an intermediate in which only the outer leaflets of the two membranes have fused, generating a so-called lipid stalk (Fig. 4d). As the generation of such hemifusion intermediates requires a strong bending of the outer leaflets, it has been attempted to prove or disprove their existence by the insertion of cone-shaped or inverted cone-shaped lipids that would be expected to either promote or impair fusion (Chernomordik & Kozlov, 2005). The results of experiments conducted with TBEV were consistent with the formation of a hemifusion stalk during fusion. At the same time, however, it was shown that the presence of inverted cone-shaped lipids impaired not only the overall fusion reaction, but also preceding steps, such as the interaction of E with target membranes and its conversion into a trimer (Stiasny & Heinz, 2004). Final proof for the involvement of a hemifusion intermediate during flavivirus fusion is therefore still lacking.

**Comparative aspects of viral membrane fusion**

**Comparison of different class II viral-fusion systems (flavi- and alphaviruses)**

Although the flavivirus and alphavirus fusion proteins (E and E1, respectively) do not exhibit any apparent sequence homology, they have a strikingly similar structure (Lescar et al., 2001). Most importantly, the E1 monomer displays the same overall domain organization (domains I, II and III) as the flavivirus E monomer and, also, the internal FP loop is located at the same relative position in DII in both cases (Fig. 5a, c) (Rey et al., 1995; Lescar et al., 2001; Modis et al., 2003, 2005; Zhang et al., 2004; Roussel et al., 2006). The identical topology of each of the three domains in E and E1 strongly suggests divergence from a common ancestor gene (Bressanelli et al., 2004). Further similarities between flaviv- and alphavirus fusion proteins include icosahedral arrangement in the viral envelope, biosynthesis as a complex with a second accessory protein (prM and p62, respectively) that acts as a chaperone for the correct folding of the fusion proteins (Andersson et al., 1997; Lorenz et al., 2002), generation of the metastable, potentially fusogenic state (Gibbons et al., 2000; Stiasny et al., 2001) through the proteolytic cleavage of this accessory protein in the TGN by furin or a related protease (Stadler et al., 1997; Zhang et al., 2003b), strict dependence on acidic pH for fusion and putative involvement of conserved histidines at the DI–DIII interfaces acting as proton acceptors for the low-pH trigger (Bressanelli et al., 2004; Roussel et al., 2006), conversion into a trimer at the pH of fusion, with a similar overall configuration in both cases (Fig. 5b, d) (Bressanelli et al., 2004; Gibbons et al., 2004b; Modis et al., 2004) and soluble forms (lacking the membrane anchors and ‘stems’) of native E and E1 (sE and E1*) that can be converted into the trimeric post-fusion conformation at acidic pH through their interactions with lipid membranes (Klimjack et al., 1994; Stiasny et al., 2002, 2004; Gibbons et al., 2004a). Based on these similarities, the overall fusion process of flavi- and alphaviruses is believed to follow very similar pathways (Kiellian & Rey, 2006).

Despite these striking similarities, there are a number of important differences that set these groups of viruses apart and caution against generalizations. Probably the most significant difference is the fact that the flavivirus E is bifunctional and responsible for both receptor binding and fusion activity, whereas the alphavirus E1 has only fusion activity (reviewed by Schlesinger & Schlesinger, 2001). In the latter case, the receptor-binding activity resides in a second protein (E2), which is derived from the precursor p62 and forms a heterodimeric complex with E1 at the surface of infectious virions. It has been speculated that the common ancestor of alpha- and flavivirus fusion proteins had an E1-like structure and that insertions in D1 led to modifications of the orientation of DIII to endow it with receptor-binding functions, thereby making the E2 protein dispensable (Bressanelli et al., 2004). Eighty trimeric E2–E1 complexes...
form spikes at the surface of mature alphaviruses in a T = 4 icosahedral arrangement (Zhang et al., 2002; Mukhopadhyay et al., 2006) – contrasting with the smooth surface of flaviviruses. It is likely that the somewhat slower fusion kinetics of alphaviruses compared with flaviviruses (summarized by Heinz & Allison, 2000) are a consequence of the more complex structural situation at the alphavirus surface, with the receptor-binding protein (E2) residing on top of the membrane-proximal fusion protein E1 in the heterotrimeric spikes. Compared with flaviviruses, alphaviruses thus have to break up additional protein scaffolds and to move E2 out of the way before the E1 protein can rearrange and mediate fusion.

The presence of E1 as a heterodimeric complex with E2 in infectious alphaviruses points to another important difference between them and the flaviviruses, pertaining to the protection of the FP loop during assembly and maturation. With flaviviruses, the FP is first protected by the prM protein in immature virions, but after the proteolytic maturation cleavage of prM there is a switch and, in the course of the rearrangements leading to the mature virion-surface lattice, the FP becomes protected by its homologous partner in the E dimer. No such switch in the protecting protein occurs through the p62 maturation cleavage of alphaviruses. The p62 cleavage product E2 remains the externally located partner of E1 in the mature form of the heterodimeric complex, burying the FP at its interface.

Although very similar in their overall structural organization, the post-fusion trimers of the flavivirus and alphavirus fusion proteins differ at an important site, i.e. the conformation at the tips of DII, which is closed in the case of flaviviruses and open in the case of SFV (Fig. 5b, d). Because the open configuration of the alphavirus post-fusion structure allows lateral interactions through both the DIII head and the FP loops, a fusion model was proposed that involves a closed ring of trimers, leading to an intermediate that has been designated ‘fusion volcano’ (Gibbons et al., 2004b). It is unclear at present whether the flavivirus E1 trimer would also have an open configuration if additional structural elements that are lacking in the sE trimer were present to allow interactions similar to those proposed in the alphavirus fusion model.

The lipid dependence of fusion and fusion-related processes exhibits both similarities and differences between alpha- and flaviviruses. In both cases, the presence of cholesterol in the target membranes promotes FP insertion and trimerization of the fusion proteins and this requirement involves the 3β-hydroxyl group of cholesterol (Phalen & Kielian, 1991; Wahlberg et al., 1992; Stiasny et al., 2003). In contrast to flaviviruses, however, alphavirus fusion is normally absolutely cholesterol-dependent and – as revealed by the analysis of cholesterol-independent mutants – the i j loop lying adjacent to the FP loop at the tip of DII (Fig. 5c) appears to play a major role in this dependence (Chanel-Vos & Kielian, 2004). The structural details of the underlying mechanism, however, are not yet known and a proposed late intermediate (Kielian, 2006) has not yet been identified.

A further requirement of the alphavirus-mediated fusion reaction, which has not been observed for flaviviruses (Corver et al., 2000), is the presence of sphingolipids in the target membrane to promote the conformational changes necessary for fusion (summarized by Kielian et al., 2000).

Comparison of flavivirus and class I viral-fusion mechanisms

Despite the structural relatedness of all class I fusion proteins, they exhibit significant conceptual heterogeneity, especially with respect to the fusion triggers. The prototypic influenza haemagglutinin (HA) requires an acidic pH – like the class II viral fusion proteins – but others are triggered at the plasma membrane through interactions with receptors and coreceptors (as with HIV) or a combination of receptor-binding and acidic pH-dependent steps (as with ASLV), or they require the binding of an additional protein to a receptor to become activated (as with paramyxoviruses) (summarized by Earp et al., 2005). In comparing class II proteins with the influenza virus HA (which is also triggered by acidic pH), it has to be emphasized that these proteins not only possess completely different molecular architectures, but also display significant differences with respect to their mode of biosynthesis and proteolytic-cleavage activation. The influenza HA (like other class I fusion proteins) is synthesized as a homotrimer of the HA0 precursor, which must be cleaved proteolytically into HA1 (amino-terminal) and HA2 (carboxy-terminal) to become fusion-active (Skehel & Wiley, 2000). Fusion activity resides in the membrane-associated HA2 part of the molecule, which carries an amino-terminal FP and is clamped in its metastable conformation by interactions with HA1. This is in striking contrast to the situation with class II proteins, which are synthesized as heterodimers with an accessory protein that protects the internal FP, and their metastable fusogenic state is generated by proteolytic cleavage of the accessory protein and not of the fusion protein itself.

With certain class I viruses, including influenza virus, it has been shown that fusion could be induced not only by the physiological trigger, but also by alternative experimental triggers, such as elevated temperature or slightly denaturing conditions (Carr et al., 1997; Paterson et al., 2000; Wharton et al., 2000; Wallin et al., 2005). The same treatments, however, did not induce fusion in the case of Semliki Forest virus (SFV) or TBEV (Gibbons et al., 2000; Stiasny et al., 2001) and, in the latter case, only led to protein denaturation. At least in the case of the influenza HA, it is likely that these different properties are related to the proposed capacity of HA2 to adopt its stable, post-fusion, hairpin-like conformation spontaneously upon the release of the external (intermolecular) HA1 clamp (Huang et al., 2002). Hairpin formation in class II fusion proteins, however, requires the relocation of DIII and thus the release from an intramolecular clamp held by the D1–DIII...
interactions in the metastable conformation. The dissociation of this domain interface apparently does not occur spontaneously at elevated temperature or upon slight denaturation, but is dependent on the specific protonation of certain, still unidentified amino acid residues.

Probably the most stunning conclusion derived from the comparative structural analysis of class I and II fusion proteins is the justified assumption that they both mediate a fusion process that is very similar in terms of its mechanistic characteristics. Irrespective of the apparent differences in molecular architecture, biosynthesis, oligomeric structure and cleavage activation, they both use the same principle of providing metastable structures at the virion surface that are primed to undergo triggered conformational changes and to adopt a more stable, hairpin-like structure as a driving force for the merger of the two membranes (Jardetzky & Lamb, 2004; Kielian & Rey, 2006).

**Perspectives**

The greatest advances in our understanding of viral membrane fusion have been due to breakthroughs in the determination of viral fusion-protein structures (or parts thereof) in their pre- and post-fusion conformations. The determination of such structures for representatives of class I and class II fusion proteins has led to the identification of a mechanistically related principle underlying viral (and certain cellular) membrane-fusion processes, despite the involvement of completely unrelated structures driving these processes. The identification of such a general and unifying mechanism in biology is certainly fascinating, but should not obscure the significant differences between fusion machineries of different viruses, even when they possess the same class of viral fusion proteins, such as flaviviruses and alphaviruses.

There are a number of unresolved and poorly defined – but very important – details of the mechanism of flavivirus membrane fusion that will be the subjects of future basic research. Such studies can be foreseen to be dominated by investigations on structural and functional intermediates, the cooperativity of multiple fusion-protein molecules for generating a fusion pore, the molecular interactions between the structural elements of fusion proteins and specific lipids and the detailed molecular basis of the low-pH trigger. It can also be expected that relatives of flavivirus fusion proteins will be detected in other virus genera and/or families and probably even cellular fusion systems, which would further stimulate an investigation of comparative and evolutionary aspects of membrane-fusion processes.

In the area of applied research, the structural information on flavivirus fusion will certainly be exploited for attempts to develop antiviral agents. Knowledge of the pre- and post-fusion structures of E has already led to strategies for the design of specific small-molecule inhibitors, proposed to target either a hydrophobic, ligand-binding pocket at the hinge between DI and DII in the pre-fusion structure of E (Fig. 2a) or other sites involved in the generation of the post-fusion trimer (Modis et al., 2003, 2004; Bressanelli et al., 2004). First attempts to develop flavivirus fusion inhibitors have yielded promising results with DenV, WNV and YFV (Kampmann et al., 2005; P. Young, personal communication) and it is hoped that the further structural refinement of such compounds will lead, in the future, to a supply of potent antiviral agents as a therapy for the most important flavivirus infections of humans.

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**References**


