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REVIEW ARTICLE

Molecular mechanisms of flavivirus membrane fusion

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Abstract Flaviviruses comprise a number of important human pathogens including yellow fever, dengue, West Nile, Japanese encephalitis and tick-borne encephalitis viruses. They are small enveloped viruses that enter cells by receptor-mediated endocytosis and release their nucleocapsid into the cytoplasm by fusing their membrane with the endosomal membrane. The fusion event is triggered by the acidic pH in the endosome and is mediated by the major envelope protein E. Based on the atomic structures of the pre- and post-fusion conformations of E, a fusion model has been proposed that includes several steps leading from the metastable assembly of E at the virion surface to membrane merger and fusion pore formation trough conversion of E into a stable trimeric post-fusion conformation. Using recombinant subviral particles of tick-borne encephalitis virus as a model, we have defined individual steps of the molecular processes underlying the flavivirus fusion mechanisms. This includes the identification of a conserved histidine as being part of the pH sensor in the fusion protein that responds to the acidic pH and thus initiates the structural transitions driving fusion.

Keywords Flavivirus · Membrane fusion · Viral fusion protein · Fusion trigger · Histidine

Introduction

Membrane fusion processes are essential for all forms of life (Martens and McMahon 2008). They are mediated by specialized proteins and consist of the apposition and subsequent merging of membranes enclosing two separate compartments. Virus-cell fusion is required for the entry of enveloped viruses into the cell, leading to the release of the genetic information into the cytoplasm. It is controlled by one or more viral surface proteins (fusion proteins) primed to undergo conformational changes that drive membrane fusion (Harrison 2008b; Kielian and Rey 2006; White et al. 2008). For these changes to occur, the fusion protein has to be activated by a specific trigger resulting in fusion at the right time and at the right place of the viral life cycle. The mode of triggering can be different: low pH after uptake by endocytosis (flaviviruses, influenza), interactions with receptor(s) (paramyxoviruses, most retroviruses), and a combination of receptor binding and low pH (alpharetroviruses) [reviewed in (White et al. 2008)]. These triggering events define the site of fusion and take place either at the plasma membrane or within endocytic compartments.

Many viruses employ only a single protein to accomplish fusion and represent relatively simple models that also contribute to a better understanding of more complex viral (Campadelli-Fiume et al. 2007; Moss 2006) and cellular membrane fusion machineries (Martens and McMahon 2008; Sollner 2004). The 3D structures have been determined for several viral fusion proteins, both in their pre-fusion and post-fusion conformations. Based on profound structural differences, three classes of viral fusion proteins can be distinguished (Weissenhorn et al. 2007; White et al. 2008). Class I fusion proteins are found in orthomyxo-, paramyxo-, retro-, filo-, and coronoviruses (White et al. 2008) and they possess analogies to cellular SNARE proteins (Sollner 2004). Class II fusion proteins have been identified in alpha- and flaviviruses (Kielian 2006; Stiasny and Heinz 2006); and class III fusion proteins in rhabdo-, herpes- and baculoviruses (Backovic and Jardetzky 2009).

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Although the classes of viral fusion proteins show different molecular architectures, the membrane fusion process follows common mechanistic principles. A specific trigger leads to the exposure of a hydrophobic structural element (fusion peptide), which interacts with the target membrane and establishes the contact between the viral and the cellular membrane. Subsequently, the fusion proteins fold into a hairpin-like structure with the membrane anchor and the fusion peptide juxtaposed at the same side of the molecule. This jack-knife-like reorganization of the fusion proteins mediates the merger of the two membranes and finally leads to the opening of a fusion pore (Chernomordik and Kozlov 2008; Kielian and Rey 2006; White et al. 2008).

Histidines and flavivirus fusion

The molecular mechanisms underlying viral fusion triggers are not well understood. In the case of low-pH-dependent viruses (found in all three classes of viral fusion proteins), histidines have been proposed as prime candidates to act as pH sensors for initiating the fusion process (Bressanelli et al. 2004; Kampmann et al. 2006; Kanai et al. 2006; Mueller et al. 2008; Qin et al. 2009; Roche et al. 2008; Roussel et al. 2006; Stevens et al. 2004). Low-pH-induced fusion occurs around a pH value of 6 which is close to the pKa of histidine (pKa ~ 6–7). At neutral pH, histidines are uncharged and become doubly protonated and positively charged at the slightly acidic pH found in endosomes. It proved difficult to answer the question whether the initial trigger is provided by the protontaion of specific residues of the fusion protein or by a cumulative effect trough the increase of positive charges. We conducted a study with recombinant subviral particles of the flavivirus tick-borne encephalitis virus (TBEV) to answer this question and identified an important residue of the molecular pH sensor for the initiation of membrane fusion (Fritz et al. 2008).

Flaviviruses comprise several important human pathogens such as yellow fever, dengue, West Nile, Japanese encephalitis and tick-borne encephalitis viruses (Gubler et al. 2006). The surface of mature flaviviruses consists of a herringbone-like assembly of 90 head-to-tail homodimers of the envelope glycoprotein E (Kuhn et al. 2002; Mukhopadhyay et al. 2003), a class II viral fusion protein. The 3D structures of soluble forms of E (soluble E [sE]) lacking the membrane anchor and the so-called "stem" region (both about 50 amino acids in length; schematically depicted in Fig. 1a) are known for different flaviviruses in their pre- and post-fusion conformations (Fig. 1) (Bressanelli et al. 2004; Kanai et al. 2006; Modis et al. 2003, 2004, 2005; Nayak et al. 2009; Nybakken et al. 2006; Rey et al. 1995; Zhang et al. 2004). The structure of all flavivirus E proteins is very similar and the crystallized part (sE) contains three domains (I, II, III) (Fig. 1a). In the pre-fusion conformation the internal fusion peptide (FP) loop is located at the tip of domain II of one monomeric subunit and hidden by the interactions with a hydrophobic pocket provided by domains I and III of the partner subunit (Fig. 1a). For the initiation of fusion—depicted schematically in Fig. 2—the FP has to be released from these

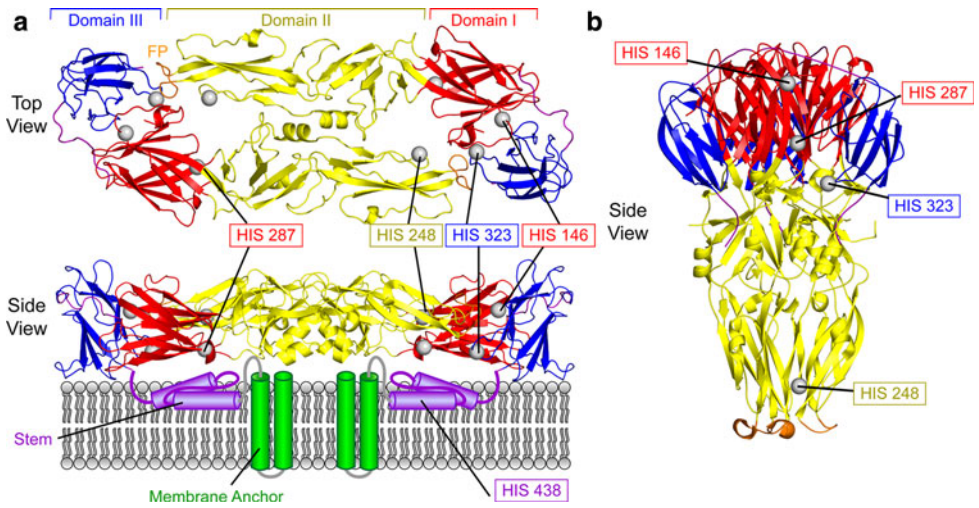

Fig. 1 Three-dimensional structure of the flavivirus fusion protein E. **a** Ribbon diagrams of the pre-fusion E dimer of the TBEV sE (*top* and *side view*) and a schematic of the stem and the membrane anchor. **b** Ribbon diagram of the post-fusion TBEV sE trimer (*side view*). The positions of the histidine residues conserved among all flavivirus E proteins are indicated by *grey* balls. E protein color code: domain I in *red*; domain II in yellow; domain III in *blue*; fusion peptide (FP) in *orange*; stem (linker between DIII and the membrane anchor) in *purple*; membrane anchor in *green*. The viral membrane is shown in *grey*. Ribbon diagrams were drawn with PyMol (DeLano 2002). PDB entry codes: 1svb (TBEV sE pre-fusion conformation), 1urz (TBEV sE trimer)

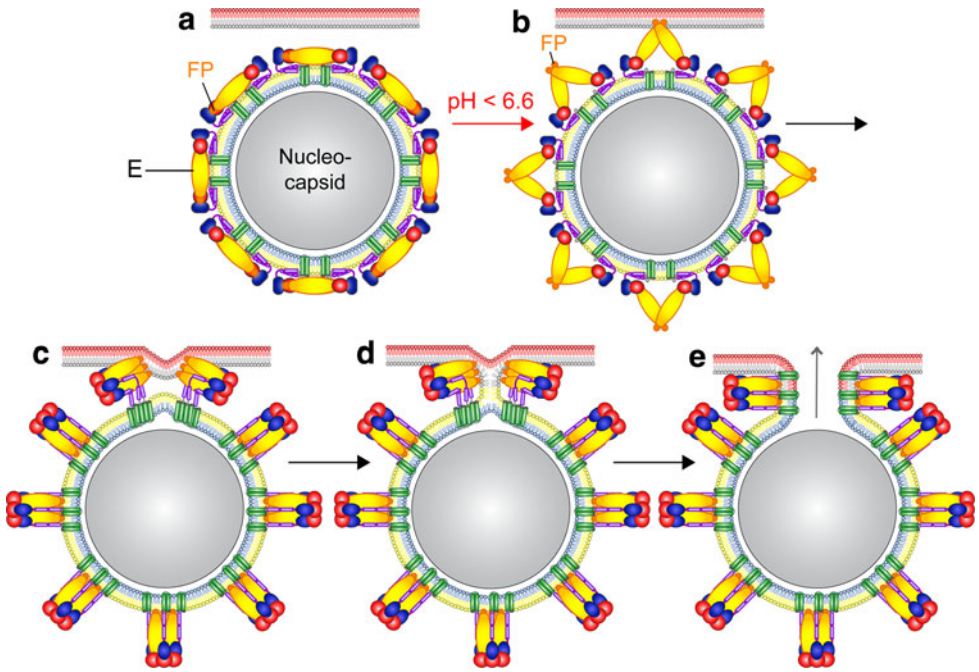

Fig. 2 Schematic of the proposed steps of the flavivirus membrane fusion process. **a** Pre-fusion E dimer on the virus surface. **b** Low-pH-induced dissociation of E dimers into monomers (pH threshold around pH 6.6), outward projection of E monomers, and interaction of the FPs with the target membrane. **c** Trimer formation, relocation of domain III, and "zippering" of the stem. **d** Hemifusion intermediate in which only the leaflets of the two membranes that face each other (outer leaflets) have mixed. **e** Formation of the final post-fusion E trimer and opening of the fusion pore. E protein color code as in Fig. 1. Viral membrane: outer leaflet in *yellow*, inner leaflet in *blue*; target membrane: outer leaflet in *black*, inner leaflet in *red*

interactions to allow its association with the target membrane (Fig. 2b). It is believed that this initial step is followed by trimerization and the relocation of domain III from the end to the side of the rod-like molecule (Fig. 2c, d). Fusion then proceeds by "zippering" of the stem (Fig. 2c, d), thus yielding a hairpin-like post-fusion trimer in which the FP and the membrane anchors are juxtaposed in the fused membrane (Fig. 2e) (Bressanelli et al. 2004; Modis et al. 2004; Nayak et al. 2009).

The E protein contains five histidines that are absolutely conserved among all flaviviruses, four in the crystallized sE (H146, H248, H287, H323) and one in the stem (H438) (Fig. 1). Two of these residues are located at the domain I/III interface: H146 in domain I and H323 in domain III (Fig. 3a). H248 is located in domain II and H287 in domain I.

Effect of mutating conserved histidines on TBEV membrane fusion

To investigate whether conserved histidines in E function as pH sensors in flavivirus fusion, we applied a mutational approach using capsid-less, non-infectious recombinant subviral particles (RSPs) of TBEV (Fritz et al. 2008). RSPs are produced by transient expression and represent excellent tools for studying fusion and the impact of mutations on fusion, because—in contrast to infectious virus—reversion to wildtype or resuscitating mutations do not occur (Allison et al. 2001; Fritz et al. 2008). On the surface of RSPs, the E proteins are organized in an icosahedral lattice (Ferlenghi et al. 2001) and are structurally and functionally equivalent to E on virions (Corver et al. 2000; Schalich et al. 1996). Most importantly, RSPs were shown to exhibit fusion properties similar to whole infectious virions (Corver et al. 2000; Schalich et al. 1996).

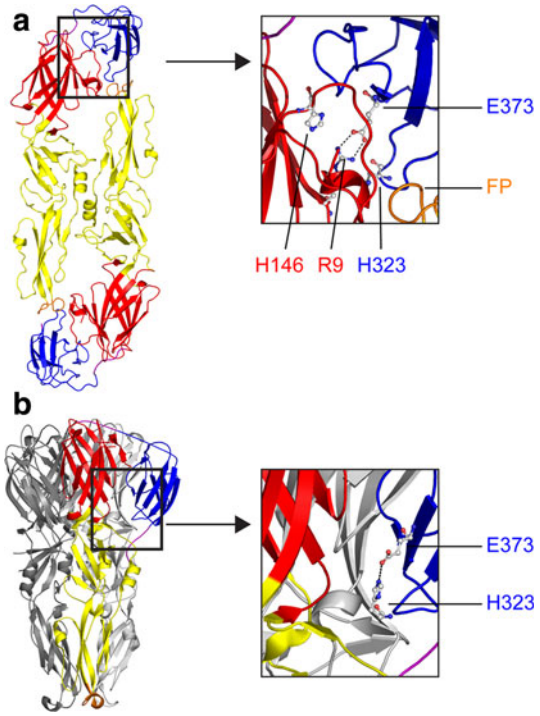

Fig. 3 Domain III interactions in the pre-fusion E dimer (**a**) and post-fusion E trimer (**b**). **a** Ribbon diagram of the sE dimer of TBEV and a zoom of the domain I/III interface highlighting the conserved histidine residues (H146, H323) and the conserved salt bridge between R9 (domain I) and E373 (domain III). **b** Ribbon diagram of the sE trimer of TBEV and a zoom of the salt bridge between H323 and E373. Ribbon diagrams were drawn with PyMol (DeLano 2002)

We mutated all five absolutely conserved histidines in TBEV RSPs (Fig. 1) and studied the effect of these substitutions on fusion and fusion-related processes (Fritz et al. 2008). The introduction of mutations at position 146 did not allow the recovery of particles. Three histidines (H248, H287, H438) could be mutated individually without any effect on fusion whereas substituting H323 strongly reduced fusion of RSPs with liposomes. As shown by additional experiments, H323 mutant RSPs were already impaired in the early steps of membrane fusion, i.e., the dissociation of the dimer, the exposure of the FP and the initial interactions with the target membrane. Due to the less efficient low-pH-induced dissociation of the dimer, the formation of H323 mutant trimers was reduced compared to wildtype. Furthermore, the H323 mutant trimers were less thermostable than the wildtype trimers. A possible explanation for this finding is probably the involvement of H323 in a salt bridge with E373 in the post-fusion trimer that might play a role in trimer stability (Fig. 3b).

Double and triple histidine mutants were also studied and RSPs with mutations at position 248 and 287 were able to undergo the low-pH-induced early stages of membrane fusion like wildtype (FP interactions with target membranes), but trimerization and trimer stability were reduced. Both of these amino acids are located in regions in the post-fusion trimer (Fig. 1b) that could contribute to the stability of this molecule (Fritz et al. 2008; Harrison 2008a).

Conclusions

Using a recombinant subviral particle system, the mutational analysis of histidines conserved among all flavivirus E proteins provided evidence that H323 is an important residue in the initiation of the low-pH-dependent multistep fusion process (Fritz et al. 2008). This amino acid is located at the domain I/III interface (Fig. 3a) which contains a network of interactions between the two domains in the E monomer of the pre-fusion dimer (hydrogen bonds, van der Waals contacts) and a salt bridge between R9 (domain I) and E373 (domain III) (Fig. 3a) (Bressanelli et al. 2004). In this conformation, domains I and III provide a pocket for the FP at the tip of the partner subunit. The destabilization of the domain I/III interface by protonation is essential for the release of the FP as well as for the relocation of domain III and thus for the entire fusion process. Understanding the details of the flavivirus membrane fusion process at a molecular level can help in the search for antiviral compounds that target structural transitions during fusion.

In a recent publication, the substitutions of histidines—in the context of single round infectious particles (SIPs) of West Nile Virus (WNV)—still allowed pH-dependent infection of cells although some of the identified mutants were less infectious than WT SIPs (Nelson et al. 2009). A possible explanation for the observed infectivity—despite the replacement of histidines—could be that other residues can take over the pH sensor function in the course of the host cell infection. It is known that the local protein environment can change the pKa of titrable side chains (Srivastava et al. 2007). Therefore, aspartates and glutamates (e.g., E373 in domain III which is part of the salt bridge with R9 in domain I in the pre-fusion conformation) could also be involved in pH sensoring. R9 and E373 as well as several additional residues (including H146 and H323) at the domain I/III interface are conserved among all flaviviruses. Further experiments—both in vitro fusion experiments with liposomes and standardized infectivity assays—are needed to clarify this issue.

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