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DISSERTATION

Molecular dissection of flavivirus membrane fusion by structure-based mutational analysis

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Verfasser:	Mag.rer.nat. Richard Fritz
Matrikel-Nummer:	9913143
Dissertationsgebiet:	Genetik & Mikrobiologie
Betreuerin / Betreuer:	Univ.-Prof. Dr. Franz. X. Heinz

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SUMMARY

Membrane fusion is an essential step in the infection cycle of enveloped viruses, occurring either at the host cell surface or – after virus uptake by receptor-mediated endocytosis – in endosomes. The merger of the viral with a cellular membrane leads to the release of the viral genome into the host cell cytoplasm and to the initiation of the viral replication cycle. This process is mediated by conformational changes of so-called viral fusion proteins.

Flaviviruses are small, enveloped viruses that comprise important human disease agents including yellow fever virus, dengue virus, Japanese encephalitis virus, West Nile virus, and tick-borne encephalitis virus. Flavivirus membrane fusion occurs in endosomes, where the acidic environment triggers irreversible conformational changes in the major envelope glycoprotein E, a class II fusion protein, that are essential for driving fusion. The molecular structure of the pH-sensor in E has not yet been resolved.

At the surface of infectious virions, E proteins form arrays of metastable homodimers. Each monomeric subunit is composed of three distinct domains. Upon contact to slightly acidic pH, the dimeric E proteins rearrange into trimeric, stable post-fusion structures. The structures of soluble forms of E (sE) have been resolved by X-ray crystallography in both their pre- and post-fusion conformations for several flaviviruses. The sE structures, however, lack the C-terminal membrane anchor and the so called stem region that connects the ectodomain with its anchor. Both are about 50 amino acids in length, contain two alpha helices and their roles in the membrane fusion process has not been investigated so far.

The main focuses of this thesis were to investigate through mutagenesis approaches (I) the structural basis of the low pH trigger for flaviviruses cell entry and (II) the role of the membrane anchor in the fusion process. As a prerequisite, quality controls of the experimental model system had to be established (III).

In the first part of the thesis, the role of specific conserved histidines as pH sensors in flavivirus fusion was elucidated. Five histidines within the E protein are absolutely conserved among all flaviviruses and – because of their pKa

around 6.6 – have been hypothesized to function as molecular sensors for initiating fusion. In this work the so-called “histidine switch hypothesis” – assuming that the flavivirus fusion protein would be activated by changing the protonation state of specific histidines – was experimentally tested for the first time in a mutagenesis study using recombinant subviral particles (RSPs) of tick-borne encephalitis virus (TBEV). The experimental results showed that the initiation of fusion requires the protonation of one highly conserved histidine located at the interface of two domains of E. Another histidine residue at the same interface may have a contributing role. The remaining three conserved histidines – located at different sites of E – however, were completely dispensable for initiating fusion.

In the second part, the functional role of one of the transmembrane domains of the E protein was investigated. The flavivirus E protein is the only known viral fusion protein with a membrane anchor consisting not only of one but of two transmembrane helices (TM1 and TM2). The functional role of TM2 in fusion was unknown and was investigated by the use of RSPs with C-terminally truncated E proteins that lack this domain. The experimental results clearly showed, that the initiation of fusion and the low pH-induced transitions of E were independent of the presence of TM2. However, in the absence of TM2 fusion was blocked at an intermediate stage, possibly related to a significantly reduced stability of the truncated E trimers formed.

The third part of the thesis describes the establishment of a suitable model system to investigate flavivirus membrane fusion by mutational analysis as has been done in parts one and two. RSPs are excellent tools for this purpose because they carry the E protein in a virus-like conformation at their surface and display fusion characteristics similar to those of the native flaviviruses. The development and use of experimental procedures to investigate specific parameters of mutant RSPs is reported.

I. GENERAL INTRODUCTION

1. Viral entry into host cells

Viruses have evolved to enter host cells and use the cellular machinery for their own replication. Based on the presence or the absence of a host cell-derived lipid bilayer membrane, viruses are broadly classified into enveloped and nonenveloped viruses. For cell entry, both groups of viruses have to recognize receptors and need to overcome a cellular membrane. The mechanisms used display similarities as well as striking differences (Harrison, 2007).

1.1. Cell entry by enveloped viruses: Membrane fusion

Whereas nonenveloped viruses deliver their genome by pore formation or a related membrane permeabilizing event (Zhang *et al.*, 2006, Harrison, 2008a; Ivanovic *et al.*, 2008; reviewed in Banerjee and Johnson, 2008), enveloped viruses fuse their membrane with a cellular membrane to release the viral genome into the host cell cytoplasm. Membrane fusion (the merging of two separate lipid bilayers) of enveloped viruses can occur either at the plasma membrane or in endosomes after virus uptake by receptor-mediated endocytosis (reviewed in Smith and Helenius, 2004). Viral membrane fusion does not occur spontaneously, but requires the activation of viral transmembrane glycoproteins, so-called viral fusion proteins (reviewed in Harrison, 2008b; White *et al*., 2008).
Membrane fusion is also a fundamental process in cell biology, including reactions such as intracellular fusion of organelles or cell to cell fusion during fertilization. All cellular fusion processes share common principles and are mediated by proteins that initiate the recognition of the membranes destined for fusion and pull them close together to initiate mixing of the lipids (reviewed in Jahn, Lang and Südhof, 2003; Sollner, 2004; Chen and Olson, 2005; Martens and McMahon, 2008).

2. Viral fusion-proteins and -mechanisms

Fusion of most viruses is mediated by a single type of fusion protein. More complex viruses use a diverse set of two (Poxviruses) or more (Herpesviruses) envelope proteins for cell entry (Spear and Longnecker, 2003; Krummenacher *et al*., 2005; Moss, 2006). Viral fusion proteins can be either monofunctional (mediating fusion only) or bifunctional (mediating receptor binding and fusion). They are often synthesized as inactive precursor proteins that need a proteolytic cleavage event to be primed for subsequent activation. Thus many fusion proteins are C-terminal fragments of their precursors. Fusion protein maturation cleavage (priming) for most viruses, including alpha-, flavi-, retro-, paramyxo- and several influenza viruses, is dependent on intracellular cleavage by furin or related proteases (reviewed in Klenk and Garten, 1994). Either the fusion protein itself is cleaved (e.g. influenza viruses) or an accompanying protein (alpha- and flaviviruses). Maturation cleavage can also occur after virus release by extracellular proteases (certain influenza strains) and after virus entry in endosomes by cathepsins (SARS virus and other Coronaviruses, Ebola virus) (Wengler and Wengler, 1989; Stieneke-Göber *et al*., 1992; Stadler *et al*., 1997; Elshuber *et al*., 2003; Chandran *et al*., 2005; Simmons *et al*., 2005; Schornberg *et al.*, 2006; Qiu *et al*., 2006; Bosch, Bartelink and Rottier, 2008).

A proteolytically primed fusion protein is typically metastable in its pre-fusion conformation, i.e. has not yet reached its lowest energy conformation. In this metastable state, the fusion peptides – conserved hydrophobic sequence elements that can insert into the target membranes and thus initiate fusion – are hidden within the oligomeric structure of the fusion proteins.

To reach the energetically lower post-fusion structure a kinetic barrier has to be overcome by activation of the proteins. Different triggers for the activation of the viral fusion proteins have been described (reviewed in Earp *et al*., 2005; White *et al*., 2008). In some cases, like human immunodeficiency virus (HIV), the triggering event is a specific interaction with cell surface based receptors of the target cell at neutral pH. In many other cases, including flaviviruses, the trigger is exposure to low pH (binding of protons) within endosomes following virus uptake via receptor-mediated endocytosis (Helenius et al., 1980). A third mechanism

that is found in alpharetroviruses requires the combination of receptor interactions at neutral pH and subsequent further activation at acidic pH (Mothes *et al*., 2000). Upon activation, the fusion proteins undergo large scale conformational changes (the pre- to the post-fusion transition) that are the driving force for the merger of the two membranes.

2.1. Structural classes of viral fusion proteins

Two different classes of viral fusion proteins have been defined on the basis of structural criteria. Recently, a third class has been introduced that combines structural features of the other two classes. Class I viral fusion proteins are found in corona-, filo-, orthomyxo-, paramyxo-, and retroviruses, class II fusion proteins in alpha- and flaviviruses. The atomic structures of the Vesicular stomatitis virus (VSV) G protein (Roche *et al*., 2006; Roche *et al*., 2007), the glycoprotein B of Herpes simplex virus 1 (HSV1) (Heldwein *et al*., 2006) and the baculovirus gp64 protein (Kadlec *et al*., 2008) showed surprising similarities between the fusion proteins of these quite unrelated viruses. They currently form the group of class III viral fusion proteins.

2.2. Characteristics of class I fusion proteins

Class I fusion proteins are synthesized as single chain precursors (Fig. 1), that subsequently assemble into homotrimers. Cleavage of the fusion-inactive precursor protein by host cell proteases is required to form a fusion competent protein. Common characteristics of class I fusion proteins are the refolding into a highly stable rod like post-fusion structure and a region dominated by alpha helical structures termed six-helix bundle (reviewed in Kielian and Rey, 2006).
The prototype of a class I viral fusion protein is the influenza virus hemagglutinin (HA) that will be used here to describe important common features of the class I proteins.

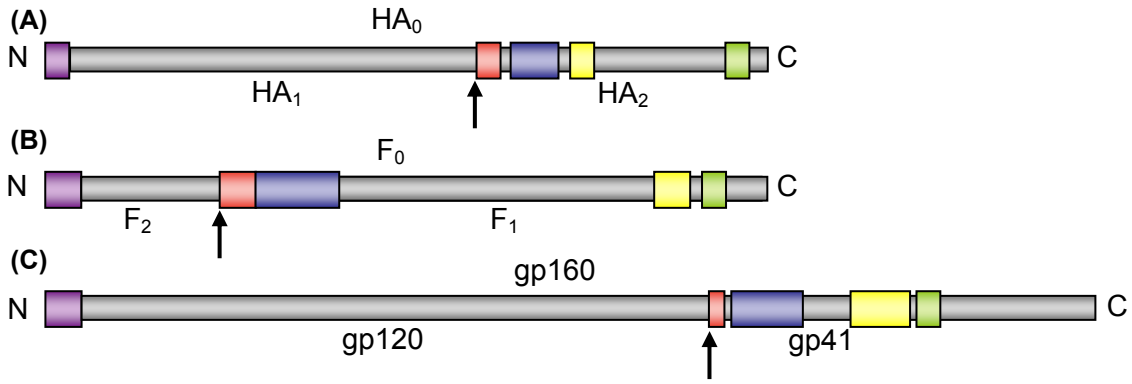


Figure 1. **Schematic representation of class I viral envelope glycoprotein precursors.** Class I envelope proteins are synthesized as single chain precursors that assemble into trimers. The precursors contain a signal sequence (purple), a proteolytic cleavage site (arrow), the fusion peptide (red) and a carboxy-terminal transmembrane anchor (green). Sequences with helix forming propensity are indicated in blue and yellow. (A) Influenza, HA. (B) Paramyxovirus F, (C) HIV gp160 (Reproduced from Colman and Lawrence, 2003).

2.2.1. The Influenza virus hemagglutinin (HA)

So far, the influenza virus HA is the most extensively studied viral fusion protein. It was the first to be structurally characterized in pioneering work by Don Wiley and John Skehel in both its pre- and post-fusion conformations (Wilson, Skehel and Wiley, 1981; Wiley, Wilson and Skehel, 1981; Bullough *et al.*, 1994).

HA is synthesized as a single-chain precursor (HA_0, Fig. 1 A) that is cleaved by host cell proteases resulting in HA_1 and HA_2 subunits that are connected via a disulphide bond. Proteolytic priming of HA_0 does not change the overall structure of the HA_1/HA_2 complex, but results in the formation of a pocket in which the first 22 amino acids of HA_2 – forming the fusion peptide (FP) – are hidden. HA_1 contains the receptor binding site. The influenza virus receptor is sialic acid which is present on many cellular membrane glycoproteins. HA_2, which forms three alpha helical coiled coils, contains the fusion peptide and is the transmembrane subunit anchoring the protein in the viral membrane (reviewed in Skehel and Wiley, 2000).

Figure 2 shows the influenza virus HA in its pre- and post-fusion conformations. In the pre-fusion conformation, the sialic acid sensing subunit HA_1 covers HA_2 (Fig. 2 A). Exposure to low pH in endosomes following sialic acid-mediated

uptake into the host cell triggers a major rearrangement of the HA_1/HA_2 complex. HA_1 dissociates and moves sideward, allowing HA_2 to adopt its post-fusion conformation that reassembles into a stable rod like structure with the fusion peptide and the transmembrane segments at the same end of the molecule (Fig. 2 B). This structure is also referred to as “trimer of hairpins” (reviewed in Kielian and Rey, 2006).

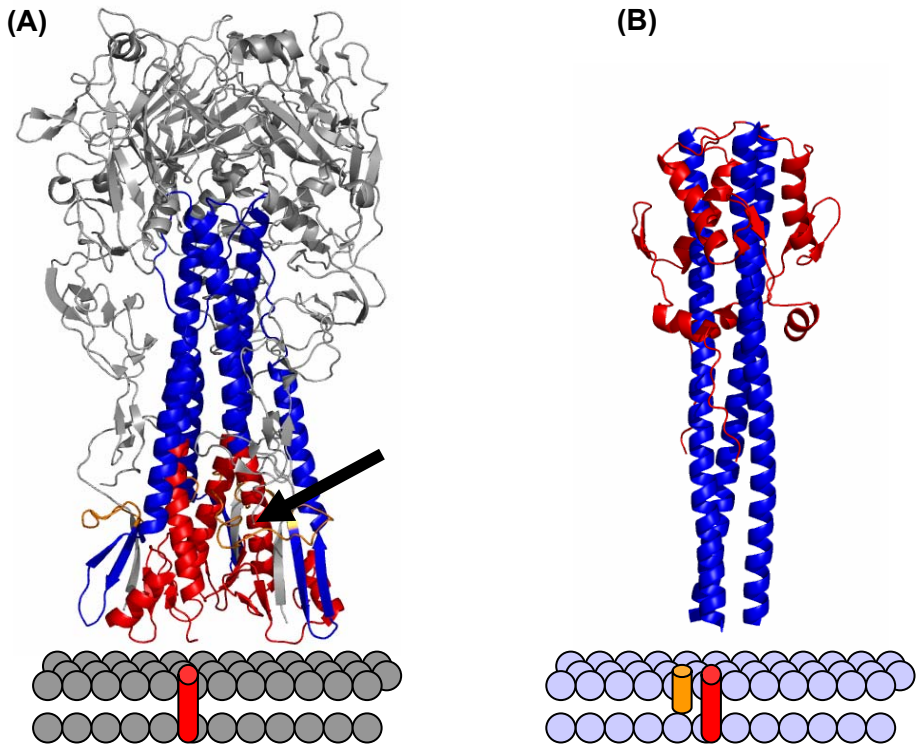


Figure 2. **Structural representations of the pre- and post-fusion conformations of influenza HA.** Fig. 2 A shows the HA_1/HA_2 complex in its native conformation as it occurs at the surface of infectious influenza virions. The receptor binding subunit HA_1 is colored grey and HA_2 is colored in red and blue. The fusion peptide (aa 1-22) is indicated by an arrow and is colored orange. Fig. 2 B shows the crystallized parts of HA_2 in its post-fusion conformation. The C-terminal part is colored red and the N-terminal part blue. The transmembrane domain of one HA subunit is indicated as a red tube (Fig. 2 A, B). One fusion peptide is indicated as an orange tube (Fig. 2 B). Drawn with PyMOL (DeLano, 2002).

2.2.2. Model of membrane fusion mediated by HA

The knowledge of the pre- and the post-fusion structures of HA and several other class I fusion proteins – together with results from biochemical studies – allowed the development of a general, but hypothetical, model of the membrane fusion mechanism mediated by class I fusion proteins. This model is depicted in Figure 3 making use of the influenza HA.

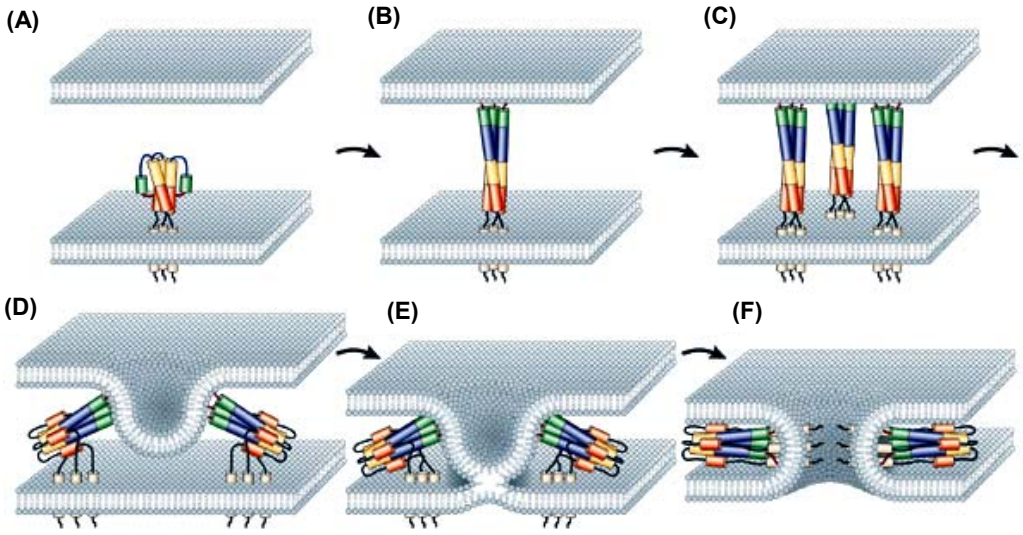


Figure 3. **Hypothetical model of the class I fusion process mediated by influenza HA_2.** (A) The metastable pre-fusion conformation of HA_2. For clarity the HA_1 trimer is not shown. The fusion peptides are buried in a pocket provided by the adjacent monomeric subunits. (B and C) After exposure to low-pH, HA_1 dissociates (not shown) and HA_2 adopts an extended intermediate conformation. The fusion peptides insert into the target bilayer. (D) The parts of HA_2 close to the C-terminus fold back along the outside of the trimeric coiled coil, thereby distorting the membranes and bringing them in close vicinity. (E) A hemifusion stalk is formed in which the outer leaflets of the lipid membranes mix. (F) HA_2 in the stable post-fusion conformation. The fusion peptides and TM domains are close to each other at the same end of the molecule and a fusion pore has formed that allows the release of the viral nucleocapsid (adapted from Mukhopadhyay *et al*., 2005).

Two key features are typical for the refolding of class I fusion proteins mediating membrane fusion: (I) The formation of an extended intermediate coiled-coil structure. In the case of influenza HA this intermediate structure is formed by a

loop-to-helix transition of a polypeptide segment of HA_2 that was previously buried underneath HA_1, leading to the exposure of the fusion peptides (Fig. 3 B, C). (II) The collapse of this intermediate to generate the post-fusion structure. In HA_2 the C-terminal end of the long helix “jackknifes” back and zips up alongside the extended core structure. This change in the HA_2 structure distorts the viral and the host cell membrane and brings them in close proximity. It also brings together the fusion peptides and the transmembrane (TM) anchors (Fig. 3 D) (Harrison, 2008b).

The proximal membrane leaflets mix first. This intermediate situation – when the inner leaflets have not mixed yet – is described as a hemifusion state (Fig. 3 E) (Chernomordik and Kozlov, 2005, 2008). When the C-terminal part of HA_2 relocates, the trimer presumably bends away from the hemifusion stalk to facilitate the mixing of the membranes (Fig. 3 E).

In the post-fusion conformation the fusion peptides and the TM domains are juxtaposed at the same end of the trimer and interactions between residues of the fusion peptides with both, residues of the coiled coil and of the N-terminal end of the TM anchors, were discussed (Harrison, 2008b). Specifically, these interactions are thought to contribute to the formation of a cap-like structure – at the N-termini of the coiled coil – that may be important for the transition from hemifusion to the opening of the fusion pore (Chen, Skehel and Wiley, 1999; Park, Gruenke and White, 2003).

2.3. Characteristics of class II fusion proteins

The three-dimensional structures of class II fusion proteins are radically different from class I proteins which are trimeric in both their pre- and post-fusion conformations. Class II fusion proteins – that are predominantly composed of beta-sheets – mediate fusion by their conversion from a metastable homo- or heterodimeric conformation to a considerably more stable post-fusion homotrimer. In addition to molecular architecture and oligomeric structure, class II differ from class I proteins in terms of maturation cleavage. In contrast to class I fusion proteins, they are not cleaved themselves during particle maturation but an associated protein with chaperone function is processed.

So far, class II fusion proteins have been found in alpha- and flaviviruses. Here flaviviruses will be used as a model to describe the properties of a representative class II viral fusion protein.

2.3.1. Flaviviruses: Assembly, maturation and structures

Flaviviruses are small, enveloped, positive-strand RNA viruses with a diameter of approximately 500 Å. They form a genus within the family *Flaviviridae* that consists of about 70 members including several important human pathogens like yellow fever (YF), dengue (Den), West Nile (WN), Japanese encephalitis (JE) and tick-borne encephalitis (TBE) viruses. Most of them are transmitted to their vertebrate hosts by arthropods (mosquitoes or ticks) and can cause fevers, hemorraghic fevers and encephalitis in humans.

The structural organization of flaviviral particles as well as the viral genome and the life cycle are shown in Figure 5. Flaviviruses consist of an isometric nucleocapsid, composed of a single protein termed C (capsid), surrounded by a lipid bilayer containing two membrane-associated proteins designated E (envelope) and M (membrane, synthesized as its precursor prM) (Fig. 5 A). The genome is about 11,000 nt in length and contains a single open reading frame (ORF) with non coding regions at its 5' and 3' ends (Fig. 5 B). The structural proteins (C, prM/M, E) are encoded in the 5' terminal third of the ORF. The rest of the ORF codes for non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (reviewed in Mukhopadhyay, Kuhn and Rossmann, 2005; Stiasny and Heinz, 2006; Lindenbach, Thiel and Rice, 2007).

Virus assembly occurs in the endoplasmatic reticulum (ER) (Mackenzie and Westaway, 2001; Lorenz *et al*., 2002) (Fig. 5 C) and results in the formation of immature virus particles containing heterodimeric complexes of prM and E proteins. These prM/E complexes give the immature particles a spiky surface (Zhang *et al*., 2007) (Fig. 6 A). Immature flavivirus particles are transported through the Golgi complex into the acidic environment of the trans-Golgi network (TGN) where low pH triggers a conformational change in the protein complex that allows cleavage of prM into pr and M by furin or a related protease (Stadler *et al*., 1997; Elshuber *et al*., 2003, reviewed in Klenk and Garten, 1994).

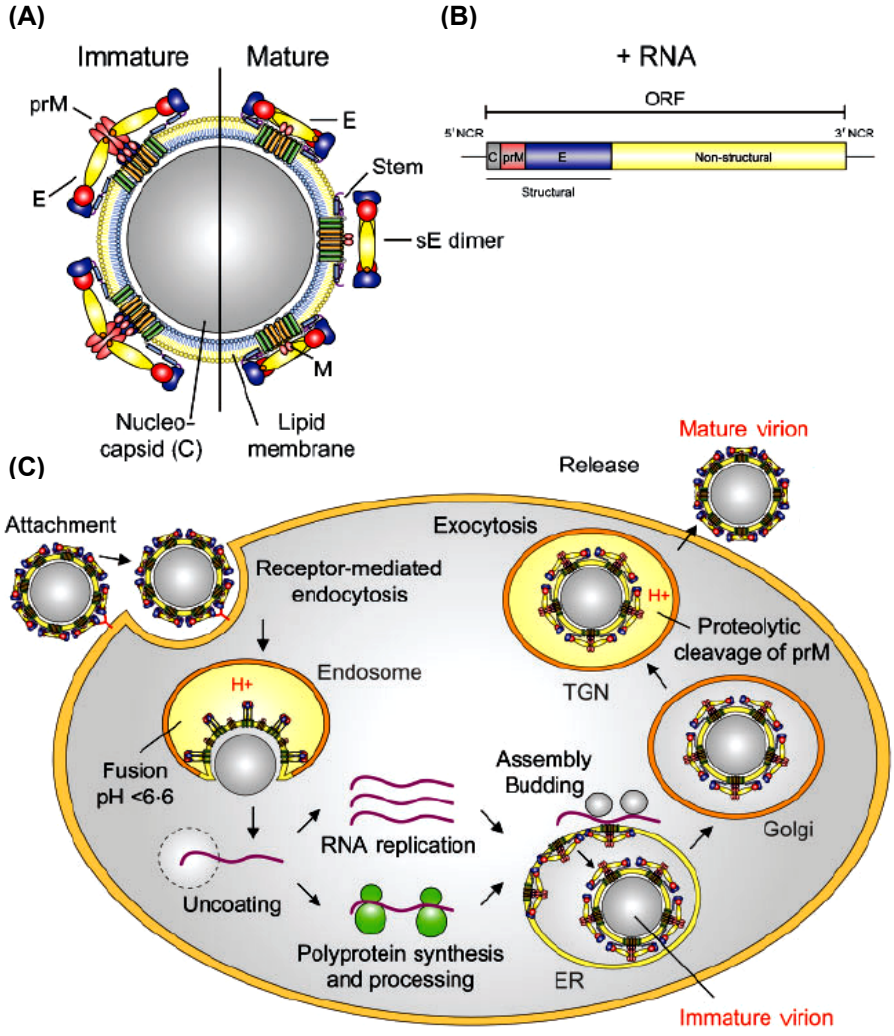


Figure 5. **Schematics of flavivirus particles (A), genome organization (B), and the viral life cycle (C).** (A): Flavivirus particle in its immature (left) and mature (right) form. Immature particles contain heterodimeric complexes of prM and E proteins. prM is cleaved during particle maturation, resulting in the reorganization of E into homodimers on the surface of mature virions. (B): The positive-stranded flavivirus RNA genome with the long ORF encoding structural proteins C, prM, E and seven non-structural proteins. (C): The Flavivirus life cycle. Infectious viruses are internalized by receptor mediated endocytosis. The acidic pH in the endosomes triggers conformational changes in E resulting in membrane fusion and the release of the nucleocapsid into the cytoplasm. After uncoating, the RNA genome is translated and viral replication begins. Assembly takes place in the ER and results in the formation of immature particles. Particles are transported through the exocytic pathway where maturation cleavage occurs. Mature virus particles are released via exocytosis (from Stiasny and Heinz, 2006).

After maturation cleavage, E is rearranged at the particle's surface and primed for subsequent activation to mediate membrane fusion. Immature (prM containing) flavivirus particles do not fuse because the prM protein has the function of a chaperone and prevents the conformational changes in E that would result in premature membrane fusion upon exposure to low pH in the TGN (Heinz *et al*., 1994). The cleaved pr peptide stays associated with the complex – after the maturation cleavage has occurred – until the virion is released into the extracellular space where neutral pH conditions induce the dissociation of pr (Li *et al*., 2008; Yu *et al*., 2008).

The structures of whole flavivirus particles have been determined by cryo electron microscopy (cryo-EM) using Dengue 2 and West Nile virus and fitting the already known structure of the envelope protein E in the EM density map (Rey *et al.*, 1995; Kuhn *et al*., 2002; Mukhopadhyay *et al.*, 2003; Kaufmann *et al*., 2006). These studies revealed the structural organization of flavivirus particles, especially of the envelope that contains 90 dimers of E. The native viral surface is smooth (Fig. 6 B) and organized in rafts of three E homodimers oriented in parallel that form a herringbone-like icosahedral lattice (Fig. 6 C) (Kuhn *et al*., 2002; Mukhopadhyay *et al*., 2003; Mukhopadhyay *et al*., 2005).

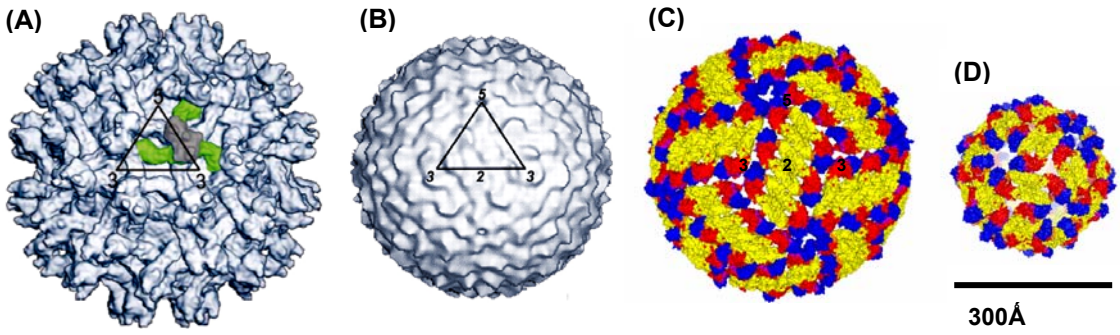


Figure 6. **Reconstructions of flavivirus particles.** (A): Cryo-EM reconstruction of an immature Dengue virus particle with one prM/E "spike" highlighted (green: E, grey: prM). From Zhang *et al*., 2003b. (B): Mature Dengue particle from Kuhn *et al*., 2002. (C): Organization of E proteins across the surface of a mature flavivirus particle. The E dimers are packed in a herringbone-like arrangement. Twofold, threefold and fivefold symmetry axes are labelled 2, 3 and 5 respectively. (D): Structural organization of a subviral particle of TBE virus. Prepared with PyMol (DeLano, 2002).

In addition to infectious virions also small non-infectious particles are released from flavivirus-infected cells, which are natural by-products of infection (Stollar, 1969; Smith *et al*., 1970). These have been termed “slowly sedimenting hemagglutinin” (SHA) because – like the virions – they are capable of agglutinating red blood cells at acidic pH conditions. Such subviral particles can be produced in recombinant form by the coexpression of E and prM. They lack the nucleocapsid but contain E and M proteins in a lipid membrane, indicating that the formation of the particles is not absolutely dependent on the presence of the C protein, but is driven by lateral interactions of E and prM (reviewed in Heinz and Allison, 2000, 2003; Stiasny and Heinz, 2006). The structure of a flavivirus recombinant subviral particle has been resolved by cryo-EM and image reconstruction (Ferlenghi *et al.*, 2001). It displays a T=1 icosahedral arrangement of 30 E dimers (Fig. 6 D).

2.3.2. The flavivirus envelope protein E

E is a prototypic bifunctional class II viral fusion protein. Soluble forms of the pre- as well as the post-fusion structures of the flavivirus envelope proteins (lacking the transmembrane anchors and a ~ 50 amino acids “stem” region) are known in atomic detail. The structure of the TBEV E protein in its pre-fusion form has been determined to 2Å resolution by X-ray crystallography of a soluble dimeric fragment (Heinz *et al*., 1991; Rey *et al*., 1995). Also the structures of the soluble E proteins (sE) of pre-fusion dimers of DenV2, DenV3 and monomers of WNV are known from truncated recombinant proteins expressed in insect cells (Modis *et al*., 2003; Zhang *et al*., 2004; Modis *et al*., 2005; Nybakken *et al*., 2006). The structures of sE post-fusion trimers have been determined for TBEV (Bressanelli *et al.*, 2004) and Dengue virus type 2 (Modis *et al*., 2004). Although the E proteins of TBE and Den viruses have less than 40% identical amino acids, the overall structures of both proteins are basically the same and it is assumed that this basic structure applies to all flavivirus E proteins.

2.3.3. Pre-fusion structure of E

E is a glycoprotein of approximately 60 kD that is gently bent corresponding to the orientation of the metastable E dimer parallel to the viral membrane at neutral pH (Fig. 7 C, D). The two monomeric subunits are oriented antiparallel to each other and each monomer is organized in three distinct domains.

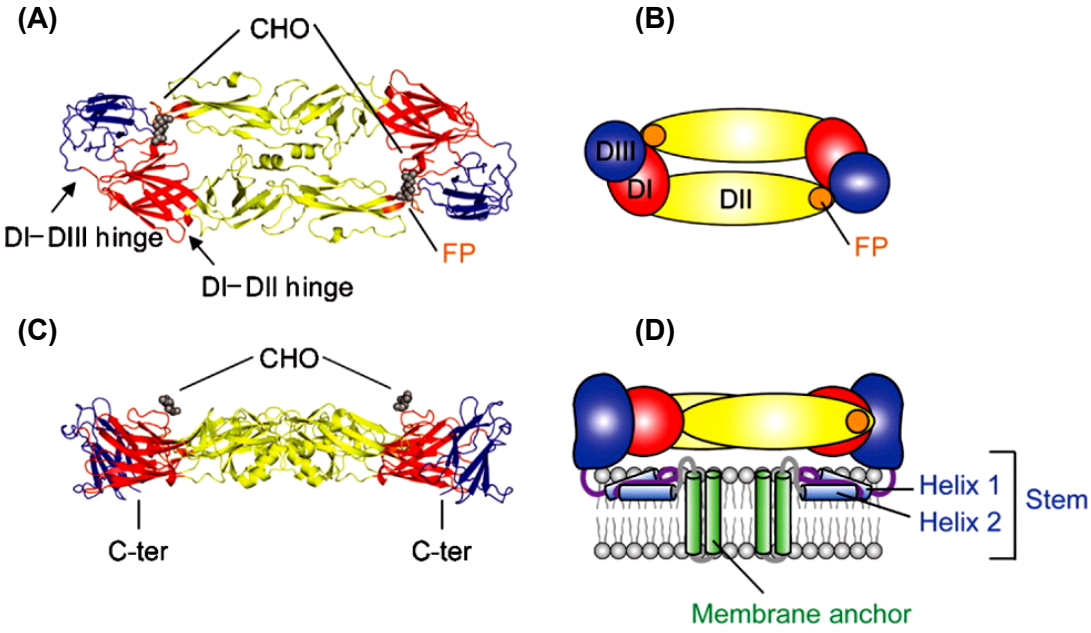


Figure 7. **Ribbon diagrams (A, C) and schematics (B, D) of TBEV E proteins in their pre-fusion conformations.** (A, B) top view. (C, D) side view. Color codes: DI, red; DII, yellow; DIII blue; FP, orange; stem helices 1 and 2, light blue; C-terminal transmembrane helices, green; lipid membrane, grey (from Stiasny and Heinz, 2006).

Domain I (DI) is the central domain composed of 120 residues and contains the N-terminus. Domain II (DII) is an elongated finger-like structure based on an antiparallel beta sheet of five short strands. Domain II is involved in most of the intersubunit contacts within the molecule (dimerization domain) and at its tip there is a highly conserved loop region that functions as internal fusion peptide (FP) (Allison *et al*., 2001). Within the dimer, the fusion peptide of one monomer is buried in a hydrophobic pocket between domains I and III (DIII) of the adjacent monomer and is thus protected from membrane-interactions in the native

conformation (Rey *et al*., 1995; Modis *et al.*, 2003). DIII is an Ig-like domain containing the C-terminus of the truncated crystallized form (sE). The soluble sE proteins lack the last ~100 C-terminal amino acids (the so-called “stem-anchor region) and did not allow investigations of structural details beyond the end of DIII. Improved techniques for reconstructing cryo-EM images made it possible to determine these parts of E in their native conformation (Zhang *et al*., 2003a). The stem (~ 50 residues) connects the ectodomain with the transmembrane anchor. It contains two amphipathic helices that lie parallel to the viral membrane and are partially buried in it via hydrophobic interactions. The flavivirus E protein has a double membrane anchor consisting of two helices (Fig. 7 D). Also the M protein has two transmembrane regions, but it is assumed that there are no interactions between the transmembrane helices of E and M in the membrane (Mukhopadhyay *et al*., 2006).

The junctions between the three domains are not rigid and allow hinge-like movements. Domains I and II are connected by four polypeptide chains, domains I and III by a single polypeptide linker (DI-DIII hinge) (Fig. 7 A) giving this region a flexibility that is important for particle maturation and membrane fusion (reviewed in Stiasny and Heinz, 2006).

DIII has been discussed to contain a receptor binding site (Bhardwaj *et al*., 2001). The receptors for flavivirus internalization have not yet been clearly identified. Most probably, there are general attachment receptors (like glycosaminoglycans), that can interact with various regions of E, contributing to specific receptor-DIII interactions. DC-SIGN and DC-SIGNR have been shown to be involved in the attachment of Dengue (Tassaneetripthep *et al*., 2003) and West Nile viruses (Davis *et al*., 2006) to immature dendritic cells. CD14 associated molecules may be essential for Dengue virus entry (Chen *et al*., 1997; Chen, Wang and King, 1999).

2.3.4. Post-fusion structure of E

In contrast to the native dimers, the low pH-induced post-fusion structures of the flavivirus fusion proteins are trimeric (Allison *et al*., 1995). While the antiparallel E dimers are oriented horizontally to the virus membrane (Fig. 7), the trimers are

oriented perpendicularly to it with the subunits arranged parallel to each other (Fig. 8). The structures of post-fusion sE trimers have been resolved for TBEV (Bressanelli *et al*., 2004) (Fig. 8 A) and for Dengue virus 2 (Modis *et al*., 2004), respectively. In the case of TBEV E these trimers have been shown to be significantly more thermostable than the E dimers (Stiasny *et al*., 2001).

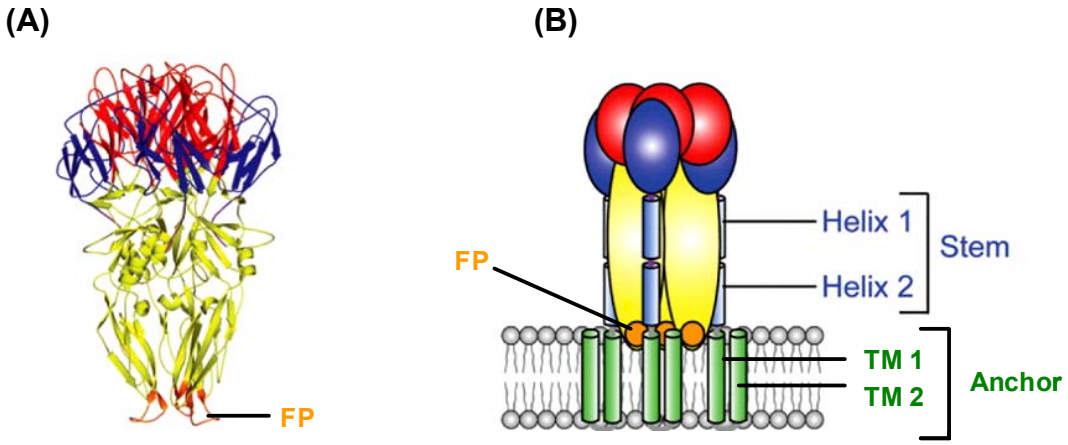


Figure 8. **Ribbon diagram (A) and schematic (B) of E in its low pH-induced post-fusion conformation.** Side view of sE (A) and the full-length E trimer (B). Color code corresponds to figure 7 above **(**from Stiasny and Heinz, 2006).

In its post-fusion conformation E forms an elongated hairpin-like structure reminiscent of class I fusion proteins. As in class I proteins, the fusion peptides and the TM regions are juxtaposed at the same end of the molecule. It is largely unknown which conformation the stem adopts in the post-fusion structure because there is no structural data available on the full-length E trimer for any of the flaviviruses. The orientation of the C-terminus in sE, however, indicates that the stem follows grooves between the domains II alongside the trimeric core body (Bressanelli *et al*., 2004) (Fig. 8 B). The lack of a structure of the stem also leaves open the question if a cap-like structure is formed as in influenza fusion. The high hydrophobicity of the stretch of amino acids N-terminal of the TM domains would make interactions with the FPs plausible (Harrison, 2008b).

2.3.5. Low pH-induced transitions of E driving fusion

The low pH-induced conversion from dimers to trimers requires the dissociation of the dimers into monomers and a subsequent reorganization into trimers. During the dimer-trimer transition the E protein domains retain their structural integrity (except some minor changes in DI), but have to rotate and rearrange with respect to each other (Fig. 9). There is a slight reorientation of DII due to a 20° rotation at the DI-II junction. DIII, on the other hand, adopts a completely new position by relocating from the end to the side of the molecule, thereby bringing the C-terminus closer to the fusion peptide (reviewed in Stiasny and Heinz, 2006).

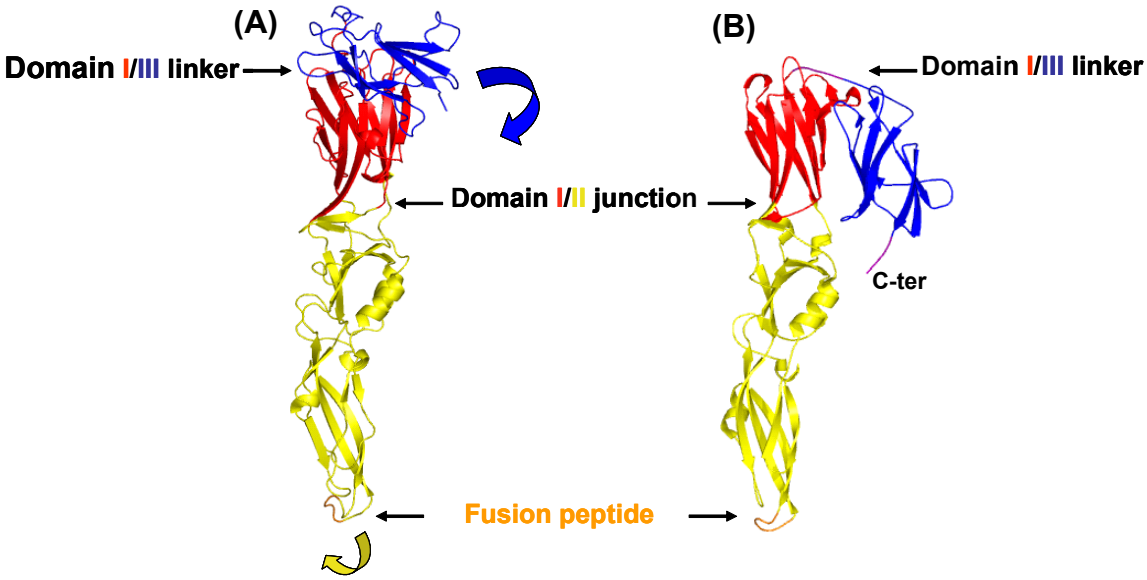


Figure 9. **Ribbon diagrams of TBE E monomers in their pre- (A) and post-fusion (B) conformations.** Color code corresponds to figure 7. The arrows indicate the low pH-induced movements of domain III (blue arrow) and domain II (yellow arrow). Drawn with PyMol (DeLano, 2002).

These rotations are made possible by the built-in flexibility of the hinge structures between domains I-II and I-III. Certain mutations at the domains I-II interface altered the pH threshold for E mediated fusion, pointing out the importance of motion at this interface (Modis et al., 2003).

The important role of the complete relocation of DIII is demonstrated by the fact that antibodies against DIII can inhibit viral entry after attachment (Nybakken *et al*., 2005) and that soluble DIII can act as a potent fusion inhibitor by binding to the polypeptide thus inhibiting the conformational changes described (Liao and Kielian, 2005).

To allow the dissociation of the monomers and the movement of DIII, several contacts at the interface of DI and DIII must be broken. These interactions, including hydrogen bonds, van der Waals contacts and a prominent salt bridge, are thought to keep the native conformation of E in place (Fig. 10).

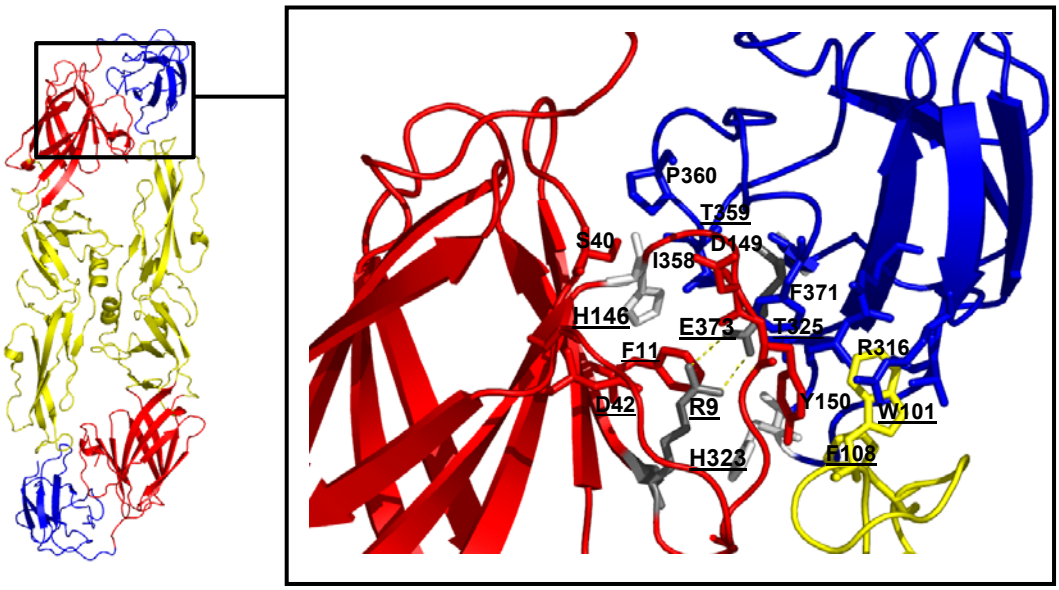


Figure 10. **Ribbon diagram showing contacts at a domains interface of native TBE E.** Shown is the top view of the interface of the domains I and III in its pre-fusion conformation. Residues involved in interactions are labelled. Two conserved histidines (His146 and His323) and a conserved salt bridge (Arg9 and Glu373) are highlighted in light- and dark-grey and bold characters. For clarity residues 1-5 are not shown (Adapted from Bressanelli *et al*., 2004). Drawn with PyMol (DeLano, 2002).

Fusion of some class I viruses is known to be inducible by unphysiological triggers – like heat – that lower the protein stability (Carr *et al*., 1997; Gibbons *et al*., 2000; Wharton, Skehel and Wiley, 2000; Wallin, Ekström and Garoff, 2005; Connoly *et al*., 2006). For class II proteins no such unspecific triggers were

identified (Gibbons et al., 2000; Stiasny et al., 2001), suggesting that the dissociation of the DI-DIII interface is tightly controlled and depends on the protonation of specific amino acids (Stiasny and Heinz, 2006). It has been proposed that protonation of key histidines within the domain interfaces is the trigger for the dissociation and the transitions described (Bressanelli *et al.*, 2004; Kampmann *et al*., 2006). This issue is discussed in detail in chapter 3.

2.3.6. Model of membrane fusion mediated by E

Like for class I fusion proteins a hypothetical model of membrane fusion mediated by class II fusion proteins – represented by the flavivirus E protein in Figure 11 – has been developed. Most of the steps are still hypothetical and build on logical deductions based on the known structures of the initial and the end states. Evidence for the existence of monomeric pre-hairpin intermediates (Fig. 11 B) has been provided by the use of alkaline pH to trigger the initial phases of fusion (Stiasny *et al*., 2007a).

The initiation of the fusion process requires the dissociation of the E dimers and the exposure of the FPs. The physiological trigger for these events is contact to low pH in the endosomes. Once dissociated, the monomeric subunits swing outwards and the exposed FPs insert into the target membrane (Fig.11 B), probably facilitating the subunit reorganization into trimers. It is not completely clear whether trimerization of the monomers occurs before the relocation of DIII (facilitated by insertion of the FPs), or afterwards probably as a consequence of it. Through the relocation of DIII the C-terminus moves close to the FP, a prominent hallmark of the post-fusion conformation. The stem is thought to zip up along the sides of domains II (Stiasny and Heinz, 2006; Harrison, 2008b). These conformational changes probably lead to the bending of the membranes involved (Fig.11 C). It is believed that fusion proceeds via a hemifusion intermediate, in which only the outer membrane leaflets have mixed (Chernomordik & Kozlov, 2005; Chernomordik, Zimmerberg and Kozlov, 2006; Harrison, 2008) (Fig.11 D). The final step is the formation of a fusion pore that allows the release of the nucleocapsid (Fig. 11 E).

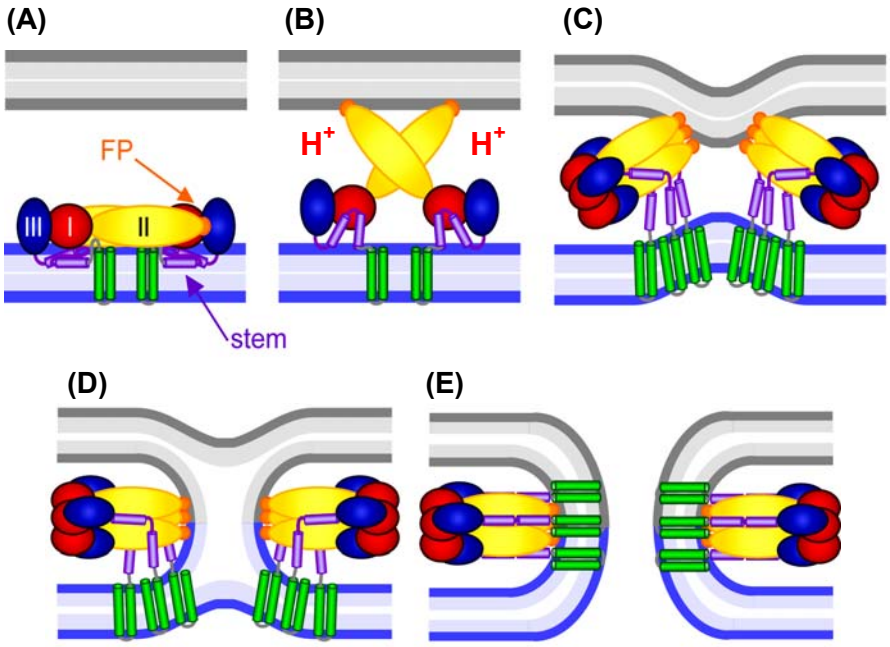


Figure 11. **Schematic model of the flavivirus membrane fusion process.** (A): Side view of one – out of 90 – metastable E dimers at the surface of a flavivirus particle at neutral pH. The FPs are buried at an interface with DIII of the adjacent monomer. (B): Low pH-induced dissociation of the E dimer and interaction of the FPs with the target membrane. (C): E trimerization and initiation of hairpin formation. DIII relocates to the side of the molecule and the stem segment zippers up along the trimeric core body. (D): Formation of a hemifusion intermediate with only the outer leaflets mixed. (E): Formation of a fusion pore. Color code for E corresponds to the figures above. Color of the membranes: viral membrane blue, host cell membrane: grey (from Stiasny *et al*., 2007b).

Class I and II viral fusion proteins display unrelated structures. Still they show the same principle of merging two membranes by leaving a state of metastability – by interactions with a host cell – and adopting a more stable “hairpin-like” post-fusion conformation. This suggests that the underlying mechanisms of membrane fusion mediated by different fusion proteins are closely related (reviewed in Jardetzky and Lamb, 2004; Schibli and Weissenhorn, 2004; Kielian and Rey, 2006).

2.3.7. Differences and shared properties of the class II fusion systems of alpha- and flaviviruses

The flavivirus class II fusion protein E has some unique characteristics, but it shares some features with the fusion protein E1 of the alphaviruses (reviewed in Stiasny and Heinz, 2006; Kielian, 2006). Figure 12 shows E1 of the alphavirus Semliki forest virus (SFV) in its pre-fusion (A) and post-fusion (C) conformations as well as a mature SFV particle at neutral pH (B) and conserved histidines in the E1 monomer in its pre-fusion conformation (D).

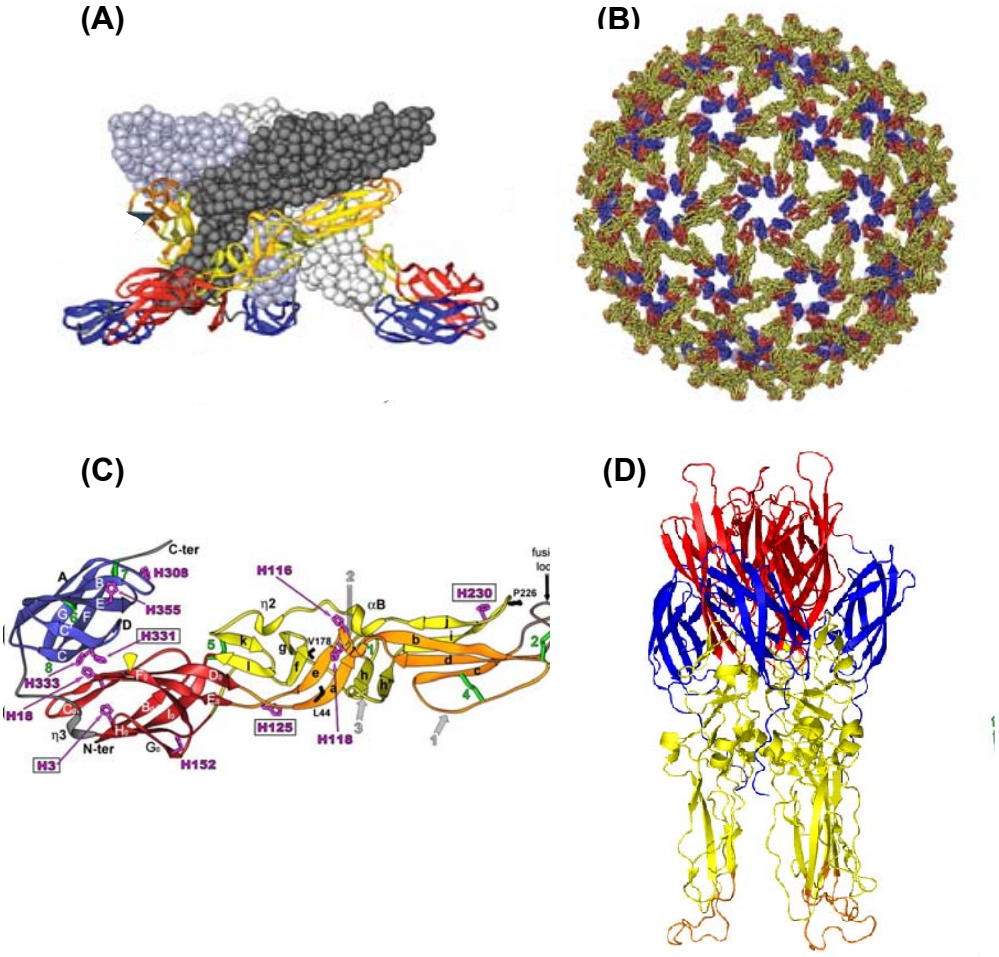


Figure 12. **The E1 protein of Semliki forest virus.** (A): Pre-fusion conformation of E1 (ribbon representation) in complex with E2 (represented as grey spheres). (B): Organization of E1 across the alphaviral surface (for clarity E2 is left out) (from Kielian and Rey, 2006). (C): Ribbon diagram of E1 in its pre-fusion conformation, showing the histidines in the monomer. Conserved residues are highlighted by boxes (from Roussel *et al.*, 2006). (D): Post-fusion E1 trimer drawn with PyMol (DeLano, 2002). Color code for (A), (B) and (D) corresponds to the flavivirus E protein above. In (C) domain II is colored yellow and orange to differentiate DII loops, the fusion loop is brown.

One similarity between E and E1 is the organization of both proteins in three domains (DI, II, III) with an internal fusion loop at the tip of DII. Similar to E in flaviviruses, the E1 proteins of alphaviruses are organized as an icosahedral lattice in the envelope (Lescar *et al*., 2001; Pletnev *et al*., 2001) (Fig.12 B). Like the flavivirus fusion protein, E1 is synthesized as a complex with an accessory protein (prM in the case of flaviviruses, p62 for alphaviruses) that has chaperone activity, and protein maturation depends on cleavage of that protein by furin. Overall, the similar structural organization and topology of the domains of E and E1, including the location of the fusion peptides, suggests a common ancestor gene (Bressanelli *et al*., 2004).

For both, the flavivirus E and the alphavirus E1 protein, the trigger for the irreversible formation of the post-fusion trimers is exposure to low pH. Like in E, conserved histidines in E1 at the interface of DI and DIII are discussed as potential proton-acceptors driving the destabilizing process causing fusion (Fig. 12 C) (Roussel *et al*., 2006; Rey, 2008). The trimers of both class II proteins were observed to form rings of six (flaviviruses) or five to six trimers (alphaviruses) when formed in the presence of membranes, suggesting cooperativity between trimers during fusion (Gibbons *et al*., 2004; Stiasny *et al*., 2004).

Despite the mentioned similarities between alpha- and flaviviruses there are a number of important differences. E and E1 do not show any remarkable sequence homology. While E mediates both, receptor binding and fusion, E1 is monofunctional, i.e. it only mediates fusion. Receptor binding is mediated by E2, a second surface glycoprotein that forms a heterodimeric complex with E1 and is derived from cleavage of the precursor p62. In contrast to the smooth surface of flaviviruses the surface of alphaviruses displays spikes formed by eighty trimers of E1/E2 heterodimers (Zhang *et al*., 2002; Mukhopadhyay *et al*., 2006).

Even though a partner protein is cleaved as in flavivirus maturation, the cellular maturation pathway of alphaviruses is different because they bud at the plasma membrane, and not at the endoplasmatic reticulum.

Another major difference between the two fusion proteins is the protection of the fusion peptides. In flavivirus E, the FP is shielded by prM before virus maturation and after maturation cleavage it is hidden in a hydrophobic pocket within the E

dimer. In the case of alphaviruses, the fusion peptide is shielded by E2 in both, the immature and the mature forms.

The alphavirus E1 trimer has an open tip, formed by domains II (Fig. 12) (Gibbons *et al*., 2004) which is in contrast to the closed tip conformation of trimeric E (Fig. 8).

Flavivirus E is anchored in the viral membrane by a double membrane anchor composed of two transmembrane domains. E1 has a single membrane-spanning helix instead.

Flaviviruses profit from cholesterol in the target membrane, because it enhances fusion peptide insertion and trimerization of E (Stiasny, Koessl and Heinz, 2003). In contrast, alphaviruses are absolutely dependent on the presence of cholesterol as well as sphingomyelin for efficient fusion (Wahlberg *et al*., 1992; Kielian *et al*., 2000).

Nevertheless, the structural similarity of E and E1 in their pre- as well as in their post-fusion conformations suggests that the overall fusion process for flavi- and alphaviruses is very similar.

2.4. A third class of viral fusion proteins

Recently the crystal structures of the HSV1 glycoprotein B (Heldwein *et al*., 2006) in its post-fusion conformation and of the VSV glycoprotein G in its neutral pH (pre-fusion) and low pH (post-fusion) conformations have been resolved (Roche *et al*., 2006; 2007). Based on their structural characteristics, these fusion proteins have been proposed to form a third class of viral fusion glycoproteins. The baculovirus gp64 glycoprotein fits into this class as well (Garry and Garry, 2008; Kadlec, 2008; Kadlec et al., 2008). In contrast to the proteins discussed so far, it has been shown for VSV G that the pH-induced shift between the conformational states is reversible (Roche and Gaudin, 2002; reviewed in Roche *et al*., 2008).

2.4.1. The Vesicular stomatitis virus (VSV) G protein

Vesicular stomatitis virus belongs to the family Rhabdoviridae, whose members are enveloped viruses with a bullet shaped structure. Cell entry, occuring via the endocytic pathway and low pH-triggered fusion with the endosomal membrane, is driven by the trimeric glycoprotein G. Before structures of G were determined, it was already known from biophysical and biochemical investigations that VSV G can adopt various conformational states (Gaudin *et al*., 1993): the native state (at neutral pH) which in contrast to class I and II fusion proteins is not metastable, an activated state interacting with target membranes and a post-fusion state that is antigenically different from the native and the activated state (Roche and Gaudin, 2002). There is a pH-dependent equilibrium between the conformations and the low pH-induced structural alterations are reversible which is different from the other two classes of fusion proteins. This reversibility is necessary to recover the neutral pH structure of G after the transport of the virus particles through the acidic Golgi apparatus during particle export (reviewed in Gaudin, 2000; Roche *et al*., 2008). The existence of a low pH, fusion-inactive state replaces the need for the chaperone molecules found in the class II systems (prM and p62) (Gaudin et al., 1995). After particle release, G adopts its native conformation which is depicted in Figure 13. In this neutral pH form, the fusion protein shows the shape of a tripod with the fusion loops pointing towards the viral membrane (Roche *et al*., 2007).

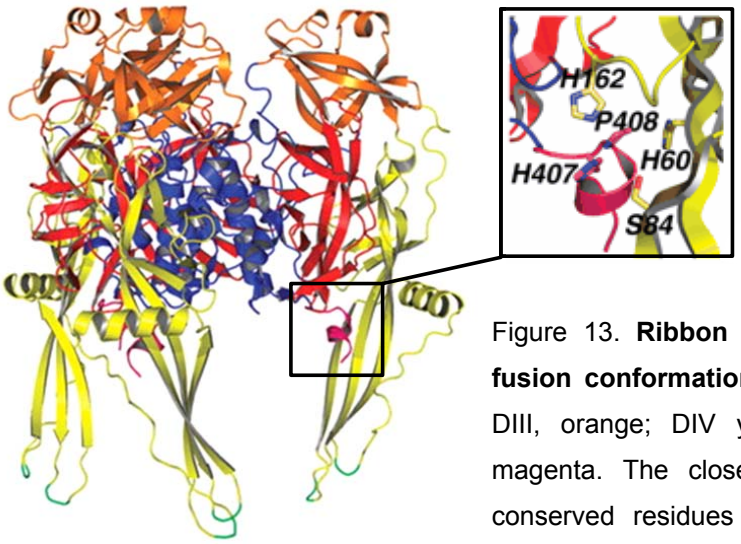


Figure 13. **Ribbon diagram of VSV G in its pre-fusion conformation.** Color code: DI, red; DII, blue; DIII, orange; DIV yellow; FPs, green; C-terminus, magenta. The close-up view shows a network of conserved residues (note the cluster of conserved histidines) between domains III and IV described closer in chapter 3 (Adapted from Roche *et al*., 2007).

The monomeric subunits within this pre-fusion trimer consist of four domains (DI-IV). Domain I – the central domain (red in Fig. 13) – and domain II – a relatively long alpha helix (blue in Fig. 13) – build most of the intersubunit contacts in the trimer. The fusion domains (DIV, yellow in Fig. 13) are set apart to separate the fusion loops that are not buried within an oligomeric structure as in class I and II fusion proteins. The subunits of the fusion proteins of the other class III members (HSV gB and baculovirus gp64) consist of five domains each.

Figure 14 C shows the structure of the glycoprotein G in its low pH (the post-fusion) form compared to class I (A) and class II (B) fusion proteins. In this fully rearranged low pH conformation G displays the classical hairpin conformation. There is a central alpha helical coiled coil (DII, colored blue) like in class I proteins. The C-terminal parts have zipped up along the fusion domains (DIV), similar to the fusion-related transition in flavivirus E. Each chain possesses bipartite fusion loops at the tip of an elongated beta sheet (domain IV) which is also comparable – but not homologous – to the situation with class II proteins.

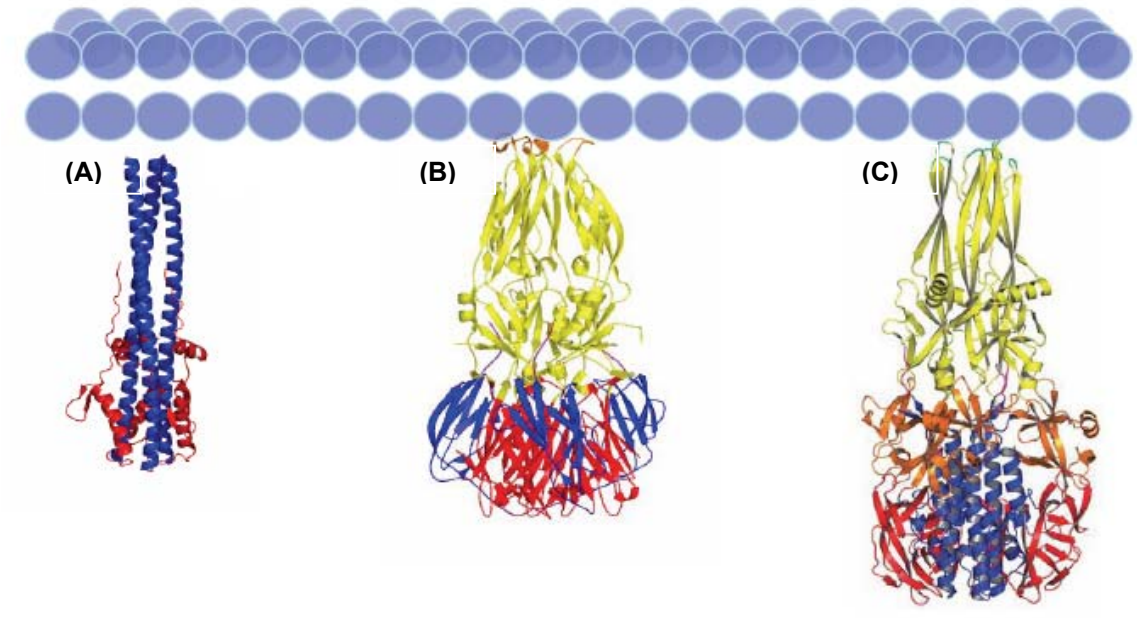


Figure 14. **Structural motifs of viral membrane fusion proteins.** Ribbon diagrams of representative structures of class I, II and III viral fusion proteins, (A) gp-41 of HIV1, (B) flavivirus glycoprotein E, (C) VSV glycoprotein G, in their post-fusion conformation (Adapted from Weissenhorn, Hinz and Gaudin, 2007).

Based on the knowledge of these structures of VSV G, the conformational changes rearranging the neutral pH form (the tripod) to the low-pH form were reconstructed (reviewed in detail in Roche *et al*., 2007; 2008). Domains I, III and IV retain their tertiary structure, but undergo large rearrangements in their relative orientation towards each other caused by changes in the hinge regions between domains III and IV. Domain II, the central domain, has to undergo major refolding to contribute to the formation of the post-fusion conformation. There is a loop to helix transition between DIV and DII creating an extended coiled coil at the trimer centre (DII, Fig. 14 C), reminiscent of the formation of the extended intermediate coiled-coil structure described for influenza HA. The part of domain II that remains unchanged, together with domain I, forms a rigid block that is the linchpin for flipping domain IV and a C-terminal segment which is the main movement of the transitions leading to the post-fusion conformation.

Refolding of G displays certain similarities to the other classes of fusion proteins. As addressed, the elongation of the central helices is a hallmark of the transitions driving class I fusion. Because of the great differences between the pre- and post-fusion conformations of G, it is possible that the whole transition process includes a transient monomeric intermediate like in class II protein mediated fusion. The low pH structure of VSV G is missing the “stem-anchor” region, but the C-terminal end of the truncated protein is oriented towards the fusion domain (Fig. 14 C). Therefore the fusion loops and the anchor elements must be in close vicinity at the same end of the trimer, suggesting hairpin formation similar to class I and II fusion proteins. A recent report compared structural motifs within the domains of class I, II and III fusion proteins and – because of the existence of structural counterparts – suggested that all three classes are more related than generally assumed (Kadlec *et al.*, 2008).

3. pH sensors in virus membrane fusion

The fusion proteins of viruses that enter cells via endocytosis sense the local proton concentration in endosomes and carry out membrane fusion in response to acidic pH. There are also numerous cellular processes that involve pH sensors (reviewed in Srivastava, Barber and Jacobson, 2007). Proton binding is extremely fast and can drive changes in protein structure, dynamics and interactions. A classical example of a proton-induced protein modification is the hemoglobin Bohr effect (reviewed in Riggs, 1988). The affinity for oxygen in human hemoglobin can be controlled by a salt bridge built by His146 and Asp94, which is broken upon deprotonation of the histidine at increased pH. The pH-dependent modification of histidines has further been reported to be the cause of structural changes in unrelated proteins and protein complexes (for example Ingram-Smith, Barber and Ferry, 2000; Nordlund *et al*., 2003; Lee *et al*., 2005; Nyarko *et al*., 2005; Astier, Bayley and Howorka, 2005; Day *et al.*, 2006; Hu *et al.*, 2006; Perrier *et al*., 2007).

Many viral fusion proteins become activated at pH ranges close to the pKa of histidines (6.5, although the effective pKa of a specific residue depends on its environment). Therefore a “histidine switch hypothesis” was developed, suggesting that – for some viruses – protonation of conserved histidine residues might be the trigger for membrane fusion (Bressanelli *et al*., 2004; Roussel *et al*., 2006; Kampmann *et al*., 2006; Mueller *et al*., 2008; Roche *et al*., 2008). Experimental evidence for this hypothesis is, however, still lacking.

3.1. Histidines as putative pH sensors in the influenza HA

Protonation of histidines in influenza HA has been proposed to play a role for initiating the membrane fusion process (Chen *et al*., 1988; Stevens *et al*., 2004; Kampmann *et al*., 2006)

Recent studies tested HA mutants for their pH requirements to undergo the structural alterations necessary for mediating fusion (Steinhauer *et al*., 1996;

Thoennes *et al*., 2008). Among these, a histidine to tyrosine mutation in residue 17 of HA_1 led to a decrease of the pH threshold for structural changes and membrane fusion compared to the wild-type. It was therefore hypothesized that protonation of H17 is triggering HA-mediated fusion (Thoennes *et al.*, 2008). H17, however, is only partially conserved but it is located close to other histidines (H18, H37, H106 and H111) and these residues may also cooperatively regulate the process. The latter hypothesis is supported by the fact that histidine mutations in influenza HA shifted the pH-dependence of fusion but did not abolish the process completely. Furthermore it was demonstrated that mutations (including mutations of H17 to arginine and glutamine) at trimer interfaces – that break during the rearrangements – also shifted the pH threshold for fusion but by affecting the trimer stability (Daniels *et al*., 1985).
In the case of influenza HA it seems that the conformational changes are triggered by a cumulative effect of positive charges resulting from unspecific protonation of a number of amino acids.

3.2. The role of histidines in the flavivirus fusion protein E

There are five conserved histidines in flavivirus E and because of the pH threshold of flavivirus fusion (6.6) they are plausible pH sensor candidates. Two are located at the interface of DI and DIII (H146 and H323 in TBEV E), one at the domains I-II interface (H287 in TBEV), one in DII (H248 in TBEV) and one in the stem (H438 in TBEV) (Fig. 15). The precise role of these residues in the membrane fusion process has not been investigated so far.
Especially the protonation of H146 and H323 in TBEV E – equivalent to H144 and H317 in E of DENV2 – has been discussed as trigger mechanism for flavivirus fusion (Bresanelli et al., 2004; Harrison 2008b). These histidines are not only located in strategic key positions, but they also interact with residues in their vicinity to clamp the structure in the pre- or the post-fusion form. At neutral pH, they interact with positively charged residues (lysines and arginines) in their vicinity. Upon protonation they become positively charged themselves. Their new charge is thought to destabilize the pre-fusion interactions and leads to the formation of salt bridges with negatively charged residues (aspartatic and

glutamic acids). These new interactions probably stabilize the post-fusion structure (Kampmann *et al*., 2006). Structure analysis revealed that in the pre-fusion dimer histidines 146 and 323 are both most likely to interact with arginine 9 of the prominent interdomain salt bridge between domains I and III (Fig. 10). In the post-fusion conformation, H146 forms a new interaction with the conserved residue D42 of domain I. H323 on the other hand interacts with E373, the other part of the salt bridge connecting DI and DIII in the pre-fusion structure.

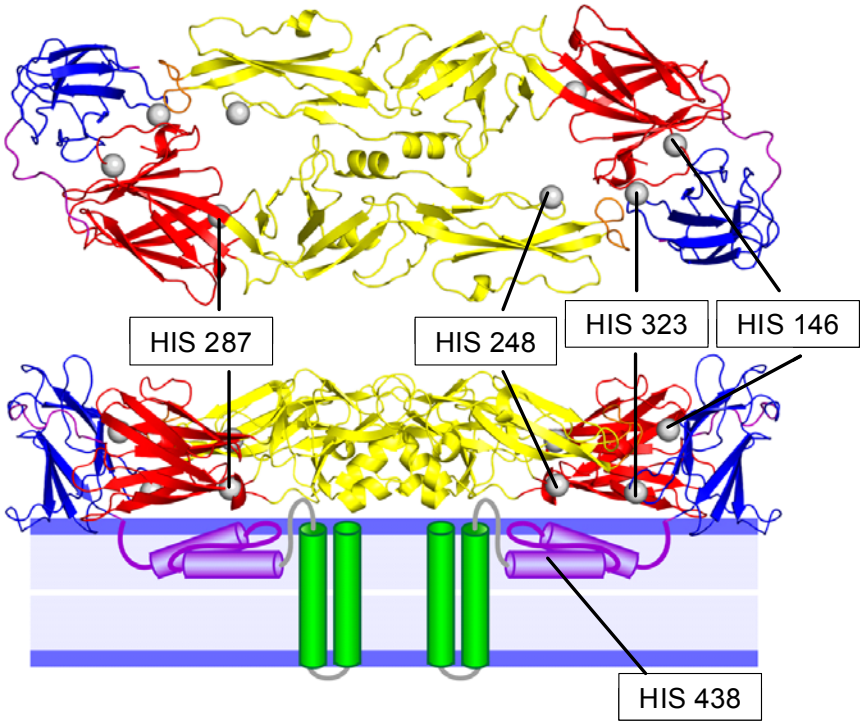


Figure 15. **Conserved histidines in the TBEV E pre-fusion dimer.** Shown are ribbon diagrams of the E dimer in top (upper part) and side view (lower part). The side view contains a schematic representation of the parts for which the aromic structure is not known. The histidines conserved among all flavivirus E proteins are labelled and highlighted as grey spheres. Color code corresponds to figure 7. Drawn with PyMol (DeLano, 2002).

Alphavirus fusion may be triggered by histidine protonation as well (Roussel *et al*., 2006) (Fig. 12), but there is only one conserved histidine at a position homologous in flavivirus E and alphavirus E1. This histidine (H248 in E and H230 in E1, respectively) was shown to be involved in a late stage of E1 mediated fusion of Semliki forest virus but not in the initial stages nor in trimer

formation (Chanel-Vos and Kielian, 2004). These results indicate that the residues involved in triggering alphavirus fusion are located elsewhere, probably between E1 domains I and III.

Conserved histidines could also be involved in other pH-dependent processes than fusion. The flavivirus membrane protein precursor prM protects the fusion protein in the TGN from early activation (see chapter 2.3.1.). The maturation cleavage of prM requires low-pH. The exposure of the furin cleavage site of prM relies on structural alterations in the prM/E complex that may also be induced by the protonation of histidines. After the cleavage, pr stays associated with E until it encounters neutral pH conditions in the extracellular environment. The recently resolved structure of prM of dengue 2 shows, that E residue histidine 244 – corresponding to conserved H248 in domain II of TBE E (Fig. 17) – is most likely interacting with the acidic residue D63 of prM (Li *et al*., 2008). This interaction may be responsible for the attachment of cleaved pr and the deprotonation of histidine 244 may be a prerequisite for releasing the pr peptide after virus secretion (Li *et al.*, 2008; Yu *et al*., 2008)

3.3. pH-sensitive molecular switches in the VSV G protein

In the class III fusion protein G of VSV, conserved residues were identified that seem to act as molecular switches for initiating the reversible structural changes described in chapter 2.4.1. (Roche *et al*., 2007): Conserved histidines in the pre-fusion conformation and acidic amino acids in the post-fusion conformation constitute two separate pH-sensitive switches. Specifically, three conserved histidines in VSV G have been discussed to form a cluster of positive charges upon protonation that might trigger the structural changes leading from the pre- to the post-fusion conformation (Roche *et al.*, 2008). These histidines (shown in the close up view in Figure 13) are residues 60 and 162 in domain IV and 407 in the C-terminal part. So far, there is no experimental evidence for their contribution to the low pH-induced structural alterations, but it was shown that treatment with diethylpyrocarbonate (DEPC) inhibits G-mediated VSV membrane fusion by unspecifically preventing the protonation of all histidine residues in the molecule (Carneiro *et al*., 2003).

In the low pH (the post-fusion) conformation, acidic residues of G (D268, D274, E276, D393 and D395) are brought into close approximity in a central bundle formed by the helices of domain II of G (Fig. 14). The low pH conformation is presumably adopted in the TGN during VSV particle exocytosis from the host cell. When the particles encounters neutral pH conditions after the release from the cell, the deprotonation of these amino acids is thought to destabilize the trimer and initiate the refolding towards the tripod-like pre-fusion state.

II. OBJECTIVES

The overall goal of this PhD thesis was to gain new insights into the structural and functional requirements of flavivirus membrane fusion by the introduction of mutations and truncations into the E protein of recombinant subviral particles (RSPs) of tick borne encephalitis virus (TBEV).
Specifically, two aspects were investigated: (I) the “histidine switch” hypothesis was experimentally tested for the first time in a class II viral fusion system and (II) the functional role of the membrane anchor of E in different stages of fusion was explored. An essential part for both subprojects was the establishment of quality controls of subviral particles to verify their maturation state and structural integrity.

Specific aims

I. Investigation of the role of specific histidines in flavivirus membrane fusion

It was the specific goal of the first part of my thesis to dissect the structural basis of the low pH trigger for flavivirus membrane fusion. Five histidines are absolutely conserved among all flavivirus E proteins and – because of their pKa – have been hypothesized to function as molecular pH sensors for initiating fusion (see chapter 3.2.). To directly investigate the role of all conserved histidines in E for acidic pH-mediated fusion, I used a mutagenesis approach and substituted them in RSPs of TBEV. The mutant RSPs were investigated for their ability to undergo low pH-mediated E dimer dissociation, target membrane binding, E trimerization and lipid mixing.

II. Dissection of the functional role of the second transmembrane helix of the flavivirus fusion protein

The purpose of the second part of the thesis was to contribute to the understanding of a so far not very well characterized part of the flavivirus fusion protein: the membrane anchor that is absent in the crystallographic structures of truncated E proteins.

The single transmembrane domains of several viral fusion proteins have been shown to be crucial for the late steps of membrane fusion and are thought to be essential for the hemifusion to fusion transition (Kemble *et al*., 1994; Melikyan, White and Cohen, 1995; Melikyan *et al*., 1997; Armstrong, Kushnir and White, 2008; reviewed in Schroth-Diez *et al*., 2000; Langosch, Hofmann and Ungermann, 2007). The role of the double membrane anchor of the flavivirus E protein has not been investigated so far.

C-terminally truncated E proteins – that lack the second transmembrane domain (TM2) – were investigated in subviral particles to clarify whether – and at which stage of the process (Binding, E dimer dissociation, E trimerization) – the second TM domain is essential for flavivirus fusion.

III. PART I

JCB: ARTICLE

Identification of specific histidines as pH sensors in flavivirus membrane fusion

Richard Fritz, Karin Stiasny and Franz X. Heinz

Institute of Virology
Medical University of Vienna
1095 Vienna, Austria

The Journal of Cell Biology, 2008, Vol. 183: 353-61.

The editors of the Journal of Cell Biology and of the Journal of Experimental Medicine highlighted the publication

Flavivirus reveals its access code

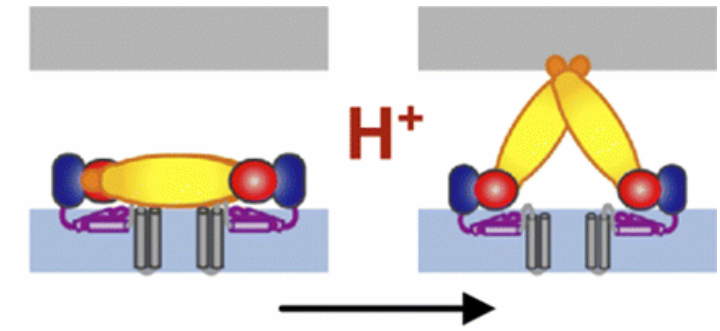
Ruth Williams The Journal of Cell Biology, Vol. 183, No. 2, 172.

Fritz et al. have identified an amino acid switch that flaviviruses flip to gain access to cells.

Flaviviruses such as tick-borne encephalitis virus (TBEV), yellow fever, and dengue are dangerous human pathogens. These membrane-encircled viruses enter cells by being gobbled up into endosomes and fusing their membrane with that of the endosome.

Fusion is triggered by the endosome's acidic environment. Low pH prompts the aptly named fusion protein, on the virus's outer membrane, to change shape and grab hold of the endosome membrane, bringing the two membranes together. In their search for possible pH sensors, researchers have focused on five highly conserved histidine residues in the flavivirus fusion protein. The chemical properties of histidines make them prime candidates—they switch from uncharged to having a double positive charge upon acidification of their environment, such as that in endosomes.

Fritz et al. replaced each of the five histidines of the TBEV fusion protein with alternative residues and observed the virus's fusion ability. Given the conservation of the five histidines, the team was surprised, that mutation of one of the histidines, His323, was sufficient to completely abolish fusion. Individual mutation of three of the others had no effect on fusion whatsoever, and mutation of the fourth led to an untestable ill-formed fusion protein. The team went on to show that mutation of the crucial His323 interfered with the pH-induced shape change of the fusion protein.



The fusion protein (yellow, red, blue) on the viral membrane (pale blue) changes shape at low pH and attaches to the endosome membrane (gray). The pH sensor for this first step of fusion is His323.

1.1. Abstract

The flavivirus membrane fusion machinery - like that of many other enveloped viruses – is triggered by the acidic pH in endosomes after virus uptake by receptor-mediated endocytosis.
It has been hypothesized that conserved histidines in the class II fusion protein E of these viruses function as molecular switches and, by their protonation, control the fusion process. Using the mutational analysis of recombinant subviral particles of tick-borne encephalitis virus, we provide direct experimental evidence that the initiation of fusion is crucially dependent on the protonation of one of the conserved histidines (His323) at the interface between domains I and III of E, leading to the dissolution of domain interactions and to the exposure of the fusion peptide. Conserved histidines located outside this critical interface were found to be completely dispensable for triggering fusion.

1.2. Introduction

The entry of enveloped viruses into host cells involves a fusion step between the viral and a cellular membrane. This process is mediated by viral fusion proteins that are associated with the viral membrane and primed to undergo structural rearrangements that drive fusion (Kielian and Rey, 2006; Weissenhorn et al., 2007; Harrison, 2008; White et al., 2008). These conformational changes are activated by specific triggers, allowing fusion to occur at the right time and at the right place in the viral life cycle. Different trigger mechanisms (and combinations thereof) have been identified, including (a) interactions with cellular receptors, leading to fusion at the plasma membrane, and (b) protonation by the acidic pH in endosomes, leading to fusion from within endosomes after virus uptake by receptor-mediated endocytosis (White et al., 2008). In the latter case, like in other protein systems of intracellular pH sensors (Srivastava et al., 2007), histidines have been discussed to play a key role as molecular switches because their protonation state changes from uncharged to doubly positively-charged at the slightly acidic pH found in endosomes (Carneiro et al., 2003; Bressanelli et al., 2004; Stevens et al., 2004; Kampmann et al., 2006; Kanai et al., 2006; Roussel et al., 2006; Mueller et al., 2008; Roche et al., 2008; Thoennes et al., 2008).

So far, two structurally unrelated classes of viral fusion proteins have been identified (class I in myxo-, paramyxo-, retro-, filo-, and coronaviruses; class II in alpha- and flaviviruses) (Kielian and Rey, 2006), together with a third class that combines features of both class I and class II (rhabdo- and herpesviruses; Weissenhorn et al., 2007; White et al., 2008). They all comprise representatives that are triggered by acidic pH. Despite the knowledge of atomic structures from all three protein classes (Weissenhorn et al., 2007; Harrison, 2008; White et al., 2008), it proved difficult, both in experimental and molecular simulation approaches, to conclusively answer the question whether the initial trigger for destabilization and conformational changes is provided by the protonation of individual histidine residues, by combinations thereof, or by a cumulative effect through the increase of positive charge (Kampmann et al., 2006; Mueller et al., 2008; Thoennes et al., 2008).

In class II fusion proteins, the molecular sensors for triggering fusion have not yet been identified. We therefore conducted a study in a prototypic class II fusion protein system (the flavivirus tick-borne encephalitis virus [TBEV]) and provide experimental evidence that the protonation of a specific histidine plays a key role for the destabilization of an intramolecular interface in the fusion protein and thus allows the initiation of the fusion process.

Flaviviruses (genus *Flavivirus* and family Flaviviridae) have an acidic pH-dependent fusion machinery (Stiasny and Heinz, 2006) and comprise several closely related important human pathogens, including yellow fever, dengue, Japanese encephalitis, West Nile, and TBE viruses (Gubler et al., 2007). The surface of mature flaviviruses is made up of a herringbone-like assembly of 90 homodimers of the E protein (Kuhn et al., 2002; Mukhopadhyay et al., 2003). The atomic structures of soluble forms of E (sE), lacking the membrane anchor and the so-called 'stem' (Fig. 1B), have been determined for different flaviviruses in pre- and post-fusion conformations (Fig. 1, B and C; Rey et al., 1995; Modis et al., 2003, 2004, 2005; Bressanelli et al., 2004; Zhang et al., 2004; Kanai et al., 2006; Nybakken et al., 2006). In the pre-fusion conformation, the internal fusion peptide (FP) loop at the tip of domain II (DII) is buried through the interaction with a hydrophobic pocket provided by DI and DIII of the second partner in the homodimer (Fig. 1 B). Exposure to acidic pH leads to the initiation of the fusion process as depicted in Fig. 1 D.

Five histidine residues, located in DI, II, and III as well as in the 'stem' region (Fig. 1, B and C), are conserved among all flavivirus E proteins and their function as pH sensors in flavivirus fusion has been discussed (Bressanelli et al., 2004; Kampmann et al., 2006; Kanai et al., 2006; Nybakken et al., 2006; Mueller et al., 2008). To support this hypothesis experimentally, we used recombinant subviral particles (RSPs) of TBEV as a model and targeted the conserved histidines in a mutational approach. As shown previously, RSPs are excellent tools for studying flavivirus fusion because they contain a lipid membrane, carry the E protein in a conformation that is indistinguishable from that on the virus and, most importantly, display fusion characteristics similar to those of infectious virions (Schalich et al., 1996; Corver et al., 2000). This system allowed us to study the effect of histidine replacements on the different steps of fusion in the absence of resuscitating mutations that would occur during virus replication.

In our work, we demonstrate that His323, located at the interface of DI and III in the pre-fusion conformation of E, has a crucial function as a pH sensor for initiating fusion. Surprisingly, 3 of the 5 conserved histidines in E were shown to be completely dispensable for the early phases of fusion but two of these residues appeared to contribute to the overall stability of the post-fusion trimer.

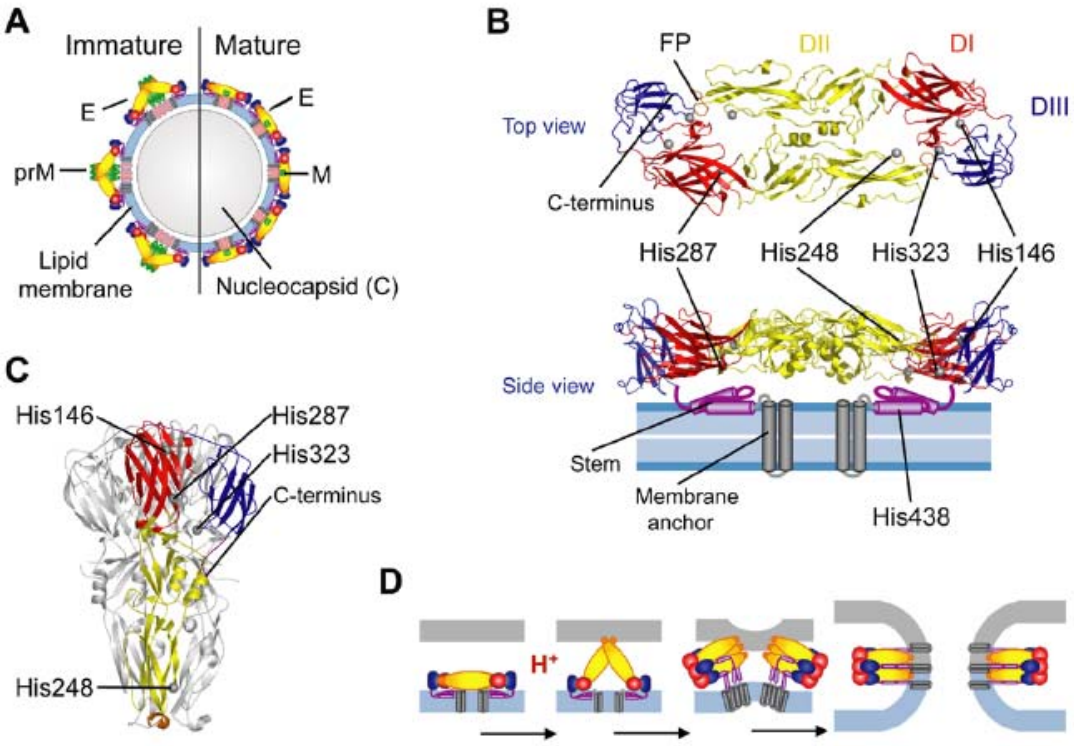


Figure 1. **Summary of the organization of flavivirus particles, the three-dimensional structures of the flavivirus envelope protein E, and a model of flavivirus membrane fusion.** (A) Schematic diagram of a flavivirus particle in its immature (prM-containing) and mature form after proteolytic cleavage of prM. (B) Schematic of the pre-fusion E dimer including ribbon diagrams of the TBEV sE ectodomain (top and side view) and those parts for which the atomic structure is not known (stem and anchor). (C) Ribbon diagram of the post-fusion TBEV sE trimer (side view). The positions of the histidines conserved in all flavivirus E proteins are indicated by grey balls. (D) Schematic of the proposed flavivirus fusion mechanism showing different steps of the fusion process. (step 1) Metastable E dimer in mature virions. (step 2) Dissociation of the E dimers at acidic pH, outward projection of E monomers, and interaction of the FP with the target membrane. (step 3) Trimerization, DIII relocation, and “zipping up” of the stem. (step 4) Formation of the post-fusion trimer and opening of the fusion pore. Red, DI; yellow, DII; blue, DIII; orange, FP; purple, stem (linker between DIII and the transmembrane anchors); gray, transmembrane anchors.

1.3. Results

1.3.1. Generation of RSPs with mutated His residues

The RSPs of TBEV used in this study display similar fusion characteristics as infectious virions and are assembled in eukaryotic cells after transfection with plasmids that coexpress prM and E. Their maturation and secretion pathway follows that of whole virions, including the cleavage of prM in the trans-Golgi network to yield mature and fusion-active particles (Schalich et al., 1996). To obtain specific information on the molecular pH sensors of fusion, we replaced each of the five absolutely conserved histidines (and combinations thereof) in the E protein of RSPs by alanine or other amino acids and investigated their effect on fusion and fusion-related properties. Any mutation introduced into this system can potentially also interfere with processes unrelated to fusion, such as proper protein folding, oligomerization, particle formation, intracellular transport, protein processing, maturation and secretion of RSPs. We therefore performed a meticulous analysis of the mutant RSPs to ensure that the amino acid substitutions had not influenced their wild-type (WT)-like properties at neutral pH. This was accomplished by a series of quality control measures, including an analysis of the amount of RSPs secreted from transfected cells, the sedimentation behavior in sucrose gradients, the extent of maturation cleavage of prM, the oligomeric state of E, and the reactivity of the RSPs with a panel of 22 monoclonal antibodies (mAbs). These mAbs recognize epitopes in each of the three domains of the mature E protein and have proven to be excellent tools for measuring conformational differences or changes (Heinz et al., 1994; Allison et al., 1995, 2001; Stiasny et al., 2005).

To replace the histidines, we used alanine as a first choice, but, if an alanine mutation did not yield RSPs conforming to all of the criteria listed in the previous paragraph, other amino acids were substituted. Table 1 displays those single and combination mutants that passed all of the quality controls and were selected for the analyses described in this work. As can be seen from the table, WT-like RSPs were obtained with mutations at four of the five positions of conserved histidines, in two cases with alanine and in two cases with asparagine

substitutions. The exception was His146; in this case replacement by any of the other 19 amino acids resulted in the abolition of proper RSP formation and/or secretion (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200806081/DC1).

Table I. **WT and mutant RSPs of TBEV used in this study**

Mutant	Location of mutations
WT	–
H248N	DII
H287A	DI
H323A	DIII
H438N	stem
H248N-H287A	DII + I
H248N-H323A	DII + III
H248N-H438N	DII + stem
H287A-H438N	DI + stem
H248N-H287A-H438N	DII + I + stem

1.3.2. Fusion activity of His mutant RSPs

Having certified that the replacement of histidines by other amino acids had not changed the overall conformation of E and its oligomeric state (nor did it impair particle formation, maturation, or secretion), we analyzed the effect of these mutations on fusion activity. For this purpose, the membranes of each of the mutant RSPs were fluorescence labeled in vivo with 1- pyrenehexadecanoic acid and subjected to an in vitro fusion assay (see Materials and methods). These RSPs were mixed with unlabeled liposomes and the decrease in pyrene excimer fluorescence (caused by dilution of the probe in the target membrane) was continuously monitored. Consistent with previous results (Corver et al., 2000), WT RSPs fused rapidly at pH 5.4 within the first seconds after acidification (Fig. 2 A), and the fusion activities of mutants lacking a histidine residue at position 248, 287 or 438 were identical to that of the wild-type (Fig. 2 B). The replacement of His323, however, resulted in the loss of fusion activity (Fig. 2, A and B) even at pH 5.0 (not depicted). We also analyzed combinations of mutations that alone did not affect fusion, i.e. H248N+H287A, H248N+H438N, and H287A+H438N. Unexpectedly, one of these combinations (H248N+H287A) impaired fusion to the same extent as did the replacement of His323 (Fig. 2, A and B). These experiments revealed the importance of individual conserved histidines for E-mediated fusion, but it remained unclear at which stage or how certain residues were involved in this multistep process.

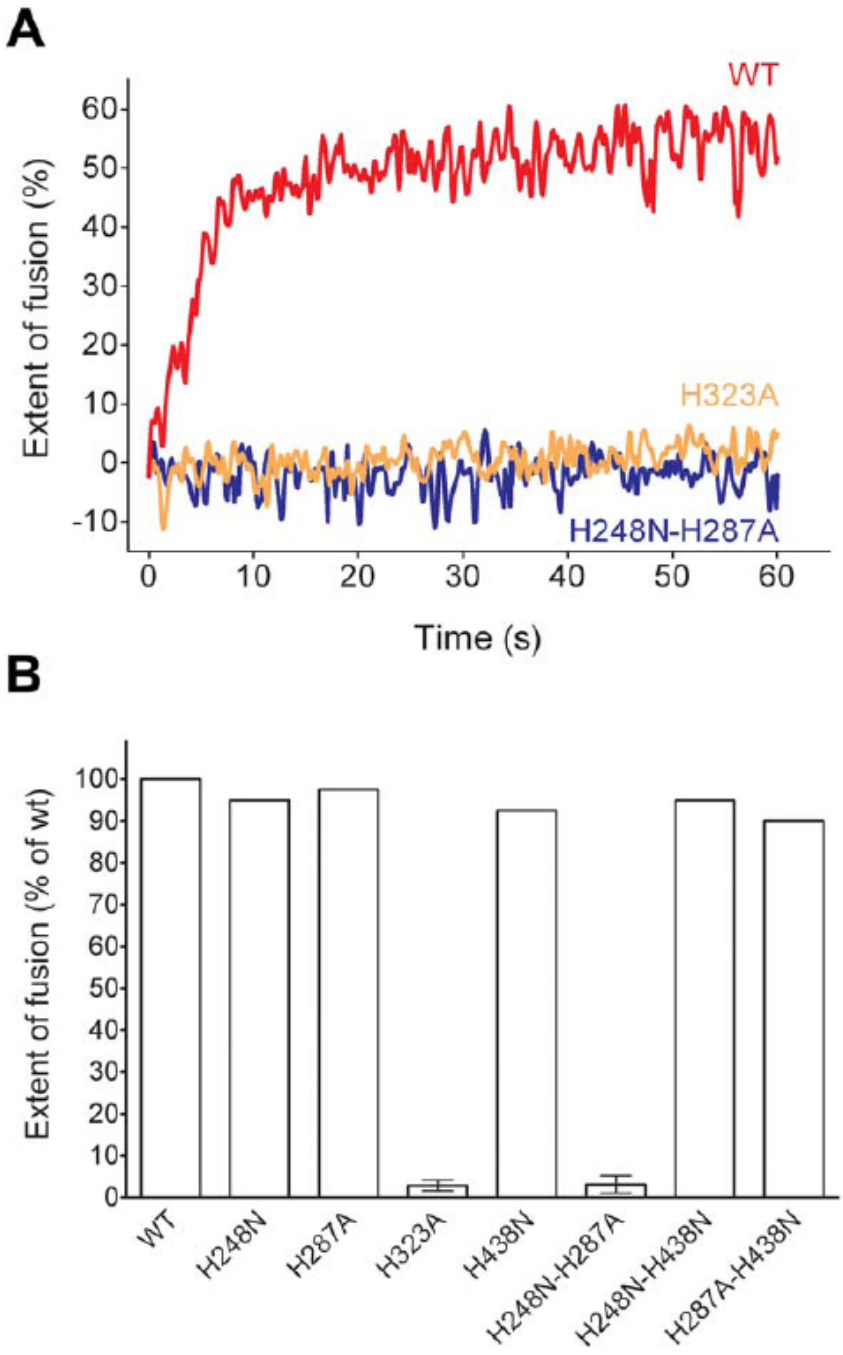


Figure 2. **Fusion activity of pyrene-labeled WT and mutant RSPs with liposomes at acidic pH.** (A) Kinetic fusion curves of RSP WT (red) and the mutant RSPs H323A (orange) and H248N-H287A (blue) at pH 5.4. (B) Extent of fusion after 60 s of mutant RSPs relative to that of the WT (set at 100%). The experiments with those mutants that were significantly different from the WT were performed at least twice and the error bars represent the standard errors of the means.

1.3.3. Effect of His mutations on fusion peptide exposure

It was the primary hypothesis of our work that histidines play a role as pH sensors and that their protonation would be the initial trigger for fusion. To analyze the very first step of the fusion process, we made use of an FP-specific mAb (A1) and developed an assay that allowed us to measure the acidic pH-induced disengagement of the FP from its protecting interactions in the E dimer and its exposure to the environment. In previous experiments, we had found that during conversion of E from the pre-fusion dimer to the post-fusion trimer the accessibility of the FP for mAb A1 was transiently increased but lost upon conversion into the final trimeric conformation (Stiasny et al., 2007). We therefore measured this transient FP exposure using an enzyme immunoassay in which the antibody was already present in the RSP samples at the time of their acidification (see Materials and methods). The results of these experiments are shown in Fig. 3. With the exception of mutant H323A, none of the three other single His mutants differed significantly from the WT, neither with respect to the extent nor to the pH threshold of FP exposure (unpublished data). The same holds true for the double and triple mutants, which all displayed a WT-like pattern (Fig. 3). In contrast, FP exposure was severely impaired in the case of the H323A mutant, but the residual activity was induced at the same pH threshold as with the WT, i.e. around pH 6.6 (Fig. 3). These results allow us to conclude that of the four histidines analyzed only His323 plays an important role as an initial fusion trigger and that the lack of fusion activity observed with the double mutant H248N-H287A was caused by an impairment of later steps of fusion.

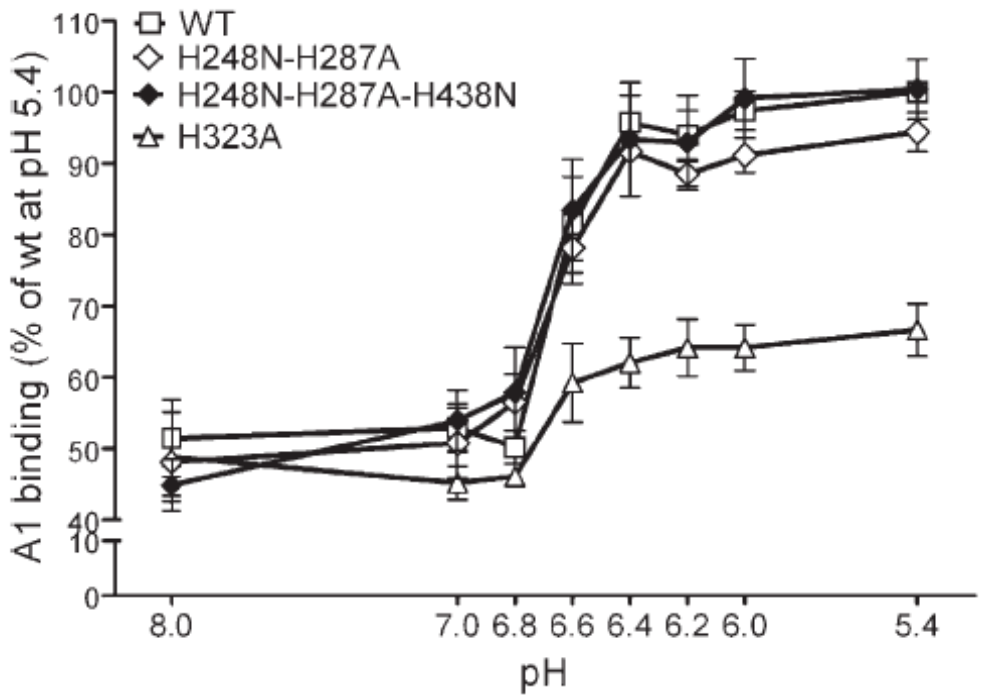


Figure 3. **Acidic pH-induced FP exposure as measured by the binding of the FP-specific mAb A1.** Results are expressed as a percentage of the maximal reactivity of A1 obtained with the WT at pH 5.4. The data are the means of three independent experiments performed in duplicate; the error bars represent the standard errors of the means.

1.3.4. Loss of membrane interactions by His mutations

According to the proposed scheme of flavivirus membrane fusion (Fig. 1 D), the exposure of the FP allows its interaction with target membranes and thus mechanistically initiates the fusion process. To assess whether the observed FP exposure indeed correlated with the proposed functional activity, we performed liposome coflotation experiments for measuring membrane binding. Preparations of WT and His mutant RSPs were mixed with liposomes, acidified, and applied to sucrose step gradients as described in Materials and methods. Coflotation of E to the top of the gradients indicates either binding to or fusion with liposomes. The results of these experiments as depicted in Fig. 4 precisely matched those of the FP exposure assay (Fig. 3). With the exception of the severely impaired mutant H323A, all of the other single and combination mutants displayed the same acidic pH-induced coflotation behavior as the WT. Because the double mutant H248N-H287A was completely fusion negative (Fig. 2), we made sure that its unimpaired coflotation was indeed only caused by binding to the liposomes, in the absence of fusion. For that purpose, coflotation assays were performed with pyrene-labeled samples of the mutant and WT RSPs, and fluorescence emission spectra were recorded with the coflotated fractions. In the case of RSP H248N-H287A, a strong pyrene excimer peak (which equals no dilution of the fluorescence probe) was observed, whereas it was strongly reduced with coflotated WT RSPs (Fig. 4, C, inset). These results thus allowed the unambiguous conclusion that the coflotation with the double mutant, in contrast to that of the WT, was caused by an interaction with liposomes in the absence of fusion activity.

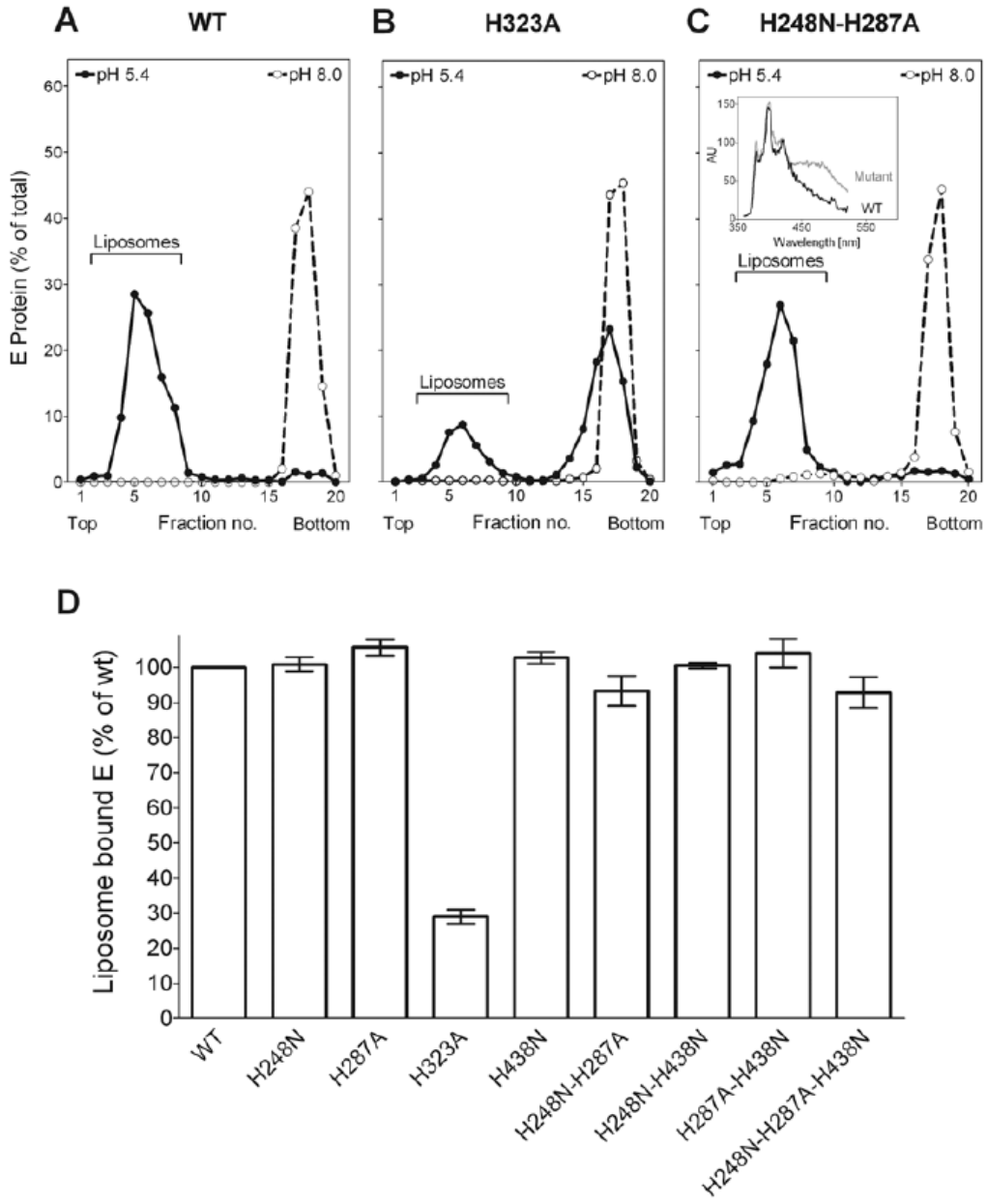


Figure 4. **Acidic pH-induced coflotation of WT and mutant RSPs with liposomes.** RSPs were incubated with liposomes at pH 5.4 (solid lines) and pH 8.0 (dotted lines), back-neutralized, and then subjected to centrifugation in sucrose step gradients. The gradients were fractionated, and the amount of E protein in each fraction was determined by a quantitative four-layer ELISA. The top fractions containing RSPs coflotated with the liposomes are indicated by a bracket. (A-C) Representative examples of the analysis of the step gradients obtained with WT (A) and the fusion-negative mutants H323A (B) and H248N-H287A (C). The inset in C shows the fluorescence spectrum of the coflotated fractions of the mutant (gray) relative to those of the WT (black) from experiments using pyrene-labeled RSPs. AU, arbitrary units. (D) Extent of acidic pH-induced coflotation of E with liposomes obtained with mutant RSPs relative to the WT (set at 100%). The data are the means from at least two independent experiments; the error bars represent the standard errors of the means.

1.3.5. Impairment of the formation and stability of E trimers

The results obtained so far indicated that only His323, but none of the other histidines investigated, was involved in the initiation of fusion. We therefore hypothesized that the lack of fusion observed with the double mutant H248N-H287A was due to an impairment of later steps, such as the formation and/or stability of the E trimer. We addressed this question by sedimentation analyses and investigated the effect of each of the His mutations on the acidic pH-induced conversion of E dimers into trimers. Like with the WT RSPs, a quantitative E dimer to trimer transition was observed with the single mutants H248N, H287A, and H438N, as well as with the double mutants H248N-H438N and H287A-H438N (Fig. 5, A and B; and not depicted). In contrast, trimer formation was impaired with H323A and H248N-H287A (Fig. 5, C and D). Similar to the situation found with the pH dependence of FP exposure (Fig. 3), the pH threshold for the oligomeric rearrangement was identical to the WT with all of the mutants (pH 6.6), irrespective of the amount of trimers formed (Fig. 6).

Because the mutations introduced into E may not only influence the formation of trimers but also their stability, we performed thermal denaturation experiments as follows. RSPs were acidified, back-neutralized, solubilized, incubated at 37 or 70°C, and subjected to rate zonal sucrose density gradient centrifugation to determine the oligomeric state of E (Fig. 7). E trimers of the single mutants H248N, H287A and H438N were as stable as WT trimers (Fig. 7 and not depicted). In contrast, not only was trimer formation with H323A and H248N-287A less efficient (Fig. 5, C and D) but also these trimers were sensitive to incubation at 70°C, as revealed by a strong reduction of the E trimer peak and an accumulation of presumably denatured and aggregated material in the pellet (Fig. 7). As expected, similar results were obtained when trimer formation was allowed to proceed in the presence of liposomes (unpublished data).

Collectively, these results allow two important conclusions: (1) the lack of fusion activity of mutant H248N-H287A is likely to be caused by an impairment of trimer formation combined with a reduction in trimer stability, and (2) H323 apparently has a double role in the fusion process and not only functions as a pH sensor for initiating fusion but also contributes to the stability of the post-fusion trimer.

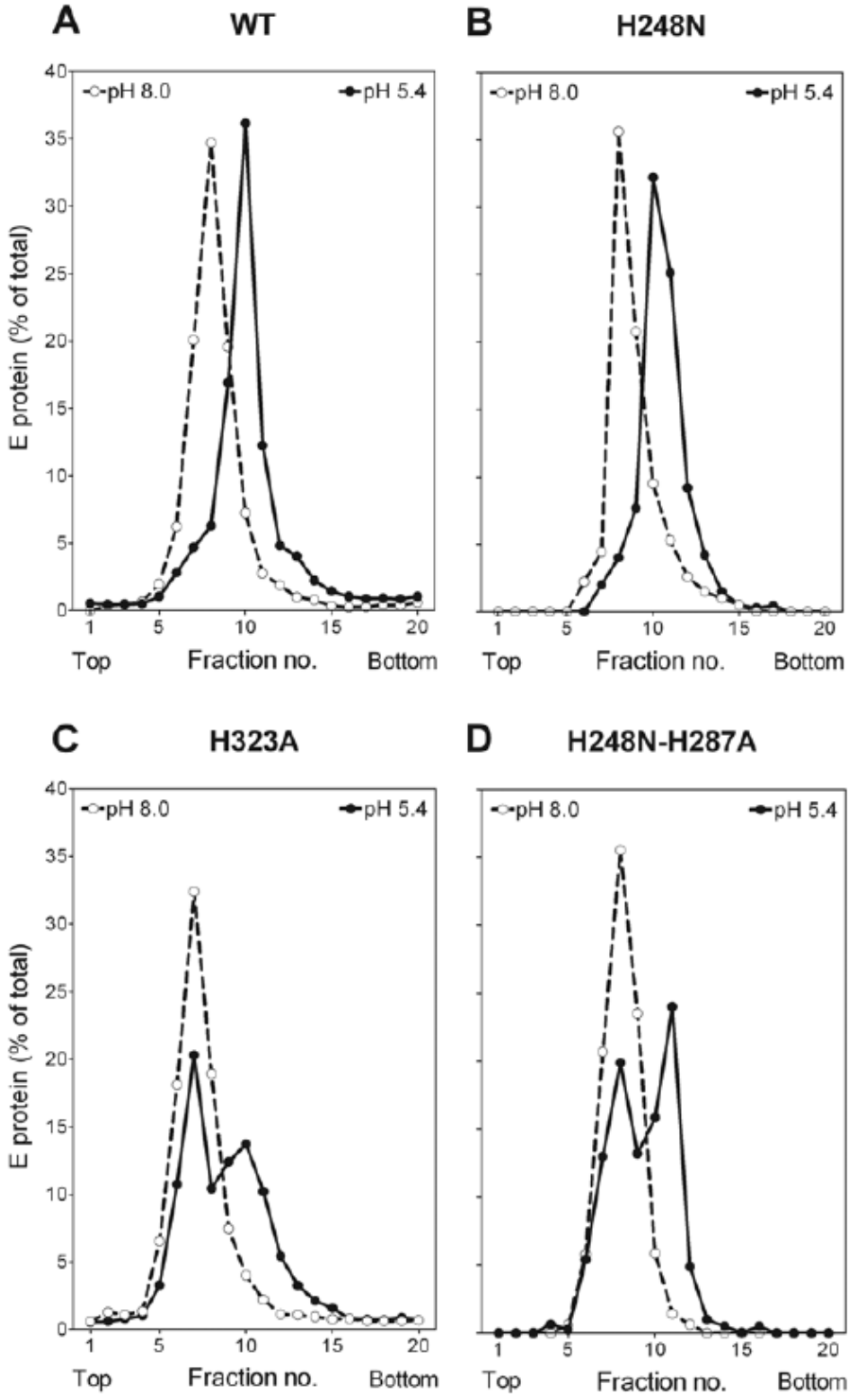


Figure 5. **Analysis of acidic pH-induced trimer formation with WT and mutant RSPs by rate zonal gradient centrifugation.** (A-D) RSPs were incubated for 10 min at pH 5.4 (solid line), or pH 8.0 (dotted line), back-neutralized, solubilized with 1% Triton X-100, and analyzed by sedimentation in 7-20% sucrose gradients containing 0.1% Triton X-100. The gradients were fractionated, and the amount of E protein in each fraction was determined by a quantitative four-layer ELISA. The sedimentation direction is from left to right. Peak of the E dimer, fraction 8; peak of the E trimer, fraction 10.

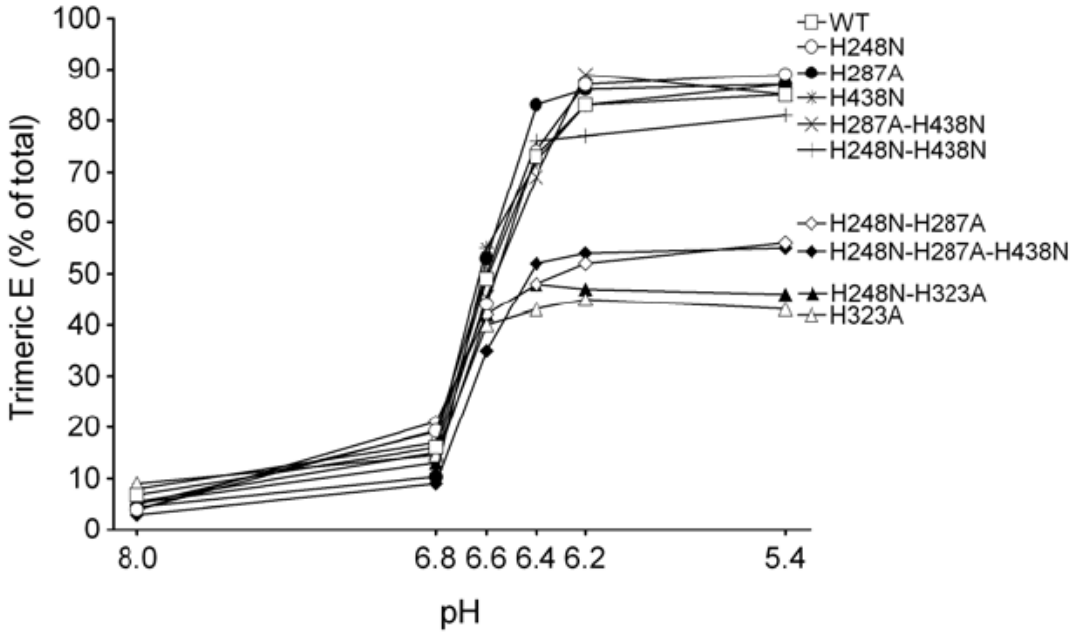


Figure 6. **pH threshold of E trimer formation of RSP WT and mutants.** Extent and pH dependence of acidic pH-induced E trimer formation with WT and mutant RSPs as determined by rate zonal sucrose density centrifugation. Results are expressed as a percentage of E found in the trimer peak fractions relative to the total amount of E in the gradient.

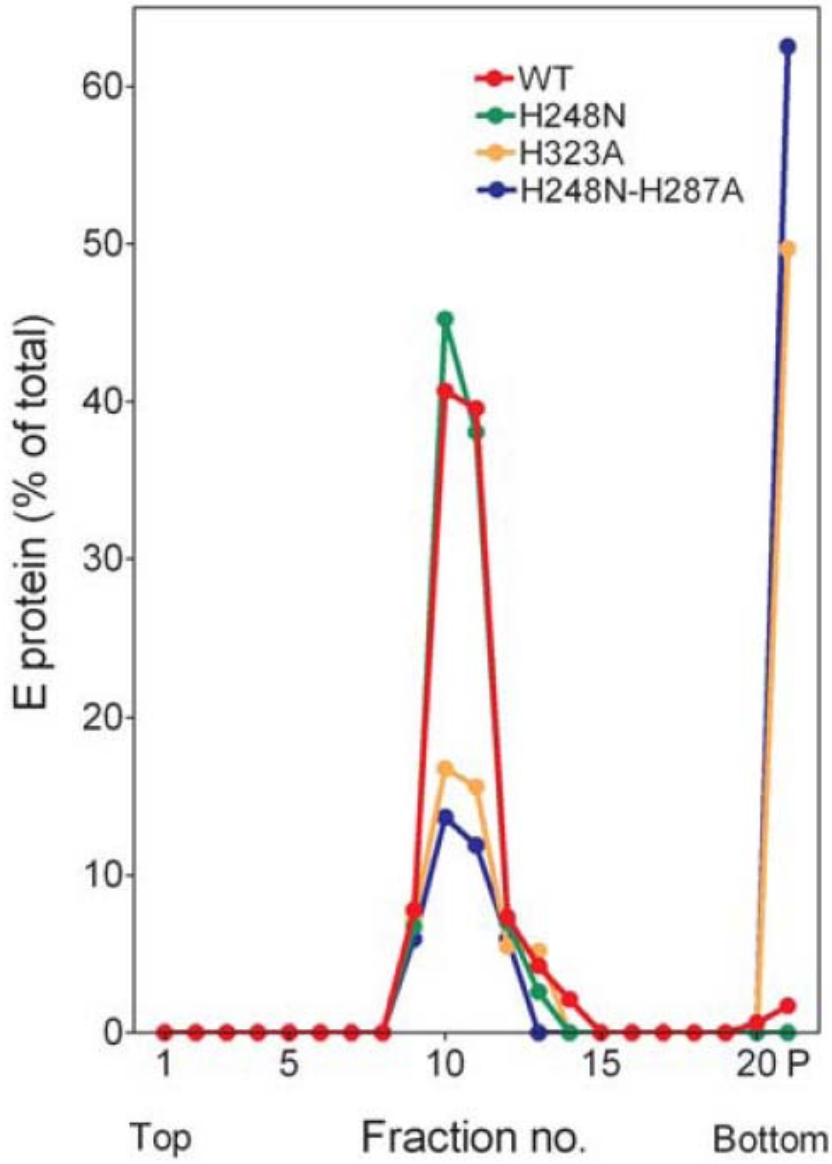


Figure 7. **Analysis of the stability of acidic pH-induced E trimers of WT and selected mutant RSPs.** Acidic pH-induced trimers of WT (red) and mutant RSPs (green, H248N; orange, H323A; blue, H248N-H287A) were exposed to 70°C and subjected to rate zonal sucrose density gradient centrifugation. The sedimentation direction is from left to right. The pellet (P) was resuspended in 0.6 ml corresponding to the volume of a single fraction.

1.4. Discussion

Although histidines have been speculated to play an important role as acidic pH sensors in class II viral fusion proteins (Bressanelli et al., 2004; Kampmann et al., 2006; Kanai et al., 2006; Nybakken et al., 2006; Roussel et al., 2006; Mueller et al., 2008), experimental evidence for such a role is still lacking. The mutational analysis presented in this work suggests that one of the five conserved hisitidines in the TBEV E protein (His323) plays a dominant role in the initiation of the multistep fusion process. This amino acid residue forms part of an intricate intramolecular network of interactions between DI and III of the E monomer in its pre-fusion conformation, consisting of hydrogen bonds, van der Waals contacts and a salt bridge between Arg9 in DI and Glu373 in DIII (Fig. 8 A; Bressanelli et al., 2004). Both of these amino acids as well as several additional residues of this contact area are absolutely conserved among flaviviruses (Bressanelli et al., 2004). The integrity of this domain interface is essential for the dimeric conformation of E in mature virions because only in this specific arrangement do the two domains together provide a protective pocket for the FP loop at the tip of DII of the second subunit (Fig. 1 B and Fig. 8 A). Through these and other DII interactions, the monomeric subunits are fixed in an antiparallel orientation in the E dimer. The release of these constraints is necessary for exposing the FP and allowing an outward projection of the E monomers as a prerequisite for target membrane interactions (Mukhopadhyay et al., 2005; Stiasny and Heinz, 2006; Harrison, 2008). Inspection of the post-fusion structure suggests that His323 may form an intradomain salt bridge with Glu373. Because in the pre-fusion conformation Glu373 is part of the central salt bridge together with Arg9, the protonation of His323 is likely to contribute to the dissolution of this salt bridge and to the concomitant destabilization of the DI-III interface. The fact that the basic architecture of these networks is conserved in the pre- and post-fusion forms of E from TBEV and the distantly related dengue 2 virus (His323 and His317 and Glu373 and Glu368, respectively) would argue for a similar mechanism of fusion initiation in all flaviviruses (Rey et al., 1995; Bressanelli et al., 2004; Modis et al., 2003, 2004).

The DI-III interface contains another absolutely conserved histidine at position 146 (Fig. 8 A), which we were not able to investigate directly because its replacement by any of the other 19 amino acids abolished the formation of native RSPs by the quality criteria applied.

Although the mutation H323A dramatically reduced the acidic pH-induced exposure of the FP, there was some residual activity that occurred at the same pH threshold of 6.6. Because all of the other His mutations as well as their combinations did not impair FP exposure, the residual activity is probably related to the protonation of His146 and suggests a possible accessory role of this residue in the destabilization of the DI-III interface. Surprisingly, and in contrast to what has been observed for the influenza virus class I fusion protein hemagglutinin (Thoennes et al., 2008), none of the histidine mutations in this work affected the pH threshold of fusion-related processes. Flavivirus variants that exhibited a pH shift for membrane fusion were described to contain mutations in the DI-II hinge region (Rey et al., 1995; Harrison, 2008), but the mechanism causing this behaviour remains to be elucidated. It has been suggested, however, that these mutations influence the stability of E (Modis et al., 2003) and thus may modulate the pH required to induce those conformational changes that drive fusion. Such “stability effects”, however, appear to be distinct from the actual pH-sensoring machinery which apparently involves His323 and probably His146.

The conversion of the E dimer to the post-fusion trimer not only requires the relocation of DIII but also a change in the orientation of DII relative to DI (Bressanelli et al., 2004; Modis et al., 2004). This is made possible by the structural flexibility of the junction between the two domains (Modis et al., 2003), which is also necessary for conformational transitions during other phases of the viral life cycle, such as virus assembly and maturation (Zhang et al., 2004; Li et al., 2008). In the context of fusion, this hinge motion may occur spontaneously upon release from dimer constraints, caused by the dissolution of the DI-III interface, but could also require an independent pH-sensoring step (Kanai et al., 2006). With respect to the conserved histidine at the DI-II junction (His287), we did not, however, find evidence for such a pH sensor function, because its replacement did not have any measureable effect, neither on early nor late stages of fusion.

It is a further finding of our work that His323 apparently has a dual role in the fusion process and contributes both to the initial pH trigger and to the stability of the post-fusion trimer. The lower stability of this mutant trimer could be due to the lack of the salt bridge between His323 and Glu373 in the post-fusion structure of DIII (Fig. 8 B) and a concomitant weakening of DIII-mediated trimer contacts and/or the proper positioning of the stem for zippering along the grooves provided by DII. None of the other conserved histidines (except His146, which was irreplaceable and therefore not amenable to analysis) seems to have a similar impact on E trimerization as His323, because their single replacements neither affected trimer formation nor trimer stability to a measurable extent (Fig. 7). An additive effect, however, was observed in the double mutant H248N-H287A which was fusion negative and as impaired in trimer formation and trimer stability as was the mutant H323A. His287 forms part of the trimer interfaces (Fig. 1 C) and His248 is located at a position at the groove, close to the FP (Fig. 1 C), that is likely to accommodate the stem during the 'zipping up' process (Fig. 1 D). Theoretically, the replacement of each of these residues alone could interfere with late stages of the fusion process, leading to the opening of the fusion pore. More specific interpretations of these results, however, will depend on the elucidation of the atomic structure of the stem in the post-fusion structure of E and assays that distinguish between lipid and contents mixing.

Although three of the conserved histidines in TBEV E (His248, His287, and His438) were ruled out as pH sensors for initiating fusion, they could be involved in other pH-dependent mechanisms, such as the conversion of immature (containing prM-E) into mature particles (Yu et al., 2008). The structure of the prM-E heterodimer of dengue 2 virus has recently been determined by X-ray crystallography and it is of special interest that His244 (corresponding to His248 in TBEV-E) is situated opposite a conserved Asp63 in prM (Li et al., 2008). It has therefore been speculated that the loss of protonation of His244 could be required for the release of the pr-peptide at neutral pH (Li et al., 2008; Yu et al., 2008). In our TBEV system, the replacement of the homologous His248, however, had no apparent effect on virus maturation and further experiments will be necessary to clarify this issue.

Despite the structural similarity of the class II fusion proteins of alphaviruses (E1) and flaviviruses (E) (Kielian, 2006), it is likely that the pH-sensoring machinery

for initiating fusion is significantly different in the two virus systems. Indeed, there is only one conserved histidine at a strictly homologous position in E and E1. This residue (His248 in TBEV and His230 in Semliki Forest Virus, respectively), however, has been ruled out as a pH sensor for both viruses and was shown to affect only a late stage of fusion (Chanel-Vos and Kielian, 2004, 2006; this study). The major difference lies in the structural details of FP protection in the pre-fusion conformations of E and E1. In flaviviruses, the FP in E is buried through homodimeric interactions, whereas in alphaviruses, the FP in E1 is protected by a heterodimeric interaction with a second, overlying protein (E2) (Kielian, 2006). Alphavirus fusion could therefore be triggered by the protonation of as yet unidentified residues in both E1 and E2.

In conclusion, our study provides experimental information on the pH trigger of flavivirus membrane fusion and the critical role of the DI-III interface in this process. Although several of the absolutely conserved histidines in the viral fusion protein were shown to be completely dispensable for fusion initiation, our study does not rule out possible roles of these residues in other pH-dependent processes of the viral life cycle. These new insights can also contribute to the design of antiviral approaches that target the structural transitions of flaviviruses during entry and morphogenesis.

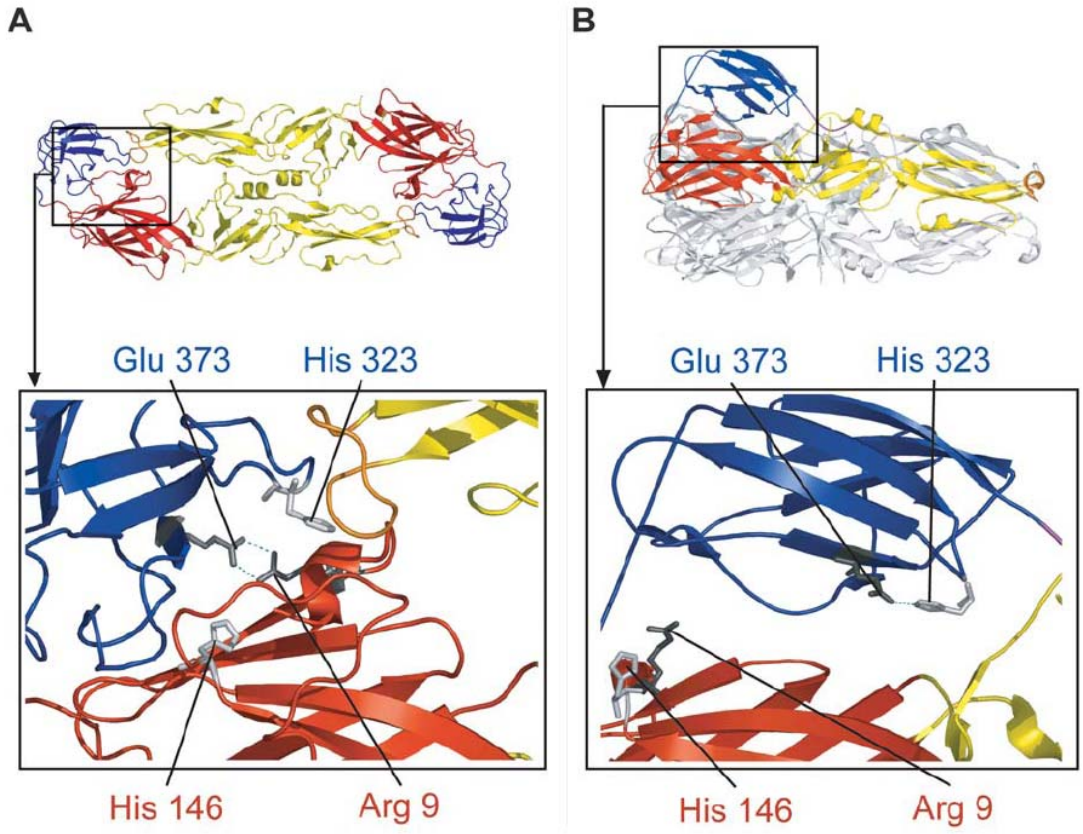


Figure 8. **Intramolecular interactions of DIII in the pre- and post-fusion structures of sE.** (A) Ribbon diagrams of the TBEV sE dimer and the details of the DI-III interface in the pre-fusion conformation with the central salt bridge between Arg9 and Glu373. (B) Ribbon diagrams of the TBEV sE trimer and the details of DIII in its post-fusion conformation revealing the possible salt bridge between His323 and Glu373.

Table S1. **Summary of histidine mutations that abolished the secretion of RSPs**

Mutant	Location of mutation
H146A	domain I
H146G	domain I
H146V	domain I
H146L	domain I
H146I	domain I
H146M	domain I
H146F	domain I
H146Y	domain I
H146W	domain I
H146S	domain I
H146P	domain I
H146T	domain I
H146C	domain I
H146N	domain I
H146Q	domain I
H146K	domain I
H146R	domain I
H146D	domain I
H146E	domain I
H248A	domain II
H248I	domain II
H287G	domain I
H287Q	domain I
H287K	domain I
H323I	domain III
H323N	domain III
H323K	domain III
H323D	domain III
H438A	stem
H438I	stem
H438K	stem

1.5. Materials and Methods

1.5.1. Mutagenesis of RSPs

Using the site-directed mutagenesis kit “Gene Tailor TM” of Invitrogen, mutations were introduced into the recombinant plasmid SV-PE WT (Allison et al., 1994), which contained the TBEV prM and E genes under the control of an SV40 early promoter, at the codon positions 146, 248, 287, 323, and 438 of the E gene. The WT and mutant plasmids were sequenced throughout the prM and E regions to confirm that only the desired mutations were present.

1.5.2. Production of RSPs

For the production of RSPs, COS-1 cells were transfected with recombinant plasmids by electroporation as described previously (Schalich et al., 1996). RSPs were harvested 48 h after transfection from cell culture supernatants, pelleted by ultracentrifugation and purified by sucrose gradient centrifugation (Schalich et al., 1996; Allison et al., 2001). For membrane fusion (lipid mixing) assays, the RSPs were metabolically labeled with 1-pyrenehexadecanoic acid (Molecular Probes) as described previously (Corver et al., 2000; Allison et al., 2001).

1.5.3. Quality controls of RSPs

The amount of RSPs secreted from transfected cells was quantified by a four-layer ELISA after solubilization with 0.4% sodium dodecyl sulfate at 65°C for 30 min (Heinz et al., 1994). The conformation of E was probed in comparison to that of the WT by epitope mapping with 22 E protein-specific mabs (Allison et al., 1995, 2001; Heinz et al., 1994; Stiasny et al., 2005) and their maturation state (presence of prM) was analyzed by Western blotting (Allison et al., 2003).

1.5.4. pH treatment of RSPs

Acidic pH incubations were carried out at 37°C and the different acidic pHs were adjusted by the addition of morpholinoethansulfonic acid (MES) ranging from 80 to 300 mM to the samples in TAN buffer, pH 8.0 (50 mM triethanolamine, 100 mM NaCl). Controls were incubated in TAN buffer, pH 8.0.

1.5.5. Lipid mixing fusion assay

Pyrene-labeled RSPs were mixed with large unilamellar liposomes (0.3-mM total lipid) consisting of phosphatidylcholine, phosphatidylethanolamine, and cholesterol (molar ratio, 1:1:2) in a continuously stirred fluorimeter cuvette at 37°C (Allison et al., 2001; Stiasny et al., 2003). Fluorescence was monitored continuously using a fluorescence spectrophotometer (Perkin-Elmer). Lipid mixing was initiated by the addition of MES to yield a final pH of 5.4. The extent of fusion was calculated by using the initial excimer-fluorescence as 0% fusion and the fluorescence after solubilization of the RSP-liposome mixture by the detergent octa(ethylene glycol)-*n*-dodecyl monoether (Sigma-Aldrich) as 100% fusion.

1.5.6. Coflotation assay

Purified RSPs were mixed with liposomes with the same composition as those used in the fusion assays at a ratio of 1µg E protein to 200 nMol of lipids. The mixture was acidified by the addition of MES to yield a pH of 5.4 and incubated for 15 minutes at 37°C. After back-neutralization by the addition of 150 mM triethanolamine to yield a final pH of 7.8, sucrose was added to the RSP-liposome mixture to a final concentration of 20% (wt/wt). This 0.6-ml sample was layered onto a 1-ml cushion of 50% sucrose in TAN buffer, pH 8.0, and overlaid with 1.4 ml of 15 % sucrose and 1 ml of 5 % sucrose (Allison et al., 2001; Stiasny et al., 2002). The step gradients were centrifuged for 2 h at 4°C (rotor SW 55; Beckman Coulter) at 50,000 rpm, and 0.2-ml fractions were collected by upward displacement using a Piston Gradient Fractionator (BioComp Instruments Inc.). E protein was quantified by four-layer ELISA after solubilization with 0.4% sodium dodecyl sulfate at 65°C for 30 min (Heinz et al., 1994).

1.5.7. Fusion peptide exposure assay

Native RSPs (at a concentration of 0.5 µg/ml of E protein) in phosphate-buffered saline, pH 7.4, containing 2% lamb serum were captured by polyclonal anti-TBEV immunoglobulin G for 1 h at 37°C as described previously (Stiasny et al., 2007). The exposure of the FP was measured by the addition of biotinylated mAb A1 (FP-specific mab) in MES buffer (50 mM MES, 100 mM NaCl), titrated to the appropriate pH by titration with 1N NaOH. After incubation for 1 h at 37°C, the bound antibody A1 was detected by using streptavidin-peroxidase (Sigma-Aldrich).

1.5.8. E trimer formation and stability

The acidic pH-induced E dimer-to-trimer transition was measured by sedimentation analysis as described previously (Allison et al., 1995, 2001). 3 µg RSPs in TAN buffer, pH 8.0, were adjusted to different acidic pHs by the addition of MES (see pH treatment of RSPs) and incubated at 37°C for 10 min. Samples were back-neutralized with 150 mM triethanolamine, solubilized for 1 h at room temperature with 1% Triton-X 100, and applied to 7-20% (wt/wt) continuous sucrose gradients containing 0.1% Triton-X 100. Centrifugation was performed for 20 h at 38.000 rpm at 15°C (rotor SW 40; Beckmann Coulter). 0.6-ml fractions were collected by upward displacement using a Piston Gradient Fractionator, and the amount of E in each fraction was determined by an E-specific four-layer ELISA after treatment with 0.4% sodium dodecyl sulfate at 65°C for 30 minutes (Heinz et al., 1994). To investigate the thermostability of the post-fusion E protein, acidic pH-pretreated and solubilized RSPs were incubated at 37 and 70°C before sedimentation analysis (Stiasny et al., 2005).

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1.7. Acknowledgements

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1.8. Abbreviations list

D, domain
FP, fusion peptide
RSP, recombinant subviral particle
sE, soluble E
SFV, Semliki Forest Virus
TBEV, tick-borne encephalitis virus
TGN, trans-Golgi network
WT, wild-type

Competing interests

The authors declare that they have no competing financial interests.

JCB: COMMENT

The pH sensor for flavivirus membrane fusion

Stephen C. Harrison

Howard Hughes Medical Institute and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

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Viruses that infect cells by uptake through endosomes have generally evolved to “sense” the local pH as part of the mechanism by which they penetrate into the cytosol. Even for the very well studied fusion proteins of enveloped viruses, identification of the specific pH sensor has been a challenge, one that has now been met successfully, for flaviviruses, by Fritz et al. (Fritz, R., K. Stiasny, and F.X. Heinz. 2008. *J. Cell Biol.* 183: 353–361) in this issue. Thorough mutational analysis of conserved histidine residues in the envelope protein of tick-borne encephalitis virus led Fritz et al. (2008) to identify a histidine at a key domain interface as the critical pH sensor; its protonation triggers the large-scale conformational rearrangement that induces fusion of viral and endosomal membranes.

The acidic pH of endosomes is one of their simplest distinguishing characteristics. Most viruses that pass through these compartments en route to productive infection have evolved to “sense” the local proton concentration as part of their mechanism for crossing into the cytosol. For enveloped viruses, fusion of their lipid bilayer with the membrane of an endosome is generally the pH-dependent molecular step, catalyzed by a “fusion protein” on the viral surface (Harrison, 2008; White et al., 2008). Although these proteins have been studied in great detail for over 30 yr, it has not been easy in any of the well characterized examples to pin down the molecular identity of the pH sensor. Histidine residues are plausible candidates, as they titrate in the relevant range, but suitably poised carboxylate pairs can have a similar pK. The long history of working out the origins of the haemoglobin Bohr effect show how tricky such a search can be (e.g., see Riggs, 1988). Moreover, charge interactions, even those with conserved physiological functions, can move around on a protein relatively easily in the course of evolution. For example, a redundant charge pair can appear by mutation, with a similar pK as that of an existing one, allowing the initial charges to disappear in some subsequent evolutionary step, without drastic change in titration properties. Exquisite stereochemistry is often not required.

Fritz et al. (2008) (see p. 353 in this issue) have taken on the challenge of determining the pH sensor for flavivirus fusion by meticulous and exhaustive mutational analysis of conserved histidine residues in the fusion protein of tick-borne encephalitis virus (TBEV). Their work builds upon elegant analyses of TBEV fusion by Heinz and co-workers over many years, including their essential contributions to structure determinations of the protein, both at neutral pH and after acidification (Rey et al., 1995; Bressanelli et al., 2004). Flaviviruses are particularly compact structures, only ~ 500 Å in diameter, tiled on their surface by 180 envelope protein (E) subunits in an icosahedral array (Zhang et al., 2003), as illustrated in Fig. 1 a . Within this outer layer is the viral membrane, a roughly spherical bilayer ~ 410 Å in outer diameter. The viral positive-strand RNA genome encodes three structural proteins — an internal, RNA packaging “core” protein (C) and two membrane-anchored proteins, prM and E (Lindenbach et al., 2007). A C protein – RNA complex buds into the endoplasmic reticulum, acquiring a membrane with 180 prM-E heterodimers in the process.

The prM protein is a specific chaperone for E (Fig. 1 b). In the trans-Golgi network (TGN), furin cleavage of prM to a membrane-anchored residual fragment (called M) allows E to settle into the regular array illustrated in Fig. 1 a and also allows it to undergo (when subsequently acidified) the low pH – induced, dimer-to-trimer reorganization shown in Fig. 2. Thus, when the mature virus particle secreted by one cell arrives in the acidic environment of an early endosome in a target cell, the large-scale molecular rearrangement of E facilitates fusion, first by exposing a hydrophobic “fusion loop”, which inserts into the endosomal membrane, and then by drawing together the viral and target membranes as the conformational change proceeds.

In the 1990’s, Heinz and co-workers (Allison et al., 1995; Schalich et al.,1996) showed that recombinant expression of TBEV prM and E in mammalian cells leads to secretion of recombinant subviral particles (RSPs), smaller than virions but still with an intact lipid bilayer. Later analysis showed that they contain just 60 copies of E (and, after passage through the TGN, the same number of copies of M), icosahedrally arrayed, and that the bilayer is only ~ 210 Å in outer diameter (Ferlenghi et al., 2001). These particles nonetheless fuse with liposomes or other target membranes, at lowered pH, in a reaction that has precisely the same characteristics as virion fusion (Corver et al.,2000). For example, as described by Fritz et al. (2008), the reactivity of E with a panel of 22 monoclonal antibodies, with quite differently positioned epitopes, changes in just the same way during viral fusion and RSP fusion. The pH dependence and the kinetics of the process are likewise the same. As RSPs can be produced by transient transfection, mutagenesis is far more straightforward than it would be with virions. Fritz et al.

(2008) could therefore pursue their hypothesis that histidines in E conserved among all flaviviruses are the likely pH sensors by an essentially complete analysis of the five such residues in TBEV. They monitored fusion by labeling the RSP membranes with 1-pyrenehexadecanoic acid, which has substantially altered fluorescence properties when diluted into the target membrane after merger of the bilayers.

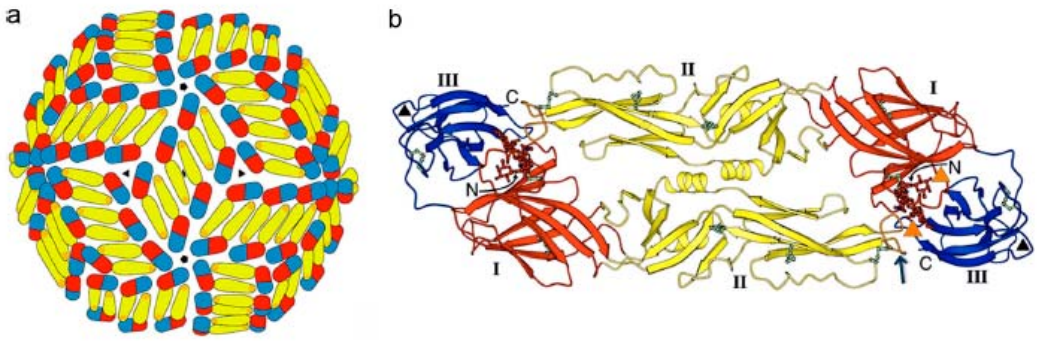

Figure 1. **Flavivirus structure.** (a) Diagram of the packing of 180 E subunits in the surface of a virion. The proteins are clustered as dimers. Each is represented by a symbol, colored to correspond to the domain representation in b. (b) The ectodomain of the E dimer, viewed as if looking toward the surface of the virion. Domains I, II, and III are labeled and colored in red, yellow, and blue, respectively. An arrow points to the fusion loop on one subunit. The locations of two histidines at the domain I – domain III interface are shown by orange triangles. His 146 is on domain I; His 323, close to the fusion peptide of the partner subunit, is on domain III. Black triangles mark a potential receptor-binding loop.

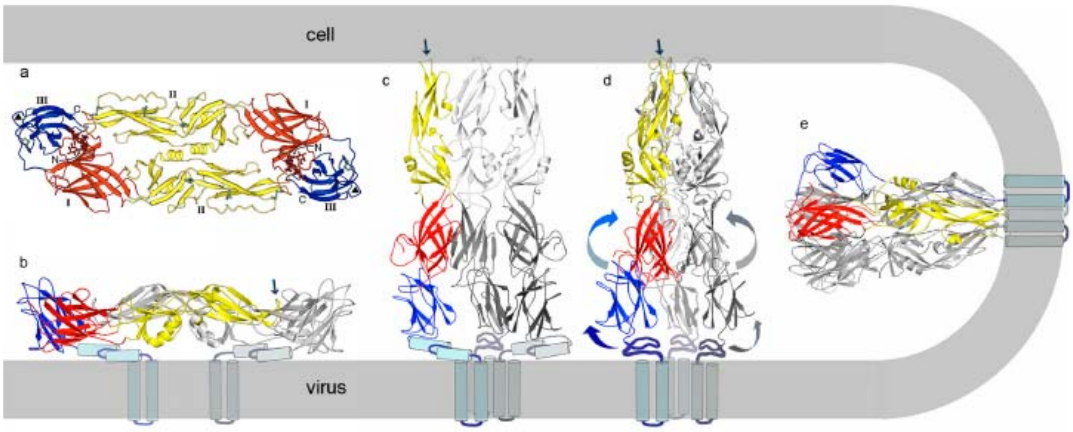

Figure 2. **Sequence of events during low pH-triggered, fusion-inducing conformational rearrangements of flavivirus E proteins.** (a) E ectodomain dimer, viewed as in Fig. 1 b . (b) Side view of the E dimer, illustrating how it is anchored in the viral membrane. A segment known as the stem connects the C terminus of domain III to the transmembrane anchor (a helical hairpin that traverses the bilayer once in each direction). (c) Low pH induces dissociation of the dimer interface and rotation outward of domains I and II, exposing the fusion loop (black arrows), which interacts with the endosomal target membrane. (d) The extended intermediate trimerizes and starts to collapse (curved arrows), so that domain III rotates back to dock against domains I and II and the stem zips up alongside the trimer-clustered domain II. (e) When the transition is complete, the two membranes have been brought together and induced to fuse. Several trimers probably participate cooperatively in this process, but only one is shown here. (This figure has been modified from Harrison, 2008.)

Two of the five conserved histidines (H146 and H323) are at a particularly "interesting" interface between domains I and III of the E protein (see Fig. 1 b), and several previous papers (Bressanelli et al., 2004; Kampmann et al., 2006) had called attention to them. This interface rearranges completely when the protein undergoes its fusion-inducing change from dimer to trimer (Fig. 2), and protonation might indeed be expected to destabilize the dimer conformation. Two of the other conserved histidines (H248 and H287) are on the protein surface; the fifth (H438) is in the so-called stem that links domain III to the transmembrane anchor. Fritz et al. (2008) show that any of these last three can be mutated individually, without effect on the fusion properties of the corresponding RSPs, whereas mutation of H323 to alanine eliminates fusion, even at pH 5.0. Mutation of H146 to any of the other 19 naturally occurring amino acid residues prevents stable expression of E and hence prevents any formation of RSPs. H323 is not only buried at a key domain interface, it is also part of the pocket that protects the fusion loop of the dimer partner. Fritz et al. (2008) convincingly conclude that H323 is the critical pH sensor, with a possible additional contribution from H146.

Fritz et al. (2008) also examine a series of double and triple mutations. One of the double mutants, H248A-H287A, gives rise to stable RSPs but prevents fusion. In earlier work, Stiasny et al. (2007) found, by using a monoclonal antibody directed against the fusion loop, that its epitope, buried in the prefusion E dimer, is transiently exposed during the fusion process and becomes again protected in the postfusion trimer. As might be expected from its location, the H323A mutation prevents even the process (presumably dissociation of the dimer interface) that makes this epitope transiently accessible after acidification. The same is not the case for the H248A-H287A double mutant, however; lowered pH allows binding of the fusion loop monoclonal, just as to wild-type RSPs. The likely interpretation is that the double mutation impairs a later step in the fusion reaction. A good candidate would be the transition from the extended, intermediate structure (Fig. 2 c) to the folded back trimer (Fig. 2 e). The trapped intermediate is probably still monomeric, as suggested by sedimentation analysis of solubilised E protein from the various RSPs. Both H248 and H287 are in locations compatible with a contribution to trimer stability. The double

mutant also binds liposomes at lowered pH, confirming exposure of its hydrophobic fusion loops.

Animating molecular structures and probing the processes in which they participate is still an arduous business. Directed mutagenesis, however well informed by structural information, is often a relatively blunt instrument. By building on nearly two decades of careful work on TBEV and its surface proteins, Fritz et al. (2008) have done more than simply identify the pH sensor that triggers E protein rearrangement, challenging as even that task has been. Together with the work of Liao and Kielian (2005) on the related alphavirus fusion proteins (and on dengue, another flavivirus), the experiments described in their paper fill in steps of the mechanism, illustrated in Fig. 2, for which we have had until now mainly the logical deductions from structures of the two end states. Methods to track the fusion of individual virus particles, by extension of the type of fluorescence assay used by Fritz et al. (2008), are likely soon to add further details and a proper time dimension. One should also recall that motivation to understand the details of this process goes beyond its considerable inherent cell biological interest. Blockade of fusion is among the mechanisms by which neutralizing antibodies prevent infection, and inhibiting viral fusion is a validated antiviral strategy, with at least one fusion inhibitor (the HIV entry inhibitor, enfuvirtide) now a licensed drug.

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IV. PART II

Role of the second transmembrane domain of E in flavivirus fusion

1.1. Abstract

The flavivirus envelope protein E is the only known viral fusion glycoprotein with a membrane anchor consisting of two transmembrane helices. To investigate the role of the second transmembrane helix (TM2) in flavivirus membrane fusion, this region of E was deleted in recombinant subviral particles (RSPs) of tick-borne encephalitis virus (TBEV). In vitro lipid mixing experiments revealed that ΔTM2 RSPs were fusion deficient. Experimental dissection of the fusion process showed that TM2 was not involved in the establishment of initial interactions with target membranes, but essential for the formation of a stable post-fusion structure of E.

1.2. Introduction

Viral fusion proteins as well as SNARE (soluble N-ethylmaleimide sensitive factor attachement protein receptors) proteins – a family of membrane-anchored proteins essential for intracellular membrane fusion – possess highly conserved transmembrane domains (TMDs). In many cases, these domains have been shown to be required for full membrane fusion (reviewed in Schroth-Diez *et al*., 2000; Langosch, Hofmann and Ungermann, 2007). Only in some reports the TMDs of viral fusion proteins were described to be functionally irrelevant (Wilk *et al*., 1996; Odell *et al*., 1997). To assess their role for fusion, the TMDs have been mutated, truncated or replaced with other anchors.

The replacement of the TM domain of the influenza hemagglutinin with a glycosylphosphatidylinositol (GPI) anchor allowed lipid mixing and fusion pore formation, but blocked pore enlargement and thus full fusion. A block before the opening of the fusion pore was also observed by the insertion of a certain point mutation into the transmembrane domain of HA or by the truncation of this region (Kemble, Danieli and White, 1994; Nuessler, Clague and Herrrmann, 1997; Markosyan, Cohen and Melikyan, 2000; Melikyan *et al*., 1997, 1999, 2000; Armstrong, Kushnir and White, 2000) (Table 1). In TMDs of other viral fusion and SNARE proteins the introduction of specific alterations had similar effects (McGinnes, Sergel and Morrison, 1993; Salzwedel *et al*., 1993; Cleverly and Lenard, 1998; Taylor and Sanders, 1999; Browne *et al*., 2003; Liao and Kielian, 2005; Xu *et al*., 2005; Miyauchi *et al*., 2006; Bissonnette *et al*., 2008; Li and Blissard, 2008) (Table 1).

These experimental analyses revealed that the initial stage of fusion i.e. the bridging of the two lipid bilayers by fusion proteins was not facilitated by the specific TMDs investigated. Many TMDs, however, were found to be required for the completion of fusion i.e. the transition from the hemifusion state to the opening of the fusion pore. The hemifusion state is a generally accepted intermediate, resulting in an hourglass-shaped stalk structure. Expansion of the stalk is thought to result in the formation of the hemifusion diaphragm. Within the

diaphragm the fusion pores are formed and their expansion leads to the fully fused state of the membrane (Fig. 6).

The concept of the existence of the hemifusion stalk was essentially supported by the investigations of viral TMDs (reviewed in Chenomordik and Kozlov, 2003, 2008; Langosch, Hofmann and Ungermann, 2007).

Table 1. **Effect of TMD alteration on viral fusion protein function**

Fusion protein	Alteration	Effect
Influenza HA	Replacement by GPI anchor	Abolished fusion, retained hemifusion
Influenza HA	Replacement by unrelated TMDs	Fusion retained
Influenza HA	G520L mutation	Abolished fusion, reduced hemifusion
Influenza HA	Shortening of TMD	Abolished fusion, retained hemifusion
VSV G	Replacement by GPI anchor	Abolished fusion
VSV G	Mutation of GxxxG motif	Abolished fusion, retained hemifusion
VSV G	Replacement by unrelated TMDs	Fusion retained
HIV gp120	Replacement by GPI anchor	Reduced syncytia formation
HIV gp120	Various mutations and truncations	Reduced syncytia formation
HIV gp120	Replacement by VSV G TMD	Reduced lipid mixing
HIV gp120	Mutation of GGxxG motif	Reduced cell-cell fusion
Measles virus F	Mutation of cysteine residues	Reduced cell-cell fusion
Paramyxovirus F	L486A, I488A mutations	Abolished syncytia formation Reduced fusion
Newcastle disease virus HN	Mutation of leucine zipper repeat	Reduced fusion promoting activity
Morony murine leukemia virus E	Mutation of P617	Reduced fusion
AcMNPV gp64	Replacement by various TMDs	Abolished fusion, retained hemifusion
Reovirus p10	Mutation of glycine motif	Reduced syncytia formation
HSV-1 gD	Replacement by GPI anchor	Reduced cell-cell fusion
HSV-1 gH	Various mutations	Reduced cell-cell fusion
SFV E1	Mutation of glycine residues	Reduced cell-cell fusion

Adapted and extended from Langosch, Hofmann and Ungermann, 2007

For several flaviviruses atomic details of both, the pre-fusion and the low pH-induced post-fusion forms of the E ectodomain are known from x-ray crystallography of sE (Rey et al., 1995; Modis *et al*., 2003, 2004; Bressanelli et al., 2004; Zhang *et al*., 2004; Modis *et al*., 2005; Nybakken *et al*., 2006). Cryo-electron microscopy images allowed the investigation of the C-terminal part of native DenV 2 E, that is absent in sE, i.e. the “stem-anchor” region (Zhang *et al*., 2003). The 56-residue stem region contains two mostly amphipathic alpha helices – half buried in the outer lipid leaflet – followed by two transmembrane alpha helices (TM1 and TM2; that travers the lipid bilayer from the external to the internal and back to the external lipid leaflet) forming the anchor (Fig. 1). The two TM helices are oriented antiparallel to each other and are linked by four to six mostly polar amino acids which are presumably buried within the inner lipid leaflet (Zhang *et al.*, 2003). Since the E protein is not exposed on the cytoplasmic side of the viral membrane, it does not possess a cytoplasmic tail found in many other viral fusion proteins. The structure and organization of the transmembrane domains of flavivirus E is unique – all other viral fusion proteins and SNAREs posses single TMDs only – and the role of the double membrane anchor in fusion has so far not been investigated.

In this study, subviral particles of TBEV were used as a model, and the second TMD of E was deleted to investigate whether this element plays a role in flavivirus membrane fusion. TM2 was found to be important for the overall fusion process, but not required during the early phases of fusion. E trimerization was not dependent on the presence of TM2, however, in its absence the trimers formed were significantly less thermostable. Overall the fusion process seems to be dependent on the formation of a stable E post-fusion trimer that requires the presence of TM2.

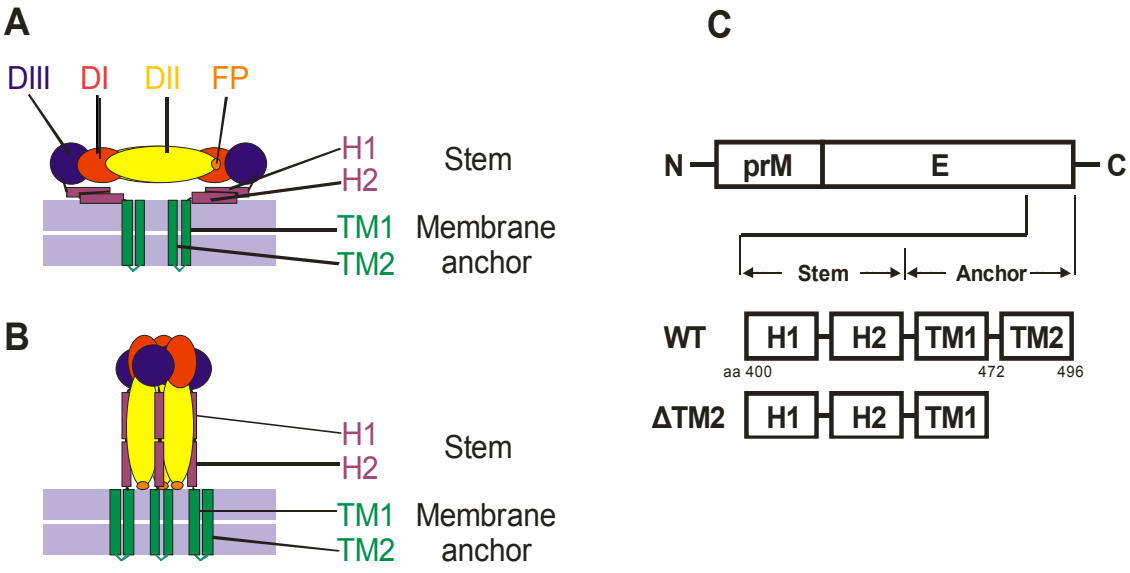

Figure 1. **Schematics of the membrane-anchored TBEV E protein (A, B) and representation of the C-terminal sequence elements of E proteins (C).** Membrane-anchored E in its pre-fusion (A) and post-fusion (B) conformations (side view). The stem and the membrane anchor both consist of two helices: H1, stem helix 1; H2, stem helix 2; TM1, transmembrane helix 1; TM2, transmembrane helix 2. Color code: E protein domain I (DI), red; DII, yellow; DIII, blue; stem helices, purple; transmembrane anchors, green; membrane, grey. (C): In ΔTM2 RSPs the second transmembrane helix of E was deleted. Numbers indicate amino acid positions.

1.3. Results

1.3.1. Generation of RSPs lacking TM2

To investigate the role of the second TMD of the flavivirus envelope protein E in membrane fusion, recombinant subviral particles (RSPs) of TBE virus containing C-terminally truncated E proteins with only the first TM helix were used (ΔTM2) (Fig. 1 C). As shown previously (Allison *et al*., 1999), TM1 is sufficient for incorporation of E into RSPs.

ΔTM2- as well as WT-particles were generated as described (Allison *et al*., 1995, 1999) (Materials and Methods) and were subjected to particle quality controls as described (Fritz, Stiasny and Heinz, 2008) (Materials and Methods), including epitope mappings with selected E protein specific monoclonal antibodies (mabs) and analysis of the particle's prM content, to ensure proper protein folding and processing. ΔTM2 RSPs displayed WT-like properties in all assays and could therefore be used to study the role of TM2 in TBE virus membrane fusion.

1.3.2. Loss of fusion activity by deletion of TM2

To assess the effect of the deletion of TM2 on the overall fusion process, an in vitro lipid mixing assay was performed as described previously (Corver et al., 2000). For this purpose, RSPs with membranes that were metabolically labelled with 1-pyrenehexadecanoic acid were mixed with liposomes. The mixture was acidified and membrane fusion was measured by continuous detection of the decrease of pyrene excimer fluorescence due to the dilution of the probe into unlabelled liposomal membranes.

As depicted in Figure 2, WT RSPs displayed fast and efficient fusion kinetics. In contrast, fusion of ΔTM2 RSPs was virtually abolished (Fig. 2 A, B).

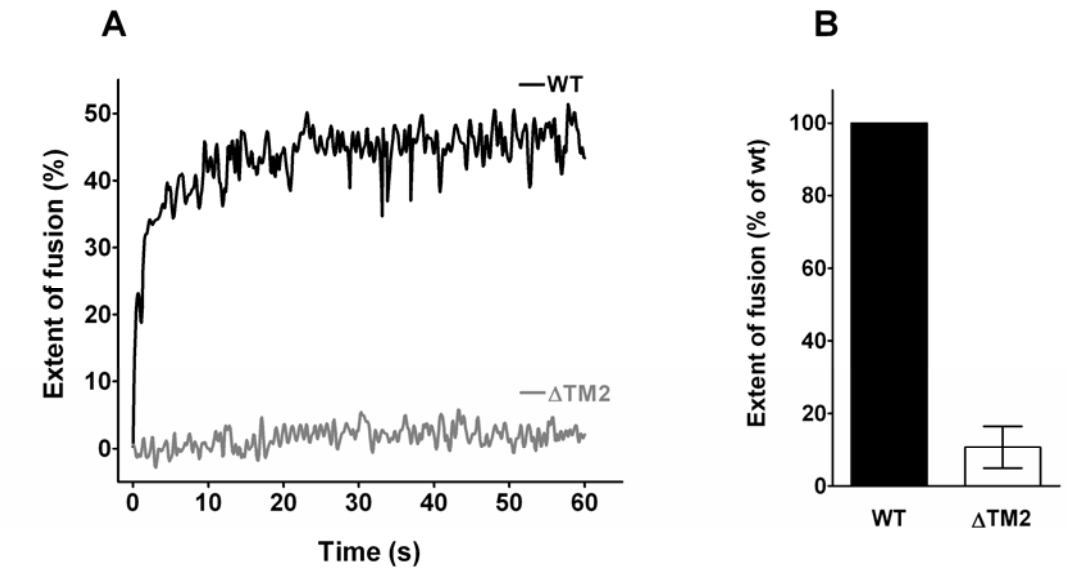

Figure 2. **Fusion activity of pyrene-labelled WT- and ΔTM2-RSPs.** (A): Fusion kinetics of WT (black line) and ΔTM2 (grey line) RSPs at pH 5.4. (B): Extent of fusion of ΔTM2 RSPs at pH 5.4 after 60 s. Three independent experiments were carried out with similar results. The error bars represent the standard error of the mean.

1.3.3. TM2 is not required for the early stages of fusion

The lipid mixing experiments revealed an important role for the second helix of the flavivirus membrane anchor in the overall fusion process. The next goal was to determine at which step of fusion TM2 is essential.

To investigate the first event of the fusion cascade, dissociation of E dimers and fusion peptide (FP) exposure, the FP specific mab A1 was used. Upon low pH-induced dimer dissociation, A1 gains increased access to its epitope as decribed previously (Fritz, Stiasny and Heinz, 2008) and in Materials and Methods.

As shown in Figure 3, no difference was found in low pH-induced FP exposure between WT- and ΔTM2-RSPs. In both cases, the increase of A1 binding occurred at the same pH threshold and to the same extent.

FP exposure allows stable interactions of the fusion protein with target membranes. To assess whether ΔTM2 E can bind to membranes, coflotation assays with liposomes were carried out as described previously (Stiasny *et al*., 2002; Fritz, Stiasny and Heinz, 2008). WT- and ΔTM2-RSPs were mixed with liposomes, acidified, and applied to sucrose step gradients (Methods). Coflotation of E with liposomes to the top of the gradients indicates either binding to or fusion with liposomes. In accordance with FP exposure, ΔTM2 RSPs were shown to bind to liposomal target membranes as efficiently as WT particles (Fig. 4 A, B).

To confirm that the observed coflotation of ΔTM2 RSPs was only due to attachement to – but not fusion with – liposomes, coflotation experiments with pyrene-labelled RSPs were performed and the pyrene fluorescence spectra were recorded as described (Fritz, Stiasny and Heinz, 2008). Figure 4 C shows that membrane fusion of WT particles with liposomes – occurring in coflotation experiments – led to a strong reduction of the pyrene excimer peak, whereas there was no such reduction for ΔTM2 RSPs. This confirms that they were only attached but not fused to the liposomes.

Taken together, the experimental data indicated that the deletion of TM2 of the flavivirus E protein membrane anchor had no effect on the initial stages of fusion.

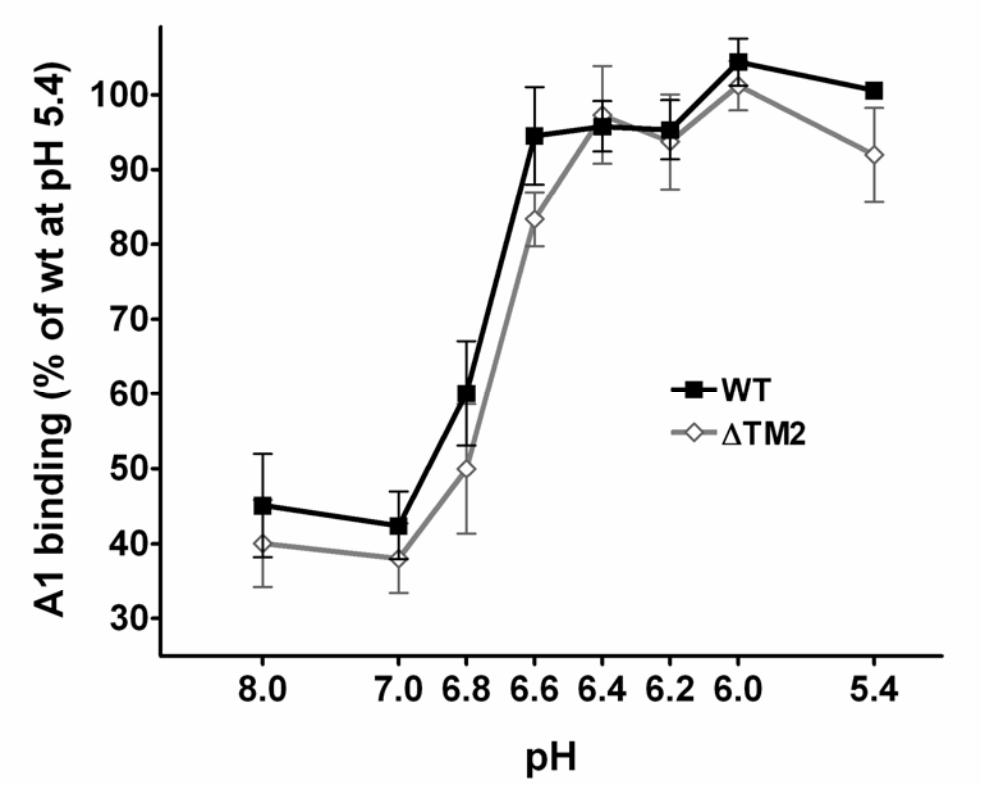

Figure 3. **Low pH-induced dimer dissociation and FP exposure measured by binding of mab A1.** Results are represented as percent of A1 reactivity with WT E at p 5.4. Data are averages of four independent experiments performed in duplicates. The error bars represent the standard error of the mean. WT: solid boxes; ΔTM2: open diamonds.

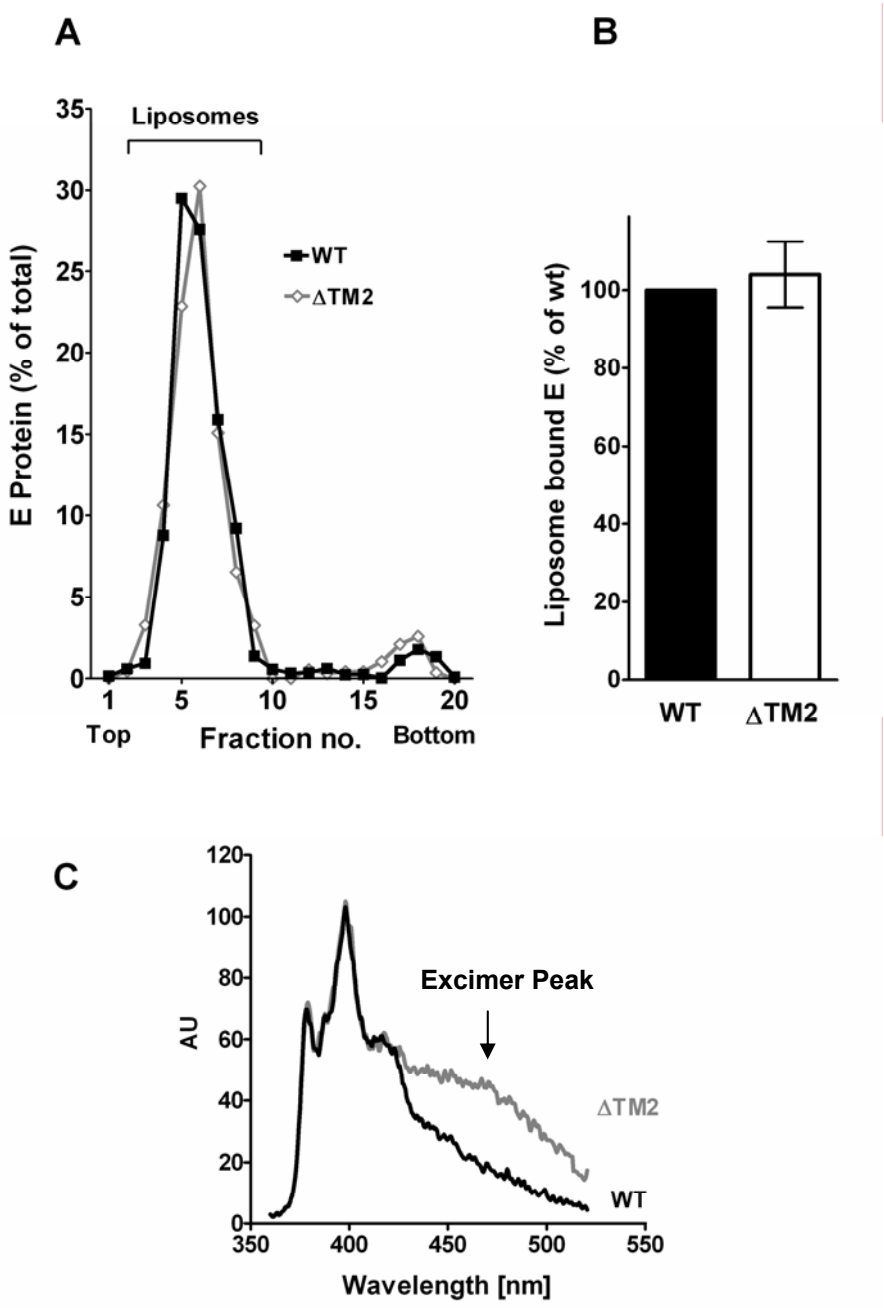

Figure 4. **Low pH-induced coflotation of WT- and ΔTM2-RSPs with liposomes.**
A: Representative example of the results obtained from coflotation experiments at pH 5.4. WT: solid boxes; ΔTM2: open diamonds. The bracket indicates the fractions containing liposomes. B: Extent of coflotation of ΔTM2 RSPs with liposomes relative to the WT derived from three independent experiments. Error bars indicate the standard error of the mean. C: Fluorescence spectrum of liposome containing fractions from coflotation experiments with ΔTM2 (grey) and WT (black line) RSPs.

1.3.4. Role of TM2 for E trimer formation and stability

Since ΔTM2 E dimers dissociated upon low pH treatment and stably interacted with target membranes via their fusion peptides like the wild-type, the block of fusion must be due to an interference with later stages of the fusion process, which includes the formation of E trimers.

To examine the effect of the TM2 deletion on E trimerization, sedimentation analyses – in the presence of the detergent TX-100 – were carried out. WT- and ΔTM2-RSPs were acidified, back-neutralized, solubilized with TX-100 and analyzed in continuous sucrose gradients as described before (Allison *et al*., 1995, 2001) and in Materials and Methods.

As shown in Figure 5, a quantitative conversion from dimers into trimers was observed for both, WT (Fig. 5 A) and ΔTM2 (Fig. 5 B), E proteins as revealed by a shift in sedimentation velocity.

However, truncated E trimers lacking the whole stem-anchor region were previously shown to be less thermostable than full-length trimers. To test whether deletion of TM2 also affects trimer stability, thermal denaturation experiments were carried out (Stiasny, Koessl and Heinz, 2005; Fritz, Stiasny and Heinz, 2008). For this purpose, preparations of trimeric E (WT and ΔTM2) were incubated at 70°C for 10 min before sedimentation analysis. As shown in Figure 5 C, the oligomeric state of WT E was not affected, but ΔTM2 trimers were found to be sensitive to increased temperatures as revealed by a strong reduction of the trimer peak and the accumulation of presumably aggregated E in the pellet.

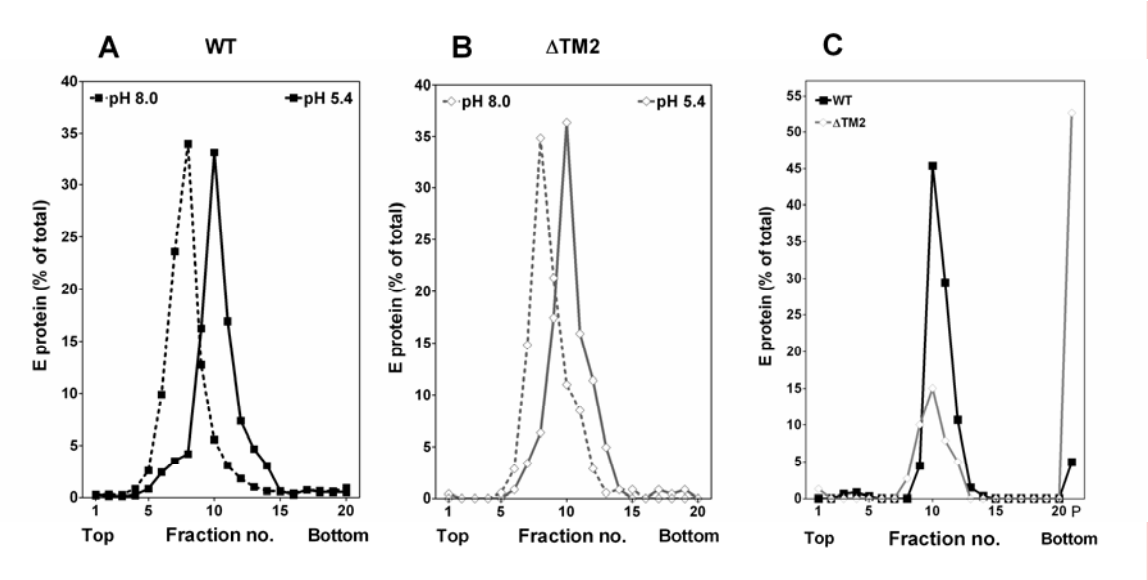

Figure 5. **Low pH-induced trimer formation and thermostability of WT and ΔTM2 E.**
A and B: RSPs (A: WT; B: ΔTM2) were incubated at pH 5.4 (solid line) or 8.0 (dotted line), solubilized, and the oligomeric state of E was analyzed by sedimentation analysis. The sedimentation direction is from left to right. C: WT (black line, solid boxes) and ΔTM2 (grey line, open diamonds) E trimers were incubated at 70°C before sedimentation analyses. The E dimer is represented by the peak in fraction 8, the trimer by the peak in fraction 10. P indicates the pellet that was resuspended in the volume of a fraction (0.6 ml).

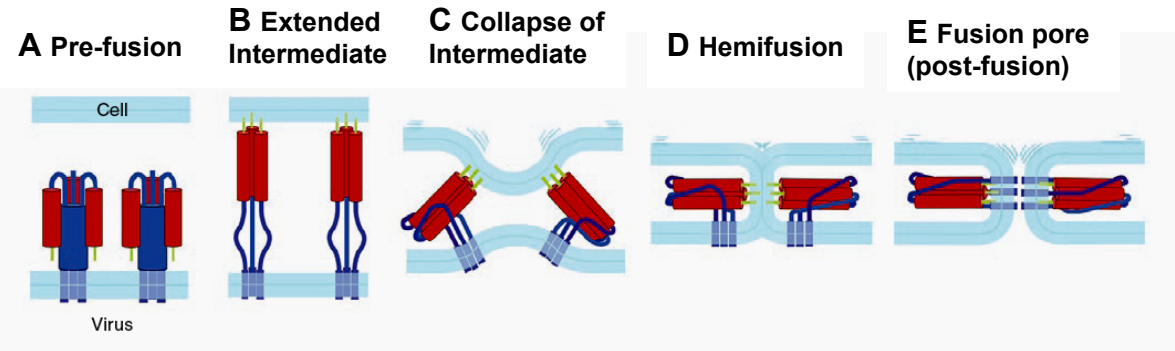

Figure 6. **Sequence of events in virus membrane fusion.** (A) A hypothetic fusion protein in its pre-fusion conformation. (B) Initial interactions with a target membrane and formation of an extended intermediate structure. (C) Collapse of the extended intermediate. (D) Formation of a hemifusion stalk. (E): Opening of the fusion pore. From Harrison, 2008b.

1.4. Discussion

The transmembrane domains of many viral fusion proteins have been shown to be required for efficient fusion, but their precise functional role – besides being a membrane anchorage domain – in this process is still unknown. Figure 6 depicts several stages of virus membrane fusion each of which could be influenced by the TMDs of viral fusion proteins.

The initial contact of the fusion protein with the target membrane (Fig. 6 B) was found to be independent of the TMD of any fusion protein studied so far (Table 1). In consistence with these findings, the experimental results presented in this study clearly show that there is no contribution of the second TMD of the TBEV E protein to the initial steps of flavivirus fusion. Early fusion events such as the dissociation of E dimers and stable interactions with target membranes were not affected by the lack of TM2.

Later steps of virus fusion involve the formation of a trimeric cluster of the fusion proteins, the formation of the hemifusion state (Fig. 6 D) and the formation of the fusion pore (Fig. 6 E). Low pH-triggered TBEV E trimerization was found to be independent of the presence of TM2, but in contrast to the full length trimers, ∆TM2 trimers were less thermostable. These results suggest a role of TM2 in late stages of flavivirus fusion. The overall fusion activity of ΔTM2 RSPs was assessed by in vitro lipid mixing assays and was found to be strongly impaired. Lipid mixing theoretically occurs during the hemifusion state. Therefore, the lack of TM2 in TBEV E apparently causes a block before the formation of the hemifusion stalk. Previously a strongly reduced hemifusion activity was only observed by the replacement of the HIV gp120 TMD (Miyauchi *et al*., 2005).

There are several hypothetical mechanisms in E-mediated flavivirus membrane fusion that could involve TM2: (I) A hypothesis was established that suggests that viral fusion glycoproteins have to cluster and form multiprotein complexes to efficiently mediate fusion. For influenza fusion with red blood cells, it was shown that at least three HA trimers are required for a single fusion event (Danieli *et al*., 1996), although this number may be dependent on the type of host cell (Harrison, 2008b). Cooperativity of fusion proteins may occur via lateral contacts between the fusion protein TMDs in a ring surrounding the fusion pore

(Chernomordik and Kozlov, 2003). Such ring complexes would be favored in overcoming the energy barrier for fusion. Clustering of fusion proteins via lateral interactions at the fusion site has been specifically proposed for the E1 protein of Semliki Forest virus (Gibbons *et al*., 2004) and for TBEV E (Stiasny *et al.*, 2004). In the case of flaviviruses, the current results in this thesis allow the speculation that interactions between the second TMDs of adjacent E proteins – probably facilitating E trimer multimerization – occur during fusion. This hypothesis has to be tested experimentally in future research. It has to be taken into account, however, that theoretically the energy barrier to a hemifusion stalk (40-50 kcal mol^{-1}) could be overcome by the refolding of just one or two trimers (Kuzmin *et al.*, 2001; Yang *et al.*, 2006; Harrison, 2008b).

(II) It has been hypothesized that fusion proteins might disturb the integrity of membranes via their TMDs. The distortion of the membranes lowers the energy barrier for fusion (reviewed in Chernomordik and Kozlov 2008; Harrison, 2008b). TM2 of TBEV E could therefore be involved in lowering the energy barrier for the membranes to form the hemifusion intermediate, whereas in the case of influenza HA, the TM could be required for lowering the energy barrier for the transition from the hemifusion state to the opening of the fusion pore.

(III) For full fusion to occur, a stable post-fusion trimer of E is necessary. The formation of this stable trimer could be dependent on TM1-TM2 interactions in the membrane. This theory, however, is in contrast to preliminary experimental results that show that the fusion activity of ΔTM2 RSPs can be rescued by the introduction of the corresponding – but not sequence homologous – TM2 of JEV (unpublished observation R. Fritz). These findings rather support a hypothesis in which a stable anchor requires the presence of an unspecific second TM element.

A combination of the mechanisms discussed is also possible. In summary, the results presented here demonstrate an active role of the TMD of TBEV E in fusion. To further clarify this role, additional analyses are required.

V. PART III

Preparatory work:
Establishment of quality controls of recombinant subviral particles for flavivirus membrane fusion studies

1.1. Introduction

Detailed investigations of viral membrane fusion – as presented in this thesis – require suitable experimental model systems. The use of replicating virions for experimental purposes is often time consuming and may be complicated by resuscitating mutations occurring during virus replication. This limitation is overcome by the use of recombinant non infectious subviral particles (RSPs), which – in the case of flaviviruses – contain the E and M proteins anchored in a lipid membrane (Fig. 1), but lack the nucleocapsid. Quality controls that help to identify RSPs suitable for membrane fusion studies were lacking and had to be established before such particles could be used in the previous parts of this thesis.

RSPs can be produced by the co-expression of the E and prM proteins of TBE virus in eukaryotic cells (Allison *et al*, 1995a). Similar particles of different flaviviruses were obtained using various eukaryotic expression systems (Mason *et al.,* 1991; Konishi *et al.,* 1992; Pincus *et al*., 1992; Fonseca *et al.,* 1994). Like the virions, RSPs are assembled at the membrane of the ER in an immature form containing a complex of E and prM. Immature RSPs (containing prM) encounter low pH conditions in the TGN that trigger a conformational change in the protein complex resulting in the maturation cleavage of prM by furin or a related protease.

RSPs were extensively characterized and shown to be ordered structures containing envelope glycoproteins with structural and functional properties very similar to those in the virion envelope (Fig. 1). The structure of RSPs of TBEV was determined by cryo-electron microscopy (cryo-EM) and image reconstruction to a resolution of 19 Å (Ferlenghi et al, 2001). Fitting the high-resolution structure of the E ectodomain into the experimental density revealed that 30 E dimers are arranged in a T=1 icosahedral lattice. The structural similarity of E on RSPs and virions has further been shown by conformation-sensitive and virus-neutralizing monoclonal antibodies. Incubation at pH <6.5 induced identical conformational changes and structural rearrangements, including an irreversible, quantitative conversion of dimers into trimers (Allison *et*

al., 1995b; Schalich *et al*., 1996). RSPs were also shown to be functionally active, inducing membrane fusion in a low pH-dependent manner (Schalich *et al*., 1996; Corver *et al*., 2000). Therefore TBE virus RSPs are an excellent model system for investigating the structural basis of viral envelope glycoprotein functions.

In this thesis several point mutations were introduced into E of TBEV RSPs (Part I) and the protein was truncated at its C-terminus (Part II). It had to be considered, however, that any mutation or truncation introduced into this experimental system could potentially interfere (A) with the formation/assembly of the particles, (B) with their maturation (prM cleavage) and (C) with their secretion from the host cell.

The presence of high amounts of uncleaved prM in mutant RSPs is very critical because the chaperone prM prevents conformational changes in E that are necessary for mediating fusion. Information on the particle's prM content can help to avoid misleading interpretations of experimental data. The amount of uncleaved prM present in RSP samples was quantitatively determined in a western blot assay.

The secretion of the recombinant particles was quantitatively analyzed by the detection of E in the supernatant of transfected cells using a four layer ELISA format.

The surface organization of every E mutant was analyzed by epitope mappings using a set of monoclonal antibodies (mabs) that recognize binding sites distributed all over the three E domains (Stiasny *et al*., 1996, 2007).

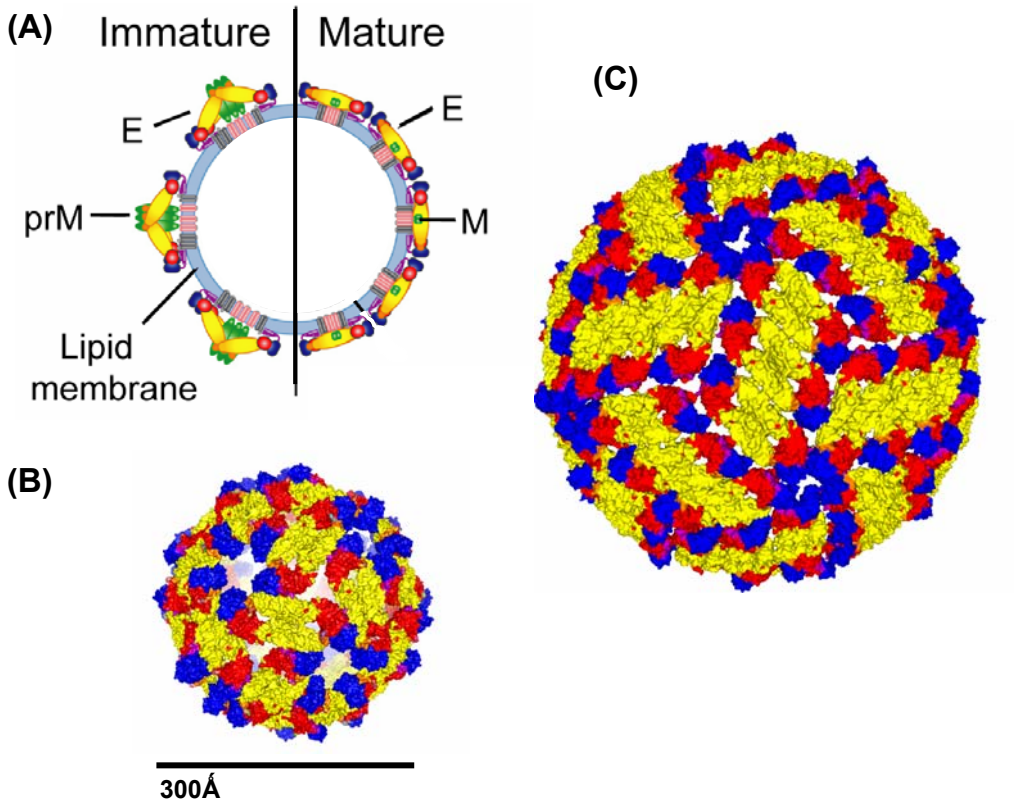

Figure 1. **Representations of the structural organization of flavivirus virions and RSPs.** (A): Schematic diagram of a subviral particle in both, its mature (right) and immature (left) form. During virus maturation prM is cleaved by an intracellular protease. This cleavage is responsible for the rearrangement of E dimers on the surface of mature virus particles. (B): Arrangement of the 30 E dimers in RSPs. (C) Arrangement of the 90 E dimers at the surface of a mature flavivirus particle. Drawn with PyMol. Color code: prM – green; E domain I – red; E domain II – yellow; E domain III – blue.

1.2. Results

1.2.1. Maturation state analysis of RSPs

The maturation state of RSPs was investigated by a western blot procedure using polyclonal sera, raised against TBEV, that detect all structural proteins (Materials and Methods). The assay was used to compare the amount of prM in mature WT RSPs with the amounts in completely immature Δ88 RSPs – that carry a mutation in the furin cleavage site and are therefore not amenable to maturation cleavage – and mutant RSPs with an unknown maturation state. The western blot detection limit of prM in RSP mutants was used as the read out. It had to be similar to WT particles to consider the mutants to be mature and unaffected in prM processing.

Preparations of TBE virus and WT RSPs were used to establish the optimal blotting conditions and to identify the dilution range in which structural proteins would be detectable. It has been reported that in all flavivirus preparations a certain amount of uncleaved prM is present (Guirakhoo *et al*., 1991; Davis *et al*., 2006; Nelson *et al*., 2008). Indeed prM was detectable in all of the WT samples (Fig. 2 A), but the degree did not vary between different preparations. Δ88 RSPs contained a significantly higher amount of prM (about 7.5 times more than the WT) and no cleaved membrane protein (M) was detectable (Fig. 2 B). The detection limit of prM in samples of Δ88 RSPs was $3 * 10^{-3}$ µg corresponding to 60 uncleaved prM molecules and a sample dilution of 1:160 with little or no deviation, whereas in all WT samples prM could be detected up to a sample dilution of 1:20, corresponding to an average of 8 molecules of prM per particle (Fig. 2). Table 1 lists the results of mutant RSPs maturation state analysis and shows that most of the mutants that secreted particles were properly processed. Interestingly several H248 mutants as well as two double mutants showed increased contents of prM.

1.2.2. Secretion of mutant RSPs

To determine whether certain alterations of E impair the assembly or the secretion of TBEV RSPs, and to investigate the specific aims described in this thesis, such particles were produced by electroporation of Cos-1 cells with plasmids encoding TBEV prM and E (WT and mutants). Cell culture supernatants that were harvested and cleared 48 hours after transfection were subjected to ultracentrifugation and the amounts of protein E in the pellet fractions was quantified in a four layer ELISA.

As shown in Table 1, substitutions that did not abolish particle formation and secretion were found for most of the target amino acids. However, not every substitution was tolerated as demonstrated by the mutations introduced at residue 323 of TBEV E (Table 1). Alanine and glutamine substitutions in this position led to the secretion of particles with the same efficiency as the wild-type, whereas in the case of exchanges to isoleucine, asparagine, lysine or aspartate no particles were found in the pelleted cell culture supernatants. Replacement of conserved histidine 146 by any of the other 19 amino acids prevented the formation and/or the secretion of RSPs (Table 1) (Fritz, Stiasny and Heinz, 2008).

To test the importance of interactions at the domain I-III interface, the residues building the prominent interdomain salt bridge, Arg9 and Glu373, as well as surrounding residues 42 and 358 were mutated. With none of these mutants particle secretion was observed (Table 1; Fig. 4).

1.2.3. Epitope mapping of mutant E

To verify the structural integrity of E on mutant RSPs, their reactivity with a set of 22 mabs was tested in epitope mappings. WT particles showed a distinct reactivity pattern displayed in Figure 3 A.

As depicted, some of the mabs – especially “A mabs” and 8H1 - showed an altered reactivity with Δ88- compared to WT-RSPs (Fig. 3 A). Most of the mutants followed the WT pattern as demonstrated in Figure 3 B and Table 1.

Certain mutations, however, shifted the reactivity profile to the Δ88 pattern as it was the case for mutant H248I (Fig. 3 C, Table 1).

In correspondence with the western blot results, these findings revealed differences in the surface organization of mutant E in RSPs indicating that in some mutants uncleaved prM was present.

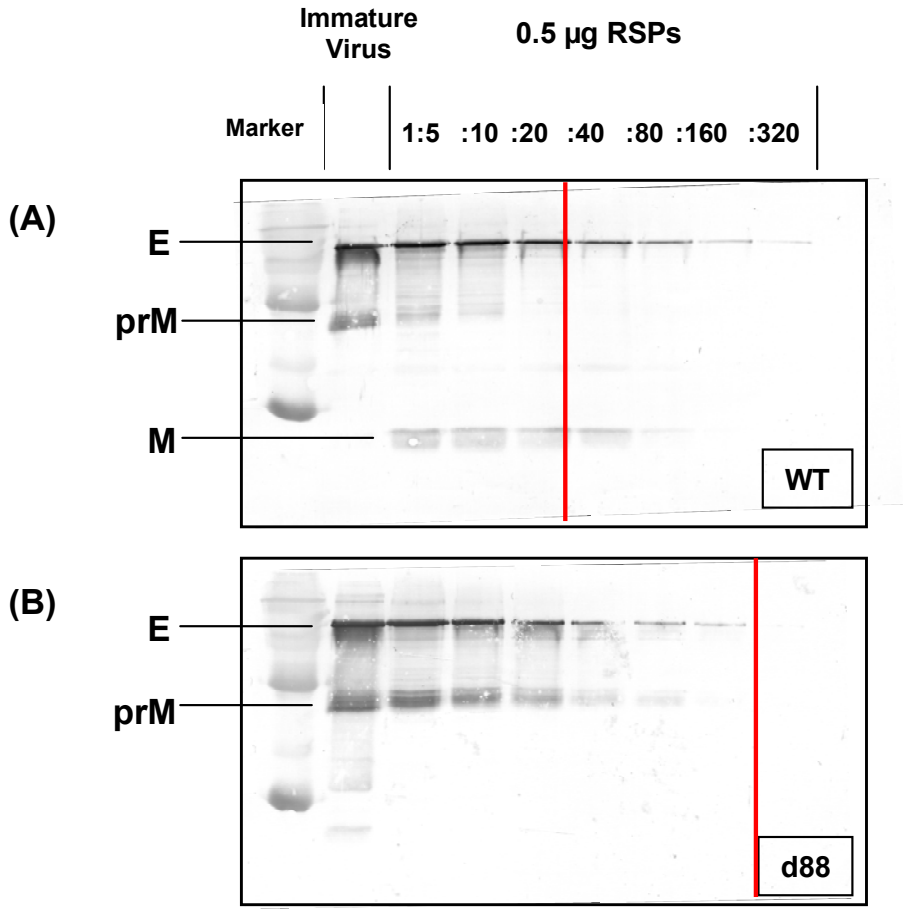

Figure 2. **Maturation state analysis of RSPs by western blotting.** Shown are dilution series of WT RSPs (A) compared to Δ88 RSPs (B). E indicates the bands of the envelope protein, prM indicates the bands of the uncleaved membrane protein precursor (M). Red bars indicate the prM detection limits. Results are examples from 8 independent experiments with similar results.

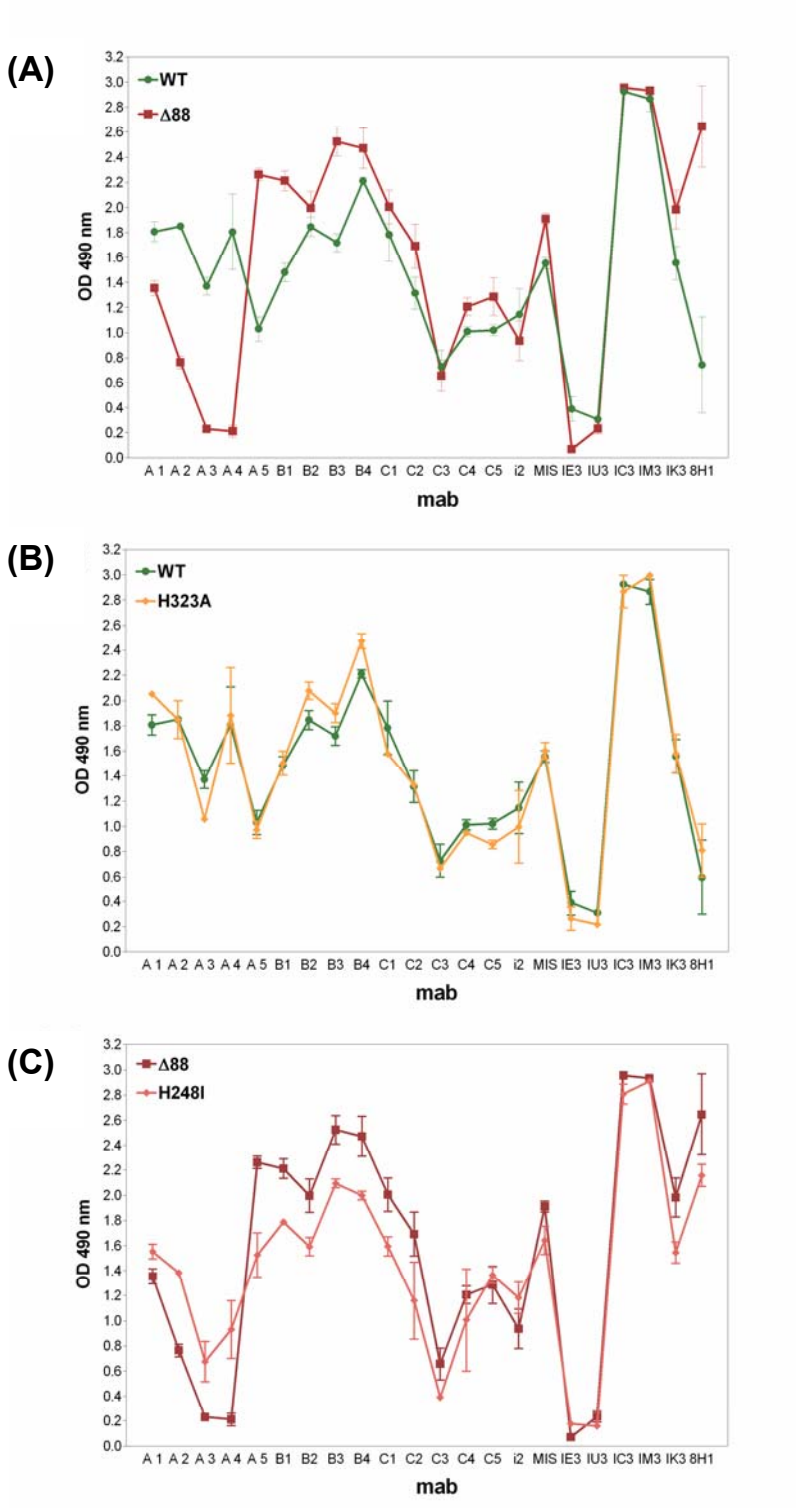

Figure 3. **Representative examples of epitope mappings obtained with mutant RSPs.** Shown is the reactivity pattern of selected monoclonal antibodies with WT RSPs (green line; A and B), Δ88 RSPs (red line; A and C), H323A RSPs (orange line; B) and H248I RSPs (salmon line; C). Error bars represent the standard deviation of the mean and are derived from 3-4 independent experiments.

Table 1 Summary of quality control analysis performed with WT and mutant TBEV RSPs

Mutant	Secretion[a]	prM processing[b]	Epitope mapping
WT	+	+	WT
Δ88	+	-	Δ88
R9A	-	ND	ND
R9E	-	ND	ND
R9G	-	ND	ND
R9I	-	ND	ND
R9K	-	ND	ND
R9P	-	ND	ND
R9Q	-	ND	ND
R9T	-	ND	ND
R9Y	-	ND	ND
D42A	-	ND	ND
D42N	-	ND	ND
H146A	-	ND	ND
H146C	-	ND	ND
H146D	-	ND	ND
H146E	-	ND	ND
H146F	-	ND	ND
H146G	-	ND	ND
H146I	-	ND	ND
H146K	-	ND	ND
H146L	-	ND	ND
H146N	-	ND	ND
H146P	-	ND	ND
H146Q	-	ND	ND
H146R	-	ND	ND
H146S	-	ND	ND
H146T	-	ND	ND
H146V	-	ND	ND

+: WT-like secretion or processing; -: negative secretion or processing;

a: mutants with negative secretion were not amenable to further invsttigation;

b: mutants defective at the processing stage were not further investigated; ND: not determined

Table 1 continued

Mutant	Secretion[a]	prM processing[b]	Epitope mapping
H146W	-	ND	WT
H146Y	-	ND	ND
H248A	+	-	ND
H248I	+	-	Δ88
H248K	+	-	Δ88
H248N	+	+	WT
H248Q	+	-	ND
H287A	+	+	WT
H287G	-	ND	ND
H287K	-	ND	ND
H287Q	-	ND	ND
H323A	+	+	WT
H323D	-	ND	ND
H323I	-	ND	ND
H323K	-	ND	ND
H323N	-	ND	ND
H323Q	+	+	WT
I358S	-	ND	ND
I358G	-	ND	ND
E373A	-	ND	ND
E373D	-	ND	ND
E373H	-	ND	ND
E373I	-	ND	ND
E373K	-	ND	ND
H438A	-	ND	ND
H438I	-	ND	ND
H438K	-	ND	ND
H438N	+	+	WT
H438Q	+	ND	ND

+: WT-like secretion or processing; -: negative secretion or processing;

a: mutants with negative secretion were not amenable to further invsttigation;

b: mutants defective at the processing stage were not further investigated; ND: not determined

Table 1 continued

Mutant	Secretion[a]	prM processing[b]	Epitope mapping
ΔTM2	+	+	WT
R9E-E373R	-	ND	ND
H287A-H323A	+	-	ND
H287G-H323Q	-	ND	ND
H248N-H287A	+	+	WT
H248N-H323A	+	+	WT
H248N-H438N	+	+	WT
H287A-H438N	+	+	WT
H323A-H438N	+	-	ND
H248N-H287A-438N	+	+	WT

+: WT-like secretion or processing; -: negative secretion or processing;

a: mutants with negative secretion were not amenable to further invsttigation;

b: mutants defective at the processing stage were not further investigated; ND: not determined

1.3. Discussion

RSPs have been shown to be valuable tools for studying flavivirus membrane fusion. Here methods are described that allow the identification of mature RSP mutants that carry the E protein in a WT-like conformation.

A western blot based assay was developed to compare the amounts of prM found in WT and mutant particles. It is an interesting finding of this work that most histidine 248 mutants were shown to contain amounts of prM comparable to immature ∆88 particles (Table 1). Two recently published reports on the structure of the prM-E complex of dengue 2 virus suggested the involvement of a homologous histidine in interactions between prM and E required for maturation (Li *et al*., 2008; Yu *et al*., 2008). A H248N mutant, however, was identified as perfectly mature in this study. Therefore, it remains a matter of speculation which residues are involved in the pH dependent processes of prM cleavage and pr peptide dissociation. The results obtained from the maturation state analyses were consistent with the epitope mappings, e.g. mutant H248I showed a mapping pattern very similar to Δ88 RSPs (Fig. 3 C) and was shown to contain the same amount of prM (Table 1).

The fact that also WT RSPs contained some uncleaved prM indicates that complete maturation is not a prerequisite for efficient membrane fusion. It is an interesting question whether the surface of native RSPs and virus particles is covered by heterogeneous E/E and prM/E complexes in varying ratios (mixed particles) or whether there are mature and immature single particles within a mixed population.

The results of the investigation of mutant RSP secretion demonstrate that the introduction of mutations in the RSP system can lead to the abolishment of particle formation and/or secretion. Specifically some residues located at the highly conserved stretch between domains I and III were found to be essential for the formation/secretion of particles. The structural integrity of this domain interface depends on an interdomain salt bridge built by residues 9 and 373 respectively. Histidine 146 is located in close vicinity at the same interface (Fig. 4). None of these residues could be exchanged without loss of particle secretion and/or formation (Table 1).

The methods established were essential for selecting those RSPs that could be used in the previous parts for dissecting the fusion process. They can also be useful in future research on flavivirus assembly and maturation.

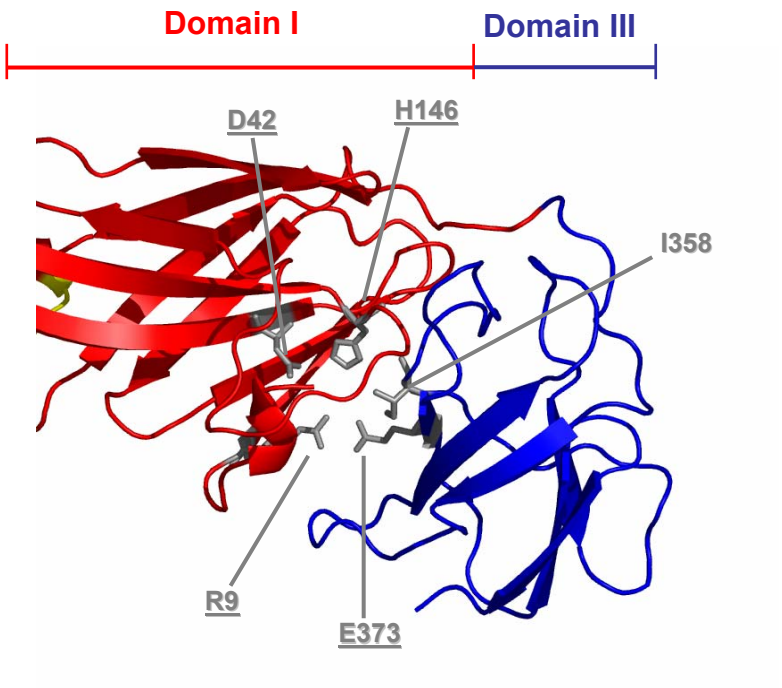

Figure 4. **Residues in TBEV E that could not been mutated without loss of RSP formation or secretion.** Ribbon diagram of the domains I/III interface of E. Residues that were identified to be critical for the assembly/secretion of RSPs are highlighted as grey sticks. Conserved ones are underlined. Drawn with PyMol.

VI. OUTLOOK

It is estimated that the number of worldwide flavivirus infections per year with severe illness symptoms exceeds 50 million. Icterus, haemorrhagic fever and encephalitis are only a few possible disease manifestations in humans caused by infections with different flaviviruses. Therefore, yellow fever virus (YFV), dengue virus (DenV), Japanese encephalitis virus (JEV), West nile virus (WNV), and tick-borne encephalitis virus (TBEV), are among the most important human pathogenic viruses worldwide. Still, effective vaccination is only available for three of the mentioned viruses, i.e. YFV, JEV and TBEV (reviewed in Pugachev, Guirakhoo and Monath, 2005; Gould and Solomon, 2008). New, innovative vaccines are in development; but additional preventive and therapeutic approaches are desirable, including the development of antiviral agents.

Among the greatest breakthroughs in studying viral membrane fusion were the structure determinations of viral fusion proteins starting with the influenza HA (Wilson, Skehel and Wiley, 1981). Since then, several structures of fusion proteins were resolved allowing their subdivision into distinct structural classes and the development of viral membrane fusion models. Enhanced knowledge of the structural basis of flavivirus cell entry can lead to the design of specific small molecules that block virus entry by selectively binding to the fusion proteins.

The only fusion inhibitor currently available, however, is for treatment-experienced HIV patients. Enfuvirtide (Fuzeon by Roche, Trimeris) was the first licensed peptide drug targeting a highly conserved pocket of the HIV-1 fusion protein, thus inhibiting extracellular fusion of HIV-1 to host cells (reviewed in Matthews *et al*., 2004). Enfuvirtide is a 36 amino acids L-peptide drug with the ability to prevent gp41 – the subunit of the HIV fusion protein functionally related to influenza HA_2 (Weissenhorn *et al*., 1997) and derived from cleavage of the gp160 precursor protein – from undergoing conformational changes that enables it to fuse the viral and cellular membranes. But the peptide is sensitive to protease degradation, expensive to produce and it has to be injected subcutaneously twice daily. These limitations were overcome by the development of D-peptide entry inhibitors – where D- instead of L-amino acids

are present – that are resistant to proteases and can be administered orally (Eckert *et al.*, 2001). Such D-peptides with antiviral potency are currently under preclinical investigation (Welch, *et al*., 2007, 2008).

In contrast to HIV, there are currently no fusion inhibitors for class II fusion proteins. The reasons, beside greater scientific and pharmaceutical interest in the HIV epidemic, are that HIV fuses at the cell membrane, while flavi- and alphavirus fusion occurs intracellularly in endosomes. A class II fusion inhibitor therefore has to get into the endosomes, either with the virus or on its own. An additional problem could be that class II fusion proteins display the fastest fusion kinetics known, making it more difficult for small molecules to interfere with this process. Nevertheless a report describes the inhibition of membrane fusion mediated by SFV E1 and dengue E by the addition of exogenous domain III. DIII was shown to stably bind to the fusion protein, thus preventing the structural alterations of the fusion proteins and blocking lipid mixing (Liao and Kielian, 2005). Soluble DIII of TBEV E lacking the whole stem did not block fusion of TBEV RSPs (unpublished observation R. Fritz). It remains to be elucidated whether a recombinant DIII plus parts of the stem can bind to the fusion protein efficiently.

Capture and study of fusion intermediates may become an important issue in the flavivirus field. The structures of E already led to the identification of potential binding sites that are at least transiently accessible for small molecules inhibitors: A ligand-binding pocket that closes when the protein undergoes low pH-induced conformational changes (Modis *et al*., 2003) and further sites involved in inter- and intra-domain contacts during trimer formation (Bressanelli *et al*., 2004). Therefore, the most promising approach for the development of class II fusion inhibitors may be the structure-based design of molecules that target the flexibility of the E protein, specifically of the junction between DI and DIII.

Beside the development of fusion inhibitors, future research on flavivirus fusion will focus on exploring potential cooperativity of fusion protein trimers for mediating full fusion and the involvement of the transmembrane domains in this process. The role of conserved histidines in the fusion protein for other purposes than initiating fusion – for example virus maturation – will be another subject of future research that is poorly understood to date.

VII. MATERIALS AND METHODS

1. 1. Plasmid Construction

All mutations were introduced into the recombinant SV-PE wt plasmid (Allison et al. 1994) - containing the TBEV prM and E genes under the control of an SV40 early promoter - by the use of the site directed mutagenesis kit Gene Tailor (Invitrogen). The ΔTM2 deletion mutant was constructed by the substitution of a *Sna*BI-*Not*I fragment, within the SV-PE wt plasmid, with a PCR generated sequence carrying a TAG codon at position 473 of E followed by a *Not*I restriction site (Allison *et al.*, 1999). Both strands of the plasmids were sequenced to confirm the presence of the desired substitutions or deletion and the absence of any undesired mutations.

1.2. Recombinant subviral particle production

RSPs were produced by electroporation of COS-1 cells with WT and recombinant plasmids as described previously (Allison *et al*., 1995; Schalich *et al*., 1996). 48 hours posttransfection particles secreted into the cell culture supernatant were harvested by ultracentrifugation for 2h at 44,000 rpm and 4°C. Pelleted RSPs were resuspended in TAN pH 8.0 buffer (50 mM triethanolamine, 100 mM NaCl). For coflotation and lipid mixing experiments the particles were purified in continuous sucrose gradients. For lipid mixing assays, the particles were labelled with 1-pyrenehexadecanoic acid (Molecular Probes) as described previously (Corver et al., 2000).

1.3. Quantification of secreted particles by ELISA

The amount of RSPs secreted from transfected Cos cells was quantitatively determined in a 4-layer ELISA (Heinz *et al*., 1994). Microtiter plates were coated

at 4°C with guinea pig anti-TBE virus IgG in carbonate coating buffer pH 9.6. Purified TBE virus – with a known protein concentration – serving as standard and the samples were denatured with 0.4% sodium dodecyl sulfate (SDS) for 30 minutes at 65°C. Standard and samples were serially diluted in PBS pH 7.4, containing 2% lamb serum and 2% Tween 20, transferred to the coated plates, and incubated for 1.5 hours at 37°C. After washing with PBS pH 7.4 (0.05% Tween 20), a polyclonal rabbit anti TBE serum was added and allowed to bind for 1 hour at 37°C. Bound antibodies were detected by 1 hour incubation with horseradish peroxidase labelled donkey anti-rabbit IgG and orthophenyldiamine/H_2O_2 as substrate. After 30 minutes the reaction was stopped by adding 100 µl 2N H_2SO_4 to each well. The absorbance was determined with an ELISA reader at 490 nm.

1.4. Epitope mapping

Epitope mappings of WT and mutated E in RSPs were performed in a four layer ELISA format using a panel of E protein specific mabs (Allison *et al*., 1995a, 2001; Stiasny *et al*., 2005). Microtiter plates were coated at 4°C with guinea pig anti-TBE virus IgG in carbonat coating buffer pH 9.6. WT and mutant RSPs were added at a concentration of 0.5 µg E / ml in a total volume of 50 µl PBS pH 7.4, containing 2% lamb serum and 2% Tween 20, and were allowed to bind for 1 hour at 37°C. Various E specific mabs were added in a predefined dilution to yield an absorbance of about 1.0 at 490 nm. After one hour incubation at 4°C, mabs bound to E were detected with horseradish peroxidase labelled rabbit anti-mouse IgG and orthophenyldiamine/H_2O_2. The reaction was stopped with 100 µl 2N H_2SO_4 per well and the absorbance was measured in an ELISA reader.

1.5. Maturation state analysis

The particle's maturation state was determined by the quantification of the relative amount of prM by western blotting. RSPs at a concentration of 0.5 µg E protein / 50 µl Laemmli sample buffer were boiled at 100°C for 5 min. Samples

were diluted 6 times 1:1 in Laemmli buffer, boiled again and 20 µl of each dilution were applied to 15% acrylamide gels. Immature virus was used as a positive prM control. SDS gel electrophoresis was carried out using Mini Protean II gel apparatuses (BioRad). After electrophoresis, gels were equilibrated for 10 min in blotting buffer (48mM Tris, 39mM Glycine, 1.3mM SDS, 20% methanol) to avoid shrinking during blotting. PVDF membranes (BioRad) were incubated in methanol for 5 min and then soaked in blotting buffer together with the filter papers used for blotting (BioRad) for 15 min. Western blotting was performed with the semidry blotter Trans-Blot SD from BioRad (18 V, 90 min). PVDF membranes were then saturated by overnight incubation in blocking buffer (PBS pH 7.4, 1% BSA, 0.1% Tween 20) at 4°C under constant shaking. Blot development was done by incubation with an anti TBEV polyclonal rabbit serum – that recognizes all structural proteins (E, prM, M, C) – in blocking buffer and bound antibodies were detetced with horseradish peroxidase labelled donkey anti-rabbit IgG and Sigma Fast™ tablets by Sigma Aldrich.

1.6. Liposomes

Liposomes were prepared as described previously (Stiasny, Koessl and Heinz, 2003; Stiasny *et al.*, 2007). Phosphatidylcholine, phosphatidylethanolamine (Avanti Polar Lipids) and cholesterol (Sigma) were mixed at a molar ratio of 1:1:2 in chloroform. The mixture was dried to a homogenous film in high vacuum and hydrated in liposome buffer (10 mM triethanolamine, 140 mM NaCl) by 5 freeze thaw cycles. Prior to use liposomes were subjected to 21 extrusion cycles through polycarbonate membranes with 200 nm pores (Avestin) using a LiposoFast-Basic extruder (Avestin).

1.7. Lipid mixing assay

Lipid mixing was measured by monitoring the decrease in pyrene excimer fluorescence caused by the dilution of pyrene-labelled phospholipids in the RSP membrane into an unlabeled liposomal membrane (Corver *et al.*, 2000). Pyrene-

labelled RSPs were mixed with liposomes (total lipid 0.3 mM) in a continuously stirred fluorimeter cuvette at 37°C in liposome buffer. Lipid mixing was induced by the addition of 50µl 300 mM morpholinoethansulfonic acid (MES) to yield a pH of 5.4. To controls the same volume of TAN pH 8.0 buffer was added. Fluorescence was continuously recorded for 120s by a Perkin-Elmer LS-50B fluorescence spectrophotometer at 480 nm. The initial pyrene excimer fluorescence before acidification was defined as 0% fusion extent and the fluorescence after solubilization of the RSP-liposome mixture by the detergent octa(ethylene glycol)-*n*-dodecyl monoether (Fluka) as 100% fusion. Based on these data the extent of fusion of each sample was calculated

1.8. Fusion peptide exposure

Microtiter plates were coated with polyclonal anti-TBE virus IgG in carbonate coating buffer pH 9.6 to capture WT and mutant RSPs at a concentration of 0.5 µg E / ml in PBS pH 7.4, containing 2% lamb serum as described previously (Stiasny et al., 2007). After 1 hour incubation at 37°C the FP specific biotinylated mab A1 was added at a concentration of 0.25 µg/ml in MES buffer (50 mM MES, 100 mM NaCl), titrated to pHs between 7.0 and 5.4 using 1N NaOH (Fritz, Stiasny and Heinz, 2008). The bound antibody A1 was detected by using a streptavidin peroxidase (Sigma-Aldrich) and orthophenyldiamine/H_2O_2. The reaction was stopped with 100 µl 2N H_2SO_4 per well and the absorbance was measured in an ELISA reader at 490 nm.

1.9. Coflotation of RSPs with liposomes

RSPs were mixed with liposomes at a ratio of 1 µg E to 200 nMol lipids and incubated at 37°C for 10 min. The RSP-liposome mixture was then acidified with 300 mM MES and incubated at 37°C for 15 min. TAN buffer pH 8.0 was added instead of MES to controls. Acidified samples were back-neutralized to pH 8.0 by the addition of 150 mM triethanolamine. Then the samples were adjusted to 20% sucrose (w/w) in a final volume of 0.6 ml. Samples were applied to a 1 ml 50% sucrose cushion and overlaid with 1.4 ml 15% sucrose and 1 ml 5% sucrose as

described before (Allison *et al*., 2001; Fritz, Stiasny and Heinz, 2008). Centrifugation was carried out at 50,000 rpm and 4°C for 2 hours in a Beckmann SW 55 rotor. 0.2 ml fractions were collected by upward displacement using a Piston Gradient Fractionator (BioComp Instruments Inc.) and the amount of E protein in each fraction was quantified by four-layer ELISA (Heinz et al., 1994).

1.10. Sedimentation analysis

The oligomeric state of E – after incubation of RSPs at acidic or neutral pH – was determined by sedimentation analysis as described previously (Allison *et al*., 1995, 2001). 3 µg subviral particles in TAN buffer pH 8.0 were incubated for 10 min at pH 5.4 or 8.0 by the addition of 300 mMES or TAN pH 8.0, respectively. Acidified samples were back-neutralized with 150 mM triethanolamine and all samples were solubilised with 1% Triton-X 100 for 1 hour at room temperature. The samples were then applied on top of 7-20% continuous sucrose gradients (w/w) containing 0.1% Triton-X 100. Centrifugation was carried out at 38,000 rpm and 15°C for 20 hours in a Beckmann SW 40 rotor. Fractions of 0.6 ml were collected by upward displacement using a Piston Gradient Fractionator (BioComp Instruments Inc.). The amount of E in the fractions was determined by a quantitative four-layer ELISA as above.

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ABBREVIATIONS

AcMNPV	Autographa californica multicapsid nucleopolyhedrovirus
BSA	bovine serum albumin
C	capsid
D	domain
DenV	dengue virus
E	envelope
ELISA	enzyme linked immunosorbent assay
FP	fusion peptide
HIV	human immunodeficiency virus
HSV	herpes simplex virus
JEV	japanese encephalitis virus
M	membrane
mab	monoclonal antibody
MES	2-morpholinoethansulfonic acid
NS	non structural
ORF	open reading frame
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
RSP	recombinant subviral particle
SFV	Semliki Forest virus
sE	soluble envelope protein
TBEV	tick-borne encephalitis virus
TGN	trans-Golgi network
TM	trans-membrane
TMD	trans-membrane domain
VSV	Vesicular stomatitis virus
WNV	West Nile virus
WT	wild-type

APPENDIX

Kurzfassung

Flaviviren sind kleine membran-umhüllte RNA Viren, die in vielen Fällen durch Stechmücken oder Zecken auf ihre Vertebratenwirte übertragen werden. Die wichtigsten humanpathogenen Vertreter sind das Frühsommer-Meningo-Enzephalitis (FSME) Virus, das Gelbfieber Virus, das West Nil Virus, das Japanische Encephalitis Virus und die Dengue Viren. Die Infektion einer Wirtszelle durch umhüllte Viren erfordert die Verschmelzung (Fusion) der viralen mit einer zellulären Membran um die genetische Information der Viren in das Zellzytoplasma freizusetzen. Im Fall der Flaviviren erfolgt diese Membranfusion mit einer endosomalen Membran – nach Aufnahme durch Rezeptor-vermittelte Endozytose – und wird durch den sauren pH-Wert im Endosom ausgelöst. Dieser Prozess wird durch das virale Oberflächenglykoprotein E – ein Klasse II Fusionsprotein – gesteuert, das auf der Oberfläche infektiöser Viren als metastabiles, dimeres Molekül vorliegt. Saurer pH löst in diesem Protein dramatische Änderungen der Konformation und Oligomerstruktur aus, die die beiden Membranen in Kontakt bringen und schließlich zu ihrer Fusionierung führen. Es wurde die Hypothese aufgestellt, dass hochkonservierte Histidine in E als molekulare pH Sensoren fungieren und durch ihre Protonierung die Fusions-relevanten Konformationsänderungen auslösen.

Im Hauptteil dieser Dissertation wurden die fünf bei allen Flaviviren konservierten Histidine des E Proteins in rekombinanten Virus-ähnlichen Partikel (RSPs) des FSME Virus mutiert und die Auswirkungen der Substitutionen auf die verschiedenen Schritte des Fusionsprozesses analysiert. Dabei stellte sich heraus, dass einem Histidin (H323) an der Grenzfläche zwischen zwei Domänen von E eine zentrale und essentielle Rolle als pH Sensor zur Fusionsauslösung zukommt, während Histidine ausserhalb dieser Region keine Rolle bei der Initiierung der Membranfusion spielen.

Weitere Experimente waren der Aufklärung der Rolle des Membranankers des E Proteins im Fusionsmechanismus gewidmet. Im Gegensatz zu allen anderen bekannte viralen Fusionsproteinen, ist das Flavivirus E Protein nicht nur über eine, sondern über zwei transmembranale Helices (TM1 und TM2) in der Virusmembran verankert. Die Deletion von TM2 hatte zwar keinen Einfluss auf die Initiation und die frühen Phasen der Fusion, aber der Prozess wurde in einem intermediären Stadium blockiert und es kam nicht zur Bildung einer Fusionspore. Die experimentellen Befunde weisen darauf hin, dass dieser Effekt auf einen Stabilitätsverlust des in späten Stadien der Fusion gebildeten E Trimeren zurückzuführen ist.

Die genaue Kenntnis der einzelnen Schritte des Fusionsmechanismus kann einen wichtigen Beitrag zur Entwicklung antiviraler Agenzien leisten, wie das Beispiel des AIDS Erregers HIV zeigt, gegen den bereits ein fusionshemmendes Medikament zur Verfügung steht.

Abstract

Flaviviruses are small, enveloped RNA viruses. Most of them are transmitted to their vertebrate hosts by mosquitoes or ticks. The most important human pathogenic flaviviruses are tick-borne encephalitis (TBE), yellow fever, West Nile, Japanese encephalitis, and dengue viruses. Infection of a host cell by any enveloped virus requires the fusion of the viral membrane with a cellular membrane to release the viral genome into the host cell's cytoplasm. Flavivirus fusion occurs at endosomal membranes – following uptake via receptor-mediated endocytosis – and is triggered by the acidic pH in the endosomes. This fusion process is mediated by the major envelope glycoprotein E – a class II viral fusion protein – that exists in a metastable, dimeric conformation at the surface of infectious virions. Acidic pH triggers dramatic rearrangements of the conformation and oligomeric structure of E that bring the two membranes in close proximity and finally result in their fusion. It has been hypothesized that conserved histidines in E function as molecular pH sensors and, by their protonation, initiate the fusion-relevant conformational changes.

In this thesis, the five histidines of E that are conserved among all flaviviruses were mutated in recombinant subviral particles (RSPs) of TBE virus and the effects of these substitutions on the various stages of the fusion process were analyzed. The results showed that one histidine (H323), located at the interface of two domains of E, has a critical and essential role as pH sensor for triggering fusion, whereas conserved histidines located outside this interface were found to be completely dispensable for the initiation of membrane fusion.

Further experimental work aimed at dissecting the functional role of the membrane-anchor of E in the fusion process. The flavivirus E protein is anchored in the viral membrane via two transmembrane helices (TM1 and TM2). This is in contrast to all other known viral fusion proteins that have only single transmembrane domains. The deletion of TM2 of E in TBE virus RSPs had no effect on initiation or the early steps of fusion, but the process was blocked at an intermediate stage, preventing the formation of a fusion pore. The experimental data indicates that this effect is due to a loss of stability of the E trimers formed at late stages of fusion.

The understanding of the details of the fusion process can contribute to the development of antiviral strategies, with one fusion inhibitor directed against HIV now a licensed drug.

Curriculum vitae

Personal information **Richard Fritz,** Mag.rer.nat.

*07.02.1980 in Graz, Austria

Clinical Institute of Virology
Kinderspitalgasse 15
A-1095 Vienna

Mobile: +43/0650/2163986
Phone: +43/01/4049079541
Email: richard.fritz@meduniwien.ac.at

University education

10/2005 – present	**PhD studies in Genetics and Microbiology** at the University of Vienna. Special emphasizes in Structural Biology and Virology.
04/2005 – present	**Studies in Medical Sciences** at the Medical University of Vienna. Focus on Tumorbiology and Molecular Medicine.
10/1999 – 10/2004	**Studies of Biology**, subject areas Molecular Biology and Zoology, at the Karl-Franzens University of Graz. Concentration on Medical Biophysics and Membrane Biochemistry. Final examinations passed with distinction and first class honours.
10/2001 – 06/2002	**Studies of Economy**.

Experience

09/2005 - present	**PhD student** at the Clinical Institute of Virology, Laboratory Prof. F.X.Heinz, Medical University of Vienna. Topic: Viral cell entry and maturation.
04/2005 – 12/2007	**Research associate** at the Department of Internal Medicine I, Division of Hematology and Haemastaseology, the Center of Excellence in Experimental and Clinical Oncology, Laboratory Prof. P. Valent, General Hospital of Vienna.
02/2005 – 04/2005	**Internship** at the Research Institute of Molecular Pathology (IMP). Protein annotation with F. Eisenhabers Bioinformatics Group.
11/2004 – 02/2005	**Internship** at the Institute for Genomics and Bioinformatics, Technical University Graz. Production and analysis of microarray data for the National Institute of Health with Prof. Z. Trajanoskis group.

10/2003 – 02/2005	**Master thesis** and subsequent research activities at the Institute for Medical Physics and Biophysics, Department of Physiological Medicine, Laboratory Prof. W. Schreibmayer, Medical University of Graz. Topic: Phosphorylation of cardiac and neuronal ion channels.
02/2003 – 12/2003	**Employment** "Technical Environment Protection, Water Pollution Control, Provincial Government of Styria. "
07/2001 – 08/2001	**Trainee** "Technical Environment Protection, Water Pollution Control, Provincial Government of Styria. "
06/2001 – 07/2001	**Employmen**t at the "Federal Research Center for Fisheries"**,** Hamburg, Germany. Investigation of redfish stocks in the northern atlantic.
07/2000 – 08/2000	**Trainee** "Technical Environment Protection, Water Pollution Control, Provincial Government of Styria. "
05/2000 – 06/2000	**Trainee** on a research vessel of the "Federal Research Center for Fisheries", Hamburg, Germany. Investigation of contaminated seafish in the Barentsea region.
01/2000 – 01/2002	Employed as barkeeper.
10/1998 – 09/1999	Community service.

List of Publications

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