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The role of the stem region of the fusion protein E in flavivirus membrane fusion

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2 Summary

Flaviviruses, comprising important human pathogens such as dengue virus, Japanese encephalitis virus, West Nile virus, yellow fever virus and tick-borne encephalitis virus (TBEV), enter cells by receptor-mediated endocytosis and low-pH-induced fusion from within the endosome. Fusion of the viral with the endosomal membrane is mediated by the major envelope protein E, a class II viral fusion protein, which is organized on the virus surface as metastable dimers forming an icosahedral shell. In the course of membrane fusion, the E protein homodimers are irreversibly converted to more stable homotrimers, thereby releasing energy for the fusion process. The external part of E, which is composed of three distinct domains (DI, DII, DIII), is connected to the double transmembrane anchor by a membrane-proximal region, the so-called stem. The current fusion models hypothesize that the stem zippers along the core of the trimer and interacts with the trimeric ectodomain in the postfusion conformation. In this work, we wanted to generate direct experimental evidence for the importance of stem-trimer core interactions and investigate the role of the stem region in flavivirus membrane fusion.

In the first part of the thesis, we addressed this question by mutagenesis of recombinant subviral particles (RSPs) of TBEV. We targeted highly conserved residues in the stem and at possible stem interaction sites in the E protein DII and assessed the effect of these mutations on different stages of membrane fusion. By this approach, we could demonstrate that the stem region is crucial for late stages of the fusion process and contributes to the postfusion trimer stability. Moreover, the substitution of the DII residue L223 by an isoleucine, drastically affected the thermostability of the E protein trimer and RSP fusion activity. This negative effect was partially compensated by additionally replacing the stem residue F403 to an isoleucine. The double mutant L223I-F403I exhibited enhanced trimer stability and fusion activity compared to the single mutant L223I, indicating a specific interaction site between the stem and the trimer core, which is important for the stability of the E protein

postfusion conformation and particle fusion.

In the second part of this thesis, we wanted to supplement the data, obtained with the RSP system, by investigating the effect of these mutations on infectivity in the infectious virus system. Both mutations (L223I and L223I-F403I) were introduced into the infectious clone of TBEV strain Neudoerfl. We discovered that both mutant viruses (L223I and L223I-F403I) were still infectious, although with a reduction in their specific infectivity in comparison to wild-type virus. Apparently, fusion data obtained with mutated RSPs cannot be readily extended to the infectious system and further studies are needed to clarify this point. A possible explanation for this discrepancy could be the difference in particle geometry. Whereas on RSPs 30 E protein dimers are organized in a T=1 symmetry, on virions 90 E protein dimers are more densely packed in a herringbone-like lattice. This special arrangement might provide cooperative effects on virions, resulting in a less dramatic effect of introduced mutations on viral fusion. In addition, we could show that mutations, which did not result in particle secretion in the RSP system, allowed the generation of infectious virions.

In this work, we were able to reveal mechanistic details on the role of the stem region in flavivirus fusion, especially through the use of RSPs. We identified an intramolecular interaction site, which clearly stabilizes the postfusion trimer and facilitates membrane fusion. The observed discrepancies between RSP and infectious virus system will be investigated in future studies.

3 Zusammenfassung

Zum Genus der *Flaviviren* zählen eine Reihe von humanpathogenen Krankheitserregern wie Gelbfieberevirus, Dengue-Virus, West Nil Virus, Japanische Enzephalitis Virus und Frühsommer-Meningoenzephalitis (FSME) Virus. Flaviviren gelangen durch Rezeptor-vermittelte Endocytose in die Zelle und der saure pH im Endosom induziert die Fusion von viraler und endosomaler Membran. Dieser Fusionsprozess wird von dem viralen Hüllprotein E, einem Klasse II Fusionsprotein vermittelt, das eine Dimer-Trimer-Umwandlung durchläuft und dabei Energie für die Fusion zur Verfügung stellt. Der externe Teil des E Proteins, der aus 3 Domänen (DI, DII, DIII) zusammengesetzt ist, wird durch eine sogenannte Stamm-Region mit einem Doppel-Transmembrananker verbunden. In derzeitigen Modellen wird vermutet, dass spezifische Interaktionen des Stammes mit der DII des Trimers die treibende Kraft für die späten Phasen der Fusion (also das Verschmelzen der beiden Lipid Bilayer) darstellen. Das Hauptziel dieser Arbeit bestand darin, einen eindeutigen Nachweis für die Bedeutung der Stamm-Trimer-Interaktion zu finden und die Rolle der Stamm-Region in der Membranfusion von Flaviviren zu untersuchen.

Im ersten Teil dieser Arbeit wurde diese Fragestellung durch Mutagenese von Rekombinanten Subviralen Partikeln (RSP) des FSME Virus behandelt. Wir mutierten konservierte Reste im Stamm und in möglichen Interaktionsregionen und analysierten den Einfluss der Mutationen auf verschiedene Schritte des Fusionsprozesses. Am Beispiel mehrerer Stamm-Mutanten konnten wir nachweisen, dass die Stamm-Region für die späten Stadien der Fusion bedeutend ist. Desweiteren war es uns möglich, eine spezifische Interaktion zwischen Stamm und Trimer-Ektodomäne zu identifizieren. Die Mutation (L223I) in DII beeinträchtigte stark die Thermostabilität des Trimers und zugleich die Fusogenität der RSP-Mutante. Dieser negative Effekt konnte durch eine zusätzliche Mutation im Stamm zumindest teilweise kompensiert werden. Im Vergleich zur Einzelmutante wies die Doppelmutante (L223I-F403I) verbesserte Trimerstabilität und Fusionsaktivität auf.

Im zweiten Teil dieser Arbeit analysierten wir diese Interaktion im infektiösen System und führten die im RSP-System charakterisierten Mutationen in den infektiösen FSME Virus-Klon ein. Überraschenderweise waren beide Virusmutanten (L223I und L223I-F403I) infektiös, obgleich sie eine Reduktion in ihrer spezifischen Infektiosität im Vergleich zum Wildtyp aufwiesen. Offensichtlich können Fusionsaktivitäten von RSP-Mutanten nicht direkt auf das infektiöse System übertragen werden, was in zukünftigen Studien genauer untersucht werden muss. Eine mögliche Erklärung für diese Diskrepanz könnte die unterschiedliche Geometrie von RSP und Virionen sein. Während an der Oberfläche von RSP 30 E Proteindimere in einer T=1 Symmetrie organisiert sind, sind an der Oberfläche von Virionen 90 E Proteindimere dicht in einer Fischgräten-ähnlichen Anordnung gepackt. Diese spezielle E Proteinanordnung auf Virionen könnte zusätzliche kooperative Effekte ermöglichen, welche Auswirkungen von Mutationen auf die Virusfusion abschwächen. Darüberhinaus stellten wir fest, dass Mutationen, welche die Sekretion von Partikeln im RSP System verhindern, die Produktion von infektiösen Viren erlauben.

Wir konnten mit dieser Arbeit mechanistische Details über die Rolle der Stamm-Region in der Membranfusion von Flaviviren aufdecken. Wir identifizierten eine spezifische intramolekulare Interaktion zwischen Stamm und DII, welche für die Stabilität der Postfusionsstruktur von großer Bedeutung ist und Membranfusion begünstigt. Das unterschiedliche Ausmaß der Auswirkungen von Mutationen auf RSP und Viren muss in weiterführenden Studien genauer untersucht werden.

4 Introduction

4.1 Flaviviruses

The family *Flaviviridae* comprises three genera: *Hepacivirus*, *Pestivirus* and *Flavivirus* (18). All members of this family have similar characteristics in morphology, genome composition and replication. The largest genus *Flavivirus* includes more than 70 viruses which are mostly transmitted by mosquitoes or ticks (Figure 4.1). For some flaviviruses the vector is unknown (42). The most important human pathogens are dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), yellow fever virus (YFV) and tick-borne encephalitis virus (TBEV) which can cause severe diseases inducing symptoms that range from mild fever and malaise to fatal encephalitis (42).

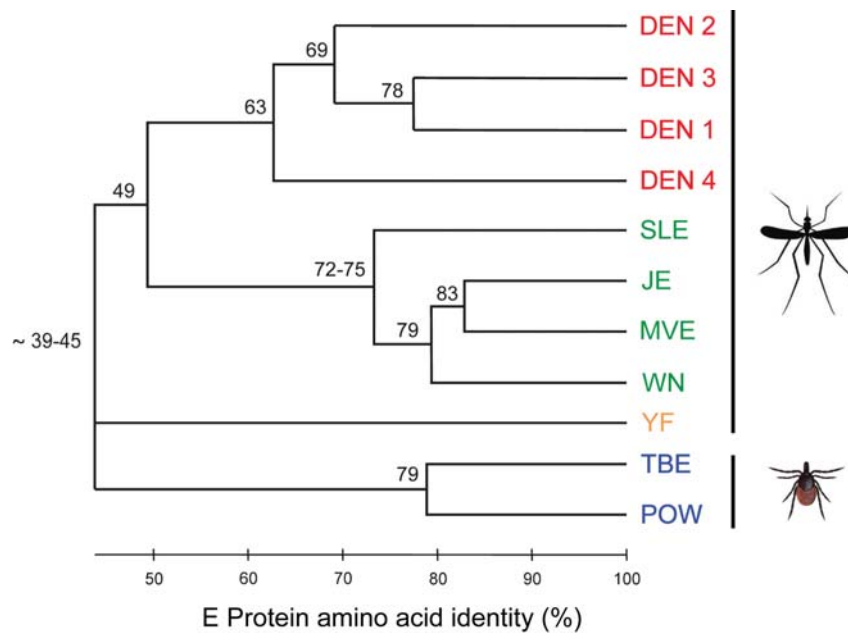


Figure 4.1: Flavivirus classification. Relationships between flaviviruses according to the amino acid sequence of the envelope protein. Serocomplexes are shown in four colors: red, dengue virus serocomplex; green, Japanese encephalitis serocomplex; orange, yellow fever virus serocomplex; blue, tick-borne encephalitis virus serocomplex. The corresponding transmission vector is shown on the right side. SLE, Saint Louis encephalitis; MVE, Murray Valley encephalitis; POW, Powassan (adapted from (67)).

TBEV is further subdivided into the three subtypes: Far Eastern, Siberian and

European subtype. Sequence analysis demonstrated a close relationship between these subtypes (15, 18). TBEV is usually transmitted by tick bites, but human infections can also occur through the consumption of unpasteurized milk from infected goat, sheep or cow (27, 31). In Europe, two vaccines are licensed, both containing highly purified, formalin-inactivated European TBEV strains (8).

4.1.1 Flavivirus particles

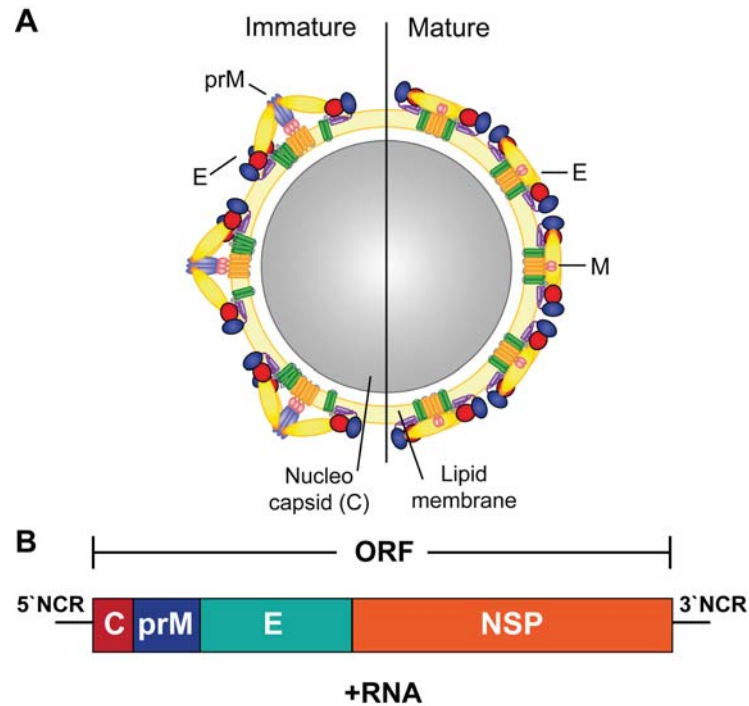


Figure 4.2: Schematic illustration of flavivirus particles and genome organization. (A) Immature virions have two membrane proteins (prM and E), which form heterodimers (left). The prM protein is cleaved during maturation, resulting in the rearrangement of E into homodimers, present on mature virions (right). The surface proteins are integrated in a host-derived lipid bilayer, which surrounds the nucleocapsid composed of protein C and the viral genome. (B) The positive-sense RNA genome consists of one open reading frame (ORF) and encodes the structural proteins C, prM and E, followed by non-structural proteins (NSP). The ORF is flanked by non-coding regions (NCR) (modified from (66)).

Flaviviruses are enveloped viruses with a dense, icosahedral outer glycoprotein shell and a diameter of approximately 50 nm. Virions are composed of a host-derived, polygonal lipid bilayer into which the viral glycoproteins E and prM/M are integrated. In the interior, a single positive-sense RNA genome of approximately 11kb in length is packaged by capsid proteins, forming the viral nucleocapsid (Figure

4.2) (42).

The viral genome has one open reading frame and encodes a single polyprotein (Figure 4.2 B). In the host cell the polyprotein is processed into 3 structural (C, prM, E) and 7 non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins, which are important for viral replication. The protein C (12 – 14 kDa) is essential in particle assembly and encapsidates the viral genome (42). Protein prM, a precursor form of M, is present on the surface of immature particles (Figure 4.2 A). Upon maturation the pr peptide is cleaved off, while the M protein remains anchored to the viral membrane. During polyprotein synthesis, prM functions as a chaperone for E protein folding and assembly (48).

4.1.2 **Flavivirus life cycle**

Flaviviruses enter the host cell via receptor-mediated endocytosis. After virus attachment to the cell and particle endocytosis, the acidic pH in the endosome triggers fusion between the viral and the endosomal membrane (48). The nucleocapsid is released into the cytoplasm where it is subsequently disassembled, thus enabling translation of the positive-stranded RNA (Figure 4.3). The viral genome is the only viral mRNA and codes for a single polyprotein, which is translocated and anchored to the membrane of the endoplasmic reticulum (ER) by several stop-transfer and signal sequences which control the compartmentalization of the individual proteins. The subsequent cleavages by cellular and viral proteases regulate the polyprotein processing and production of the individual structural and non-structural proteins (Figure 4.4) (48, 66).

Viral particles assembled in the lumen of the ER are immature. They are composed of the nucleocapsid, surrounded by an ER-derived lipid bilayer, carrying the glycoproteins prM and E (Figure 4.2 A) (48). These particles are not infectious. Maturation occurs in the trans-Golgi network (TGN) at low pH, where the cellular protease furin cleaves the precursor protein prM (63). Upon cleavage the prM/E heterodimer rearranges to form E homodimers. The N-terminal pr peptide remains attached to the M protein until the particle is released from the cell by exocytosis

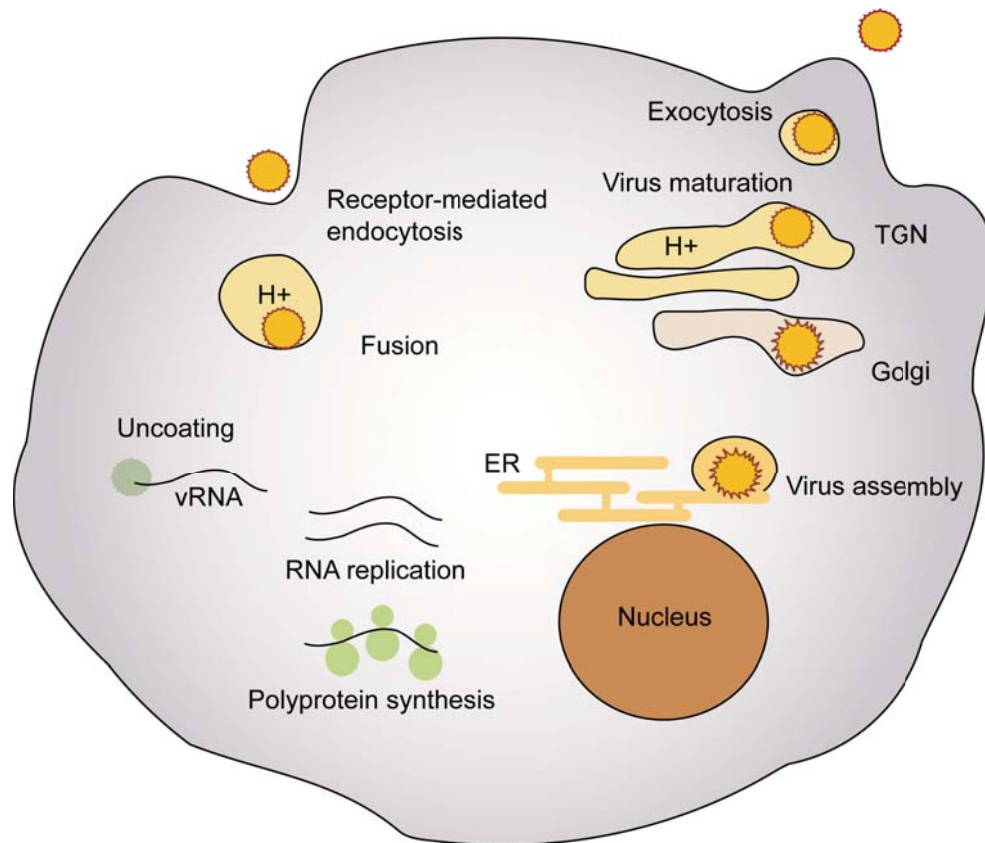


Figure 4.3: Flavivirus life cycle. Flaviviruses enter the cell via receptor-mediated endocytosis. Fusion between viral and endosomal membrane is triggered by acidic pH in the endosome and results in the release of the viral genome into the cytoplasm, where viral translation and replication takes place. Polyprotein processing and virus assembly happens in the ER, from which immature particles are released. The maturation cleavage of the precursor protein prM occurs in the TGN, by the cellular protease furin. Viral particles are released from the host cell by exocytosis (modified from (48)).

(Figure 4.3), thus preventing the premature fusion of the particle during exocytosis. Release of the pr peptide is triggered by a change to neutral pH, present in the extracellular environment (76).

A characteristic feature of flavivirus infection is the production of non-infectious subviral particles (SVP) in addition to the production and release of infectious virions. SVPs are capsidless, enveloped particles that contain the two viral membrane-associated proteins M and E (61, 70).

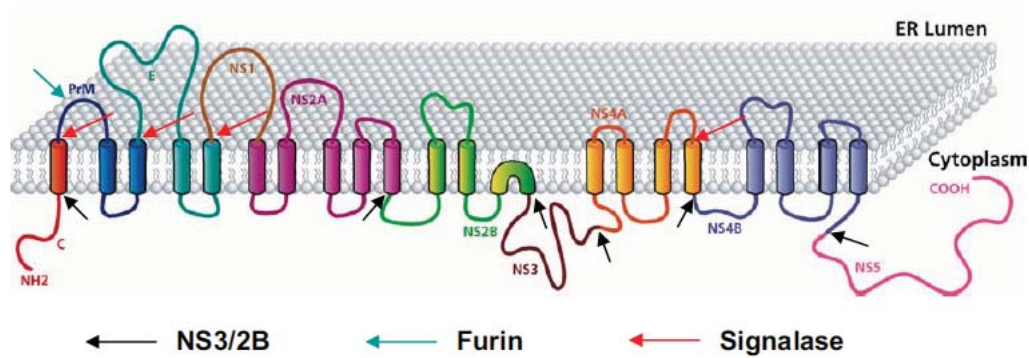


Figure 4.4: Topology of flavivirus structural and non-structural proteins in the ER membrane. Cleavage sites are indicated by arrows. Color code: capsid protein, red; prM, blue; E protein, cyan; NS1, brown; NS2A, magenta; NS2B, green; NS3, dark brown; NS4A, orange; NS4B, purple; NS5, light pink (adapted from (71)).

4.2 Entry of enveloped viruses

Enveloped viruses enter cells by fusion of the viral membrane with a cellular membrane after virus attachment to the cell. Fusion can occur either directly at the plasma membrane or after endocytosis, with membranes of intracellular compartments, e.g. endosomes. The process is mediated by specialized fusion proteins, which are present on the viral surface. In many cases, these proteins have additional functions, such as receptor binding. This interaction, along with changes in physico-chemical parameters, such as pH, trigger large conformational rearrangements of the fusion proteins, which provide the main energy for membrane fusion (23, 74).

Flaviviruses bind to the host cell surface by interaction of the viral glycoprotein E with cellular receptors. Flavivirus particles can interact with diverse surface molecules on different cell types, but only a few receptors have been identified. Glycosaminoglycans have been shown to function as low-affinity receptors (38). More specific interactions are supposed to take place between cellular surface proteins and the protruding domain III of protein E (Figure 4.7) (29). The carbohydrate side chains of E have been shown to interact with the lectin receptor DC-SIGN in DENV and WNV attachment to immature dendritic cells (66). Prior to fusion, the attached virion is internalized by receptor-mediated endocytosis and transported by clathrin-coated pits to a pre-lysosomal compartment (11, 42). In the endosome, the acidic

pH induces the structural rearrangements of the fusion protein E, which cause the merging of the two opposed bilayers and allow the release of the viral genome into the host cytoplasm (42).

4.2.1 Structural classes of viral fusion proteins and mechanisms of fusion

Three different structural classes of viral membrane fusion proteins have been described (23, 74). Fusion proteins found in retro-, filo-, corona-, paramyxo- and orthomyxoviruses have been typed as class I fusion proteins (57). Based on their structure and function, the fusion proteins of alpha- and flaviviruses represent the second class (33). Fusion proteins combining characteristics of both classes have been identified in rhabdo-, baculo- and herpesviruses. They have been classified as class III fusion proteins (7).

4.2.1.1 Class I viral fusion proteins

Class I viral fusion proteins are present on the surface of viral particles as homotrimers with an α -helical coiled-coil structure in the centre. In many cases, these proteins are synthesized as precursor proteins, which require a proteolytic maturation cleavage, generating a carboxy-terminal fragment. This fragment finally represents the actual fusion-active subunit with a hydrophobic, glycine-rich “fusion peptide” at its N-terminus (23). The rod-like structure resembles the architecture of cellular SNARE fusion proteins (62).

The fusion protein hemagglutinin (HA) of influenza virus serves as the prototype of a class I fusion protein (57).

Pre-and postfusion conformation of influenza HA

HA was the first structure of a viral fusion protein which was solved in its native and in part, in its postfusion conformation (74). HA is synthesized as a trimeric HA₀ precursor protein, C-terminally anchored to the viral membrane. HA₀ is proteolytically processed into the two polypeptide chains HA₁ (receptor binding) and HA₂,

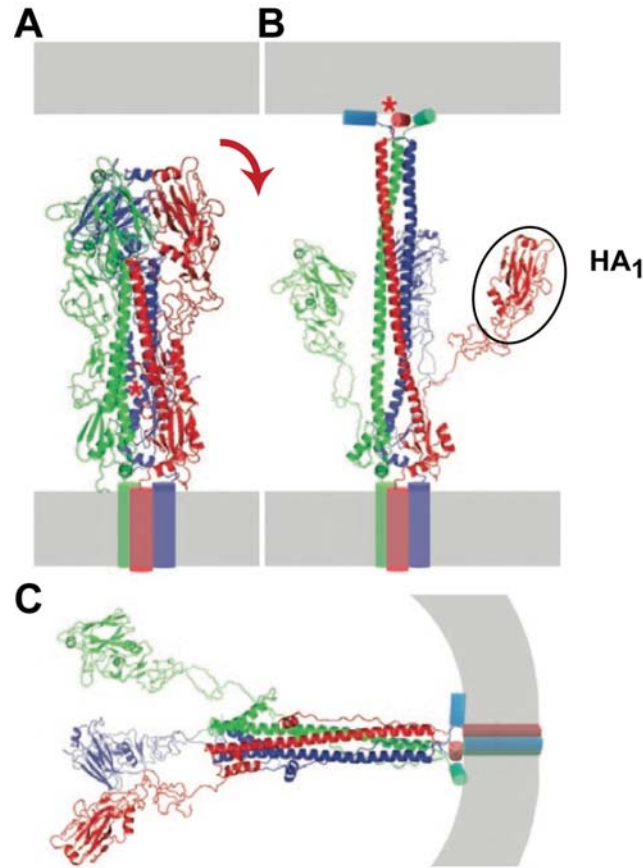


Figure 4.5: Structures of influenza HA. Each monomeric subunit is displayed in a different color. The monomers are C-terminally anchored in the viral membrane. (A) Prefusion structure of influenza HA. The red asterisk indicates the shielded fusion peptide. (B) Proposed extended intermediate of influenza HA. HA₁ domains have dissociated from each other and the fusion peptide is translocated towards the target membrane for interaction. (C) Postfusion conformation of influenza HA. The extended intermediate of HA₂ has collapsed and the typical hairpin conformation is formed (adapted from) (23)).

which mediates fusion. A disulphide bridge covalently associates HA₁ and HA₂ (60). The proteolytic cleavage of HA₀ does not induce major conformational changes (23).

The native metastable HA is a homotrimer, composed of two structural regions (Figure 4.5 A): a fibrous region combining structures of HA₁ and HA₂ and a globular (HA₁) region, which is positioned on the top of the elongated segment. The centre of the trimer is built by a triple-stranded, α -helical coiled-coil (HA₂). The backbone of each monomer consists of two antiparallel helices connected by a bent loop. On top of the central coiled-coil the receptor-binding domain HA₁, built up by an eight-stranded- β -sheet-structure, stabilizes the HA₂ forming a clip around the

centre (75). In the native prefusion conformation the hydrophobic fusion peptide at the N-terminus of HA₂ is buried in the trimer interface (28). Influenza virus enters the cell via receptor-mediated endocytosis. The acidic pH in the endosome induces a conformational change in the HA protein. Two sequential structural alterations are significant for the refolding process of the protein, facilitated by the dissociation of the HA₁ domains (Figure 4.5 B) (23). First, a non-structured loop converts into a helical segment, bringing up the previously buried fusion peptide for target membrane interaction. Then, this segment turns back and zips up along the triple-stranded coiled-coil core, resulting in the juxtaposition of the fusion peptide and the transmembrane region in the fused membranes. This postfusion trimer resembles the shape of a hairpin (figure 4.5 C) (57).

Proposed class I membrane fusion mechanism

Structural data are only available for the pre-and postfusion conformations of class I fusion proteins. However, based on mutational, biochemical and biophysical studies general characteristics of a class I fusion pathway with intermediate structures have been proposed (Figure 4.6) (23). Upon exposure to the low pH of the endosomes, the HA₁ subunits move away, allowing HA₂ to undergo a conformational change that drives the fusion reaction. On the native viral particle, HA₂ is present as a metastable trimer burying the fusion peptide. Low pH triggers the extension of the central helix and exposure of the fusion peptide for target membrane interaction (48). Subsequently, the extended chain folds back, facilitating the bending of the two membranes. A hemifusion intermediate in which just the outer leaflets have merged is believed to lead over to the helical postfusion hairpin structure in which the inserted fusion peptide and the transmembrane anchor are next to each other (23, 48). The opening and dilation of the fusion pore allows the entry of the nucleocapsid into the cell.

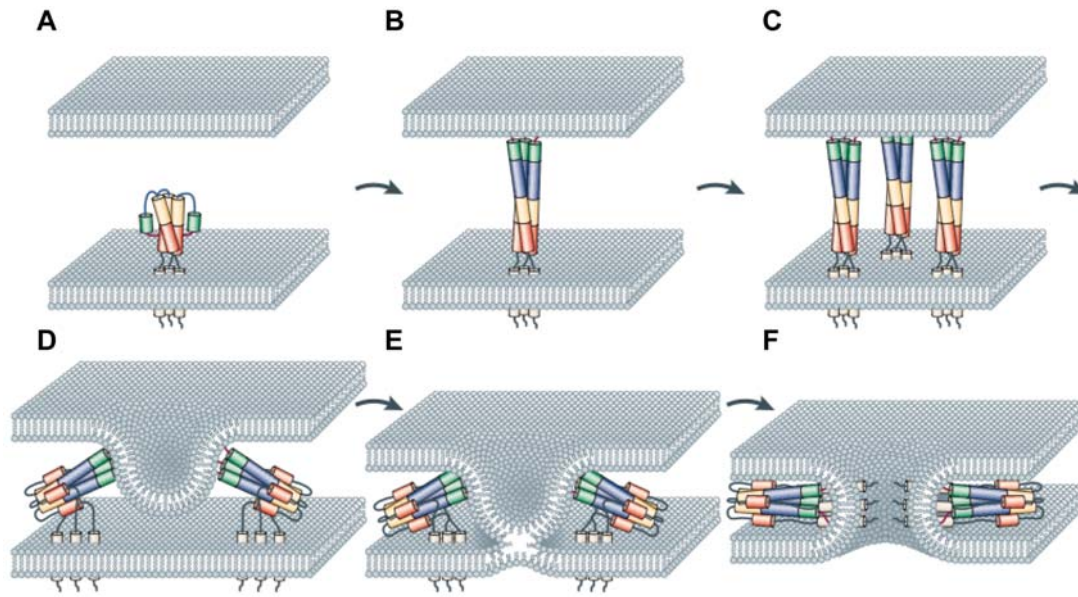


Figure 4.6: Proposed class I fusion process. (A) Native, trimeric class I fusion protein with the fusion peptide in red. (B,C) Upon exposure to the initial fusion trigger, the fusion protein converts into an extended intermediate conformation and the fusion peptides interact with the host membrane. (D) Conformational change in the fusion protein drive the bending of the viral and the target membrane towards each other. (E) Hemifusion intermediate. (F) Postfusion, hairpin conformation with the fusion peptides and the transmembrane anchors juxtaposed in the same membrane (adapted from (48)).

4.2.1.2 Class II viral fusion proteins

The fusion machineries of alpha- and flaviviruses consist of class II fusion proteins. They convert from a metastable hetero- or homodimer into an energetically favourable postfusion homotrimer and are mainly composed of beta-sheets (Figure 4.7). The activation of class II fusion machineries also requires a proteolytic cleavage, but in contrast to class I fusion proteins not the fusion protein itself but a second, associated glycoprotein is proteolytically processed. Moreover, the fusion peptide of class II proteins is located internally and not at or proximal to the N-terminus (Figure 4.7) (23).

Flavivirus protein E

The receptor-binding and membrane-fusion protein E of flaviviruses is a well characterized example for class II viral fusion proteins. Conformational changes in E drive the fusion reaction that is essential for virus entry. Crystal structures of the E

protein ectodomain in its pre- and postfusion conformation have provided insights into the flavivirus fusion process (48, 66).

Prefusion conformation of E

Structures of recombinant and truncated E protein dimers of DENV 2 and DENV 3 and E monomers of WNV have been published (45, 47, 52, 79). The crystal structure of the soluble fragment of protein E (sE), released by trypsin treatment of TBE virions and lacking the around 100 carboxyterminal amino acids, has been solved at 2 Å resolution (52). The atomic structure of the flavivirus E protein ectodomain in its prefusion conformation will be explained in detail on the basis of the TBEV E dimer (Figure 4.7).

At neutral pH the E protein forms head-to-tail homodimers lying flat on the viral membrane (Figure 4.7). The crystal structure of the external, crystallized part (residues 1 – 395) lacking the stem-anchor region shows a significantly different architecture compared to class I fusion proteins. The gently curved dimer is oriented parallel to the membrane and spans the overall dimensions of approximately 150 Å x 55 Å x 30 Å (52). The polypeptide chain of each monomer of sE is composed of 3 individual domains, with a high β -sheet content (Figure 4.7 A, C). Domain I lies at the centre of the protomer structure. It forms a β -sandwich, stabilized by two disulphide bridges. The axis of the β -sandwich lies nearly parallel to the viral membrane (52). Two protruding loops are stabilized by three disulphide bridges and build the elongated dimerization domain II. At the tip of this elongated, finger-like domain a glycine-rich hydrophobic sequence is located. This highly conserved region has been identified as the fusion loop which is important for target membrane interaction during fusion. The C-terminal domain (DIII) of the soluble E structure has an immunoglobulin-like fold with a single disulphide bridge. DIII is linked to the central domain I by a 15 residue-linker-segment. This domain protrudes away from the outer protein surface and has thus been implicated in receptor-binding (52, 66). In different crystal forms, slightly variable angles between the distinct domains have been observed, indicating a significant degree of DIII-flexibility.

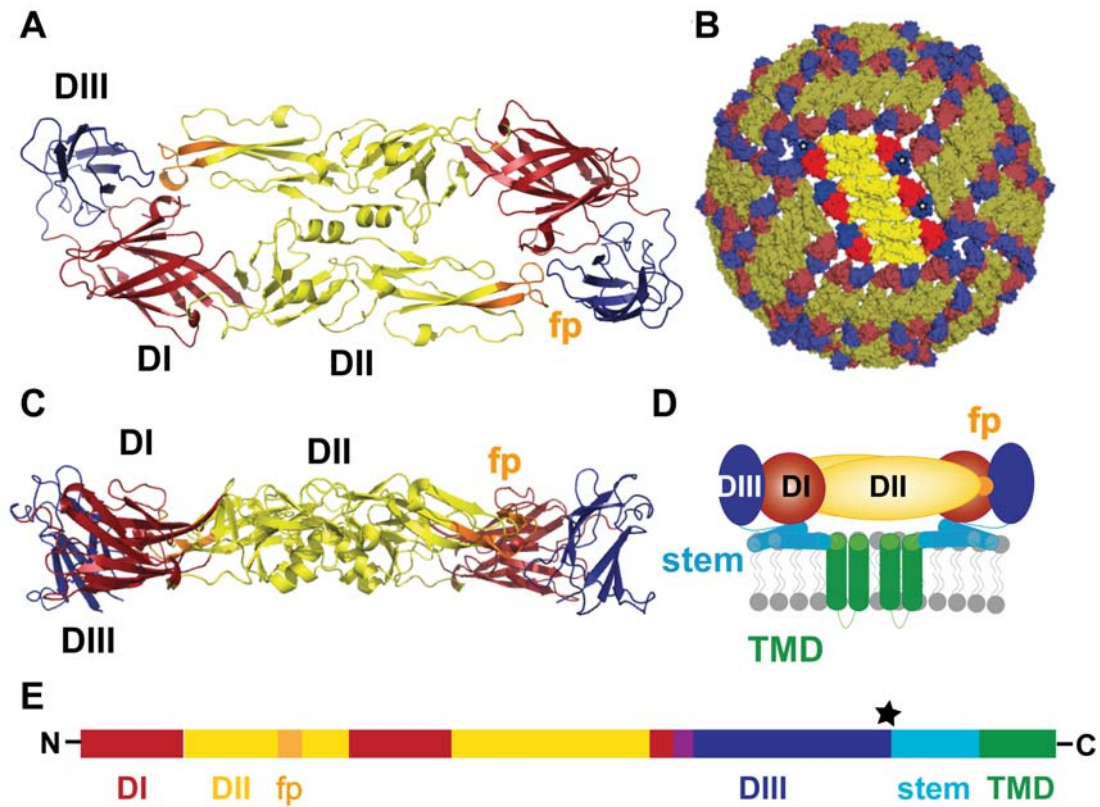


Figure 4.7: Prefusion conformation of flavivirus E protein. (A) Top view of dimeric, prefusion structure of TBEV E protein, residues 1-395 (52), (pdb ID 1SVB). (B) Schematic representation of E protein organization on mature flavivirus particles (adapted from (34)). (C) Side view of dimeric, prefusion structure of TBEV E protein, residues 1-395. (D) Schematic representation of full-length E protein dimer (side view). (E) Linear representation of E protein architecture. The black star indicates the C-terminus of the crystal structure. Individual domains are labeled in one monomer, color code: domain I (DI), red; domain II (DII), yellow; fusion peptide (fp), orange; domain III (DIII), flexible linker, purple; blue; stem, cyan and transmembrane domain (TMD), green; The membrane is colored in grey.

The dimer is largely stabilized by interactions between two opposite DII domains. The conserved fusion peptide dips with a tryptophan into a hydrophobic cave, provided by the DI/III interface of the opposite subunit (Figure 4.7). This arrangement prevents the fusion peptide from membrane interactions in its neutral pH conformation. The fusion loop is additionally covered by an oligosaccharide, which is positioned above the DI/III crevice (22, 45, 52, 79).

E protein homodimers form the outer layer of a mature virion, where 180 copies of protein E are organized in a smooth, icosahedral shell of metastable homodimers.

Three parallel dimers are arranged in 30 rafts in a herringbone-like orientation (39). The lipid bilayer is largely inaccessible and covered by E (Figure 4.7 B). Cryo-electron microscopy (EM) image reconstructions have shed light on the conformation of the C-terminal part of the E protein, which is absent in the sE crystal structures. The EM data indicate, that the stem, which connects the sE with the transmembrane region is composed of two α -helices linked by a loop. In the prefusion conformation of E, this stem is cooped between the ectodomain and the viral membrane (78). The transmembrane domain spans the viral bilayer twice, resembling the shape of a helical hairpin (Figure 4.7 D) (78).

Postfusion conformation of E

Upon exposure to low pH, the flavivirus E protein undergoes a dimer-to-trimer transition. The irreversible trimerization of the soluble protein fragment could only be achieved by exposing the sE dimer to acidic pH in the presence of liposomes (65). The crystal structure of the solubilized sE trimer has been solved and characterized in detail for TBEV, DENV 1 and DENV 2 (Figure 4.8) (9, 46, 49).

In the postfusion trimer, the monomers are oriented perpendicular to the bilayer. The conformational change of E includes no major refolding of the individual domains but rather a rearrangement of the domains relative to each other (66). Relative to domain I, domain II rotates 19° around the DI/II junction, resulting in a long rod, composed of DI and DII (9). The residues of the fusion loop, which are exposed to the solvent, are supposed to interact with the lipid heads of the outer leaflet of the target membrane. A more dramatic relocation is observed for domain III, enabled by the flexible linker between DI and DIII. Compared to the neutral pH conformation, DIII relocates from the top of the monomer to the side of the trimeric core and flips against the interface of two protomers. As a consequence, the C-terminus of the crystallized fragment is directed towards the viral membrane (9). In the full length protein the distance between the DIII/stem linker and the bilayer is about 50 Å, which is proposed to be bridged by the stem region (50 aa). With regard to membrane fusion, the E protein membrane anchor and the target membrane-inserted

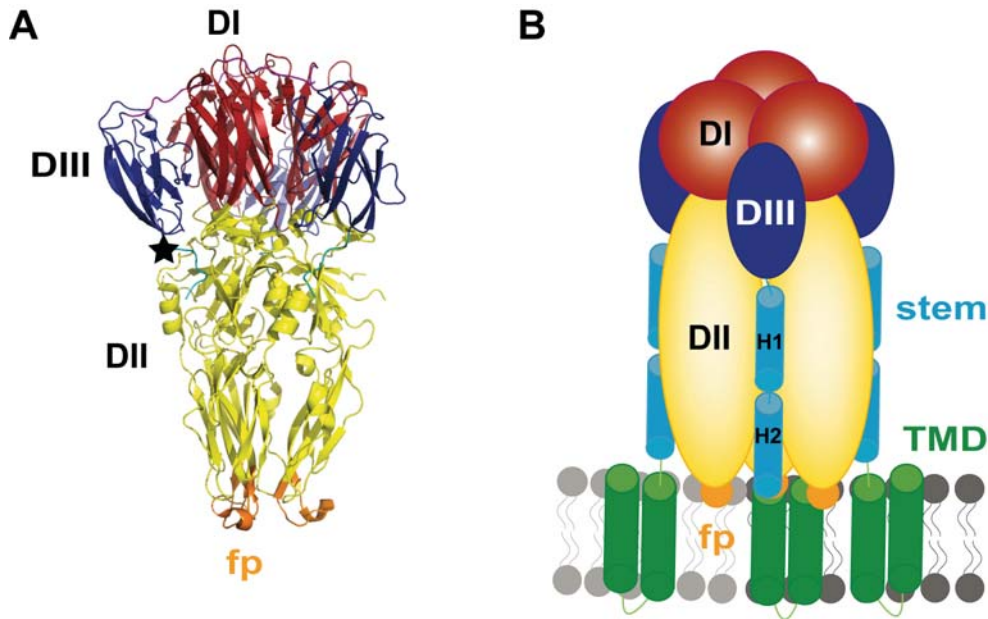


Figure 4.8: Postfusion conformation of flavivirus E protein. (A) Side view of trimeric, low pH structure of TBEV E protein, residues 1-401 (9), (pdb ID 1URZ). The carboxy-terminus of the crystallized part is indicated by a black star. (B) Schematic representation of full-length E protein trimer (side view). Helix 1 and helix 2 of the stem are labeled with H1 and H2, respectively. Individual domains are labeled in one monomer: DI, red; DII, yellow; fp, orange; DIII, blue; stem, cyan and transmembrane domain, green; viral membrane, light grey; host membrane, dark grey.

fusion peptide would be juxtaposed in the postfusion structure. The overall trimer resembles the hairpin-like arrangement of class I fusion proteins, suggesting a similar fusion mechanism (9).

Proposed class II membrane fusion mechanism

Flavivirus membrane fusion is a fast and efficient process (66). X-ray crystallography data and cryo-EM structures of E in its pre- and postfusion conformation in addition to biochemical studies have provided insight into the class II membrane fusion mechanism. Based on these results a class II fusion model has been developed (Figure 4.9) (66).

Flaviviruses enter the host cell via receptor-mediated endocytosis. The acidic pH in the endosome causes the dissociation of the E protein homodimers into monomers and the exposure of the previously buried fusion peptide for interaction with target

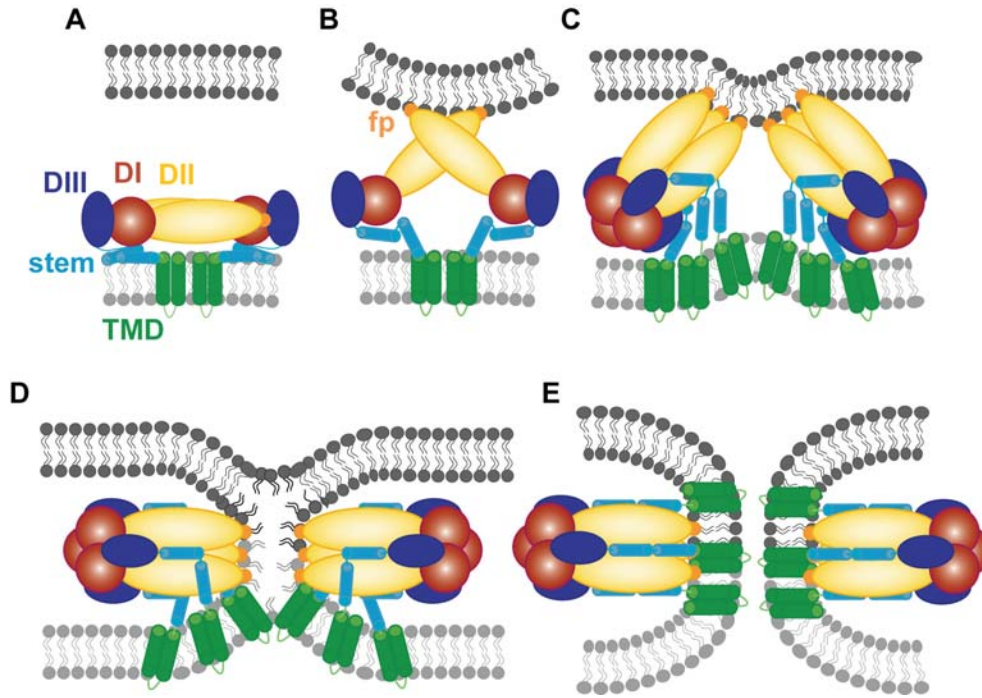


Figure 4.9: Proposed class II fusion process. (A) Prefusion dimer, oriented parallel to the viral membrane. (B) Low-pH-induced dimer dissociation and target membrane interaction of exposed fps. (C) Trimerization and zippering of the stem along the trimer core. (D) Hemifusion intermediate. (E) Trimeric, hairpin structure of postfusion protein with fusion peptides and TMDs juxtaposed in the fused membranes (modified from (66)).

membranes (Figure 4.9 B) (66). At this stage, the viral and the host membrane are bridged by the fusion protein. The extension of the stem is thought to facilitate the insertion of the fusion peptide into the target membrane (32). Further structural changes involve the relocation of domain III from the top of the rod-like structure to the side of DI, the zippering of the stem along DII and the formation of stable postfusion trimers with the membrane anchors and the fusion peptides juxtaposed in the fused bilayers (Figure 4.9 C-E) (66). This model illustrates the transition of the E protein homodimer to a postfusion trimer, including a transient hemifusion intermediate in which just the outer leaflets have merged (66).

4.2.1.3 Class III viral fusion proteins

Glycoproteins from rhabdo-, baculo- and herpesviruses have been assigned to the third class of viral fusion proteins (7). These proteins have a different structural organization, in comparison to class I and II proteins (53). Class III fusion proteins

combine structural features of the first two previously identified classes (53). The proteins are trimers, built up by multidomain protomers. The α -helical coiled-coil core of the trimer is a characteristic attribute of class I fusion proteins. In contrast, the presence of extended β -hairpins with hydrophobic tips are features of class II fusion proteins (26). Vesicular stomatitis virus is an enveloped, bullet-shaped virus, assigned to the family Rhabdoviridae. The virus enters the cell through the endocytic pathway, mediated by the viral glycoprotein G (54). The crystal structure of the vesicular stomatitis virus glycoprotein G (ectodomain) has been solved in its pre- and postfusion conformation (53, 54). In the prefusion state, the trimeric G protein exhibits the shape of a tripod with the fusion loops at the tip of each leg, directed towards the viral membrane. Each protomer carries two fusion loops, which are not buried, but separated from each other (54). The protomers are composed of four individual domains (Figure 4.10 A). Domain I is an elongated β -sheet-rich domain; domain II is composed of antiparallel β -sheets; domain III has a long, central α -helix, involved in trimerization at the top of the molecule, whereas domain IV consists entirely of β -sheets (53).

Upon exposure to low pH, the G protein undergoes dramatic structural rearrangements to an inverted cone-shape like organization. Domain I, carrying the fusion loops, flips to the opposite side of the molecule for target membrane interaction (Figure 4.10 B). The refolding of the trimerization domain II drives the projection of the C-terminal part, resulting in the postfusion conformation with transmembrane region and fusion domain at the same side of the molecule (Figure 4.10 C). Domain II undergoes a dramatic change in fold, whereas domain I, III and IV maintain their folds and rather change their orientation relative to each other. It has been shown that VSV G can be present in at least three different conformations: the native prefusion state, an activated intermediate state (target membrane interaction) and the postfusion structure. In contrast to class I and class II fusion proteins, the conformational change of VSV G is reversible. There is an equilibrium between these conformations, depending on the present pH (Figure 4.10) (54).

Despite the structural diversity among the viral fusion protein classes, in all cases,

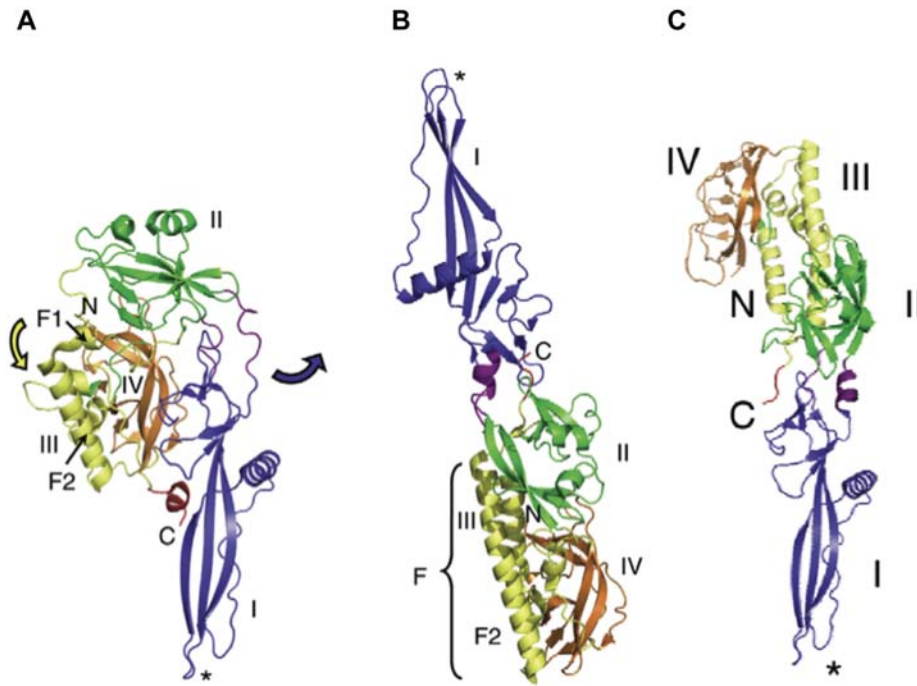


Figure 4.10: Conformational changes in VSV G class III fusion protein. Individual domains of the protomer are labeled with different colors: domain I, purple; domain II, green; domain III, yellow; domain IV, orange. Fusion loops are indicated by an asterisk. C- and N-termini are labeled with C and N, respectively. (A) Prefusion structure of VSV G ectodomain. (B) Extended intermediate structure of VSV G ectodomain. (C) Postfusion conformation of VSV G ectodomain. C-terminus and the fusion loops are oriented towards the same direction (adapted from (7)).

known so far, fusion protein transition results in a hairpin structure with the viral membrane anchor and target membrane-inserted fusion peptide at the same end of the postfusion trimer (73).

4.3 Membrane-proximal regions of viral fusion proteins

Viral fusion proteins of all three classes are composed of oligomeric ectodomains anchored to the viral membrane by transmembrane domains (TMD). A striking characteristic of many viral fusion proteins is a long sequence stretch, rich in aromatic residues, which precedes the TMD (43). This external part of viral fusion proteins is defined as the membrane-proximal external region (MPER). MPERs of several viral fusion proteins share common features. Secondary structure analyses of these regions with respect to the distribution of hydrophobic patches have often revealed

an amphipathic character. Such structural elements have been predicted to promote hydrophobic interactions between different protein elements or tend to appear at water-membrane intersections (43). Studies on different viruses have shown that MPERs of viral fusion proteins can promote membrane fusion. They can transfer the energy of conformational changes in proteins towards membranes or participate in membrane destabilization (43).

The membrane-proximal region of the class I fusion protein gp41 of human immunodeficiency virus (HIV) has been studied extensively. It has been shown that the helical element contributes to the thermostability of the trimer and is thought to be involved in membrane interactions (10). Mutations of aromatic MPER residues in gp41 inhibited fusion in a late stage of the fusion process (55). A peptide derived from the HIV envelope glycoprotein (env) MPER has been identified as an effective fusion inhibitor (16). This synthetic peptide, named T20 binds to intermediate structures of the env ectodomain and thereby inhibits HIV entry (35).

The MPER of the VSV class III fusion protein G has been shown to increase the fusion activity of heterologous viral fusion proteins, by inducing lipid-mixing of the outer leaflets (30).

The MPER of alphavirus class II fusion protein E1, the so-called E1 stem, has been shown to pack against the core of the trimeric ectodomain in a late stage of the fusion process. The stem of the E1 class II fusion protein contributes to the stability of the trimer and enhances the fusion inhibition effect of exogenous domain III (40). These examples clearly demonstrate the importance of MPERs in viral membrane fusion.

Membrane-proximal region of the flavivirus E protein

The membrane-proximal region of the flavivirus E protein, the stem, connects the ectodomain with the double transmembrane anchor (Figure 4.7 D). The stem comprises approximately 50 residues and forms two amphipathic alpha helices (H1 and H2), connected by a short linker (64, 78). Secondary structural elements of flavivirus E proteins of mature dengue virus particles, including the stem-anchor region, have

been visualized by cryo-EM of DENV 2 (Figure 4.11) (78). These images have illustrated that in the dimeric prefusion conformation of E the two stem helices are sandwiched in the interface of the E protein ectodomain and the viral membrane. The helices are arranged parallel to the viral surface and partly buried in the outer lipid bilayer. The hydrophobic patches of the helices are directed towards the membrane, allowing an interaction with the lipid phosphate head groups (66, 78).

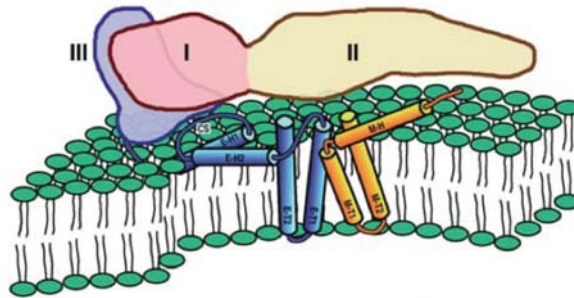


Figure 4.11: Diagram of the full-length dengue virus protein E in the prefusion conformation. Helix 1 (H1) and helix 2 (H2) of the stem are oriented in parallel to the viral membrane, interacting with the outer leaflets of the bilayer (adapted from ((78))).

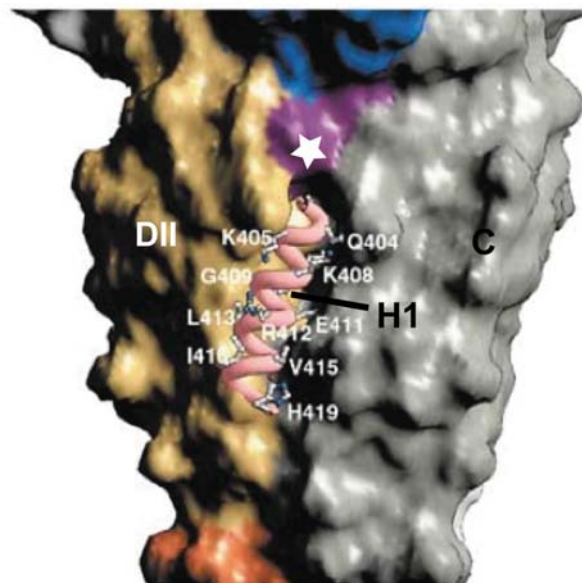


Figure 4.12: Helix 1 (H1) of the stem modeled into postfusion structure. H1 was modeled into the groove of the trimer interface. The conserved residues are directed towards DII of the same polypeptide chain. The carboxy-terminus of the crystallized part is labeled with a white star (adapted from (9)).

The structure and orientation of the crystallized postfusion sE trimer indicates,

that the stem region would follow the sE C-terminus and bridge the gap (50 Å) to the viral surface (Figure 4.8 B) (9). The stem helices would run along the vertical groove provided by neighbouring subunits of the trimer, bringing TM anchor and target membrane-inserted fusion peptides into close proximity (Figure 4.8 B) (9). During membrane fusion the stem is supposed to zipper along domain II forcing the bending of the two opposing membranes (Figure 4.9 C) (9, 66). Biochemical studies have shown that the stem contributes to the stability of the postfusion trimer (69). The presence of stem-H1 was crucial for trimerization in the absence of target membranes (5).

H1 of the stem was modeled into the DII/DII* interface of the truncated trimeric postfusion structure (Figure 4.12) (9). In this conformation, specific intramolecular interactions between highly conserved stem residues and the domain II have been proposed (9). The conserved, aromatic residue F403 is predicted to interact with a highly conserved pocket, formed by the domain II residues W219 and L223. Further contacts are thought to form between the stem and the postfusion trimer core (9).

5 Objectives

The general aim of this work was to gain more detailed information about the mechanism of flavivirus membrane fusion. Crystal structures of the pre- and post-fusion conformation of soluble truncated forms of the fusion protein E (sE), lacking the stem-anchor region, provided important information about viral class II fusion proteins (9, 45–47, 49, 52, 79). High-resolution structures of the E protein stem, which connects the crystallized part to the transmembrane anchor are not available, so far. Structures of fusion intermediate stages of E are also not known. The currently existing flavivirus fusion model is therefore hypothetical and based on the truncated pre- and postfusion structures and on biochemical data. According to this flavivirus fusion model, the stem is hypothesized to zipper along domain II in the course of hairpin formation (66), but direct experimental evidence for stem-trimer core interactions does not exist. In this thesis, we want to gain new insights into the role of the stem region in flavivirus fusion, using mutational analyses of viruses and recombinant subviral particles (RSP) of tick-borne encephalitis virus.

Specific aim part I

Bressanelli et al. used the trimeric postfusion conformation of the truncated E protein and modeled the first helix of the stem into the existing structure. H1 was fitted into a vertical groove, located at the oligomer interface. In this model, conserved stem residues oriented towards DII of the same protomer, supporting the idea that the stem might interact with the trimer core in the postfusion conformation (9). To investigate possible stem-trimer core contact sites, we will mutate residues of the stem and DII, which were predicted to interact with each other in the postfusion trimer. To examine these mutations and their effect on possible stem-interactions and flavivirus fusion, we will use the experimental system of TBEV RSPs. RSPs are noninfectious particles, which exhibit fusion properties similar to virions and are an established model to examine flavivirus fusion and fusion-related processes (12).

Specific aim part II

Although the RSP system is an elegant system to study flavivirus fusion and fusion-related processes, it cannot provide data about infectivity. It was therefore the objective of the second part of the thesis, to extend the findings obtained in the RSP system to the infectious virus system and to investigate the impact of mutations on virus infectivity and fusion. For that purpose, mutations with an effect on RSP fusion activity and additional substitutions at the proposed DII/stem interaction site will be introduced into the infectious clone of TBEV. Infectivity of virus mutants will be analyzed to supplement the RSP data.

6 Part I (RSP system)

6.1 Introduction

A characteristic feature of flavivirus infection is the production and release of non-infectious subviral particles (SVP) - also designated Slowly-Sedimenting Hemagglutinin (SHA) - along with the infectious virions (61, 70). SHA are capsidless particles which consist of a lipid bilayer in which the two viral glycoproteins M and E are integrated. Subviral particles can also be produced in a recombinant form by transfecting mammalian cells with a recombinant plasmid, which allows the coexpression of the two TBE envelope proteins E and prM (4). Two size classes of RSPs have been characterized (6). The larger particles have similar dimensions as virions. The smaller and prevalent species, which is approximately 30 nm in diameter has been characterized in detail. 60 copies of E protein are present as homodimers arranged in a $T = 1$ icosahedral symmetry (Figure 6.1) (19). It was shown, that E in RSPs has the same antigenic and oligomeric structure as that in infectious virions (56). RSPs are assembled in the ER as immature particles and traverse the same maturation and secretion pathway as virions (12, 56). The fully functional E proteins are capable of undergoing an irreversible dimer-to-trimer transition and the associated conformational and structural changes could also be observed (56). Cover et al. used fluorescently-labeled RSPs and measured particle fusion with liposomes after acidification, in order to examine the fusion characteristics of RSPs. The data showed, that RSPs have a rate and extent of low-pH-induced fusion that is similar to viral particles (12). Thus, RSPs provide a useful non-infectious experimental system to investigate the flavivirus fusion mechanism. Using a mutational analysis on RSPs of TBEV, the conserved loop of E at the tip of domain II has been identified as the fusion peptide (2). In this project we want to make use of RSPs to obtain information about the role of the E protein stem in flavivirus fusion.

The flavivirus E protein stem is hypothesized to be important in early and late stages of fusion. Cryo-EM studies on West Nile virus with antibody fragments

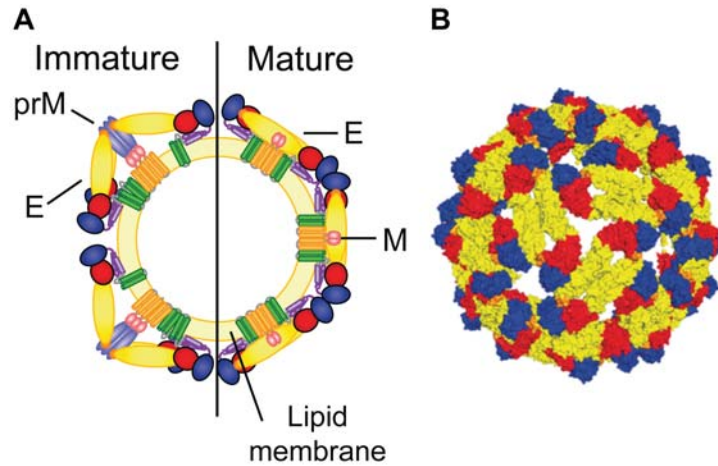


Figure 6.1: Flaviviral recombinant subviral particles (RSP). (A) Schematic representation of a RSP cross-section. RSPs are composed of a lipid-bilayer with the glycoproteins prM/M and E on the particle surface. Heterodimeric prM/E arrangement of immature RSPs (left). After particle maturation in the TGN, E protein homodimers cover the particle surface (modified from (66)). (B) Schematic representation, demonstrating the icosahedral symmetry of the E protein arrangement in the mature RSP (adapted from (34)).

demonstrated that the stem probably supports fusion peptide insertion into target membranes by extending from the viral membrane upon dimer dissociation (32). Later in the fusion process, the stem is speculated to zip along the body of the trimer after relocation of domain III (58, 66) and might thereby provide part of the energy for membrane merger (Figure 4.9). Consistent with this hypothesis, it has been shown that the addition of dengue virus recombinant DIII alone was not sufficient to inhibit dengue virus fusion, whereas DIII together with H1 of the stem (Figure 4.8) inhibited fusion of dengue viruses (40). Moreover, peptides derived from the stem region of the dengue virus E protein have been shown to bind to the truncated stem-less sE trimer and thereby function as fusion inhibitors at a stage prior to hemifusion and fusion-pore formation (58). Using the RSP system, we introduced mutations into the stem and into the parts of domain II located at the predicted stem-trimer core interface and investigated the effect of these mutations on fusion. An established control system was implemented to guarantee the quality and maturity of the used RSPs.

6.2 Materials and Methods

6.2.1 Mutagenesis of RSPs

Mutations were introduced into the SV-PEwt plasmid by site-directed mutagenesis, using the Gene Tailor Kit of Invitrogen. Mutagenesis primers are listed in table 6.1. The plasmid contained the TBEV prM and E genes under the control of the SV40 early promoter (1). To confirm the presence of only the desired mutations at codon positions 223, 403, 406, 421 and 434 the whole prM/E sequence of each plasmid was verified by sequencing.

Table 6.1: Oligonucleotide sequences.
F = forward primer, R = reverse primer.

Oligonucleotide name	Sequence ^a
L223I-F	CAT AGG GAC TGG TTC AAT GAT ATA GCT CTG CCA T
L223I-R	ATC ATT GAA CCA GTC CCT ATG GAC CTG CC
F403I-F	GG AGC AGC ATC GGA AGG GTT ATC CAA AAG ACC
F403I-R	AAC CCT TCC GAT GCT GCT CCC TTT TTG G
T406A-F	C GGA AGG GTT TTC CAA AAG GCC AAG AAA GGC A
T406A-R	CTT TTG GAA AAC CCT TCC GAT GCT GCT CCC
W421I-F	CA GTG ATA GGA GAG CAC GCC ATA GAC T TC GGT T
W421I-R	GGC GTG CTC TCC TAT CAC TGT CAG TCT TTC
G434N-F	GA GGC TTT CTG AGT TCA ATT AAC AAG GCG GTA C
G434N-R	AAT TGA ACT CAG AAA GCC TCC AGC AGA ACC

^a Nucleotides were synthesized by VBC BIOTECH.

6.2.2 Production of RSPs

The recombinant wild-type (WT) and mutant plasmids were electroporated into COS-1 cells for RSP production as described previously (56). COS-1 cells were grown in Dulbecco's modified eagle medium (DMEM), supplemented with 5% PSG (penicillin-streptomycin-glutamine) and 10% fetal calf serum and maintained in DMEM with 5% PSG. 48 hours post transfection, RSPs were harvested from cell culture supernatants and pelleted by ultracentrifugation. For coflotation assays, RSPs were further purified by sucrose gradient centrifugation (56). For membrane fusion assays, RSPs were metabolically labeled with 1-pyrene hexadecanoic acid (Invitrogen), as described previously (12).

6.2.3 Quality control of RSPs

Secretion of RSPs into the supernatant of transfected cells was quantified with a four-layer enzyme-linked immunosorbent assay (ELISA) after sodium dodecylsulphate solubilization at 65°C for 30 min (25).

The maturation state (presence of prM) of RSPs was analyzed by Western blotting (20). Equal amounts of RSPs (standardized to E protein) were serially diluted and loaded onto a 15% polyacrylamide gel. Proteins were separated under SDS denaturing conditions and blotted onto a polyvinylidene difluoride membrane with a semidry blotting apparatus (Bio-Rad). The content of prM protein was detected with a polyclonal anti-TBEV serum and anti-rabbit-immunoglobulin G- alkaline peroxidase (Amersham). Proteins were visualized using SigmaFast DAB tablets (59).

E protein conformation and folding was investigated by epitope mapping with a set of E protein specific monoclonal antibodies (mabs). WT and mutant RSPs (0.5 μ g/ml E protein) were captured on solid phase by a guinea pig anti-TBE virus immunoglobulin (Ig) and tested with a predetermined dilution of each mab. A peroxidase-labeled rabbit anti-mouse Ig was used to detect the reactivity of the preparations to the mabs in a four-layer ELISA (6, 56).

6.2.4 Fusion assay

Fusion activity of WT and mutant RSPs was tested with an *in vitro* fusion assay. Unilamellar liposomes (pore size: 200 nm) composed of phosphatidylcholine, phosphatidylethanolamine and cholesterol (molar ratio 1:1:2) were mixed with pyrene-labeled RSPs in a fluorimeter cuvette at 37°C (2, 68). Fluorescence was continuously monitored for 60 s with a fluorescence spectrophotometer (LS-50B; Perkin Elmer) at a wavelength of 343 nm. Fusion of RSPs with liposomes was initiated by acidification with MES to a final pH of 5.4. Fusion activity was determined by recording the decrease in the pyrene-excimer-fluorescence, due to dilution of the probe into unlabeled liposomes. The fusion extent was calculated by defining the initial fluorescence as 0% fusion and the signal of the solubilized RSP-liposome mixture as 100% fusion. Solubilization was achieved by the addition of the detergent octa(ethylene glycol)-n-dodecyl monoether (Sigma-Aldrich) (20, 68).

6.2.5 Coflotation assay

Purified RSPs were incubated with liposomes at a ratio of 1 μ g E to 200 nMol lipids (composition as described above). The mixture was acidified to pH 5.4 by the addition of MES and incubated for 15 min at 37°C. The samples were back-neutralized with 150 mM triethanolamine to pH 7.8 and mixed with sucrose to yield a concentration of 20% (wt/wt). This sample (0.6 ml) was put onto a 1ml 50% sucrose cushion and further overlaid with 1.4 ml 15% sucrose and 1 ml of 5% sucrose. All sucrose solutions were prepared in TAN buffer, pH 8.0 (20, 65). The gradients were centrifuged for 2 hours at 4°C at 50000 rpm in a SW 55 Beckman Coulter rotor. Fractions of 200 μ l were collected by upward displacement with a Piston Gradient Fractionator (BioComp Instruments Inc.) and the E protein in each fraction was determined in a four-layer ELISA after treatment with 0.4% sodium dodecyl sulphate at 65°C for 30 min (25).

6.2.6 Dimer-trimer transition assay and trimer stability assay

For E protein trimerization, RSPs (3 μ g E protein) were acidified by the addition of MES to a final pH of 5.4 (2, 3). After 10 min at 37°C the sample was back-neutralized with 150 mM triethanolamine and solubilized with 1% Triton X-100. The mixture was incubated for 1 hour at room temperature and subjected to a 7%-20% (wt/wt) continuous sucrose gradient containing 0.1% Triton X-100. The gradients were centrifuged for 20 hours at 38000 rpm at 15°C in a SW 40 Beckman Coulter rotor. Fractions of 600 μ l were collected and analyzed by a quantitative four-layer ELISA. To test the thermostability of trimers, low-pH-treated and solubilized RSPs were incubated for 10 min at 70°C before subjected to sedimentation analysis, as described above.

6.3 Results and Discussion

6.3.1 Generation and quality control of mutant RSPs

To investigate the role of the protein E stem region in flavivirus membrane fusion, we introduced mutations into the stem and into the part of domain II, which was predicted to form the interface with the stem in the postfusion trimer (9). We targeted highly conserved residues in both helices of the stem, in the linker between the two helices, and in domain II. The targeted residues are illustrated in a flavivirus E protein alignment in Figure 6.2 A.

We wanted to investigate the impact of the introduced mutations on flavivirus fusion and fusion-related properties, but the introduced mutations could theoretically also interfere with other processes than fusion, e.g. correct E processing, folding and oligomerization and particle formation, maturation and secretion (20). In order to assure, that the mutations do not affect a wild-type (WT)- like character at neutral pH, we subjected the mutant particles to a set of quality control experiments (20). To assess the structural integrity of the mutant E proteins, their reactivity with monoclonal antibodies (mabs) was analyzed by a four-layer ELISA and compared to the WT binding pattern (20). The used mabs react with E protein epitopes on domain I, domain II or domain III and are suitable to detect conformational differences (20, 34). The binding profiles of two mutants and WT are shown in Figure 6.3. All mabs recognized mutant and WT particles similarly, as shown by the comparable absorbance-values obtained in the ELISA.

The maturation state of mutant RSPs was further analyzed by Western Blotting, using a polyclonal serum raised against TBEV, as described previously (20). The amount of prM protein on flaviviral particles is an indication for the maturation state of the particles. The detection limit of prM in serially diluted RSP samples (standardized to E protein) was compared to the prM content of WT RSPs and immature RSPs (20). As shown in Figure 6.4 with mutant L223I, the prM content was similar to WT, whereas in immature RSPs, carrying a mutation in the furin cleavage site of prM, a significantly higher amount of the precursor protein is present.

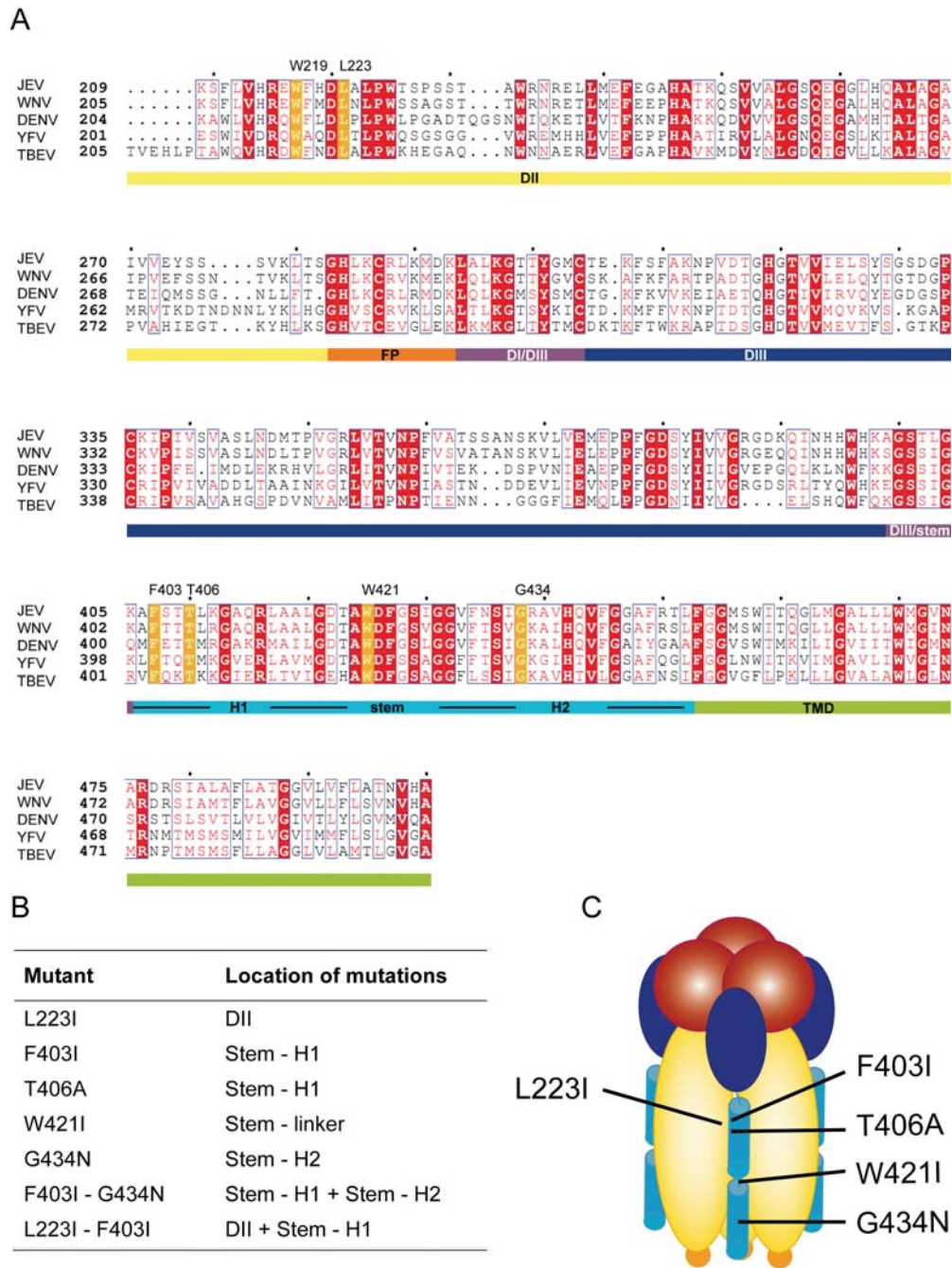


Figure 6.2: E protein sequence alignment and residue substitutions. (A) The amino acid sequences of JEV, WNV, DENV, YFV and TBEV E proteins were aligned. Conserved regions are marked in red. Mutated residues are highlighted in orange and labeled. (B, C) E protein residue substitutions and their position.

Immature TBE virus was applied as a prM standard in the first line of mutant and WT RSP blots. The mutants, which passed the quality control and were subject

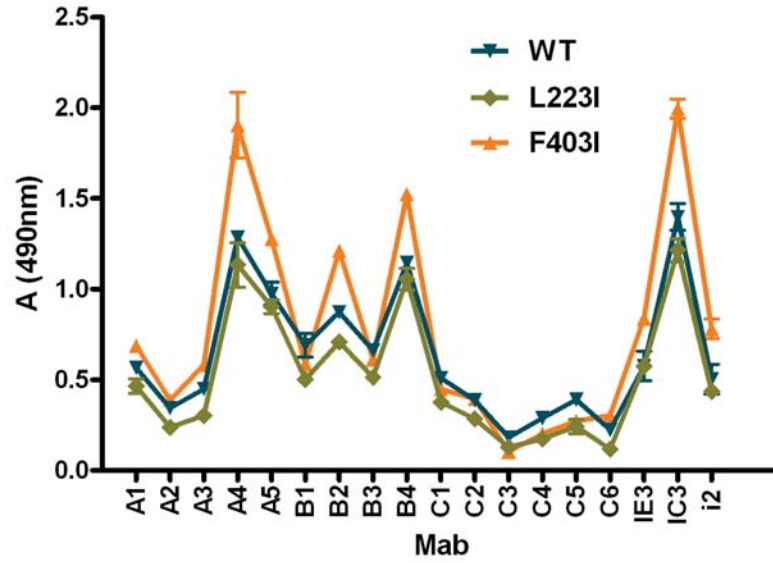


Figure 6.3: Binding profiles of mabs in a four-layer ELISA. Determination of reactivities of E protein specific mabs with WT and mutant RSPs. The absorbance at 490 nm is plotted on the y-axis.

to further investigation are depicted in Figure 6.2 B and C. Substitution of residue W219 (A, V, I, Y, N) completely abolished particle secretion. At position L223, several amino acid exchanges have been performed (A, I, V, Y). Only the mutation to isoleucine resulted in the release of RSPs. The secretion of L223I mutant RSPs was reduced compared to the WT (approximately 20 - 35% of WT), but the secreted particles were mature (Figure 6.4), with WT-like E protein conformation at neutral pH (Figure 6.3).

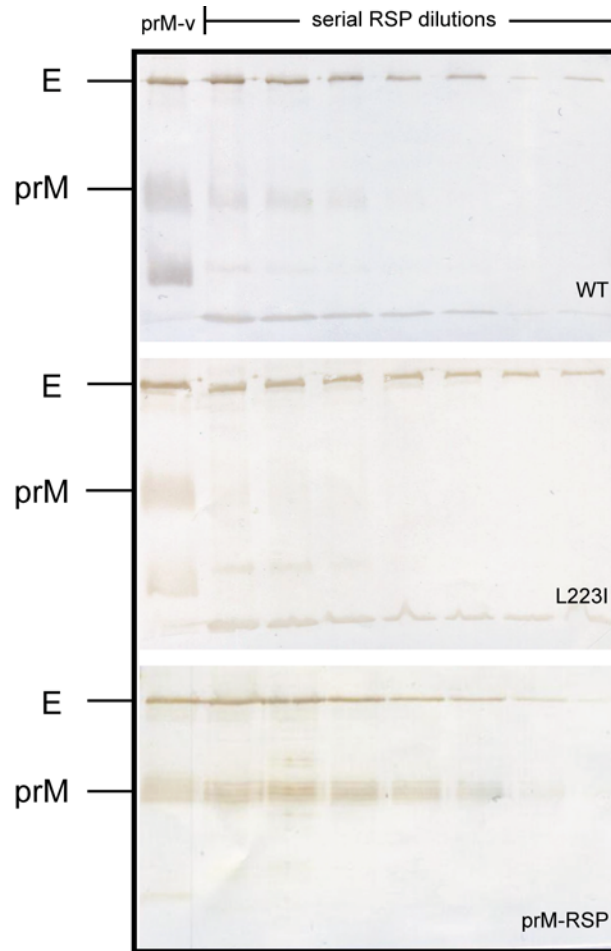


Figure 6.4: RSP maturation state analysis by Western Blotting. The precursor protein prM is present on immature particles. The maturation state (prM content) of RSPs was detected by Western Blot analysis. RSPs were serially diluted in two-fold steps to determine the prM detection limits. Immature virus (prM-v) was applied into the first lane, used as a prM standard. PrM content of mutant RSPs (here L223I, middle blot) was compared to WT RSPs (upper blot) and immature RSPs (lower blot).

6.3.2 Characterization of mutant RSPs

6.3.2.1 Fusion activity of mutant RSPs

To test the effect of the mutations in the stem and domain II on the overall fusion activity, WT and mutant RSPs were metabolically labeled with 1-pyrene hexadecanoic acid and tested in an *in vitro* fusion assay (Figure 6.5). Fluorescence-labeled WT and mutant RSPs were incubated with unlabeled liposomes, exposed to neutral or acidic pH and fluorescence was continuously monitored for 60 seconds

(12). As shown in Figure 6.6 A, at pH 5.4 WT RSP fusion occurred within seconds, whereas the L223I mutant displayed a strong reduction of fusion. All other mutants exhibited the same extent and rate of fusion as WT. Fusion extents are shown in Figure 6.6 B.

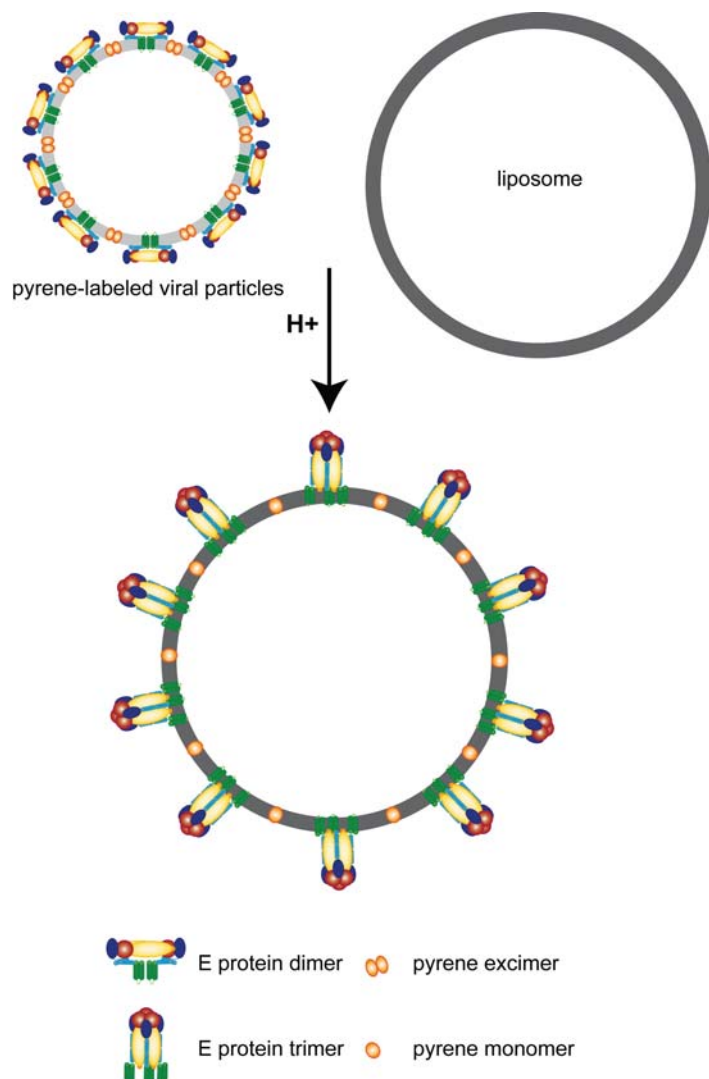


Figure 6.5: Schematic of fusion assay using pyrene-labeled RSPs and unlabeled liposomes. 1-pyrene hexadecanoic acid is a fluorescent dye which integrates into membranes, thus allowing to detect lipid-mixing. In labeled RSPs, pyrene is present as excimers. Upon fusion of the RSP membrane with the liposomal membrane, the fluorescent dye is diluted, resulting in a decrease in the pyrene-excimer fluorescence.

The residue L223 was predicted to be involved in the interaction of domain II with helix 1 of the stem region in the postfusion conformation (9). To determine at which stage the fusion process is interrupted we used different experimental tools

that allowed us to analyze the effect of mutations on the fusion process step by step.

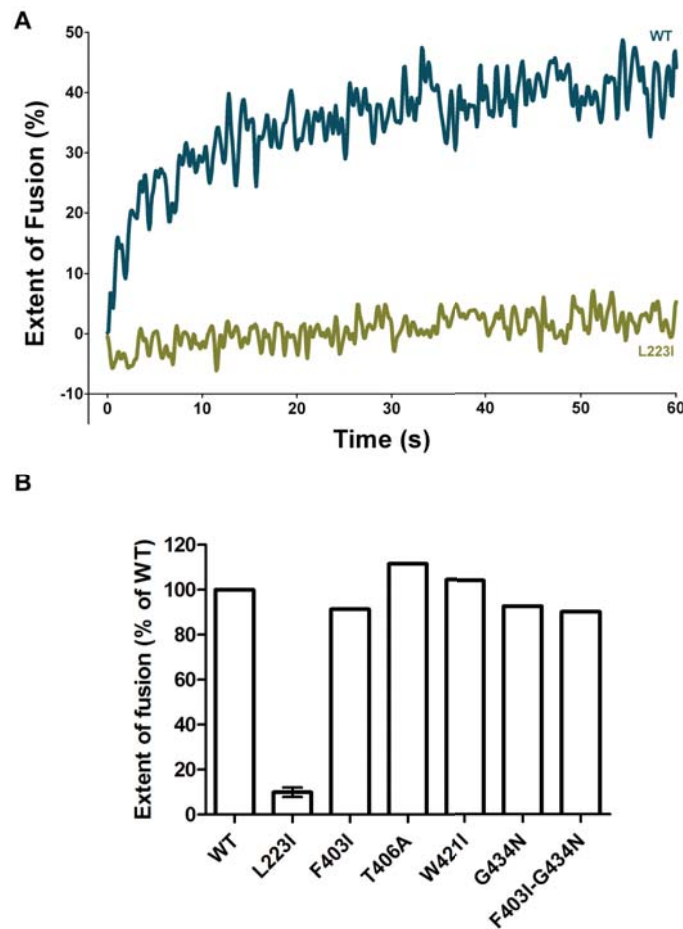


Figure 6.6: Fusion of pyrene-labeled WT and mutant RSPs with liposomes at acidic pH. Pyrene-labeled subviral particles were mixed with liposomes. The decrease in pyrene-excimer-fluorescence after acidification was monitored continuously for 60 s. (A) Fusion curve of WT (blue) and L223I mutant (green) RSP at pH 5.4. (B) Extent of fusion of mutant RSPs compared to WT (100%) after 60 s.

6.3.2.2 Target membrane interaction of mutant RSPs

To investigate the early steps of membrane fusion (dimer dissociation, fusion peptide exposure and interaction with target membranes) we tested all mutant RSPs and the WT in a liposome coflotation assay. With this experiment, we measured the interaction of RSPs with target membranes after exposure to acidic pH, resembling the initial step of the fusion process. Purified RSPs were mixed with liposomes. After exposure to acidic pH, samples were back-neutralized and applied to sucrose step gradients. The appearance of protein E at the top of the gradient, indicating

liposome binding, was determined by a four-layer ELISA (25, 65). As shown in Figure 6.7, acidification of WT and mutant RSPs in the presence of liposomes led to a quantitative association with target membranes. These data indicate that the initiation of membrane fusion was not disturbed by any mutation investigated in this work. This is consistent with the existing fusion model, supporting the idea that stem-zippering occurs after fusion peptide exposure and target membrane interaction (58, 66).

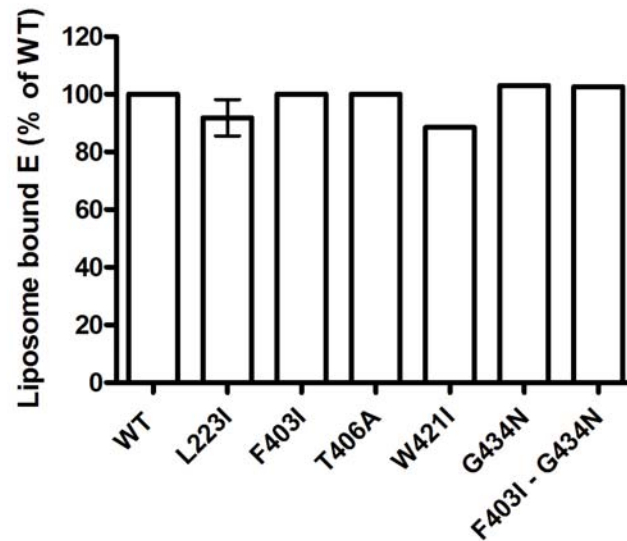


Figure 6.7: Low-pH-induced coflotation of RSPs with liposomes. WT and mutant RSPs were mixed with liposomes and exposed to acidic pH. After back-neutralization the mixture was subjected to sucrose step gradient centrifugation. The gradients were fractionated and the amount of E protein in each fraction was quantified in a four-layer ELISA. The extent of low-pH-induced coflotation of protein E with liposomes is plotted relative to the WT (100%).

6.3.2.3 Low-pH-induced trimerization of E protein mutants

Since the L223I mutation does not influence the initial steps of fusion, the impairment, observed in overall fusion (Figure 6.6 A) has to occur at a later stage of the fusion process, such as the trimer formation of E. To investigate the effect of mutations in E on low-pH-induced trimerization, WT and mutant RSPs were exposed to acidic pH, back-neutralized and solubilized. The oligomeric state of E was analyzed by sedimentation in sucrose gradients (3). At neutral pH, E sedimented as a dimer. All mutants, including the fusion-impaired L223I mutant were able to convert into

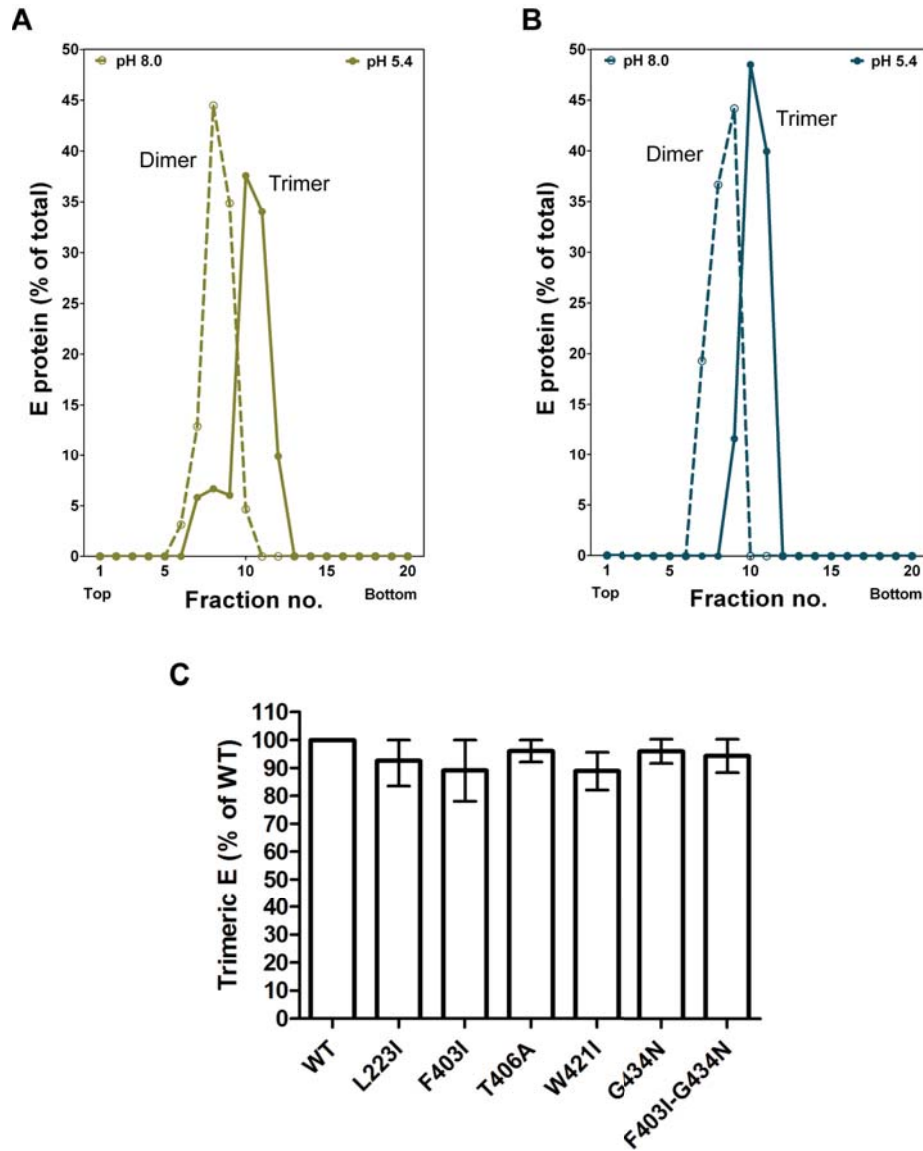


Figure 6.8: Low-pH-induced trimerization of protein E in WT and mutant RSPs. WT and mutant RSPs were exposed to pH 5.4 or pH 8.0, back-neutralized, solubilized and the oligomeric state of the E protein was analyzed by rate zonal sucrose gradient centrifugation. The sedimentation direction is from left to right. At pH 8.0, E sediments as a dimer (dashed lines). In all cases (WT and mutants) quantitative E trimerization was observed at low pH (solid lines). (A) Quantitative E protein trimerization of L223I mutant. (B) Low-pH-induced E protein trimerization of WT RSP. (C) Extent of trimer formation of all mutant RSPs, compared to WT (100%)

trimers at low pH (Figure 6.8). Although previous studies with truncated forms of E have shown that the stem-H1 is required for trimerization in the absence of target membranes (5), the introduction of single and double mutations into the stem in the context of full-length E did not affect this process.

6.3.2.4 Thermostability of E protein mutant trimers

It was shown previously that the stem-anchor region contributes to the thermostability of E protein trimers (69). In order to assess whether the introduced mutations had an effect on the stability of the mutant trimers, we performed thermal denaturation experiments. For this purpose, RSPs were exposed to acidic pH, back-neutralized, solubilized, heated for 10 minutes to 70°C and then subjected to sedimentation analysis. Under these conditions, the sedimentation of two mutants (T406A and W421I) was comparable to the WT and not affected by high temperature (Figure 6.9). In contrast, the four mutants L223I, F403I, G434N and F403I-G434N were partially denatured at this temperature as indicated by a reduced trimer peak and the accumulation of aggregated material in the pellet (Figure 6.9). These findings confirm the important role of the stem in the overall stability of the postfusion trimer, as single residue substitutions can impair the stability of the postfusion trimer.

Interestingly, mutant trimers containing the L223I and F403I substitution showed a higher amount of aggregated E compared to the other mutants with reduced stability (Figure 6.9). These two residues have been suggested to be interaction partners in the trimeric postfusion conformation (9). Bressanelli et al. predicted, that according to their model the stem residue F403 neatly fits into the hydrophobic pocket on the DII surface, formed by W219/L223 in the postfusion structure (9). This intramolecular interaction is predicted to be at the initial contact site of DII and stem-H1 (Figure 6.10), based on the model (Figure 4.12). Replacement of the pocket residue L223 by isoleucine led to reduced thermostability of the trimer and impaired fusion activity (Figure 6.6). Amino acid exchange of the interaction partner in the stem (F403I) also resulted in reduction of trimer stability, but did not affect fusion with liposomes (Figure 6.6 B).

We therefore hypothesized, that the L223I mutation disturbs the interaction between the DII pocket and stem residue F403, as a consequence of an altered pocket geometry or steric hindrance. The bulky phenylalanine (F403) might not fit into the altered DII pocket anymore. The disturbed interaction between the trimer core and

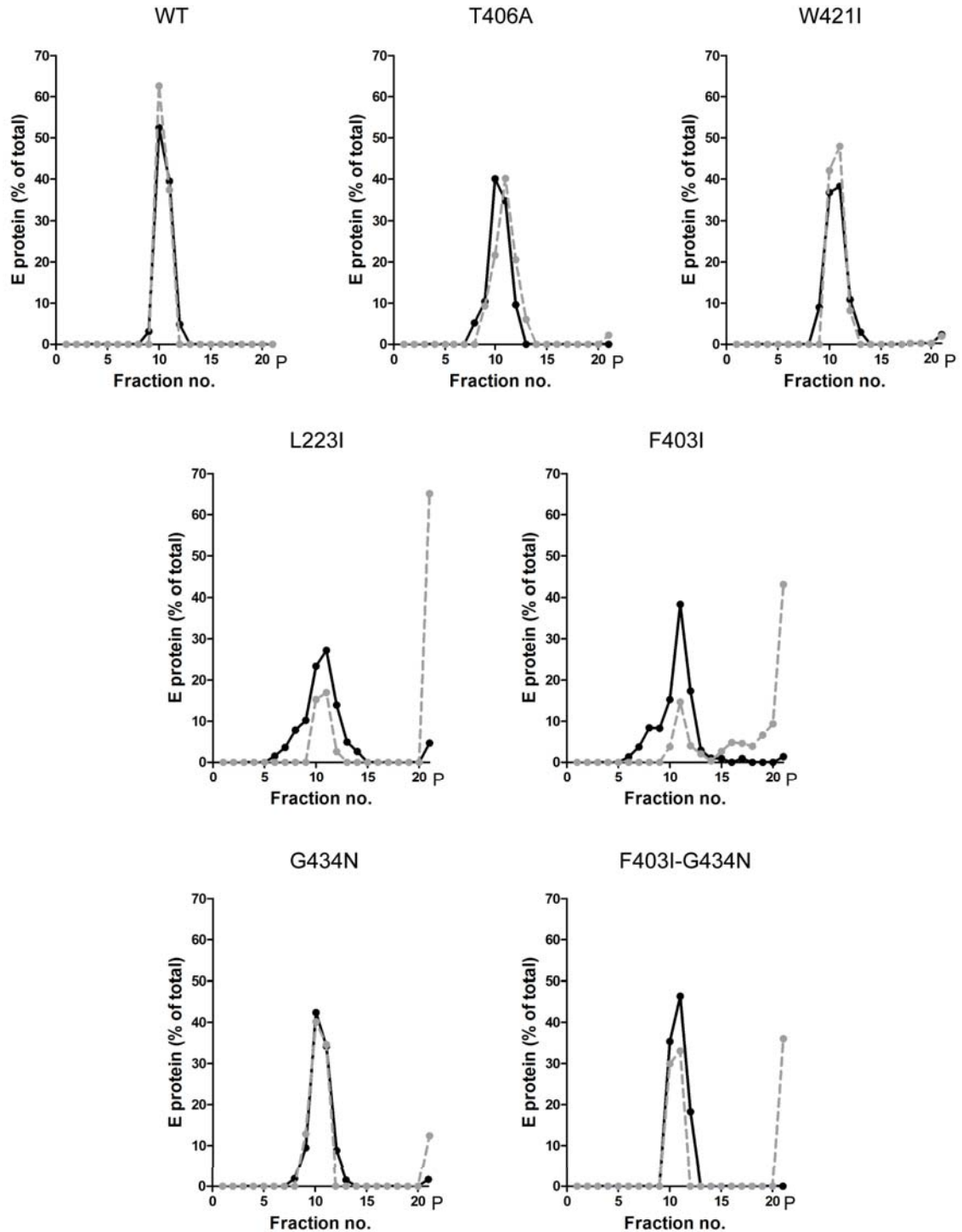


Figure 6.9: Thermostability of WT and mutant protein E trimers. Low-pH-induced and solubilized E trimers were heated to 70 °C for 10 min and subjected to sedimentation analysis. The gradients were fractionated and the amount of E protein in each fraction was determined by a quantitative four-layer ELISA. The sedimentation direction is from left to right. The last fraction represents the pellet (P), resuspended in 0.6 ml buffer, according to a single fraction volume. Trimers incubated at 37 °C are shown in black solid lines. Trimers heated to 70 °C are shown in grey dashed lines.

the N-terminus of the stem might prevent the correct positioning of the stem during zippering. This could lead to the observed impairment in trimer stability and fusion activity. In order to find out if residues L223 and F403 actually interact with each other in the trimer and if this interaction site is important for trimer stability we constructed a double mutant (L223I-F403I), combining the altered DII-pocket (L223I) and a smaller, already characterized substitution of the phenylalanine (F403I). We wanted to test whether deficiencies in trimer stability and fusion, obtained with the L223I mutant, can be compensated by an additional substitution in the stem (F403I). We hypothesized that the smaller, yet hydrophobic isoleucine might allow interaction.

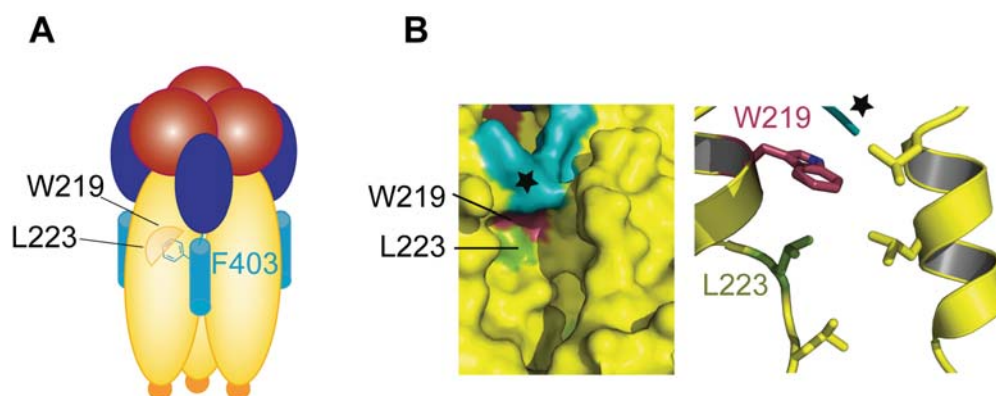


Figure 6.10: Schematic representation and structures of predicted DII/stem interaction site. (A) The residues W219 and L223 form a pocket, predicted to interact with the stem residue F403 (9). (B, left) Surface view of DII pocket. (B, right) Side chains of pocket residues are shown in sticks. Color code: W219, magenta; L223, green (pdb 1URZ). C-terminus of crystallized part is indicated with a black star.

6.3.3 Characterization of double mutant L223I-F403I

We wanted to characterize the constructed mutant in fusion and fusion-related processes and compare it to the L223I single mutant, to find out if there is an interaction between the residues in the trimer and if the additional stem mutation can compensate the reduced trimer stability and fusion deficiency, observed for the L223I mutant. We therefore analyzed the fusion activity of the L223I-F403I mutant with liposomes and compared it to the single mutants. Consistent with the hypothesis, the double mutant exhibited enhanced fusion activity compared to the single mu-

tant L223I, but was still lower than the WT, as shown in Figure 6.11 A. Despite this apparent compensatory effect, the double mutant still displayed less efficient fusion than the WT. This could be due to an impairment of early fusion stages (target membrane interaction) or later steps, such as trimerization and lipid-mixing. In order to find out, if the early stages of the fusion process are affected in the double mutant, we investigated the interaction with target membranes in the coflotation assay. WT and mutant RSPs were mixed with liposomes, acidified and subjected to sedimentation analysis. Target membrane interaction was detected by ELISA (Figure 6.11 B). E protein found in fractions 4 to 9 corresponded to membrane-associated E protein. Like all other mutants investigated in this work, the L223I-F403I mutant coflotated like WT. To investigate low-pH-induced trimer formation and trimer thermostability, E protein trimerization was performed as described before. The mutant RSPs showed complete dimer-to-trimer transition (Figure 6.11 C), but after exposure to 70 °C the trimer peak was reduced and E protein accumulated in the gradient pellet (Figure 6.11 D). In good agreement with the fusion results, the L223I-F403I mutant trimer was more stable than the L223I mutant (Figure 6.11 D). Consistent with our hypothesis, the L223I-F403I double mutant exhibited enhanced fusion activity compared to the single mutant L223I in the *in vitro* fusion assay and intermediate trimer thermostability. Apparently, the double mutation in both DII pocket and stem significantly enhances binding, thereby increasing the thermostability of the trimer and fusion activity of the particle.

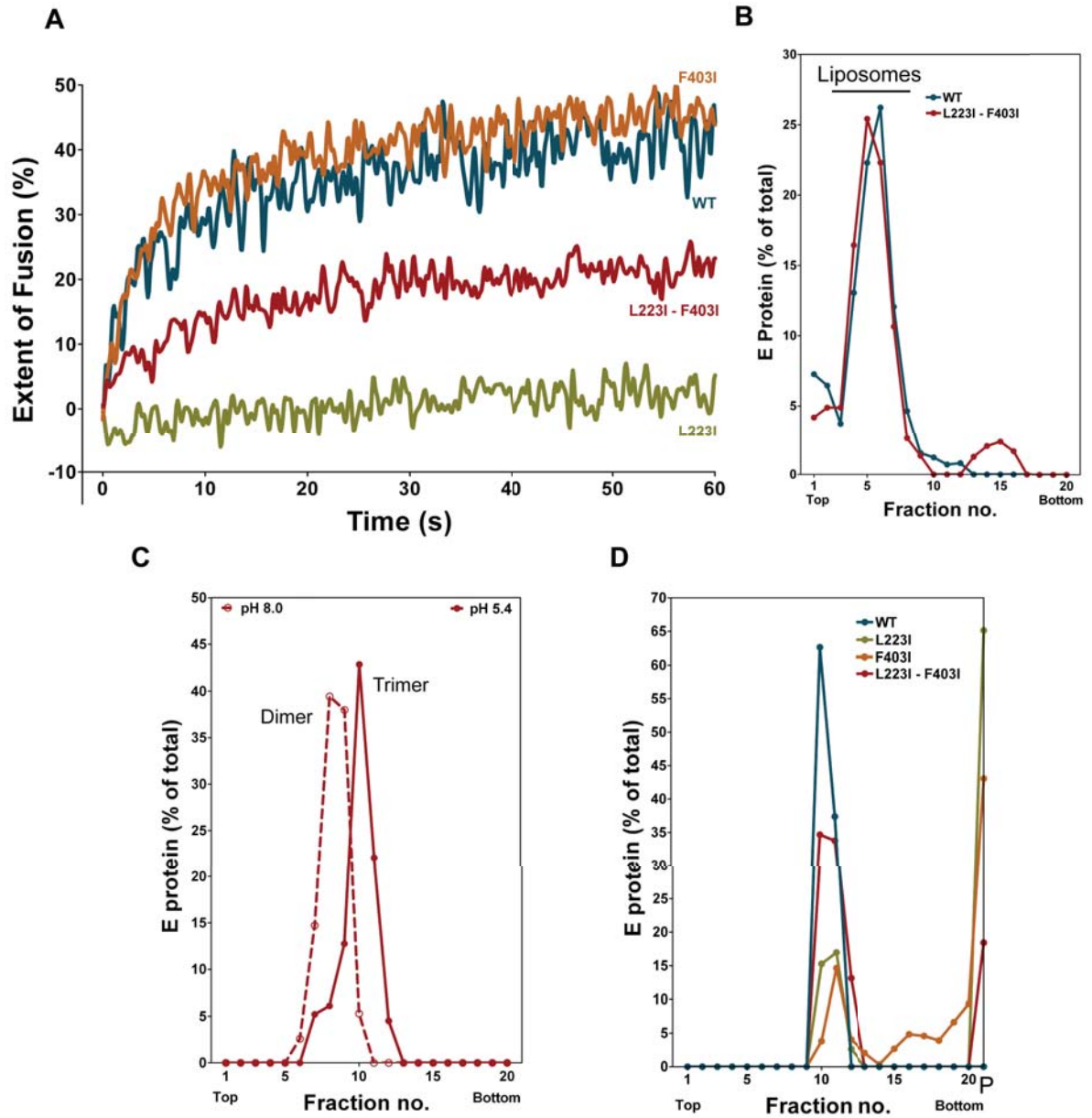


Figure 6.11: Characterization of L223I-F403I mutant RSP. (A) Fusion kinetic curve of L223I-F403I mutant RSP (red) compared to WT (blue) and single mutants L223I (green) and F403I (orange). (B) Acidic-pH-induced coflotation of WT (blue line) and L223I-F403I (red line) mutant RSP. E protein found in fractions 4 to 9 represents RSPs coflotated with liposomes. (C) Sedimentation analysis of low-pH-induced trimer formation of L223I-F403I mutant. The sedimentation direction is from left to right. At neutral pH L223I-F403I mutant E sedimented as a dimer. Quantitative trimer formation was observed at acidic pH. (D) Sedimentation analysis of L223I-F403I E protein trimers, exposed to 70°C in comparison to WT and single mutant trimers.

Taken together, our data provide the first evidence that modifications in domain II and the stem can result in decreased fusion activities. It has been argued, that zippering of the stem along domain II is important to provide the energy for membrane merging (66). Our data imply that structural alterations in the DII/stem interface by single-residue substitutions, lead to decreased trimer thermostability that subsequently provides less energy to drive the fusion process. Consistent with these results, thermal denaturation studies on the class I fusion protein gp41 of HIV showed, that the presence of the membrane-proximal external region (MPER) of gp41, like the stem of class II proteins, also significantly increases trimer stability (10). This is in good agreement with fusion inhibition studies using exogenous domain III + stem-H1 of dengue virus E protein. Dengue virus 2 fusion inhibition was not observed with DIII alone, but in the presence of DIII together with stem-H1 (40). However, for the closely related alphavirus Semliki Forest virus (SFV), DIII alone was sufficient to inhibit membrane fusion (40). Although both proteins are classified as class II viral fusion proteins and are highly similar in structure, the stem of flavivirus E is composed of two α -helices, whereas the alphavirus stem is shorter and rearranges from an unstructured state into an α -helical element only upon trimerization (21). Mutational analysis of SFV E1 stem demonstrated that neither a specific stem residue, nor the conserved length is essential for membrane fusion (41), indicating a difference in the requirement of this structural element between alpha- and flaviviruses.

By interfacial hydrophobicity analysis MPERs of several viral fusion proteins were characterized. Functional studies showed that both, the MPER of gp41 (class I) and VSV G (class III) contribute to the fusion reaction (43), indicating a similar function to the flaviviral E protein stem. The gp41 MPER was shown to contribute to the thermostability of the trimer and the x-ray structure indicates an involvement in membrane interactions during membrane fusion (10). The MPER of class III VSV G can increase the fusion activity of heterologous viral fusion proteins. This region induces lipid-mixing of the outer leaflets (30).

For flaviviruses, it was shown that stem-derived peptides bind to stem-less trimers

and thus trap the structure in a fusion intermediate state (58). Moreover, in a recent study, peptides derived from dengue 2 virus E, containing the proposed domain II pocket sequence, inhibited cell entry (13). Peptides have been investigated as fusion inhibitors and their effects have been described for class I fusion proteins, as well (16). Peptides which bind to intermediate structures of the fusion proteins are used to inhibit viral entry, such as HIV (35). Fusion inhibitors in general display an interesting tool for antiviral therapy, as they could effectively inhibit viral replication at an early stage. Based on the present study, it would be interesting to synthesize and target small molecules to the domain II pocket. A more extensive analysis of the residues involved in the zippering of the stem along DII would allow the development of further strategies to prevent stem-driven trimer stabilization.

A manuscript with the title *The role of the stem region in flavivirus membrane fusion*, describing these data is in preparation.

7 Part II (infectious system)

7.1 Introduction

We used recombinant subviral particles (RSPs) to show, that the interaction between the pocket-forming residues W219 and L223 of DII and F403 of the stem is important for E protein postfusion trimer stabilization and fusion activity. The significance of these findings was further tested using an infectious virus system.

Despite the fact that RSPs are an established model to study viral fusion, there are important differences between RSPs and virions. RSPs are smaller and exhibit a different quaternary structure compared to virions. The major difference is the particle geometry. Whereas on virions 90 E protein dimers are organized in a herringbone-like icosahedral symmetry, on RSPs 30 E protein dimers are arranged in a T=1 icosahedral lattice (19). RSPs have a higher curvature and E protein dimers are less densely packed on the particle surface (34). Fusion of RSPs with liposomes is a very elegant, yet simplified method. Virus infection is a complex process including different environmental factors, which cannot be employed in the RSP system. TBEV enters the cell by receptor-mediated endocytosis followed by fusion from within the early endosome (42). Fusion-impaired virions, which are not able to completely fuse with the membrane of early endosomes, might be trafficked to late endosomes with different environmental parameters, such as a lower pH and different lipid compositions (77).

Infectious cDNA clones of TBEV provide the possibility to introduce specific mutations into the viral genome and investigate the effect of these mutations on virus infectivity and other processes of the viral life cycle. The flavivirus genome of strain Neudoerfl was stably inserted as cDNA into a plasmid vector suitable for bacterial expression (44). A full-length TBEV cDNA clone and two shorter plasmids, carrying either the 5' or 3' region of the TBEV genome allow the recombinant introduction of mutations into the TBEV genome. By site-directed mutagenesis, mutations can be engineered into the smaller partial clones and then transferred into the full-length

clone by restriction and ligation. Stable, full-length cDNA clones can then be transcribed to viral RNA *in vitro*. It has been shown, that WT TBEV RNA transcripts from the cDNA clone are infectious and induce the production of infectious progenies, when transfected into suitable cells. Virus obtained from cDNA clones has been characterized according to its antigenic structure, growth and virulence, and did not reveal any difference compared to the parental virus strain (44). Using this approach, infectivity of mutant viruses were analyzed and compared to the data obtained for RSPs.

7.2 Materials and Methods

7.2.1 Cells and virus

BHK-21 cells (ATCC CCL10) were grown at 37°C in 5% CO₂ in Eagle's minimal essential medium, supplemented with 5% fetal calf serum (FCS), 1% glutamine and 0.5% neomycin (growth medium) and maintained in Eagle's minimal essential medium, supplemented with 1% FCS, 1% glutamine, 0.5% neomycin and 15 mM HEPES (maintenance medium). For all experiments, the western subtype TBE virus prototypic strain Neudoerfl (GenBank accession number U27495) (44) was used as a template for mutagenesis and wild-type (WT) control.

7.2.2 Mutant construction

Table 7.1: Oligonucleotide sequences.
F = forward primer, R = reverse primer.

Oligonucleotide name	Sequence ^a
W219A-F	CT TGG CAG GTC CAT AGG GAC GCG TTC AAT GAT C
W219A-R	GTC CCT ATG GAC CTG CCA AGC CGT TGG AAG
L223A-F	CA TAG GGA CTG GTT CAA TGAT GCG GCT CTG CCA T
L223A-R	ATC ATT GAA CCA GTC CCT ATG GAC CTG CC
L223I-F	CAT AGG GAC TGG TTC AAT GAT ATA GCT CTG CCA T
L223I-R	ATC ATT GAA CCA GTC CCT ATG GAC CTG CC
F403I-F	GG AGC AGC ATC GGA AGG GTT ATC CAA AAG ACC
F403I-R	AAC CCT TCC GAT GCT GCT CCC TTT TTG G

^a Nucleotides were synthesized by VBC BIOTECH.

All plasmids used for cloning were derived from cDNA of the TBEV strain Neudoerfl. Mutations in the structural protein E of TBEV were first engineered into

the plasmid pTNd/5', containing the 5' part of the viral genome in a pBR322 plasmid vector. The mutated insert was cut and transferred into the plasmid pTNd/c, carrying a full-length cDNA insert of TBEV. Mutagenesis was performed with the Gene Tailor System (Invitrogen).

Briefly, the template plasmid pTNd/5' was methylated and mutations were introduced by site-directed mutagenesis. Mutagenesis primers are listed in table 7.1. The mutations were transferred into the full-length cDNA clone pTNd/c using the restriction sites SalI and SnaBI (for substitutions at positions 219 and 223) or SnaBI and ClaI (for substitution at position 403) (44). All plasmids were amplified in the *Escherichia coli* strain HB101 and plasmid preparations were made by the use of the Qiagen purification system. The constructs were checked by sequence analysis with an automated DNA sequencing system (PE Applied Biosystems, GA3100) (37).

7.2.3 RNA transcription

The engineered pTNd/c plasmids were used as templates for *in vitro* transcription with the T7 Megascript Kit (Ambion) according to manufacturer's protocol. The template DNA was digested by incubation with DNase I. RNA was purified by the use of the RNeasy Mini Kit (Qiagen) and quantified by spectrophotometric measurement (37).

7.2.4 Virus stock production in BHK-21 cells

For production of virus stocks, equal amounts of RNA were electroporated into BHK-21 cells with a Gene Pulser (Bio-Rad) applying previously described settings (17). Virus stocks were harvested from cell culture supernatant 48 hours post transfection, cleared by centrifugation at 10000 rpm for 20 min at 4°C in a Beckman JA-12 rotor and stored at -80°C. For sequence analysis, RNA was isolated from the stock and the region encoding for the structural proteins was checked after reverse transcription with a cDNA synthesis system (Roche Applied Science) (37).

7.2.5 Immunofluorescence

Expression of viral proteins was determined by immunofluorescence staining with a polyclonal serum, predominantly recognizing viral E protein. BHK-21 cells transfected with viral RNA were disseminated into 24-well plates, containing microscope coverslips. Transfection efficiency and protein expression was detected 1 day post electroporation. Cells were permeabilized by acetone-methanol (1:1) fixation and serially incubated with a rabbit polyclonal anti-TBEV and a fluorescein-isothiocyanate-conjugated anti-rabbit antibody (Jackson Immune Research laboratory) (59).

7.2.6 Focus assay

Infectious titers of viral stocks were determined by infection of BHK-21 cells with serial dilutions of virus in maintenance medium. Confluent monolayers of BHK cells were incubated for 3 hours with virus dilutions. Virus was removed and cells were covered with 3% carboxymethyl-cellulose-overlay in maintenance medium. Fifty-three hours post infection the overlay was removed and cells were fixed with acetone-methanol (1:1). Cells were further treated by successive incubations with a polyclonal rabbit anti-TBEV serum and with goat anti-rabbit IgG-alkaline phosphatase. SigmaFast Red TR/Naphtol AS-MX tablets were used as substrate to visualize the foci (59).

7.2.7 Quantification of E protein secretion

The concentration of E protein in virus stock preparations was determined by a quantitative four-layer ELISA. Microtiter plates (Nunc) were coated with 2.5 µg/ml anti-TBEV IgG (guinea pig) for at least 48 hours at 4°C. The samples and the TBEV standard were solubilized with 0.4% sodium dodecyl sulphate (SDS), serially diluted and transferred onto the coated plates. A polyclonal rabbit anti-TBEV serum was used as a second antibody and a horseradish peroxidase-linked donkey anti-rabbit IgG served as a detection antibody. The reaction with the substrate

orthophenyldimine/H₂O₂ was stopped by the addition of 2N H₂SO₄. The optical density was measured with an ELISA reader. Wells lacking the antigen served as blank (25).

For particle quantification, 3 μ g total E in virus stock were applied to 5-30% (wt/wt) sucrose gradients and centrifuged for 70 min at 38000 rpm at 4°C in a Beckman SW 40 rotor. Fractions of 600 μ l were taken by upward displacement and the E protein in each fraction was determined again in a four-layer ELISA (described above).

7.2.8 RNA isolation and quantification

Cell culture supernatants of primary transfected cells were cleared 48 hours post-transfection and RNA was isolated using a QIAamp vial RNA Mini kit (QIAGEN), according to manufacturers protocol (37). RNA was directly transcribed into cDNA by the use of the iScriptcDNA Synthesis Kit (BioRad), according to the supplied protocol. The cDNA was quantified under real-time PCR conditions (PE applied Biosystems). Serial dilutions of an already quantified and *in vitro* transcribed RNA were used for standard curve generation.

7.2.9 Liposomes

Liposomes, composed of phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL), phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL) and cholesterol (SIGMA) in a 1:1:2 molar ratio in chloroform were dried to a thin film with a rotary evaporator in a high vacuum. The lipid film was hydrated in liposome buffer (10 mM triethanolamine, 140 mM NaCl, pH 8.0) and subjected to 5 cycles of freeze and thawing. Liposomes were extruded (21 passes) through two 200 nm polycarbonate membranes (pore size: 200 nm) by the use of a Liposofast syringe type extruder (Avestin, Ottawa, Canada) (68). For the content-mixing assay, liposomes, containing trypsin were produced by the use of hydration buffer supplemented with trypsin (6000U/liposome production) (SIGMA).

7.2.10 Production of pyrene-labeled viruses

Metabolically labeled WT and mutant TBE virus preparations were produced by infection of primary chicken embryo cells with WT or mutant virus stock from BHK-21 cells. Cells were grown and maintained in medium, supplemented with 1-pyrene hexadecanoic acid. Characterized viral stocks, produced in BHK-21 cells were used for infection. Virus was harvested 48 hours post infection and purified by two cycles of sucrose density gradient centrifugation (12).

7.2.11 Quality control of pyrene-labeled viruses

The maturation state of virus preparations was analyzed with two mabs, B4 and 8H1, which are directed to E protein and prM, respectively. Microtiter plates (nunc) were coated with 2.5 μ g/ml anti-TBEV IgG (guinea pig) for at least 48 hours at 4°C. The samples and in addition immature and mature TBEV preparations, which were used as controls were put onto the coated plates in a 0.5 μ g/ml concentration. Mabs B4 and 8H1 in serial dilutions were used as second antibodies and a horseradish peroxidase-linked rabbit anti-mouse IgG was used for detection with the substrate orthophenyldimine/H₂O₂. The reaction was stopped by the addition of 2N H₂SO₄. The optical density was measured with an ELISA reader. Wells with antigen, but lacking the mab served as blank.

7.2.12 Virus fusion with plasma membrane

Plates with confluent monolayers of BHK-21 cells containing coverslips, were washed with cold binding medium (TCM Hank's medium, supplemented with 0.5% neomycin, 1% glutamine and 20 mM NH₄Cl, pH 7.5). TBEV (1 μ g E protein/well) was diluted in binding medium and bound to BHK-21 cells for 100 min on ice. Unbound virus was removed by washing with binding medium and fusion was induced by exposure to acidic pH for 2 min/37°C using binding medium, supplemented with MES. Subsequently, cells were incubated for 3 hours in MEM, supplemented with 2% FCS and 50 mM NH₄Cl. For further 24 hours, the NH₄Cl concentration was

reduced to 20 mM (adjusted from (40)). Viral protein expression was detected by immunofluorescence staining as described above.

7.2.13 Content-mixing assay

Virus fusion with liposomes, containing trypsin was detected by Western Blot. TBEV (1 μ g E protein) was mixed with 2 mM of trypsin-containing liposomes in liposome buffer. The reaction volume of 240 μ l was divided into 3 equal aliquots (acidic pH, neutral pH, solubilized). The samples were adjusted by the addition of MES, TAN and Triton X-100 respectively, and incubated for 5 min at 37°C. After neutralization with TEA the mixture was incubated for 75 min at 37°C for trypsin digestion. The reaction was stopped by the addition of 5x Laemmli sample buffer and heated to 95°C for 5 min before applied to SDS-PAGE (adjusted from (51)). The integrity of viral capsid protein was analyzed by immunoblotting after applying 20 μ l of sample onto SDS-PAGE under denaturing conditions. The separated protein bands were transferred to a polyvinylidene difluoride membrane with a Bio-Rad Trans-Blot semidry transfer apparatus. Proteins were detected by consecutive incubation with a rabbit anti-TBEV C protein serum, directed against the capsid protein and anti rabbit immunoglobulin G-alkaline peroxidase (Amersham).

7.3 Results and Discussion

7.3.1 Generation of E protein (L223I and L223I-F403I) mutants of TBE virus

By the use of recombinant subviral particles of TBE virus, we were able to identify an interaction site between the stem and DII. We generated E protein mutants which exhibited a reduction in E trimer thermostability and were strongly impaired in fusion activity, as detected in an *in vitro* fusion assay with liposomes. Since RSPs are capsidless, noninfectious particles, they do not allow the examination of particle infectivity. To investigate the effect of mutations on virus infectivity and fusion, we engineered the respective mutations (L223I and L223I-F403I) into the TBE virus genome, by site-directed mutagenesis. The presence of the mutations was confirmed by sequencing. Viral full-length RNAs were transcribed *in vitro* and quantified. Equal amounts of viral RNA were introduced into BHK-21 cells by electroporation. Cell culture supernatants after primary transfection were harvested 48h post electroporation and clarified by centrifugation.

7.3.2 Characterization of L223I and L223I-F403I mutant viruses

To confirm the genomic sequence of the virus in the cell culture supernatants after transfection, RNA was isolated and used as a template for cDNA synthesis and sequence analysis. The entire structural-protein-region was checked by sequencing of the cDNA to exclude additional mutations. Apart from the introduced mutations, no additional mutations were found. The cell culture supernatants were further used to characterize mutant virus infectivity.

7.3.2.1 Infectious properties of virus mutants

The RSP mutants L223I and L223I-F403I exhibited reduced fusion activity. Since fusion is a prerequisite for infection, we aimed to test whether the mutations that impair fusion of RSPs, have an inhibitory effect on infection, when introduced into viral particles. We thus transfected BHK-21 cells with mutant viral RNA and subsequently measured the quantity of intracellular viral proteins by immunostaining

(Figure 7.1, upper panel). Cells transfected with mutant RNAs exhibited similar immunofluorescence signals in comparison to cells transfected with WT TBEV RNA. Detection of intracellular viral protein is dependent on RNA replication and protein translation (36). Thus, mutant and WT RNAs were transfected into cells with comparable efficiencies and were competent to perform RNA replication and protein translation.

To see whether the transfected cells produced infectious progenies, the supernatants were transferred onto fresh cells and investigated by immunostaining 24 hours post infection. As shown in Figure 7.1 (lower panel), both mutants revealed a positive immunofluorescence staining, indicating that both mutant viruses (L223I and L223I-F403I) are infectious. To exclude the possibility of additional mutations that might account for the infectious phenotype, RNA was isolated from the supernatant of infected cells (1. passage) and sequenced after reverse transcription. No additional mutations were found.

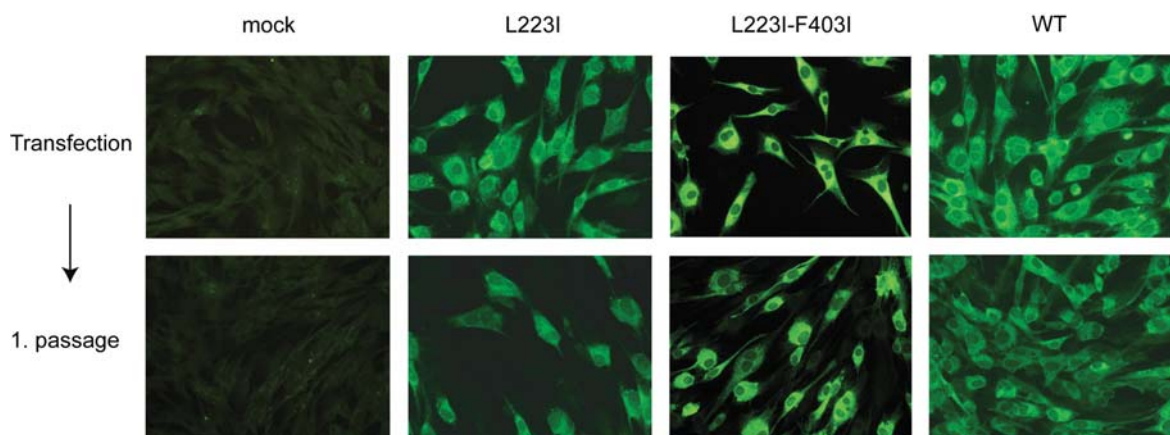


Figure 7.1: Immunofluorescence analysis. E protein expression was visualized in transfected and infected BHK-21 cells by immunofluorescence staining.

The same supernatants, investigated by immunofluorescence after primary transfection, were analyzed by focus forming assays (described in Materials and Methods) to determine the infectious titers of mutant and WT viruses. The infectious units are shown in Figure 7.2. Both mutant viruses exhibited similar infectious titers as the corresponding WT production. The titer of the L223I mutant virus, in the supernatant of transfected cells was slightly lower compared to WT. These analyses

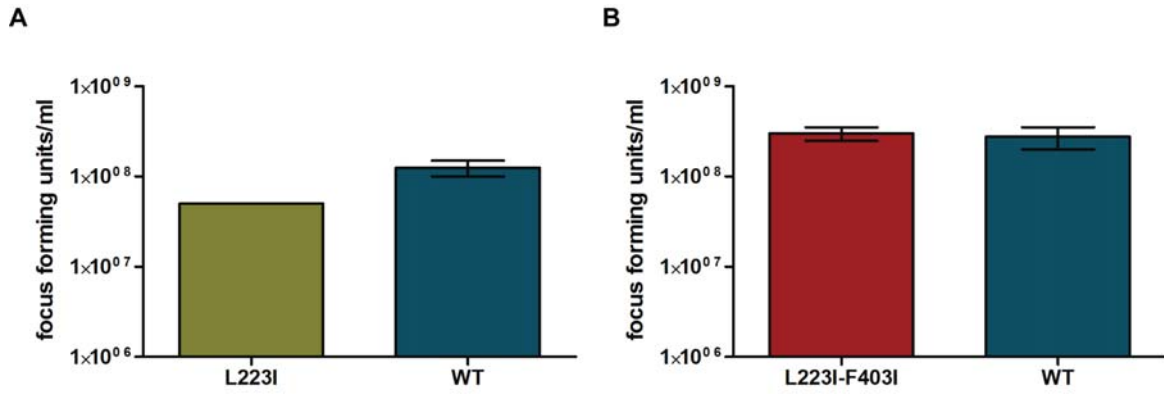


Figure 7.2: Infectivity of supernatants of primary transfected cells. The infectious units were determined in a focus assay.

revealed, that cells transfected with RNA were able to produce infectious progenies.

7.3.2.2 Specific infectivities of virus mutants

In order to directly compare infectious properties of mutant and WT viruses, it was necessary to determine their specific infectivities, by quantifying the number of viral particles and relating these to the respective virus titers (Figure 7.2). We used two different approaches to quantify viral particles.

E protein quantification

The first approach to quantify viral particles was based on E protein quantification by an E protein-specific four-layer SDS-ELISA. The E protein concentrations in the supernatants of cells transfected with mutant RNA are shown in Figure 7.3 A, B.

E protein could be present in particulate and soluble form. In order to quantify viral particles, it was necessary to assess the proportion of soluble and particle-associated E protein in the cell culture supernatants after transfection, by rate gradient centrifugation. For that purpose, equal amounts of total E protein were subjected to a continuous sucrose gradient centrifugation and fractionated. The amount of E protein in each fraction was quantified by ELISA. Two pairs of mutant and WT preparations had sufficient E protein concentrations to be subjected to sedimentation analysis (Figure 7.4 A and B). In all investigated cell culture supernatants, a large

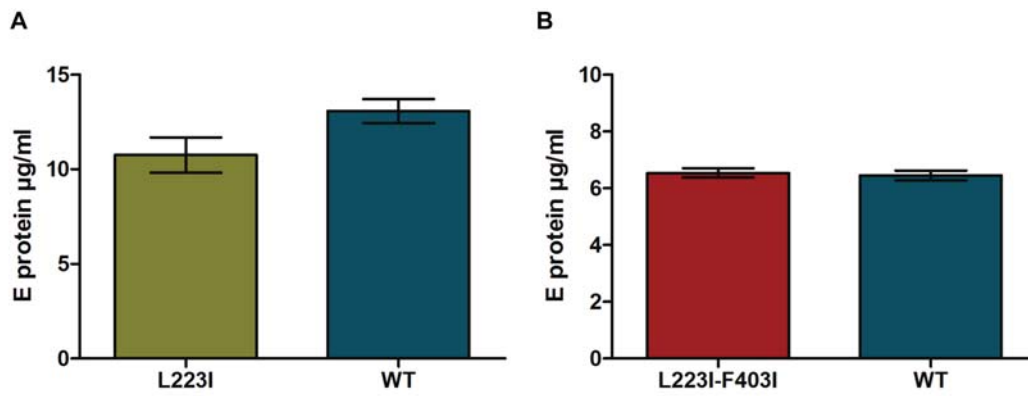


Figure 7.3: E protein concentration in supernatants of cells transfected with viral RNA. The amount of E protein was determined in a quantitative four-layer ELISA.

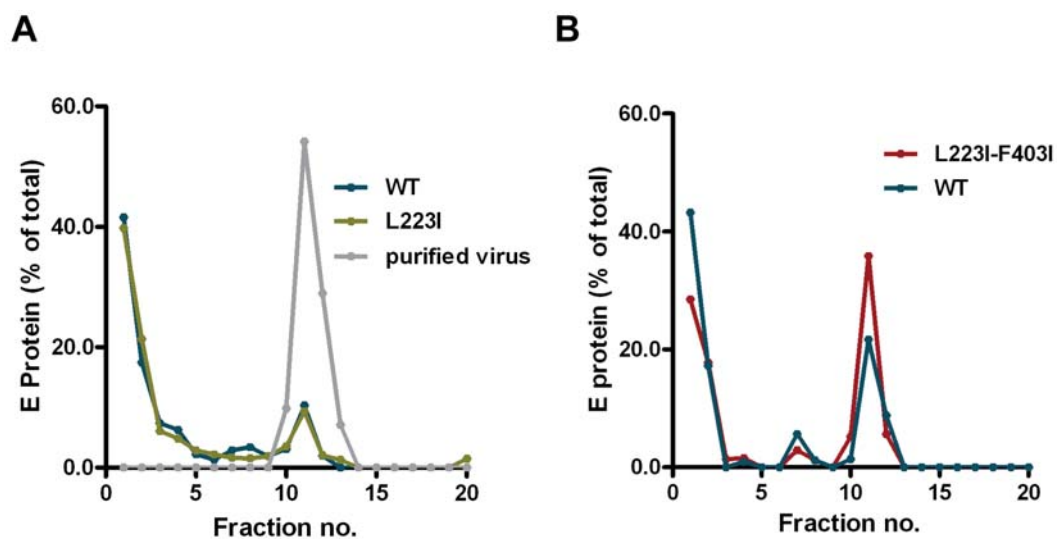


Figure 7.4: Analysis of E protein distribution in cell culture supernatants of cells transfected with viral RNA. 3 μg total E protein was subjected to sedimentation analysis.

proportion of secreted E protein was not incorporated into viral particles and was thus detected in the upper fractions. Most likely these fractions represent soluble and lipid-associated E protein. E protein associated with viral particles was detected in smaller amounts in fractions 10 - 14, where it co-localizes with the control sample of purified virus. A small peak in fractions 7 and 8 most probably represents sub-viral particles secreted from transfected cells. Mutant L223I showed a distribution pattern similar to WT (Figure 7.4 A). The E protein distribution pattern of the L223I-F403I mutant indicated a slightly higher proportion of particulate E protein

compared to WT (Figure 7.4 B). Since the E protein concentration was not high enough in all cell culture supernatants to be analyzed by gradient centrifugation, we used an additional approach to quantify viral particles.

Quantification of viral RNA

An alternative way to determine specific infectivity is to quantify viral RNA, which is incorporated into viral particles, present in the supernatant of transfected cells. For this purpose, RNA was isolated from such supernatants and detected by quantitative PCR (qPCR). RNA copies/ml of both mutants and the corresponding WT are shown in Figure 7.5. The amount of RNA copies in the supernatant of transfected cells was similar for both mutant viruses and WT virus, indicating that similar amounts of viral particles were present.

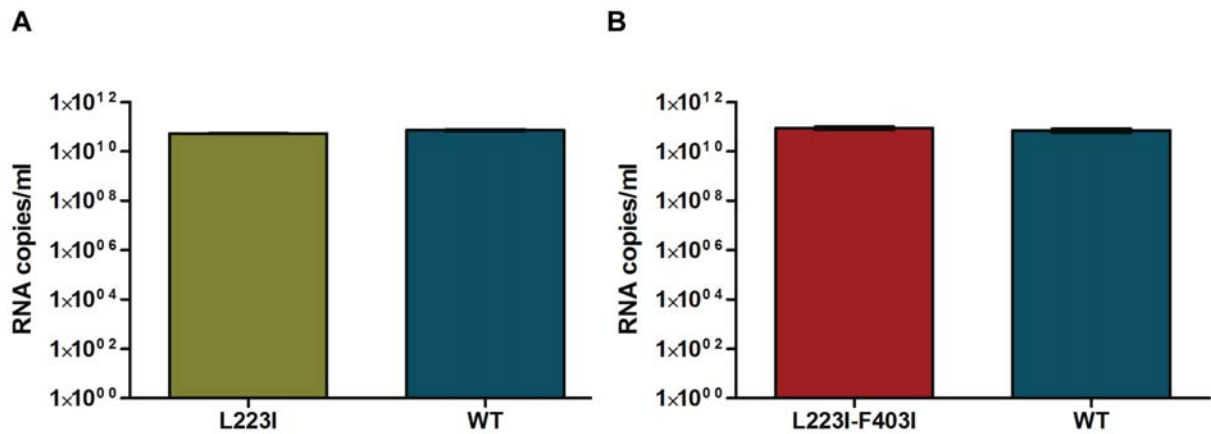


Figure 7.5: Quantification of viral particles in the supernatant of primary transfected cells. RNA copies were quantified by quantitative PCR analysis.

To determine specific infectivities, the values obtained from particle quantification were put into relation to the infectivity titers (ffu) of the same supernatants, determined by focus forming assays (Figure 7.2). In Figure 7.6, specific infectivities of the mutants are depicted relative to the corresponding WT preparation. Figure 7.6 A shows the results deduced from particle-associated E protein measurements and Figure 7.6 B those from determinations of RNA.

With both approaches, the L223I mutant virus exhibited a reduced specific infec-

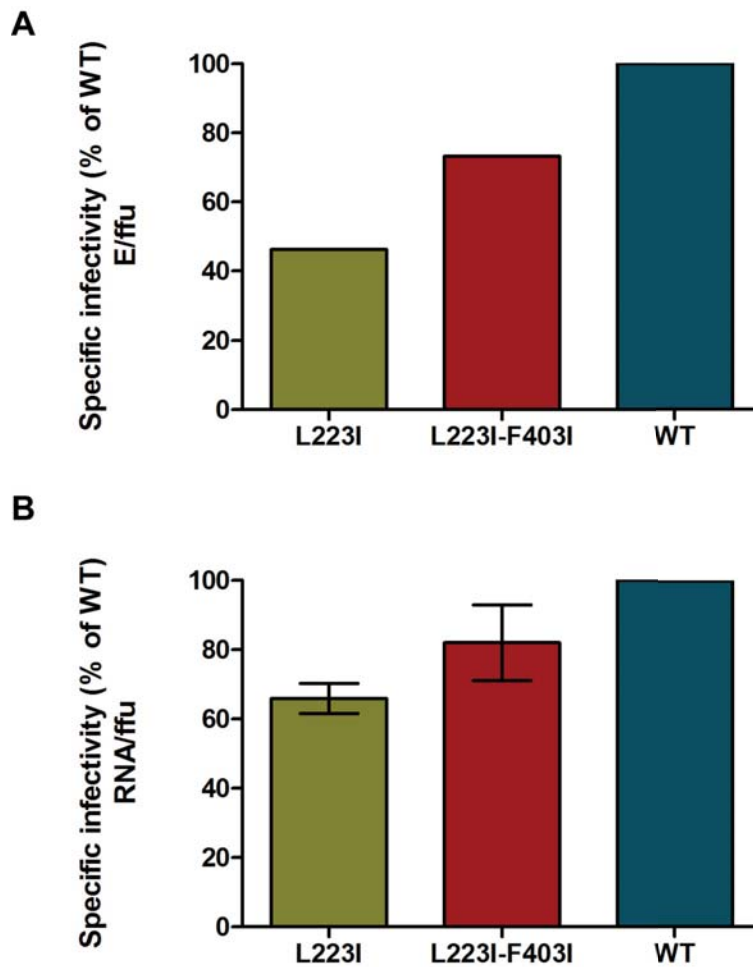


Figure 7.6: Specific infectivity. The specific infectivities of mutant viruses are depicted relative to the WT, produced in parallel. (A) Calculation of specific infectivities based on the ratio of particle-associated E protein per focus forming unit (results of a single experiment). (B) Specific infectivities based on the ratio of RNA copies per focus forming unit (mean of 3 independent experiments).

tivity relative to the WT. However, the observed difference in infectivity was lower than expected, given that the same mutation in the RSPs almost completely abolished fusion activity. A possible explanation could be that mutations affected fusion activity of the virus much less dramatically than that of the RSP, perhaps due to differences in particle geometry of RSPs and virions. Therefore, it would be important to investigate in which way these mutations affected virus fusion in the same *in vitro* fusion assay performed with RSPs.

7.3.2.3 Production and quality control of labeled viruses

To investigate the corresponding fusion characteristics of viruses carrying mutations with an effect on RSP fusion, pyrene-labeled virions were produced in chicken embryo fibroblasts as described previously (12, 24). Supernatants of transfected BHK-21 cells were used for infection of pyrene-labeled chicken embryo cells. In order to determine the maturation state (prM content) of the virus preparations, we tested their reactivity with serial dilutions of two mabs (B4 and 8H1) in a four-layer ELISA. Mab B4 is directed to E protein DIII, whereas mab 8H1 reacts with the prM protein, which is present on immature particles. As controls, well-characterized mature and immature virions were used (Figure 7.7 A). The DIII specific mab exhibited a higher reactivity with mature virions than the prM-specific mab 8H1, whereas the binding pattern was reversed with immature particles (Figure 7.7 A). The pyrene-labeled WT and mutant virus preparations displayed a binding profile more similar to the immature than the mature virus control (Figure 7.7 B). Since the presence of uncleaved prM affects fusion, no conclusive data could be obtained with these preparations. The reason for the production of immature particles has to be investigated and further attempts will be made to produce mature, pyrene-labeled mutant viruses.

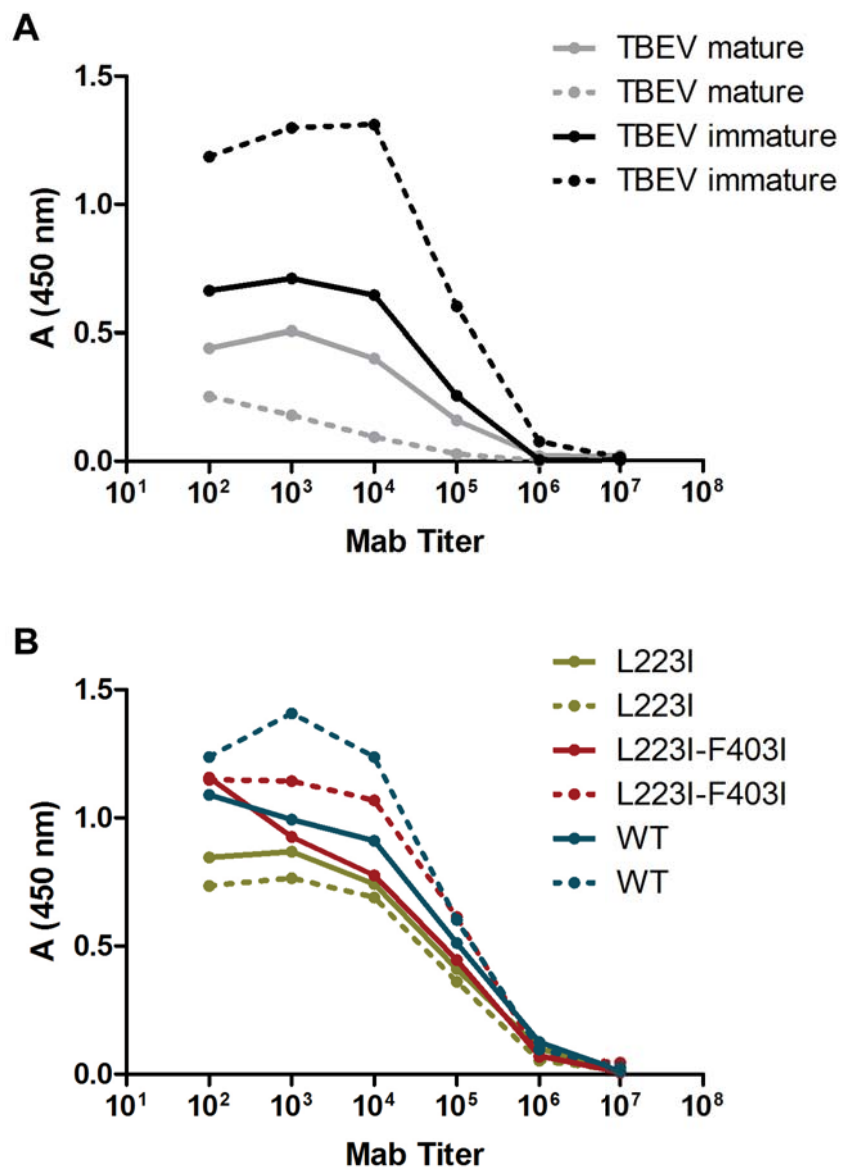


Figure 7.7: Maturation state analysis of pyrene-labeled virus preparations. The reactivity of two mabs B4 and 8H1, directed to E protein and prM, respectively in serial dilutions with pyrene-labeled virus productions was determined in a four-layer ELISA. (A) Binding profiles of mabs with mature and immature virus controls. (B) Reactivities of mabs with pyrene-labeled virus preparations. The absorbance at 450 nm is plotted on the y-axis. Binding profile of E protein directed mab B4 is shown in solid lines. Binding profile of prM directed mab 8H1 is drawn in dashed lines.

7.3.3 Generation of E protein (W219A and L223A) mutants of TBE virus

Utilizing the RSP system, we were able to identify an interaction between residues L223 in DII and F403 in the stem in the trimeric postfusion conformation. Together with W219, L223 forms a hydrophobic pocket, into which the residue F403 fits (Figure 6.10). The amino acid exchange L223I was the only replacement of leucine 223, which resulted in the release of mature and properly folded RSPs. In the case of residue W219, all introduced substitutions of residue W219 (A, V, I, Y, N) completely abolished particle secretion. The RSP system thus did not allow the investigation of other mutations at this site. However, it has been shown, that mutations, that abolished particle secretion in the RSP system, allowed the generation of infectious virions (20, 50). Moreover, the infectious virus system provides the possibility of the occurrence of resuscitating mutations that might appear during mutant virus passaging. The location of such compensatory mutations could provide additional information about stem-trimer core interactions.

Therefore, additional pocket substitutions were introduced into the infectious clone of TBEV (W219A and L223A) and the mutants were characterized with respect to their infectivity and genetic stability.

7.3.4 Characterization of W219A and L223A mutant viruses

7.3.4.1 Infectious properties of virus mutants

BHK-21 cells, transfected with viral RNA were analyzed by immunofluorescence staining 24 hours post electroporation (Figure 7.8). Positive signals in immunofluorescence staining were comparable for both, mutant and WT viruses, indicating a similar RNA replication and viral protein expression level (Figure 7.8, upper panel).

In order to investigate whether the transfected cells released infectious virions, the supernatants were harvested and transferred onto fresh BHK-21 cells (Figure 7.8, middle panel) and analyzed by immunofluorescence 24 hours post infection. Transfer of cell culture supernatant from W219A transfected cells did not result in infection, indicated by the negative immunofluorescence staining. Infection with the

supernatant of cells, transfected with L223A viral RNA was dramatically reduced compared to the WT.

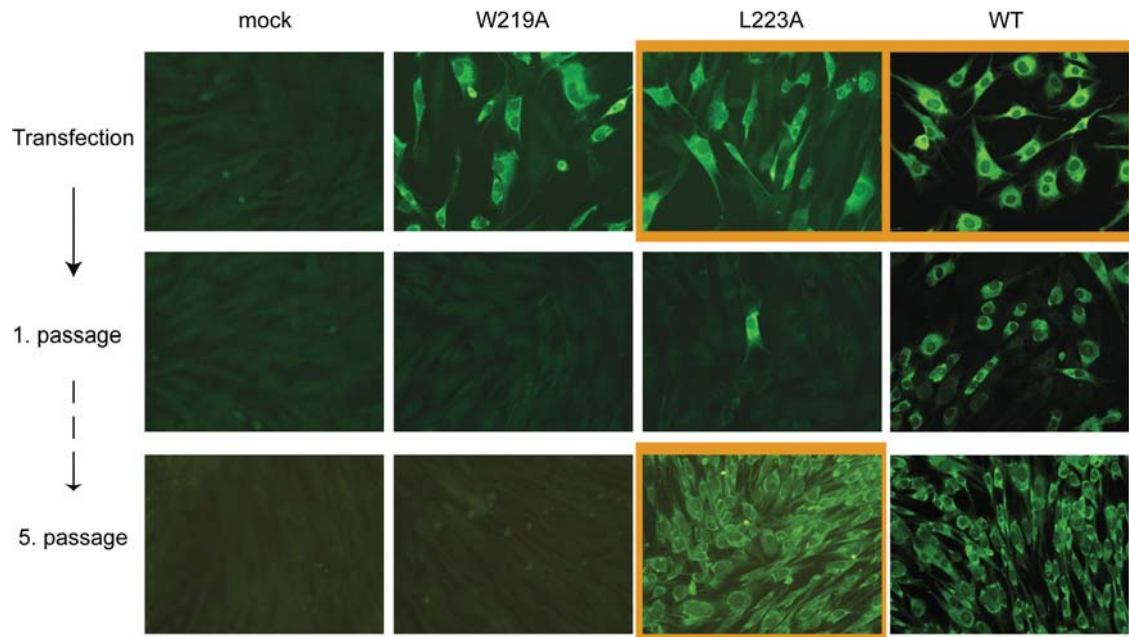


Figure 7.8: Immunofluorescence analysis. E protein expression and virus infectivity was visualized by immunofluorescence staining. Supernatants, which were subject of further analyses are highlighted in orange.

Cell culture supernatants were further passaged five times in BHK-21 cells to see whether resuscitating mutations might occur. After the fifth passage, cells were fixed and immunofluorescence staining was performed (Figure 7.8, lower panel). In the case of the W219A mutation, viral protein expression was still negative, whereas the infectivity of the fifth L223A virus passage was substantially improved (almost 100% infected cells), indicating that either resuscitating mutations or a reversion to WT have occurred.

7.3.4.2 Specific infectivities of virus mutants

In order to compare the infectious properties of mutant and WT viruses, we determined the specific infectivity of cell culture supernatants of cells transfected with L223A and WT RNA and the supernatant harvested after L223A virus passaging (Figure 7.8, highlighted in orange). Specific infectivities were determined by relating viral RNA to the respective infectivity titers, determined in focus forming assays (Fi-

gure 7.9 A). The infectious titer of L223A virus was dramatically reduced compared to the WT, whereas the supernatant of the fifth L223A passage displayed infectious units similar to WT.

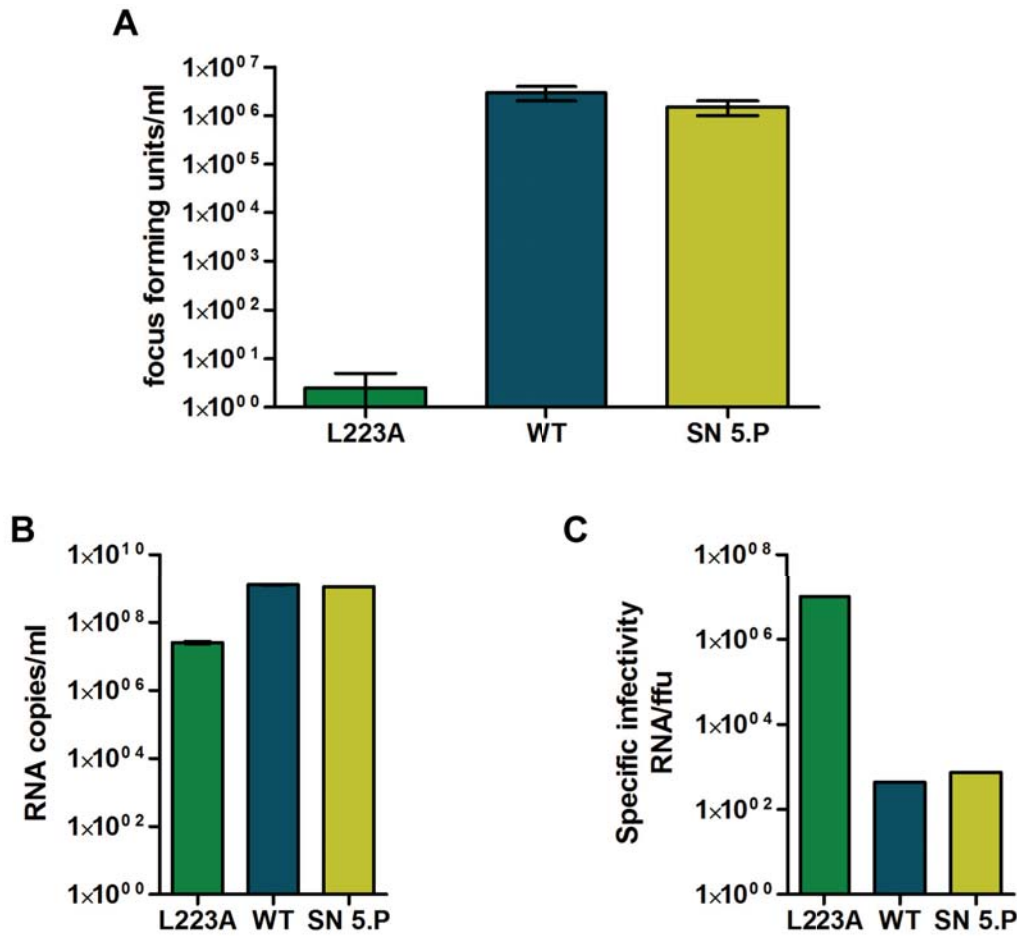


Figure 7.9: Specific infectivity determination of supernatants of transfected cells (L223A, WT) and supernatant after L223A passaging (SN 5.P). Viral particle quantification and specific infectivity. (A) Virus titers were determined in focus forming assays. (B) Viral particles were quantified by RNA isolation and quantification. (C) The specific infectivities were calculated based on the ratio of RNA copies per focus forming unit.

The number of RNA copies in the supernatant of transfected cells (L223A, WT) and in the supernatant harvested after L223A virus passaging are shown in Figure 7.9 B. To determine the specific infectivity, the number of viral RNA copies was put into relation to the respective titer (Figure 7.9 C). The specific infectivity of the supernatant harvested after L223A virus passaging was significantly improved compared to that of the engineered mutant L223A and similar to WT.

In order to find the reason for the significant increase in infectivity, we checked the sequence of viral RNA in the supernatant harvested after L223A passaging. For that purpose, RNA was isolated from the cell culture supernatant of the fifth L223A passage. Sequencing of cDNA showed that passaging of L223A virus had resulted in a mutation at position 223 to valine (by a single base exchange) (Figure 7.10).

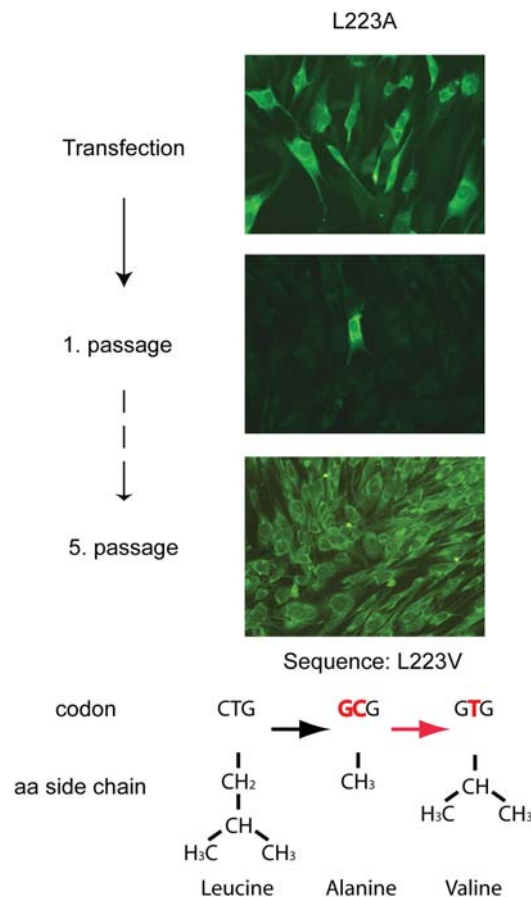


Figure 7.10: Resuscitating mutation at E protein amino acid 223. The original residue L223 was replaced by alanine. After virus passaging a resuscitating mutation at the same position to valine occurred.

Taken together, substitution of the DII pocket residues W219 and L223 to an alanine dramatically impaired the formation of infectious particles. The reduced specific infectivity observed for mutant L223A could be the consequence of either a deficiency in virus assembly, or an impaired fusion activity. The data indicate, that the mutation L223A had an effect on both processes. The number of viral RNA copies in the supernatant of transfected cells was reduced compared to the

WT (Figure 7.9 B), suggesting that the mutation had an effect on particle assembly. This is to some extent consistent with the RSP data, where introduction of the L223A mutation completely abolished particle secretion. But a deficiency in virus assembly alone cannot explain the observation, that viral particles, which are present in the supernatant of transfected cells (Figure 7.9 B) are drastically reduced in their infectivity (Figure 7.9 A). The significantly reduced specific infectivity suggests an effect of the L223A mutation on virus fusion, which might be a consequence of the alteration in the DII pocket. Data obtained with the RSP system demonstrated an interaction of the pocket forming residues W219 and L223 with the stem residue F403. Substitution of L223 by an isoleucine had a strong effect on RSP fusion, which was partially compensated by additionally replacing the proposed stem interaction partner F403 by an isoleucine, suggesting that the isoleucine at position 223 changed the DII pocket in that way, that the bulky F403 cannot bind to the pocket anymore. The smaller isoleucine at 403 might allow some interaction with the pocket. There was also an effect of the L223I mutation on virus infectivity, albeit lower than on RSP fusion. The replacement of the same residue L223 by an alanine showed a drastic impact on the specific infectivity. The smaller alanine might not allow the interaction with the stem, which might affect zippering of the stem along DII and prevent the correct positioning of the stem to obtain a stable postfusion trimer. This deficiency in virus fusion might be the reason for the provoked resuscitating mutation of A223 to a valine, which was possible by a single base change. Valine is a hydrophobic amino acid with two methyl groups, very similar to the original amino acid leucine (Figure 7.10). L223V seems to restore the DII pocket and might thus improve the interactions with the stem residue F403 (Figure 6.10), demonstrated by the strongly increased specific infectivity in comparison to the L223A mutant.

7.3.5 Establishment of full fusion assays with virus

The current flavivirus fusion model includes a transient hemifusion state in which just the outer leaflets have merged (Figure 4.9). In this thesis, we used a lipid-mixing assay with fluorescence-labeled RSPs and liposomes (Figure 6.5), but this assay cannot distinguish between hemifusion and full fusion, in which a fusion pore has been formed. In order to allow a differentiation between these two stages of fusion, two full fusion assays were established. Since RSPs are capsidless and do not possess the properties (nucleocapsid, infectivity) required for these assays, infectious virus particles were used for the full fusion test establishment.

7.3.5.1 Virus fusion with the plasma membrane

The acidic pH in the endosome triggers fusion of the viral with the endosomal membrane after virus uptake via receptor-mediated endocytosis (42). It has been shown, that full fusion can be measured by infection after artificial low-pH-induced fusion at the plasma membrane (40). For that purpose, virions were pre-bound to BHK-21 cells at 4°C. The samples were then acidified for 2 minutes at 37°C and incubated for 24 hours at 37°C in the presence of 20 mM NH_4Cl to prevent secondary infections through the endocytic pathway. Infected cells were visualized by fluorescence microscopy using polyclonal anti-TBEV sera. The experimental strategy is demonstrated in Figure 7.11 and in detail explained in Materials and Methods. Cells without virus (mock) and cells incubated with virus, but maintained at neutral pH were used as controls for pH-dependent fusion.

As shown in Figure 7.12, full fusion of the viral with the plasma membrane of BHK-21 cells could be induced by acidification of virions, which were pre-bound to cells, as indicated by a positive immunofluorescence signal (Figure 7.12 upper panel). Only a single infected cell was detected, when virus was incubated with cells and maintained at neutral pH, indicating that NH_4Cl almost completely abolished natural virus infection (Figure 7.12 lower panel). We therefore conclude, that low-pH-induced virus fusion with the plasma membrane and subsequent detection of viral protein expression can be used as an experimental approach to study full fusion of

TBE virus.

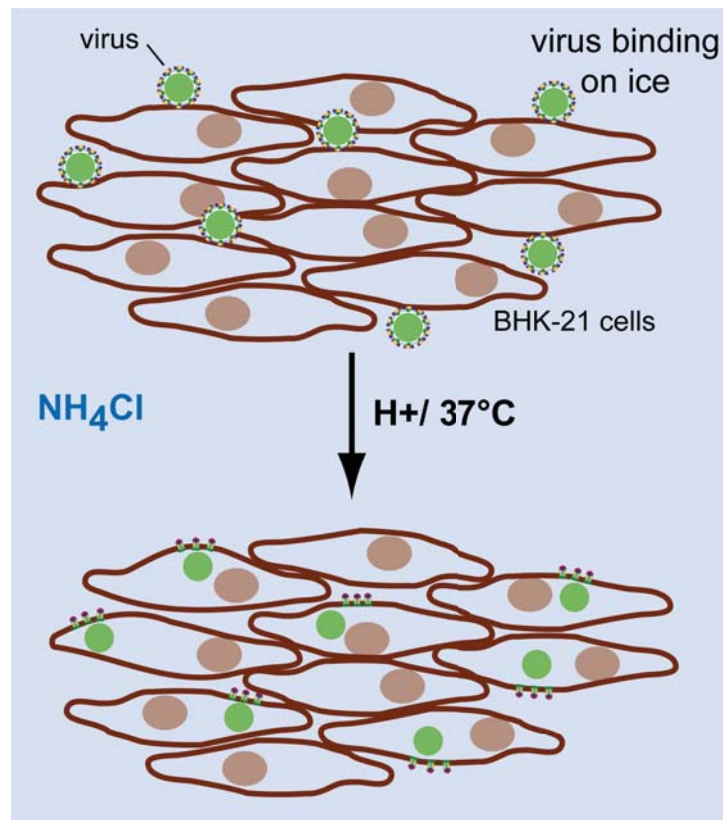


Figure 7.11: Schematic of full fusion assay. Virus fusion with the plasma membrane of BHK-21 cells was induced by acidic pH. Viral protein translation was detected by immunofluorescence.

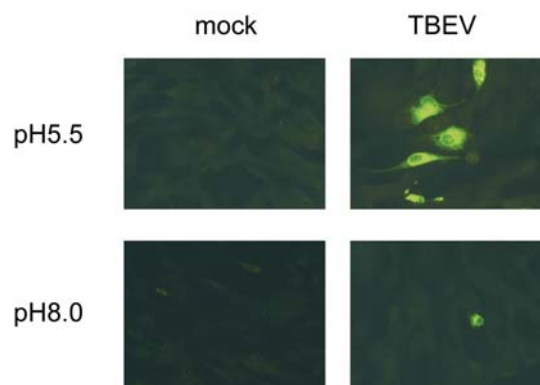


Figure 7.12: Full fusion activity of TBE virus. Fusion of virus with the plasma membrane, induced by acidic pH was detected by immunofluorescence staining of viral protein expression.

7.3.5.2 Content-mixing assay

An alternative assay to measure full fusion was based on content-mixing (51). For that purpose, trypsin-containing liposomes were mixed with virions. In the intact virus, the capsid is protected by the viral membrane against external trypsin. Upon acidification of the liposome-virus mixtures and low-pH-induced membrane fusion the nucleocapsid will enter the liposome and trypsin will then be able to degrade the capsid proteins. The degradation of C can be measured by SDS-PAGE and Western blot using a C protein specific polyclonal serum.

For that purpose, liposomes were loaded with trypsin and mixed with purified TBE virus. The mixture was split into three aliquots and exposed to acidic pH, maintained at neutral pH and solubilized with Triton X-100 (lysis of membranes and C protein degradation without fusion), respectively. After five minutes at 37°C, the acidified sample was back-neutralized. The aliquots were maintained at 37°C for further 75 minutes for trypsin digestion. The reaction was stopped by the addition of sample buffer and subjected to SDS-PAGE and subsequent immunoblotting with a TBEV C protein specific rabbit serum. The experimental procedure is illustrated in Figure 7.13 and described in detail in Materials and Methods.

Figure 7.14 shows that at low pH, the capsid protein has been degraded completely. Lysis of membranes with Triton X-100 also resulted in capsid degradation. At neutral pH, the capsid protein band is still detected in the blot, indicating that content-mixing is dependent on low-pH-induced fusion.

With these fusion assays it will be possible to study full fusion with virus and in combination with the pyrene lipid-mixing assay, we have the experimental tools to differentiate between hemifusion and full fusion. These approaches can be employed in future studies to dissect the late stages of flavivirus membrane fusion.

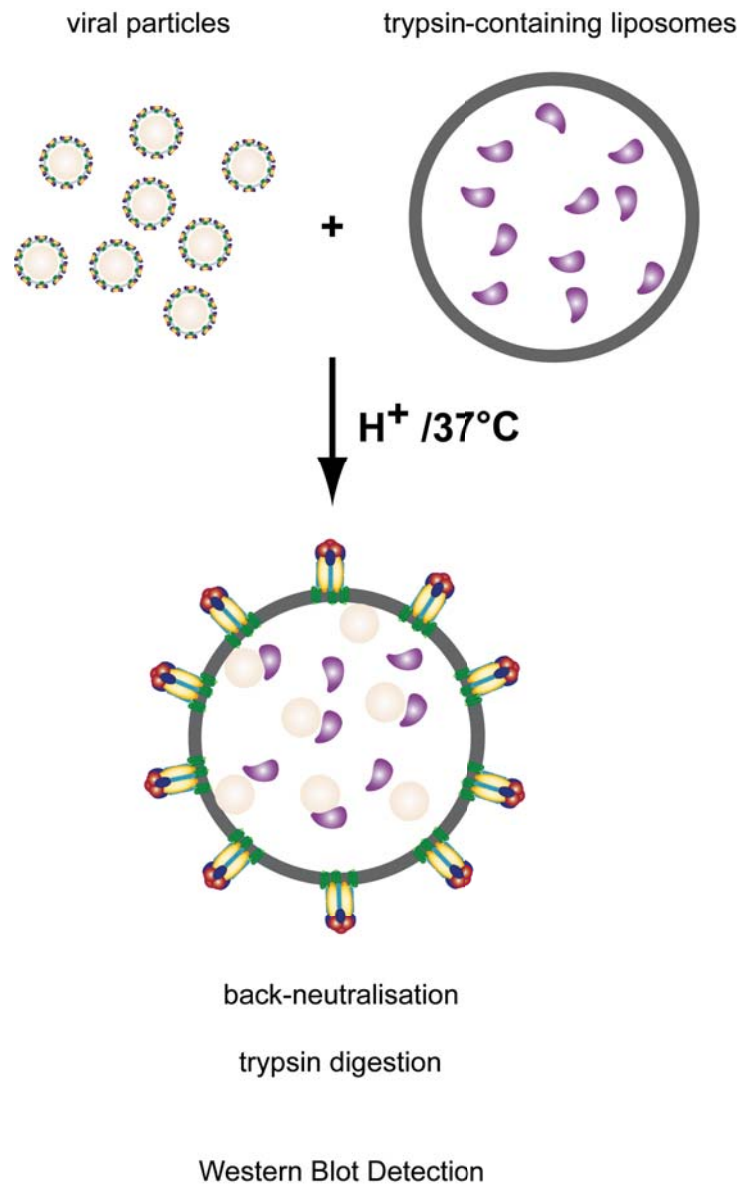


Figure 7.13: Schematic representation of content-mixing assay. Fusion of virus and trypsin-containing liposomes was induced by acidic pH.

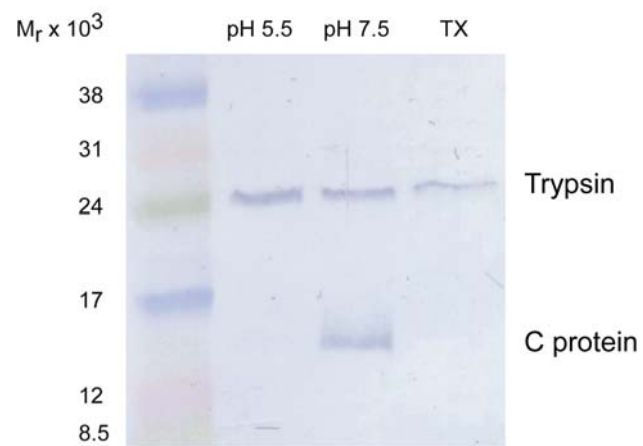


Figure 7.14: Western Blot analysis of content-mixing assay. Fusion of TBEV with trypsin-containing liposomes was detected by Western Blot. Digestion of the capsid protein by trypsin at acidic pH was detected with a C protein directed serum.

8 General Discussion and Conclusions

In this work, we were able to gain new insights into the mechanism of flavivirus membrane fusion and the role of the stem region of E in the fusion process. We investigated the effect of mutations in the E protein stem and DII on fusion and fusion-related processes in two different experimental systems.

Using the RSP system, we could show, that the early steps of the fusion process were not affected by mutations at the DII/stem interaction site (coflotation data). However, reduced E protein trimer stabilities of stem mutants revealed that the stem contributes to the overall stability of the postfusion conformation. The strongly impaired fusion activity of the L223I mutant RSP in the *in vitro* fusion assay implied, that the single mutation in DII crucially affected the late stages of fusion. The trimers of this mutant RSP were also clearly reduced in thermostability, indicating that these trimers cannot apply sufficient energy to drive the fusion process. By additionally replacing the interacting stem residue F403 by an isoleucine, the negative effect could be partially compensated, most likely facilitated by intramolecular interactions between DII and the stem, which might be important for the correct positioning of the stem during stem-zippering along the trimer core.

Despite their strong effects on RSP fusion, the same mutations introduced into the virus still allowed the production of infectious virions, albeit with a reduction in specific infectivity compared to WT virus. There are several, not mutually exclusive explanations for these findings:

One explanation for the discrepancy between RSP fusion and virus infectivity might be due to the different experimental set up. A cellular system provides different environmental factors, which might facilitate virus entry. Fusion usually occurs from within the early endosome but can also occur later in the endocytic pathway (77). The pH continually decreases from early endosomes to late endosomes up to lysosomes. In addition to the high pH variability in living cells, the lipid composition and thus biophysical properties also differ in the vesicles of the endocytic pathway

(72). In contrast, the *in vitro* fusion assay directly analyzes membrane fusion, mediated by the E protein, under defined conditions.

Another explanation for the discrepancy in mutant RSP fusion and mutant virus infectivity could be the different particle architecture of RSPs and virions. RSPs are 30 nm in diameter and thus about two thirds smaller than whole virions (Figure 8.1). Based on their size they have a higher curvature and are covered by 30 E dimers compared to 90 E dimers on virions (19). The E protein dimers on viral particles are more densely packed and in sets of three parallel dimer rafts arranged in a herringbone-like structure (39). The difference in number and arrangement of E proteins on the particle surface might be a reason for a stronger impact of mutations on RSP fusion. The E protein transition into stable trimers provides the energy for membrane fusion. Due to the dense E protein assembly on the virus surface, more E proteins are present at the fusion site and can thus contribute to the energy-driven fusion process. Hence, this special surface architecture of virions might favor the compensation of deficiencies derived from mutations, which destabilized the trimer through cooperative interactions of multiple E proteins at the fusion site, which is not possible to the same extent on RSPs.

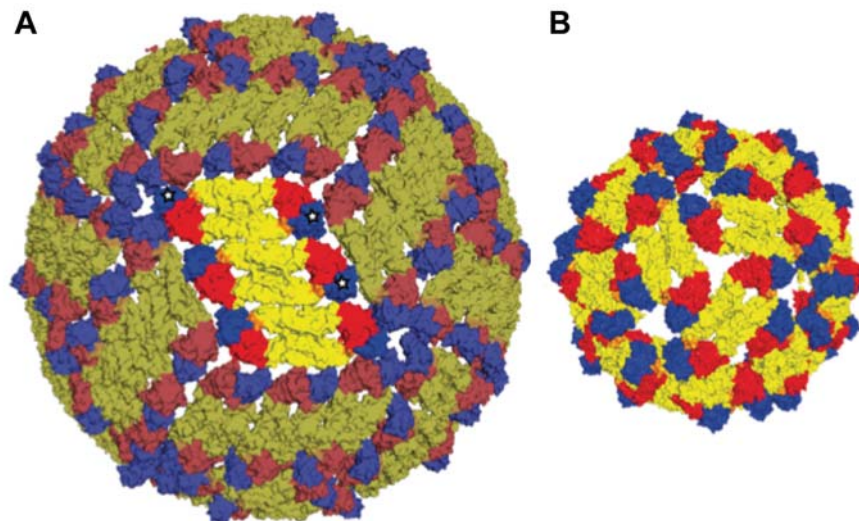


Figure 8.1: Schematic representations of TBE virion and RSP. (A) Pseudo-atomic structure of a flavivirus virion. Three E protein dimers are arranged in a raft, indicated by lighter colors. Stars are labeling DIIs, which belong to an icosahedral asymmetric unit. (B) Pseudo-atomic structure of a RSP of flavivirus (adapted from (34)).

Moreover, the positions of E proteins relative to each other and the particle curvature might be important for possible supramolecular assemblies. It has been hypothesized that five E protein trimers with their fusion peptides inserted into the target membrane might act together in a ring formation to induce a nipple-like deformation in the target membrane, which leads to hemifusion (9). As proposed for the SFV E1 the stem is hypothesized to function as a spacer between DIIs of different subunits to keep them in an open conformation and facilitate lateral interactions between adjacent trimers via the fusion peptides (9). If there is a supramolecular cooperation and the stem functions as spacer, RSPs might be more susceptible to the disruption of stem/DII contacts than densely packed virions, since the arrangement of E proteins relative to each other is different. A model of supramolecular interaction between fusion proteins, working together to mediate fusion has also been described for other viral fusion proteins, for example the influenza class I fusion protein HA. It has been shown that influenza virus fusion requires at least three cooperating HA trimers. Moreover, it has been observed that the density of the fusion proteins on the surface of HA expressing cells influences the kinetics of the fusion process (14, 22).

To resolve the observed discrepancies, we attempted to directly compare mutant RSPs and virions in the same fusion assay. Unfortunately, the production of pyrene-labeled viruses resulted in immature forms and could not be used for reliable fusion assays.

In this work, we have gained important new insights into the flavivirus fusion mechanism, especially through the use of RSPs as a model system. Unfortunately, significant difficulties were encountered when transferring the observations made with RSPs to the infectious virus system. These problems included different effects of mutations on particle assembly and fusion, respectively infection. The data, obtained with the two systems were not contradictory, but indicated a stronger effect of specific mutations on RSP assembly and fusion, compared to virus. Future investigation will be necessary to resolve this issue and to obtain a complete picture of the mechanism of flavivirus membrane fusion.

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”Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.”

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