Molecular mechanisms involved in the early steps of flavivirus cell entry

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Abstract

Flaviviruses enter their host cells by receptor-mediated endocytosis, a well-orchestrated process of receptor recognition, penetration and uncoating. Recent findings on these early steps in the life cycle of flaviviruses are the focus of this review.

Keywords

FLAVIVIRUS; CELL ENTRY; RECEPTOR; ENDOCYTOSIS; ENVELOPE PROTEIN; PH ENVIRONMENT; STRUCTURE

1. The flavivirus lifecycle

The basic stages of the flavivirus life cycle include attachment to the cell surface, internalization into the host cell, transfer of the viral RNA genome into the cytoplasm, translation of the viral proteins, replication of the genomic RNA, assembly and maturation of the virions, and ultimately the release of progeny viruses from the cell.

Flaviviruses are lipid-enveloped viruses (Fig. 1A). After recognition and attachment to specific receptor molecules on the surface of the cell, the virus is internalized into the host...
cell by clathrin-dependent endocytosis. Uncoating is induced by the low pH environment of the endosomes, where the viral proteins enter into a fusion-active state and initiate the merging of the viral envelope with the endosomal membrane, thereby releasing the viral RNA genome into the cytoplasm.

Flaviviruses have a single-stranded, positive-sense, ~11-kb RNA genome with a single open reading frame that is directly translated into a polyprotein precursor [1]. The polyprotein is subsequently glycosylated by cellular glycosyltransferases and cleaved by a combination of viral and host proteases to release three structural (C, prM, and E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). NS3 and NS5 are the best-characterized non-structural proteins that assemble with several other viral and host proteins to form the replication complex. NS3 combines helicase/NTPase, serine protease, and RNA triphosphatase activity [3,4]. The functional domains of NS5 include S-adenosyl methytransferase and RNA-dependent RNA polymerase activities [5]. The replication complex is responsible for the synthesis of a negative-sense RNA that acts as the template for the synthesis of the viral genomic progeny RNA. The viral structural proteins are the capsid (C) protein that associates with the RNA genome to form the nucleocapsid core, the envelope (E) protein that functions in receptor binding, membrane fusion, and viral assembly, and the pre-membrane (prM) protein that facilitates the folding and trafficking of the E protein during virus particle biogenesis [6,7] (Fig. 1).

The assembly of virions occurs in the endoplasmic reticulum when the newly synthesised viral proteins and nucleic acid combine to form immature, fusion-incompetent virus particles [1] (Fig. 1C). The budding into the endoplasmic reticulum is also responsible for the gain of the host-derived lipid envelope. After assembly, immature virions undergo a protease- and pH-dependent maturation during their transit from the endoplasmic reticulum through the trans-Golgi network to the cell surface [8–11]. During maturation, the E proteins go through a significant positional reorganization while the prM protein is cleaved by the cellular subtilisin-like endoproteinase furin [9]. The proteolytic cleavage releases the glycosylated, N-terminal pr fragment from the small, membrane-anchored M portion that will remain virion-associated. The pr peptide is subsequently shed from the virion during the exocytotic release of virus progeny [11]. The removal of the previously covalently attached pr peptides from the virions primes the viral particles in their mature, metastable structural state (Fig 1B) ready for the low-pH triggered fusion events during the upcoming cell entry [12,13].

2. The structure of the flavivirus particle

Mature, fusion-competent flavivirions are roughly spherical with a diameter of about 500 Å (Figs. 1A and 1B). Their nucleocapsid core, formed by the RNA genome and multiple copies of the highly basic, largely α-helical C protein [14], is surrounded by a host-derived lipid bilayer and a smooth, spikeless outer glycoprotein shell [1]. The outer protein shell has icosahedral symmetry and is composed of 180 copies, each, of the membrane-anchored M and E glycoproteins.

The E protein has an important role in the replication and maturation process of the virus, being involved in receptor binding, membrane fusion and virus assembly. On the viral surface, sets of three E head-to-tail homodimers are tightly associated into 30 rafts that form a herringbone pattern [15] (Figs. 1B and 2A). Each E monomer is structurally divided into a two-helix transmembrane region and an elongated ectodomain that stretches out along the viral lipid envelope (Fig. 2B). An α-helical stem region connecting the C-terminus of the E ectodomain with its transmembrane anchor lies essentially flat against the lipid bilayer underneath the ectodomain, half-buried in the outer lipid leaflet, and might alleviate
The early stages in the life cycle of flaviviruses involve entry into susceptible cells by receptor-mediated endocytosis with the ultimate goal to release the RNA genome from the protective envelope and protein shell into the cytoplasm. Receptor recognition, internalization and uncoating involve the formation and release of numerous specific molecular interactions and large-scale re-organization of the structural proteins and their domains on the viral surface. In flaviviruses, these seemingly simple, but not well understood mechanisms are mediated by the membrane-anchored E protein that combines both receptor binding and fusogenic activity.

### 3.1 Receptor recognition and attachment

The entry of flaviviruses into their target cells is mediated by the interaction of the E glycoprotein with cellular surface receptor molecules. Receptor recognition and attachment is likely to be a process in which multiple cellular receptor molecules are used in...
combination or consecutively to gain entry. Receptor usage is both cell-type and virus-specific and contributes to host range, tissue tropism and viral pathogenicity.

After vector inoculation of flaviviruses, the initial round of viral replication is considered to be primarily supported in dendritic cells (DC) of the skin [1,32,35–38]. Infected DCs migrate to draining lymph nodes, and a second round of replication occurs in lymphoid tissues leading to the viremic stage, when virus enters the circulation and spreads to internal organs through unknown mechanisms. Monocytes and macrophages are thought to be the major target cells in DENV infection and replication, but studies have further suggested a wide variety of cells as potential targets: B lymphocytes, T lymphocytes, hepatocytes, endothelial cells, epithelial cells, and fibroblasts [37,39–45]. For WNV, neurons or macrophages are proposed to be the physiologically relevant target cell types [46,47]. Various putative attachment factors and/or receptor elements have been implicated for flaviviruses, most of which are poorly characterized and are of unclear physiological relevance.

Highly sulfated, negatively charged glycosaminoglycans, such as heparan sulfate, can be utilized by several flaviviruses as low-affinity attachment factors that concentrate the virus on the cell surface, mainly mediated by DIII of E [48–55]. Similar to observations made for other Flaviviridae, low-density lipoprotein receptor may play a role as an attachment receptor of non-heparan sulfate adapted JEV strains in mammalian cells, and might be responsible for the neurovirulence of the virus [56]. Another cell surface carbohydrate determinant identified for DENV interaction with mammalian cells is the terminal disaccharide of a glycosphingolipid, neolactotetraosylceramide [57].

Putative primary protein receptors for flaviviruses are DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3 (ICAM3)-Grabbing Non-integrin) (or CD209) on monocyte-derived dendritic cells and DC-SIGNR on microvascular endothelial cells [32,37,58,59]. These tetrameric C-type lectins bind mannose-rich N-linked glycans with high affinity and have been shown to mediate DENV and WNV infection as attachment factors, presumably requiring the existence of additional entry receptor molecules for virus internalization. For DENV, cryoEM studies on the virus-receptor complex showed that DC-SIGN preferentially utilizes carbohydrate moieties at Asn67 in DII, a site unique to DENV [60]. Another Ca$^{2+}$-dependent lectin, the mannose receptor, that carries a ligand specificity different from DC-SIGN and is constitutively internalized by clathrin-mediated endocytosis, has been shown to bind DENV, JEV and TBEV and is proposed to be an internalization receptor of DENV in human macrophages [61]. The glycosylation pattern (the number, distribution, and structure of carbohydrate moieties) on the viral surface varies among different flaviviruses or even among different strains of the same virus and influences cell/tissue tropism, infectivity and infection efficiency [38,58,62–67].

The presence of a classic RGD motif in DIII of the YFV-17D envelope protein and similar motifs in other flaviviruses suggested a mechanism for interaction with integrins [68]. However, DC infection with the YFV-17D vaccine strain does not depend on the RGD motif, nor on DC-SIGN or on various integrins [38]. For WNV and JEV, $\alpha_\text{v}\beta_3$ integrin has been documented as a functional receptor in permissive mammalian cells apparently mediated by DIII and likely in a non-RGD-dependent manner [23]. However, the cellular receptors on neurons or macrophages remain unknown. Similarly, it has been suggested that RGD-dependent integrin binding is not essential for entry of Murray Valley encephalitis virus and that multiple and/or alternative receptors may be involved in its cell entry [69]. Recent studies have reported that WNV entry into embryonic mouse fibroblasts and hamster melanoma cells is independent of $\alpha_\text{v}\beta_3$ integrin, suggesting alternative receptor molecules for different cell types or strain differences [70]. Another cell adhesion molecule, the 37/67-
kDa high-affinity laminin receptor has been described as a DENV-1 specific binding protein on human liver cells [71]. However, DENV serotypes 2 and 3 have also been shown to interact with this receptor protein extracted from porcine kidney cells [72]. These contrasting results suggest that differential receptor usage by flaviviruses might depend on cell-specific posttranslational modifications of the receptor molecule itself and not only on viral factors [73]. In mosquito cells, a closely related laminin-binding protein may be involved in DENV-3/4 infection [73].

Hsp90 and Hsp70, heat shock proteins associated with membrane microdomains (or lipid rafts), participate in DENV entry as a receptor complex in human neuroblastoma and monocytic cell lines as well as in human monocytes/macrophages [74], but do not play a role for internalization into liver cells [75]. A related stress protein, the glucose-regulated protein GRP78 or BiP, involved in the unfolded protein response, has been identified as a minor receptor element for DENV-2 internalization into human liver cells [75,76]. Hsp70 might serve as the putative receptor of JEV in mouse neuroblastoma cells [77]. Two heat shock related proteins (gp45 and a 74-kD protein) were indicated as part of the DENV receptor complex in mosquito cells [78], while others hypothesize that heat shock cognate protein 70 (HSC70) mediates JEV entry into mosquito cells as a penetration receptor [79]. Apart from the above, several proteins of varying molecular mass and of unknown identity have been described as putative flavivirus receptors in different cell lines and cell types.

Alternately to direct attachment of the E glycoprotein to a receptor, flaviviruses can infect monocytes and macrophages in an antibody-dependent manner by binding as virus-antibody complexes to immunoglobulin Fcγ receptors that recognize the constant region of IgG molecules [30,80,81].

3.2 Internalization and fusion

Flaviviruses enter their target cell by receptor-mediated, clathrin-dependent endocytosis [82,83]. The receptor-bound virus is internalized via pre-formed clathrin-coated pits that bud into the cytosol and deliver their cargo to early endosomes. Single particle-tracking in living cells showed that these early endosomes mature to late endosomes, in which membrane fusion seem to take place predominantly [82]. However, the requirement for endocytic trafficking, specifically the maturation stage of the endosome at which virus fusion occurs, appears to vary between different flaviviruses and/or virus strains [82–84], possibly dependent on the pH threshold for fusion of the particular virus.

Upon exposure of the virion to mildly acidic pH in endocytic vesicles a series of molecular events leading to membrane fusion is evoked that have currently been detailed or inferred using biochemical and structural approaches (Fig. 3). The E proteins on the viral surface rearrange from a pre-fusion homodimeric array (Figs. 1B, 2A and 2B) to fusion-active homotrimers. Large-scale conformational changes in the E domain arrangement, made possible by the flexible hinges between the three E domains, bring the viral and the target membrane in close proximity and facilitate the fusion of the two bilayers [12]. The formation of a fusion pore results in the release of the viral RNA genome into the cytoplasm for initiation of replication and translation (Fig. 3).

Fusion of flaviviruses with liposomes occurs on a time scale of a few seconds, is strictly dependent on mildly acidic pH (pH threshold ~6.6) and does not require a protein or carbohydrate receptor in the target lipid membrane [29,85,86]. Exposure of the virus alone to low pH results in inactivation of its fusion activity. The lipid composition of the target membrane influences fusion efficiency and pH threshold [13,29,87,88]. Studies have suggested that flaviviruses utilizes cholesterol-rich lipid rafts (membrane microdomains enriched with cholesterol and glycosphingolipids) during initial stages of internalization.
presumably by interacting with raft-associated proteins for attachment or signaling [70,74]. However, while WNV fusion activity is strongly promoted by the presence of cholesterol in the target membrane, the presence of lipid rafts might not be essential for fusion [29].

The molecular mechanisms prompting viral fusion are not well understood, but histidine residues have been hypothesized to function as sensors of pH that trigger conformational changes in viral fusion proteins. The binding of protons is to lower the energetic barrier separating the pre-fusion from the post-fusion conformation or to provide activation energy [12]. All E domains contain histidine residues that could potentially function as pH sensors [18], either by specifically or cumulatively increasing the surface net charges upon acidification. Studies of TBEV subviral particles identified a conserved histidine at the interface between DI and DIII of E as the critical pH sensor required for the initiation of viral membrane fusion [89], but mutational studies on WNV do not support a requirement for any single histidine of E and suggest that non-histidine residues of the E protein might be involved in initiating the pH-dependent exposure of the hydrophobic fusion loop [90].

The flavivirus E glycoprotein with its three domains, each largely adopting β-sheet folds, has been assigned as a class II fusion protein [91] (Fig 2). Triggered by low pH, the E proteins undergo extensive conformational and positional transformations that drive membrane fusion, expose the fusion loop to interact with the target membrane, and establish a close positioning of viral and target membranes leading to fusion pore formation [13] (Fig. 3). The pre-fusion conformation of E at neutral pH (Figs. 2A and 2B) and the structure of the E ectodomain at low pH, after fusion completion (Fig. 2C), is known for several flaviviruses [18]. The post-fusion structure is a trimeric arrangement of E, with the E monomers parallel to one another and with their long axis oriented perpendicular to the plane of the membrane [13] (Fig. 2C). The fusion peptides are exposed on one end of the trimer, suitable for penetrating into the outer leaflet of the associated lipid bilayer.

Hydrophobic grooves, in which the stem regions are predicted to bind, are generated by the trimerization and extend along the interface of two neighboring DIIs towards the fusion loop. Compared to the pre-fusion dimer (Fig. 2B), DIII of E has undergone a major rotational displacement to the side of DI and towards DII (Fig. 2C). The fold-back of DIII along the sides of the trimer brings the transmembrane domains in the viral envelope spatially close to the fusion peptide at the tip of DII that is embedded in the target membrane (Fig. 3). At the same time, the membranes are locally deformed and destabilized.

The oligomerization state of the post-fusion E structure suggests the presence of a pre-fusion trimeric arrangement of E. The formation of such fusogenic E trimers has to be preceded by the protonation-dependent disruption of the raft arrangement on the surface of the virion, and the dissociation of E dimers into monomers [92] (Fig. 3B). The resultant exposure of the fusion loop on the tip of the outward projected DII allows its insertion in the outer leaflet of the target membrane, thereby anchoring the E protein simultaneously in viral and target membrane, followed by the reassociation of E molecules into trimers [13] (Figs. 3B and 3C). Trimerization has been shown to be essential for fusion activity of synthetic fusion peptides derived from TBEV [93]. The large-scale reorganization of E molecules is aided by the release and extension of the E stem region away from the viral membrane [94] supporting the idea of formation of an extended “pre-hairpin” intermediate [12] before its collapse into a fold-back conformation (Fig. 3). The release of the E stem region from the viral lipid envelope is a pre-requisite for the conversion into the post-fusion E trimer, in which the stem is stretched out along the newly generated groove between associated DII domains (Fig. 2C). The irreversible fold-back conversion of the E protein into the post-fusion hairpin-like structure requires the rotational repositioning of DIII and zipping up of the stem bringing the E transmembrane helices in the viral membrane in juxtaposition with the target membrane-anchored fusion loop [19,20] (Fig. 3). The fusion process progresses via a
hemifusion intermediate to the formation of the post-fusion E trimer and opening of the fusion pore [95]. The free energy liberated by the rearrangement of the viral surface proteins from a metastable homodimer to a more stable homotrimer formation is likely used to overcome the kinetic barrier hindering fusion of the two membrane bilayers [12,13]. The number of E trimers necessary for the cooperative formation of the fusion pore is still a matter of debate [13,95,96].

4. Concluding remarks

Significant advances towards understanding flavivirus cell entry have been made and a series of conformational events can be inferred from available structures and biochemical data. However, further studies will be necessary to dissect the kinetics of the virus-cell fusion pathway and to understand the order of the large, conformational changes at a molecular level. The information obtained from structure can provide insight for the development of preventive and therapeutic means against the spread and effect of pathogenic flaviviruses, allowing the design and targeting of antiviral agents or vaccines.

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References


Fig. 1.
The flavivirion structure. (A) 3D rendering of a cryoEM density map of DENV (kindly provided by Wei Zhang, Institute of Molecular Virology, University of Minnesota, USA). The cut-out window allows the view inside the virion at the nucleocapsid core (magenta/orange) composed of the C protein and the RNA genome. The core is surrounded by a host-derived lipid envelope (green). The outer icosahedral shell of the virus (blue) is formed by two membrane-anchored glycoproteins, E and M. (B) Prefusion arrangement of the E glycoprotein on the surface of the mature flavivirion, viewed down an icosahedral twofold axis. Domains DI, DII, and DIII of each E monomer are colored red, yellow, and blue, respectively. The fusion loop is shown in green. A total of 180 E monomers are associated in 30 rafts of three, nearly parallel E homodimers that form a distinct herringbone pattern. One icosahedral asymmetric unit (ASU), the smallest unit from which the particle can be generated by applying icosahedral symmetry, is outlined by a black triangle. The positions of the neighboring icosahedral five-, three- and twofold symmetry axes are marked with symbols (pentagons, triangles, and ovals, respectively). Each ASU contains the equivalent of three E monomers. (C) Arrangement of the E glycoproteins and pr peptides on the surface of an immature, fusion-incompetent flavivirion, viewed down an icosahedral twofold axis. Domains DI, DII, and DIII of each E monomer and the fusion loop are colored as in (A). The pr peptide is shown in magenta. One ASU is outlined by a black triangle and the positions of the symmetry axes are indicated. The E proteins are icosahedrally arranged in 60 trimeric spikes, composed of prM/E heterodimers. The pr peptide of the prM protein covers the fusion peptide in DII of E (on the tip of each spike) to prevent premature fusion.
Fig. 2.
The oligomeric structure of the E glycoprotein. (A) Raft organization of three E homodimers as observed for mature flavivirions. The relative positions of the neighboring icosahedral symmetry axes are indicated by symbols. The elongated E ectodomain has three distinct, structurally defined domains that are joined by flexible hinges. DI, DII, and DIII are colored red, yellow, and blue, respectively. The fusion loop is shown in green. Two E monomers forming the central homodimer at the icosahedral twofold axis are emphasized by shadows. (B) The prefusion head-to-tail homodimer arrangement of the E glycoprotein shown from the top (looking towards the center of the virus) and from the side after a 90° rotation around its long axis. One monomer is rendered as a ribbon diagram, showing its three domains largely adopting β-sheet folds. The other monomer is represented as a surface shaded volume. The fusion loop of one E protein is buried in a hydrophobic pocket at the DI–DIII interface of the other E molecule. The side view shows that each E monomer is structurally divided into the ectodomain that elongates parallel to the viral lipid envelope and a two-helix transmembrane anchor (dark green). The α-helical stem region (purple), half-buried in the outer lipid leaflet, connects the C-terminus of the E ectodomain with the transmembrane helices. The M protein (orange) also contains two antiparallel transmembrane helices and is essentially buried under the E protein layer. The stem-anchor region is only shown for the E monomer depicted as a ribbon diagram. Similarly, only one M protein is illustrated. (C) The postfusion homotrimer arrangement of the E glycoprotein shown from the top and from the side after a 90° rotation. The E monomers are oriented parallel to one another with their fusion peptides exposed on one end of the trimer. DIII of E has undergone a major rotational displacement to the side of DI and towards DII. The stem region (dashed purple line) is predicted to bind in a hydrophobic groove that extends along the interface of two neighboring DIIIs of the E trimer towards the fusion loop. The irreversible fold-back conversion of the E protein into the post-fusion hairpin-like structure and zipping up of the stem brings the E transmembrane helices (connected via the stem to DIII of the ectodomain) in juxtaposition with the membrane-embedded fusion loop at the tip of DII.
Fig. 3.
Schematic of the proposed steps of the flavivirus membrane fusion process, modified from the original figure drawn by Stiasny et al. (2009) [18]. Domains DI, DII, and DIII of E are colored red, yellow, and blue, respectively. The fusion loop at the distal end of DII is indicated in orange. The stem region is shown in purple and the transmembrane helices in green. The outer/inner leaflets of the viral lipid envelope and the target membrane are colored yellow/blue and gray/red, respectively. (A) Pre-fusion E homodimers on the virus surface. (B) Low endosomal pH weakens inter- and intramolecular E contacts and induces the outward extension of the stem region, followed or accompanied by the dissociation of the E dimers into monomers. The outward projection of the E monomers allows the interaction of the fusion loop with the endosomal target membrane. (C) E trimer formation, back-folding of DIII, and ‘‘zipping-up’’ of the stem. (D) Hemifusion intermediate in which only the outer leaflets of viral and target membranes have mixed. (E) Formation of the post-fusion E trimer and opening of the fusion pore allows the release of the C protein-associated RNA genome (nucleocapsid) into the cytoplasm.