Review

Closing the door on flaviviruses: Entry as a target for antiviral drug design

Rushika Perera, Mansoora Khaliq, Richard J. Kuhn *

Markey Center for Structural Biology and Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, United States

ABSTRACT

With the emergence and rapid spread of West Nile virus in the United States since 1999, and the 50–100 million infections per year caused by dengue virus globally, the threat of flaviviruses as re-emerging human pathogens has become a reality. To support the efforts that are currently being pursued to develop effective vaccines against these viruses, researchers are also actively pursuing the development of small molecule compounds that target various aspects of the virus life cycle. Recent advances in the structural characterization of the flaviviruses have provided a strong foundation towards these efforts. These studies have provided the pseudo-atomic structures of virions from several members of the genus as well as atomic resolution structures of several viral proteins. Most importantly, these studies have highlighted specific structural rearrangements that occur within the virion that are necessary for the virus to complete its life cycle. These rearrangements occur when the virus must transition from immature, to mature, to fusion-active states and rely heavily on the conformational flexibility of the envelope (E) protein that forms the outer glycoprotein shell of the virus. Analysis of these conformational changes can suggest promising targets for structure-based antiviral design. For instance, by targeting the flexibility of the E protein, it might be possible to inhibit required rearrangements of this protein and trap the virus in a specific state. This would interfere with a productive flaviviral infection. This review presents a structural perspective of the flavivirus life cycle and focuses on the role of the E protein as an opportune target for structure-based antiviral drug design.

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* Corresponding author. Tel.: +1 765 494 1164; fax: +1 765 494 1189.
  E-mail address: kuhnr@purdue.edu (R.J. Kuhn).

1. The need for antivirals against flaviviruses

In the last decade, the flaviviruses have re-emerged as aggressive human pathogens causing an increased number of infections worldwide (Gould and Solomon, 2008; Kuhn, 2006). There are more than 70 related members of the flavivirus genus, a subgroup of the *Flaviviridae* family. Many of these viruses produce significant human disease and include yellow fever virus (YFV), West Nile virus (WNV), dengue virus (DENV), Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV). Vaccines are currently available for JEV, YFV, and TBEV. However, disease outbreaks caused by these three viruses continue to be a serious problem in many developing countries. Several promising candidates for WNV vaccines are in progress but may take several years for clinical evaluation (Martin et al., 2007; Monath et al., 2006). Conventional vaccine development for DENV has been challenging. For instance, prevention of antibody-dependent enhancement (ADE) is of ultimate importance when designing vaccines and requires efforts aimed at eliciting an appropriate immune response against all four serotypes of the virus. Antiviral therapies for these viruses are at a very early stage of development (for a review see Ray and Shi, 2006). Thus, the flaviviruses have huge disease burdens, and require new approaches to preventing virus replication, pathogenesis, and transmission.

Antivirals previously designed against flaviviruses have primarily focused on inhibition of viral RNA replication. Although these efforts are ongoing, new opportunities for antiviral design have recently emerged based on advances in our knowledge of flavivirus virion structure. These advances include obtaining the pseudoatomic structures of TBEV subviral particles (Ferlenghi et al., 2001) and of immature and mature DENV (Kuhn et al., 2002; Zhang et al., 2003a, 2004, 2007) and WNV (Mukhopadhyay et al., 2003) under different physiological conditions, as well as mature DENV and WNV virus complexed with antibodies and cell-surface attachment molecules (Kaufmann et al., 2006; Lok et al., 2008). X-ray crystallographic analyses and nuclear magnetic resonance (NMR) spectroscopy studies have provided atomic resolution structures of the three flavivirus structural proteins: capsid (C) (Dokland et al., 2004; Ma et al., 2004), pre-membrane (prM) (Li et al., 2008) and envelope (E) (Bressanelli et al., 2004; Heinz et al., 1991; Huang et al., 2008; Kanai et al., 2006; Modis et al., 2003, 2004, 2005; Mukherjee et al., 2006; Nybakken et al., 2006; Rey et al., 1995; Volk et al., 2007; Yu et al., 2004; Zhang et al., 2004). These studies have invited an alternative focus from inhibition of RNA replication towards blocking structural transitions required for efficient virus spread. Similar strategies were previously employed for antiviral design against the rhinoviruses (Badger et al., 1988; Hadfield et al., 1999; Heinz et al., 1989; Rossmann, 1994; Rossmann et al., 2000) and enteroviruses (Padalko et al., 2004; Rossmann et al., 2000), and have recently gained momentum in other fields such as HIV (Copeland, 2006; Veiga et al., 2006; Wang and Duan, 2007) and influenza (Hsieh and Hsu, 2007).

In this review, we will describe the structural transitions that occur in the flavivirus E protein during the life cycle of the virus, and discuss how these transitions can be targeted for inhibition by antiviral compounds. Specifically, recent advances in the structure determination of the flaviviruses and their component proteins are described with special emphasis on the conformational and translational changes of the E protein as it transitions between the immature, mature and fusion-active forms of the virus. Specific surfaces on the E protein are described as potential targets for structure-based antiviral drug design and alternate strategies for viral inhibition are discussed based on the interaction of the E protein with receptor molecules and neutralizing antibodies.

2. Flavivirus replication cycle

Like other positive-strand RNA viruses, flaviviruses replicate in the cytoplasm of susceptible cells (Fig. 1). A specific receptor for internalization of these viruses into host cells has not yet been identified. Several cellular molecules capable of mediating virus attachment are known, but none has been conclusively shown to function as virus receptors (Barba-Spaeth et al., 2005; Chu et al., 2004; Jindadamrongwech et al., 2004; Krishnan et al., 2007; Lozach et al., 2005; Miller et al., 2008; Navarro-Sanchez et al., 2003; Pokidysheva et al., 2006; Tassaneetrithep et al., 2003).

The flavivirus virion consists of an outer glycoprotein shell and an internal host derived lipid bilayer that surrounds the capsid and viral RNA. During virus entry, envelope (E) proteins forming the glycoprotein shell bind to cell surface receptors that assist in internalizing the virus through clathrin-mediated endocytosis (Gollins and Porterfield, 1984; Ishak et al., 1988; van der Schaar et al., 2007). Following internalization, the low pH of the endosome triggers structural rearrangements in the viral glycoproteins that drive fusion of the viral and endocytic membranes to release the viral RNA into the cytoplasm (Bressanelli et al., 2004; Modis et al., 2004). This RNA (~11 kb) is directly translated as a single polyprotein that is processed by viral and cellular proteases into three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A/B, NS3, NS4A/B and NS5) (Lindenbach and Rice, 2001). The non-structural proteins actively replicate the viral RNA in replication complexes associated with cellular membranes (Mackenzie et al., 2001). Newly synthesized RNA and capsid protein are enveloped by glycoproteins prM and E to assemble immature virus particles that bud into the ER. These immature particles are transported through the secretory pathway to the Golgi apparatus. In the low pH environment of the trans-Golgi, furin-mediated cleavage of prM to M drives maturation of the virus. Maturation is also accompanied by significant structural rearrangements of the glycoprotein shell (Elshuber et al., 2003; Kuhn et al., 2002; Li et al., 2008; Modis et al., 2003, 2005; Mukhopadhyay et al., 2003; Stadler et al., 1997; Yu et al., 2008; Zhang et al., 2003a). Following maturation, virus particles migrate along the surface of naïve cells until they encounter clathrin-coated pits that assist in virus entry (van der Schaar et al., 2007). Because of differences in the cellular environments and the non-lytic nature of the infections, the entry, replication and assembly of these viruses may differ in mosquito cells versus vertebrate cells.

3. The flavivirus E protein as a target for antiviral therapy

Current efforts to develop antivirals against flavivirus entry are focused on the E protein. This protein assumes different conformations in the immature, mature and fusion-activated forms of the virus and plays an important role during virus assembly, maturation and entry (reviewed in Mukhopadhyay et al., 2005; Rey, 2003). E also functions as a receptor-binding ligand, as well as the fusion engine that carries out mixing of viral and cellular membranes. Furthermore, it is a target for most of the antibodies that neutralize the virus.

The E protein forms the glycoprotein shell of the virus (Fig. 2). It is a class II fusion protein that shares about ~40% amino acid identity among the flaviviruses. The atomic structure for the ectodomain of E has been solved for DENV-2, DENV-3, WNV and TBEV (Kanai et al., 2006; Modis et al., 2003, 2004, 2005; Nybakken et al., 2006; Rey et al., 1995; Zhang et al., 2004). The protein consists of three β-barrel domains. Domain I (DI) contains the N-terminus, but is centrally located in the molecule. Domain II (DII) is rather elongated, mediates dimerization of E and also includes the hydrophobic and well conserved fusion peptide at its distal end (Allison et al., 2001).
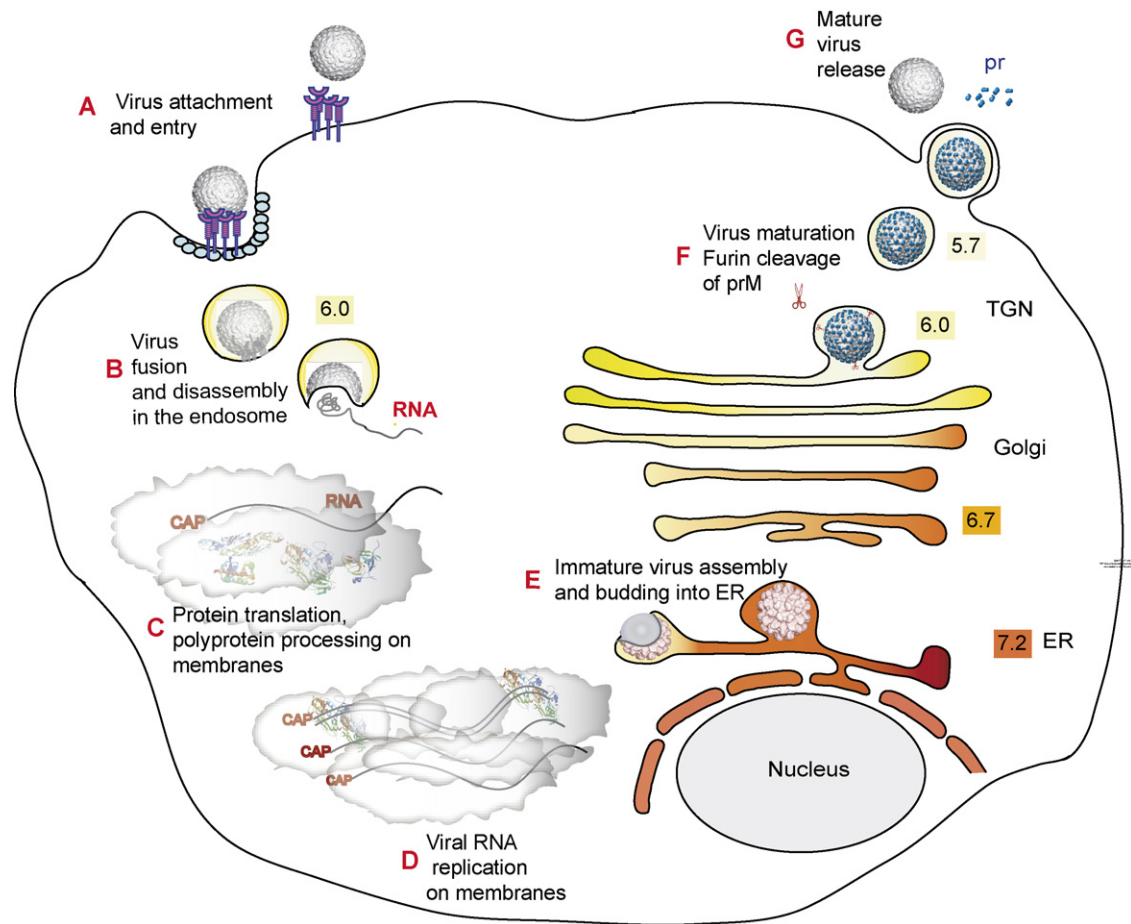
Fig. 1. The flavivirus life cycle. (A) Virions bind to cell-surface attachment molecules and receptors and are internalized through endocytosis. (B) In the low pH of the endosome, viral glycoproteins mediate fusion of viral and cellular membranes, allowing disassembly of the virion and release of RNA into the cytoplasm. (C) Viral RNA is translated into a polyprotein that is processed by viral and cellular proteases. (D) Viral non-structural proteins replicate the genome RNA. (E) Virus assembly occurs at the ER membrane, where capsid protein and viral RNA are enveloped by the ER membrane and glycoproteins to form immature virus particles. (F) Immature virus particles are transported through the secretory pathway. In the low pH of the trans-Golgi, network (TGN) furin-mediated cleavage of prM drives maturation of the virus. (G) Mature virus is released into the cytoplasm. Numbers shown in colored boxes refer to the pH of the respective compartments.

Domain III (DIII) is an immunoglobulin (Ig)-like domain that is predicted to be involved in receptor binding (Bhardwaj et al., 2001; Rey et al., 1995) and antibody neutralization (Beasley and Barrett, 2002; Crill and Chang, 2004; Crill and Roehrig, 2001; Halstead et al., 2005; Li et al., 2005; Pierson et al., 2007; Stiasny et al., 2006; Sukupolvi-Petty et al., 2007).

Domains I and II are connected by four polypeptide chains. The hinge angle between these two domains varies by approximately 20° in the various structures of E (Fig. 2B). Domains I and III are connected by a single polypeptide linker. Hinge motion at both DI–DII and DI–DIII play an important role during the structural rearrangements of the E protein as it transitions between immature, mature and fusion-active forms of the virus. The DENV-2 crystal structure showed the presence of a N-octyl-β-D-glucoside (β-OG) molecule that was located in a hydrophobic pocket between DI and DII of selected E protein monomers (Modis et al., 2003). This observation sparked much interest in this pocket as a potential site for binding small molecules that might inhibit conformational changes in the E protein (Li et al., in press; Modis et al., 2003; Zhou et al., submitted for publication). The C-terminal region of E, absent in the above structures, is directed towards the viral membrane and consists of a "stem" in the form of two helices connected by a highly conserved sequence, and an "anchor" comprised of two transmembrane antiparallel coiled-coils (Allison et al., 1999; Stiasny et al.,

1996; Zhang et al., 2004). The stem-anchor region also undergoes structural transitions during the fusion activation of E.

3.1. Structural transitions of the E protein

During the flavivirus life cycle, the virion assumes three main conformational states: immature, mature and fusion-activated. The conformation of the E protein differs in these three states (Fig. 2C–E) and therefore, structural rearrangements must occur within the glycoprotein shell to achieve these endpoints. These rearrangements stem from the need to transition between prM–E heterodimers in the immature particle to E homodimers in the mature particle, and finally to E homotrimers in the fusion-activated particle. Understanding these transitions is necessary when considering the inhibition of the E protein and its activity during virus entry. The E protein in each of these conformations is described below.

3.2. Conformation of E in the immature virus

Virus particles that bud into the ER are termed "immature" due to the presence of a pre-membrane protein (prM) that must be proteolytically processed during virion maturation. Recent studies by Yu et al. have indicated that there are two forms of the imma-
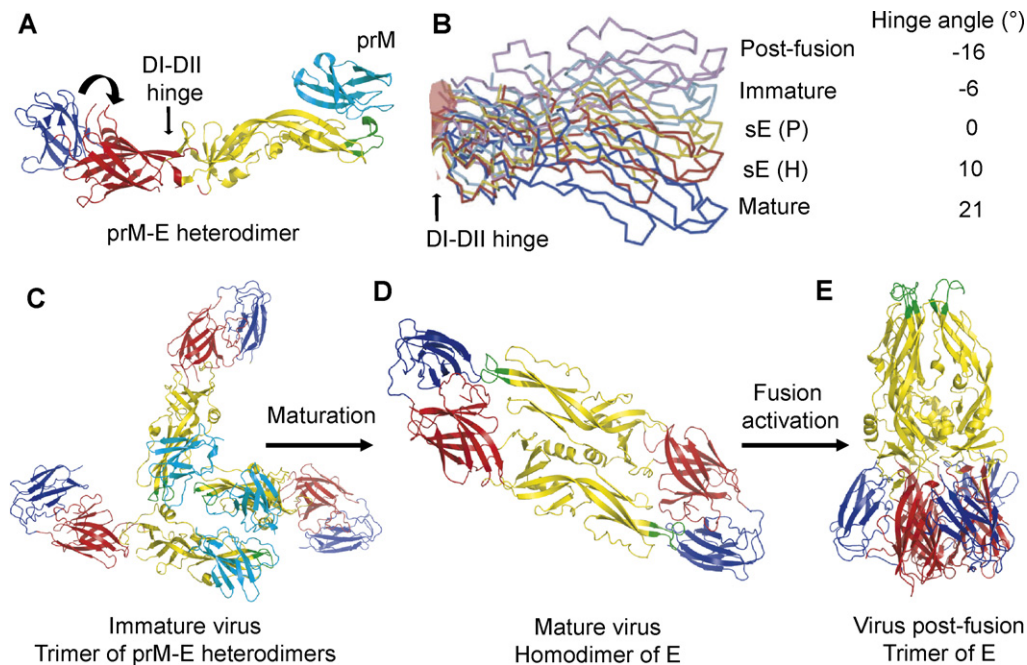
Fig. 2. Structure of the flavivirus E protein and its various oligomeric states. (A) A ribbon diagram of the atomic structure of the prM–E heterodimer (Li et al., 2008). The E protein is colored as follows: domain I (red), domain II (yellow), domain III (blue) and the fusion peptide (green). The prM protein (cyan) is only found in the immature virus and is shown in its role as a cap structure that protects the fusion peptide. The icosahedral asymmetric unit is shown as a white triangle with the five-fold and three-fold axes labeled. The DI–DII hinge is shown with a straight arrow. The movement of DIII with respect to DI during fusion activation is indicated with the circular arrow. (B) DII of the E protein is shown as represented by the different structures of E. sE(P), sE(H) and the post-fusion confirmation represent the known crystal structures of E (Modis et al., 2003, 2004; Zhang et al., 2003b; Bressanelli et al., 2004), while the mature and immature conformations represent the fit of the atomic structure of E into the cryo-EM density of the virus (Kuhn et al., 2002; Zhang et al., 2003a, 2004). The structures were superimposed with respect to DI. Flexibility at the hinge between DI and DII (indicated with an arrow) gives rises to varying hinge angles along the direction of the hinge axis and is suggested to be important in the structural transitions of E during the flavivirus life cycle. (C) Oligomeric state of the E protein in the immature particle, (D) mature particle, (E) fusion-active conformation. In the immature particle the E protein forms a trimer of prM–E heterodimers, which must dissociate and form E protein homodimers during maturation. These then undergo further reorganization to form E homotrimers prior to fusion and entry of the virus. Transitions of the E protein are supported by hinge motions that occur between DI–DII and DI–DIII (Modis et al., 2003, 2004; Zhang et al., 2003a, 2003b).

ture virus ("spiky" and "smooth") based on the oligomeric state and arrangement of E proteins on the surface of these particles (Yu et al., 2008). The oligomeric state of the E protein is controlled by the pH of the cellular environment and the presence or absence of prM.

Immature virus particles have a diameter of ∼600 Å (Fig. 3A and C). They have a spiky surface protein shell consisting of prM and E proteins, which form 180 heterodimers that are arranged as 60 trimeric spikes. The three E proteins within each spike are tilted such that the long axis of the protein forms a ∼25 Å angle with the surface of the particle (Zhang et al., 2003b, 2004). This arrangement places the fusion peptide on DII at the furthest point from the viral surface and DIII in close proximity to the viral membrane. Recently, the structure of the prM–E heterodimer was solved by X-ray crystallography (Li et al., 2008). Fitting of this prM–E structure into the 12.5 Å cryo-electron microscopy (cryo-EM) map of immature (spiky) DENV-2 (Zhang et al., 2004) indicates that the pr peptide portion of prM extends linearly along the E protein surface remaining on the inside edge of the spike. This places the M protein along the dimerization interface on DII, a location where it would prevent homodimerization of E (observed in the mature particle). The prM protein forms a cap-like structure that protects the fusion peptide of E and prevents premature fusion of the virus with host cell membranes (Guirakhoo et al., 1992; Zhang et al., 2003b). This orientation also allows the carbohydrate moieties on prM proteins to form a hydrophilic surface on the immature virus particle. Conserved histidine residues within the prM and E proteins are appropriately located within the interface of the heterodimer and suggest a pH-mediated interaction between the two proteins (Li et al., 2008).

When these immature "spiky" virus particles transit through the Golgi apparatus, they encounter a low pH environment that triggers significant rotational and translational movements within the glycoprotein lattice. These movements result in the transition of the E proteins from prM–E heterodimers (Fig. 3C) to antiparallel E homodimers (Fig. 3D) that lie flat against the viral membrane. The resulting particle has a "smooth" morphology, but is still considered to be immature, due to the presence of prM protecting the fusion peptide on E. The transition from spiky to smooth morphology is reversible, becoming irreversible only when furin cleavage of prM (maturation) has occurred. Furin cleavage occurs in the trans-Golgi and results in the processing of prM to pr and M. The cleaved pr portion remains associated with E and is only released from the virus particle following exit into a neutral pH environment (Yu et al., 2008). Therefore, following maturation, release of the particle into a neutral pH environment does not cause any further re-arrangement of the E proteins, but instead drives the release of the pr peptide from the virion (Yu et al., 2008). The 'mature' particles are competent for fusion and entry into new host cells.

3.3. Conformation of E in the mature virus

Mature flavivirus particles (diameter ∼500 Å) have a relatively smooth surface with the lipid bilayer membrane completely covered by the envelope (E) and membrane (M) protein shell (Fig. 3B and D). This shell consists of 180 copies of the E protein arranged as 90 homodimers forming a herringbone pattern or so-called protein rafts that lie flat on the viral surface. The E protein homodimers within these rafts consist of two monomers associated in
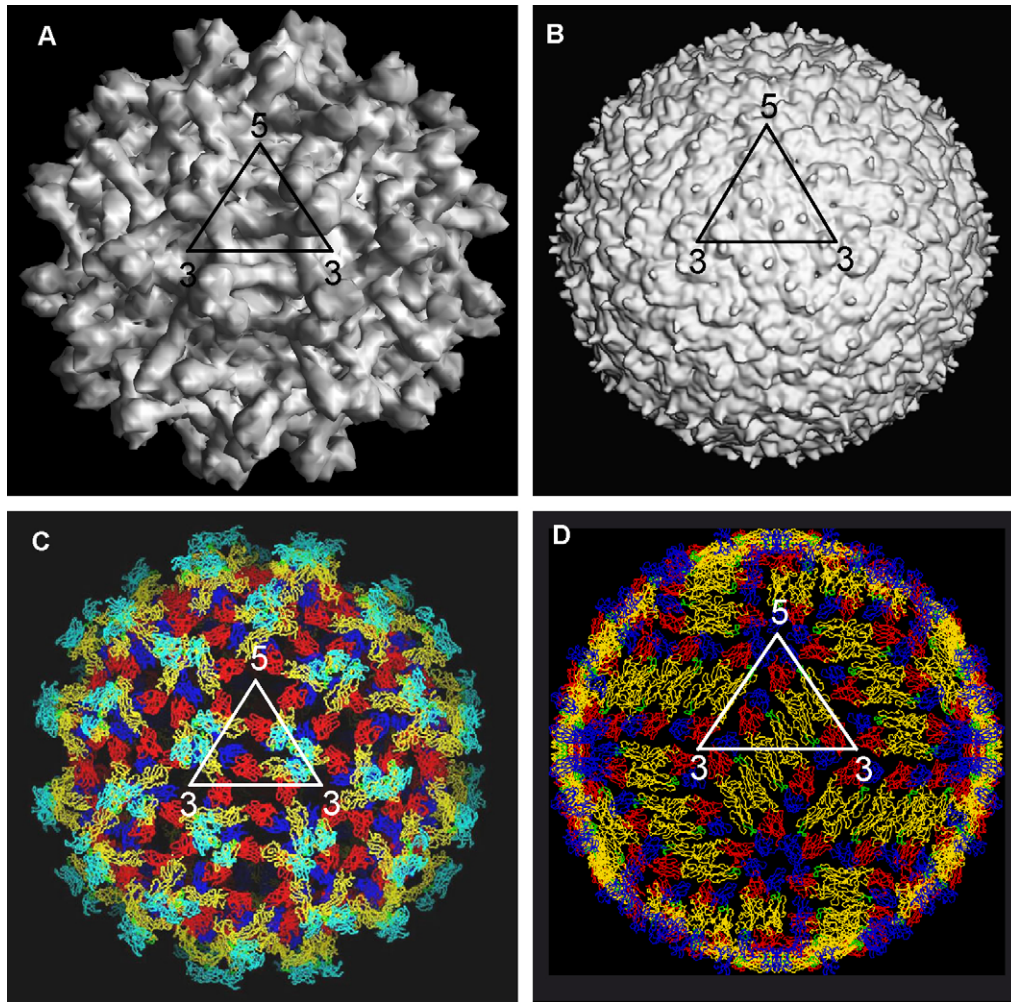
Fig. 3. Structure of flaviviruses. (A) A surface shaded view of the cryo-EM reconstruction of immature DENV-2 showing the spiky surface features (Zhang et al., 2004). The map is calculated to 12.5 Å. (B) A surface shaded view of the cryo-EM reconstruction of mature DENV-2 showing the relatively smooth surface features (map is calculated to 14 Å resolution) (Zhang et al., 2003a). (C) Fit of the atomic coordinates of the E protein C$_\alpha$ residues into the immature virus (Li et al., 2008) and (D) mature virus (Zhang et al., 2003a), showing the arrangement of the E proteins on the surface of the virion. The E proteins are colored as in Fig. 2. Note the difference in orientation of the E proteins as well as the surface packing of the E proteins in the immature and mature viruses.

an antiparallel orientation with DII forming the primary dimerization interface (Zhang et al., 2004; Modis et al., 2003, 2005; Kanai et al., 2006; Nybakken et al., 2006). The fusion loop at the distal end of DII (now missing its pr cap) is buried in a pocket between DI and DIII (see Fig. 2D, where the fusion loop is shown in green). Comparison of the E protein in the mature and immature viruses indicates that flexibility at the hinge between DI and DII (angular difference of 27°) primarily underlies the ability of E to adopt distinct conformations in these two particles (Fig. 3).

3.4. Flavivirus entry and the post-fusion conformation of E

Several distinct events contribute to flavivirus entry into target cells. Initially, viral glycoproteins interact with molecules on the surface of host cells as a point of attachment. Following attachment, specific cell-surface receptors mediate endocytosis of the virus. In the endosome, the fusion peptide of the virus becomes exposed at the distal end of the E protein and is inserted into the host membrane. Low pH-induced structural rearrangements within E then bring the transmembrane domains anchored in the viral membrane closer to the fusion peptide, forming a hairpin structure that promotes fusion of the viral and host membranes. The flavivirus fusion mechanism is similar to that of other viruses, such as the

alphaviruses, with class II fusion mechanisms and is attributed to their very similar secondary and tertiary fusion protein structure (reviewed in Kielian et al., 2000; Heinz and Allison, 2001; Stiasny and Heinz, 2006; Mukhopadhyay et al., 2005; Earp et al., 2005; Harrison, 2005; Schibli and Weissenhorn, 2004; Cohen and Melikyan, 2004; Jardetzky and Lamb, 2004).

Flavivirus fusion has a maximum pH threshold of 6.6–6.8 (Ueba and Kimura, 1977; Gollins and Porterfield, 1986; Summers et al., 1989; Randolph and Stollar, 1990; Guirakhoo et al., 1991, 1993; Despres et al., 1993; McMinn et al., 1996; Corver et al., 2000; Stiasny et al., 2003). In the late endosome, the E homodimers within the mature virion dissociate and re-arrange into fusion-active homotrimers (Allison et al., 1995; Stiasny et al., 2002, 2007). In this conformation, the E proteins are in a parallel orientation to one another within the trimer, extending vertically away from the virion surface. As a result, the fusion loop on DII that was previously buried in the DI–DIII pocket in the homodimer becomes exposed and available to insert into a target host cell membrane.

The post-fusion structures of the DENV and TBEV E protein have been solved by X-ray crystallography (Modis et al., 2004; Bressanelli et al., 2004). The DENV post-fusion E structure is shown in Fig. 2E. The structures suggest that the E protein trimers differ markedly from the mature homodimers due to the rotation and translation of

the three domains relative to one another. In the E protein of DENV-2, domain II rotates approximately 30° relative to domain I through the movement of a hairpin that resides between the two domains. This hairpin induces a switch between an "open" (β-OG bound) and "closed" conformation of a hydrophobic pocket that was observed in the selected crystal structures of E. Residues within this pocket have been shown to influence the pH threshold of fusion (Modis et al., 2003).

The largest displacement is found in DIII, which folds over DI, rotating approximately 70° to bring its C-terminus closer to the fusion loop in DII. This displacement is mediated by a 10-residue linker (residues 290–299 in DENV-2) that was previously found to be disordered in the E homodimer, but assumes a short β-strand configuration in the trimer. Furthermore, the aromatic residues (W101 and F108, TBEV) that are buried in the pocket between DI–DIII of the dimer become exposed in the trimer, suggesting an interaction of this region with the aliphatic groups in the lipid bilayer during fusion. The fusion peptide loops exposed at one end of the trimer still maintain the conformation observed in the dimer, but several polar groups in the loop are exposed, suggesting that the fusion loop only interacts with the polar head groups of the lipid outer leaflet and does not penetrate much deeper (only ∼6 Å) (Modis et al., 2004; Stiasny et al., 2004; Bressanelli et al., 2004). These post-fusion conformations of E suggest that in the final fusogenic form of the E trimer, the fusion peptide loops are juxtaposed with the transmembrane domains of E, forming a hairpin-like structure. These structural transitions of E present ideal targets that can be explored as surfaces for structure-based antiviral design.

3.5. Inhibition of protein–protein interactions

Several groups have identified molecules that interfere with flavivirus entry. Liao and Kielian (2005) demonstrated that a recombinant form of DENV-2 DIII that included helix I of the E protein blocked flavivirus entry by specifically inhibiting virus fusion. However, DIII from Semliki Forest virus, an alphavirus, did not inhibit DENV-2 entry. The alphaviruses were also blocked at the fusion step by their own DIII proteins. Therefore, the authors suggest that exogenous DIII proteins could function as inhibitors of class II fusion mechanisms. Their study also revealed that DIII functioned by binding to fusion intermediates following low pH-induced trimerization and prevented hairpin formation (Liao and Kielian, 2005).

Chu et al. have also shown that a recombinant form of WNV DIII blocked entry of WNV into Vero and mosquito cells while it only effectively blocked DENV-2 entry into mosquito cells (Chu et al., 2005). The corresponding DIIIs of DENV-1 and DENV-2 could inhibit the entry of the respective viruses into HepG2 and mosquito cells. Murine polyclonal antibodies generated against these soluble DIII proteins were capable of neutralizing these viruses in plaque reduction assays (Chin et al., 2007). Several peptides derived from a murine brain cDNA library were shown to inhibit WNV *in vitro* at a concentration of 2.6–67 M. These peptides reduced viremia and fatality in mice during a challenge with WNV, and some were also found to cross the blood–brain barrier (Bai et al., 2007).

Other molecules that have been explored as entry inhibitors include sulfated polysaccharides, polyoxotungstates, and sulfated galactomannans (Talarico et al., 2005; Talarico and Damonte, 2007; Pujol et al., 2002; Ono et al., 2003), sulfated glycosaminoglycans, heparin and suramin (Chen et al., 1997; Marks et al., 2001; Lee et al., 2006; Lee and Lobigs, 2000, 2002; Goto et al., 2003; Mandl et al., 2001; Lin et al., 2002). The biological effects of these molecules that block flavivirus entry may be explained and potentially improved by analysis of the vast array of structural data currently available on these viruses and their component proteins. These structural stud-

ies provide insight into the role of the E protein in the flaivivirus life cycle and present several promising targets for the design of entry inhibitors. Three specific regions on the E protein have emerged from structural studies as targets for the rational design of antivirals against these viruses. They include the β-OG pocket, the E-protein rafts in the mature virus and the E-protein homotrimer.

3.6. The β-OG binding pocket

The discovery by Modis and colleagues of a ligand binding pocket buried at the hinge between DI and DII and its movement in the fusion activation of E made it a prime target for the design of compounds that might inhibit required structural transitions of the virus (Modis et al., 2003). Several groups including ours have undertaken a targeted drug discovery search for biologically active compounds that bind in this pocket and inhibit virus assembly and entry (Li et al., in press; Zhou et al., submitted for publication).

The β-OG pocket forms a channel with open access at both ends (Fig. 4), allowing linear molecules of varying lengths to be accommodated. The channel is lined by hydrophobic residues that have been shown through mutagenesis studies to influence the pH threshold of fusion (Modis et al., 2003). The movement of a loop (kl), that resides between DI and DII (shown in grey in Fig. 4) controls access to the channel for small hydrophobic molecules. In the β-OG-bound structure of DENV-2 E, the kl loop formed a salt bridge and hydrogen bond with the ij loop (shown in pale yellow) in the dimer partner (within the homodimer of E) forming an "open" conformation of the pocket. This conformation exposes the hydrophobic core and accommodates binding of a single β-OG molecule (Fig. 4C). In the absence of β-OG, a "closed" conformation of the pocket was observed, with the kl loop burying the underlying hydrophobic residues (as seen in the crystal structures of TBEV, DENV-3, WNV and selected structures of DENV-2). It has been proposed that the varying conformations of this pocket induced by the movement of the kl loop play an important role during fusion. Specifically, the loop assists in the movement of DII and allows the fusion peptide at the distal end of DII to be directed towards the host cell membrane. The β-OG pocket therefore may prove to be an ideal target for the inhibition of viral fusion and entry. Binding of small molecules that "pry open" the pocket may trigger conformational changes similar to those induced by low pH and induce premature fusion. Alternatively, inhibitors binding in the pocket may also prevent the structural transitions necessary for maturation and fusion activation of the virus (Modis et al., 2003).

Observations from the picornaviruses provide precedence for these hypotheses (Pevear et al., 1989; Rossmann, 1994; Rossmann et al., 2000; Badger et al., 1988; Heinz et al., 1989; Smith et al., 1986; Fox et al., 1986; Kim et al., 1993; Reisdorph et al., 2003). The picornavirus virion consists of four structural proteins (VP1–4), with VP1–3 forming the external protein shell and VP4 remaining inside the capsid (Rossmann et al., 1985). VP4 is released from the capsid upon uncoating. In these viruses, a deep canyon is formed at the junction between VP1 and VP2/3 that functions as a receptor-binding site as well as an antigenic site for neutralizing antibodies. This canyon is found around the five-fold vertices in the capsid. A pocket within VP1 in the canyon floor was found to bind antiviral compounds (WIN drugs) (Smith et al., 1986; Badger et al., 1988; Kim et al., 1993; Reisdorph et al., 2003). Binding of these compounds inhibited uncoating and entry of the virus by preventing "breathing" of the capsid. It was also observed that conformational changes occurred in residues forming the canyon floor upon binding the compounds (up to 4 Å movement in C_α positions) that could potentially prevent receptor binding and attachment to host cells.

To identify small molecules that directly bind the β-OG pocket and functionally inhibit the virus, we have used a hierarchical
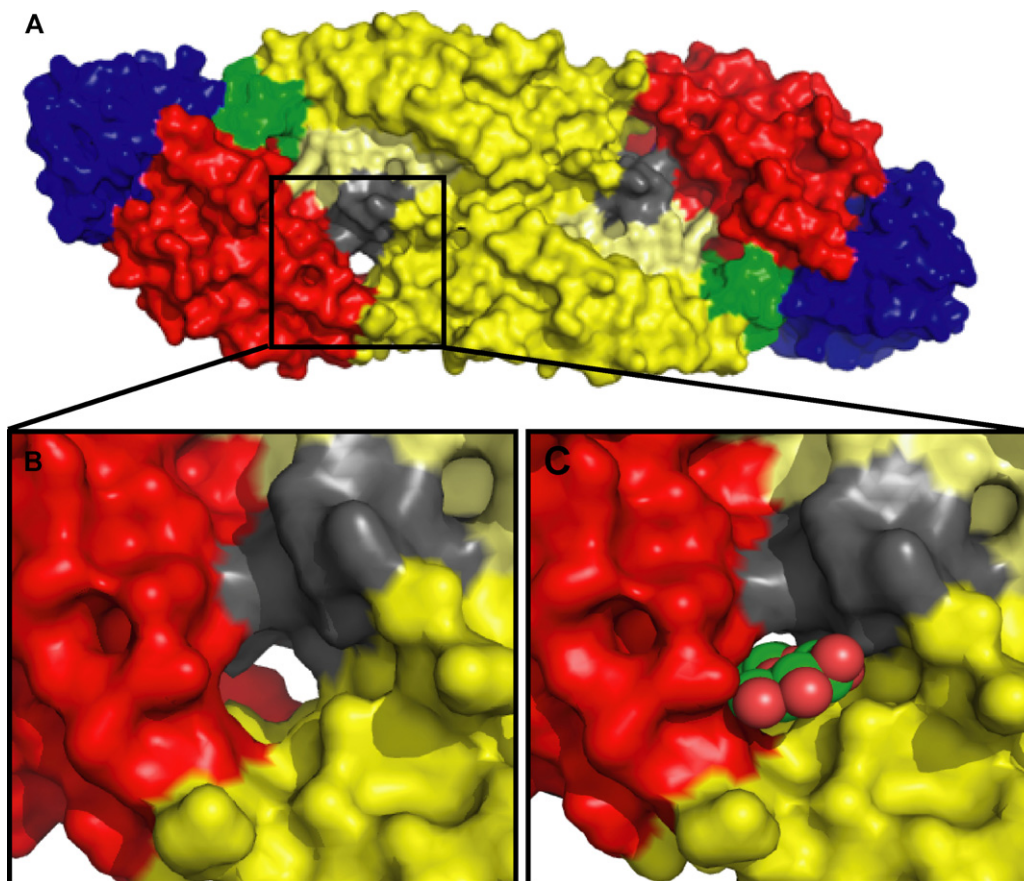
Fig. 4. β-OG binding pocket in the E protein. (A) A space-filled representation of the atomic structure of the E protein homodimer (Modis et al., 2003; Zhang et al., 2003a, 2003b). The E proteins are colored as in Fig. 2, with the exception of the kl loop (residues 268–280 in DENV-2) (shaded in grey) that is suggested to control the 'open' and 'closed' confirmation of the β-OG pocket (boxed). This kl loop interacts with the ij loop in DII (residues 237–253 of DENV-2 and shaded in pale yellow) during these conformational changes. (B) An enlarged view of the β-OG pocket in its 'apo' conformation. Note that the pocket forms a channel with open access at both ends. (C) An enlarged view of the pocket with β-OG occupying the pocket.

computational approach to screen three compound libraries (a total of 143,000 compounds) from the National Cancer Institute (Zhou et al., submitted for publication; Li et al., in press). The 45 top-scoring compounds selected from the computational docking approach were further visually screened for drug-like properties and ideal structural characteristics for interacting with the β-OG pocket. A group of 23 compounds were tested for cytotoxicity (CC50) in baby hamster kidney (BHK) cells and inhibitory activity against YFV (Zhou et al., submitted for publication; Li et al., in press).

Three approaches were used to assess virus spread. Initially, the effect of the compounds was monitored using a YFV expressing the fire-fly luciferase gene. BHK cells were infected at low multiplicity and levels of luciferase activity in infected cell lysates was measured at various compound concentrations. Nine compounds inhibited virus spread at inhibitory concentrations (IC50) between 20 and 500 μM. In a second approach, pseudo-infectious particles (PIPS) obtained using a replicon system were produced in cells either treated with compounds or untreated. These PIPS are generated through sequential transfection of BHK cells with two RNA transcripts independently expressing the viral non-structural proteins and the viral structural proteins. The particles produced by this method are only capable of a single round of infection. Therefore, PIPs provide a direct measure of virus assembly and release, as well as virus entry, requiring all of the structural transitions of E to be completed. As originally designed, five of the nine compounds inhibited virus maturation/entry and/or fusion.

In a third approach, a replicon expressing the viral non-structural proteins and the *Renilla* luciferase gene was used to monitor the effect of these compounds on viral RNA replication. The replicon is capable of autonomous replication but cannot spread from cell to cell due to the lack of the structural proteins (Jones et al., 2005). Luciferase activity of cells transfected with this construct provides a direct measure of the replication of the viral RNA. Therefore, it should identify compounds that may influence only viral RNA replication. Of the nine compounds tested, four affected RNA replication. Promisingly, there was no overlap between the compounds that affected the structural transitions of E and those affecting viral RNA replication (Zhou et al., submitted for publication; Li et al., in press).

NMR studies show that one of the compounds directly binds the DENV E protein and competes with β-OG (Zhou et al., submitted for publication). In the DENV-2 E structure, the β-OG molecule is oriented with the glucosyl head group in the channel's mouth and the hydrocarbon chain projecting deep into the channel's cavity. Hydrogen bonds between the pocket residues and the β-OG molecule fix its orientation in the cavity (Modis et al., 2003). Similarly, several of the compounds mentioned above are predicted to form hydrogen bonds with pocket residues that lie on the top of the channel (Zhou et al., submitted for publication). These interactions potentially contribute to the binding free energy, as well as specificity, as observed with HIV-1 reverse transcriptase inhibitors (Zhou et al., 2004, 2005).

Second- and third-generation compounds were designed from these initial lead compound hits. Promising reactive groups such as thiazole rings and aromatic rings were maintained while eliminating cytotoxic groups such as α,β-unsaturated ketones. Tighter binding was explored by increasing the number of potential hydrogen bonds between the pocket residues and the compounds. These efforts were rewarded by the design and synthesis of two compounds that showed IC_{50} values ranging from 1 to 5 μM. Computational docking indicated that they bound into a channel in the pocket that increased in hydrophobicity with increasing depth from the entrance to the bottom. Three electrostatic/steric cavities within this channel accommodated various parts of the two compounds. The most potent compound was smaller and bound very deeply into the channel and interacted with a cavity surrounded by hydrophylic residues (Zhou et al., submitted for publication; Li et al., in press).

3.7. E-protein rafts

The E-protein rafts (Fig. 3D) that densely pack against the viral membrane in the mature virus present another promising target for the design of flaviviral entry inhibitors. These rafts provide ideal protein surfaces for docking small molecules that might interfere with protein–protein interactions. Molecules that stabilize the dimers in the mature virus and prevent downstream structural rearrangements could also be effective. Such inhibitors could prevent the transformation of E into the low pH-induced trimer conformation required for fusion and entry into naïve cells.

A similar computational docking protocol as mentioned above has been used to identify potential binding surfaces that would accommodate such inhibitors. Two pockets within the rafts have currently been identified and used for high throughput screening of compounds from the same NCI library. Based on initial screens, 14 out of 42 potential compounds have been classified as inhibitors of virion morphogenesis (i.e. they do not affect viral RNA replication). Further analyses of these compounds as potential entry inhibitors are ongoing (La Bauve, Zhou, Kuhn and Post, unpublished data).

3.8. Fusion-active trimer of E

The fusion-active state of viruses has long been a target for inhibition as observed in viruses with class I fusion mechanisms (Copeland, 2006; Eckert and Kim, 2001; Veiga et al., 2006; Rusconi et al., 2007; Esté and Telenti, 2007). The active fusion core of viruses such as influenza and the human immunodeficiency virus constitutes a six-helix bundle consisting of two helices from each fusion protein in the trimer (Skehel and Wiley, 2000; Melikyan et al., 2000; Russell et al., 2001; Chang et al., 2008). Its formation can be prevented by antiviral peptides that mimic the helices and compete with the protein–protein interactions that give rise to the fusion core (Hsieh and Hsu, 2007; Stevens and Donis, 2007).

Similarly, the fusion core of the flaviviruses could be targeted to prevent virus entry. As discussed above, preliminary studies have been carried out using exogenous DIII proteins to trap fusion intermediates (Liao and Kielian, 2005; Chu et al., 2005; Chin et al., 2007). The DIII proteins bound a fusion intermediate following trimerization indicating that a trimer intermediate with a relatively prolonged lifetime existed and could be blocked prior to fusion (Liao and Kielian, 2005). These studies could be further extended to prevent the formation of the trimer, by sterically inhibiting the movement between DI and DIII and preventing the folding over of DIII (Fig. 2). The existence of intermediates prior to trimer formation has been previously observed (Stiasny et al., 2002, 2007). These studies have indicated that a monomeric E intermediate capable of interacting with membranes in a pre-hairpin conformation does

exist prior to the formation of E homotrimers. In addition, Stiasny et al., also showed that DIII relocation and trimer formation were concomitant and occurred after the membrane binding of the monomeric E pre-hairpin. These intermediates are targets that could be considered during the design of entry inhibitors.

Although class I and II fusion proteins are structurally distinct, the end result remains the same. The former undergoes a refolding step prior to forming its fusion-active conformation, and the latter re-orients domains with limited refolding. Ultimately, a hairpin structure is formed with the fusion loop and transmembrane regions juxtaposed in the same membrane. Therefore, lessons learned from class I fusion proteins could be adapted to inhibit flavivirus fusion and entry.

4. Other potential sites for the design of flaviviral entry inhibitors

Apart from the binding pockets within the E protein that are obvious targets for the design of antivirals, several other interactions involving the E protein provide promising targets for the design of entry inhibitors. Studies have shown that attachment molecules are required for virus entry. The interaction of E with these molecules have been studied in detail using structural and biochemical techniques and can be pursued as an option for interrupting virus entry. In addition, the interaction of the E protein with neutralizing antibodies has been studied extensively. Structural information gleaned from these interactions can be utilized to design novel entry inhibitors with increased efficacy.

4.1. Attachment molecules

Several attachment molecules important for flavivirus entry have been identified. The C-type lectin, dendritic cell-specific ICAM3 grabbing nonintegrin (DC-SIGN) has been shown to be essential for DENV infections through its interaction with carbohydrate moieties on the E protein (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003). Depending on the virus from which it is derived, one or two N-linked glycosylation sites are found on the E protein. Asn153 is conserved among all flaviviruses while Asn67 is unique to DENV (Rey, 2003). N-linked glycosylation of Asn67 is required for DENV growth in mammalian cells (Bryant et al., 2007; Mondotte et al., 2007). The above-cited studies by Navarro-sanchez et al. and Tassaneetrithep et al. have demonstrated that both soluble DC-SIGN and antibodies against DC-SIGN inhibit DENV infection. However, Lozach et al. (2005) showed that internalization of DC-SIGN was not necessary for DENV infectivity. It therefore probably does not function as a specific receptor, but allows for virus attachment and concentration on the cell surface.

Structural insight into the interaction of DENV E with DC-SIGN has been obtained through a cryo-EM reconstruction of DENV-2 in complex with the carbohydrate recognition domain (CRD) of human DC-SIGN (Podishevskaya et al., 2006). The CRD bound the Asn67 residue in the DENV E protein. Interestingly, binding did not induce conformational changes in the E protein on the mature virus, although such changes may occur when full-length DC-SIGN binds to E. The stoichiometry of binding between the CRD and the E proteins on the virion surface left one E molecule in the asymmetric unit unoccupied and the putative DIII receptor-binding domain of each E molecule free to bind the receptor (on the icosahedral five-fold and three-fold axes). Based on the number of DC-SIGN molecules that interact with the virus, the oligomeric state of DC-SIGN on the surface of a cell is a tetramer, so binding of DC-SIGN could therefore promote clustering of the virus on the cell surface and assist in receptor binding. The authors suggested that the binding of the carbohydrate moieties to DC-SIGN mimics normal

cellular processes and therefore functions to protect the receptor-binding domain from immune surveillance and neutralization. By contrast, WNV binds the related C-type lectin, DC-SIGNR, during dendritic cell infections (Davis et al., 2006), while YFV does not have any glycan modification on E and therefore attaches to cells in a lectin-independent manner (Barba-Spaeth et al., 2005).

Another C-type lectin receptor, the mannose receptor (MR), has recently been shown to bind DENV, JEV and TBEV through a mechanism similar to that of DC-SIGN. However, the ligand specificity of MR (terminal mannose, fucose and N-acetyl glucosamine) differs from that of DC-SIGN (high-mannose oligosaccharides and fucosylated glycans). The authors propose that MR has the potential for being a DENV receptor (rather than just an attachment molecule), because it is constitutively internalized and found mainly in the endocytic pathway, in contrast to DC-SIGN, which is mainly localized in the plasma membrane (Miller et al., 2008).

Other molecules that have been implicated in assisting flavivirus entry include heparan sulfate (Hung et al., 1999; Kroschewski et al., 2003; Liu et al., 2004), $\alpha_v\beta_3$ integrin (Chu and Ng, 2004), Rab5 (Krishnan et al., 2007), HSC70 (Ren et al., 2007) and BiP (Jindadamrongwech et al., 2004). $\alpha_v\beta_3$ integrin is an endothelial cell receptor that is implicated in WNV and JEV entry of vertebrate cells. Integrin binding has been predicted for the flaviviruses, because domain III of most flavivirus envelope proteins has an RGD-type motif important for integrin-ligand interactions (van der Most et al., 1999). Other viruses, including foot-and-mouth disease virus, coxsackie virus and adenovirus, also interact with integrins in an RGD-dependent manner (Roivainen et al., 1991; Rieder et al., 1996; Bai et al., 1993). However, the binding of WNV to $\alpha_v\beta_3$ integrin was independent of the RGD motif. Antibodies against this integrin, as well as soluble forms of the protein, inhibited WNV and JEV entry into permissive cells. RNAi studies also supported the observation that this particular integrin might serve as a receptor for WNV (Chu and Ng, 2004). Rab5 GTPase is a key regulator of traffic to the early endosome and has been implicated in DENV and WNV entry. Dominant negative inhibition or RNAi-based inhibition of Rab5 (but not Rab7, a late endosomal GTPase) significantly reduced DENV and WNV entry and replication in Hela cells (Krishnan et al., 2007).

4.2. Neutralizing epitopes on E

The humoral immune response plays an important role during flavivirus infections and several antibodies that are effective in neutralizing these viruses have been identified (Halstead, 1989; Halstead et al., 1980; Kaufmann et al., 1987, 1989; Phillpotts et al., 1987; Johnson and Roehrig, 1999; Mathews and Roehrig, 1984; Roehrig et al., 1998, 2001; Roehrig, 2003; Diamond et al., 2003; Sukupolvi-Petty et al., 2007). The interaction of the E protein with these antibodies provides insight into epitopes that might be accessible during structural transitions of the virus and presents novel avenues of antibody-mediated therapeutic intervention (Crill and Chang, 2004; Crill and Roehrig, 2001; Halstead et al., 2005; Li et al., 2005; Pierson et al., 2007; Stiasny et al., 2006; Sukupolvi-Petty et al., 2007; Kaufmann et al., 2006; Lok et al., 2008; Beasley and Barrett, 2002; Lin et al., 1994; Lin and Wu, 2003; Oliphant et al., 2005, 2006; Roehrig et al., 1998; Sanchez et al., 2005). Specifically, antibodies or Fabs can interfere with virus attachment, membrane fusion, and internalization mediated by the E protein, and they can also trap intermediates during the structural transition between mature and fusion-active forms of the virus. Although the primary neutralizing epitopes lie on DIII, cross-reactive epitopes have also been identified on DI and DII (Oliphant et al., 2006; Crill and Chang, 2004; Goncalvez et al., 2004; Ledizet et al., 2007).

A pseudo-atomic structure of the neutralizing monoclonal antibody E16 Fab in complex with WNV was recently determined (Nybakken et al., 2005; Kaufmann et al., 2006). This structure suggests that E16 interferes with virus entry by blocking conformational rearrangement of E at a step following receptor attachment. In this structure, E16 only partially obscures the surface of the particle by binding to 120 of 180 DIII domains, leaving those on the five-fold axes of the particle available for receptor binding. This preferential binding has been attributed to steric hindrance that prevents complete occupancy of all DIII epitopes. These observations were predicted from previous *in vitro* studies that indicated that E16 only partially prevents attachment of the virus to cell-surface receptors and blocks infection at a step following receptor binding (Nybakken et al., 2005; Oliphant et al., 2005). It is plausible that E16 may function at a step following receptor binding by inhibiting these rearrangements necessary for the mature virus to transform to its fusion-active state. This structure also suggests a combination therapeutic strategy that could utilize both E16 antibodies and antivirals designed against the co-receptors (exposed at the five-fold axes) to block flavivirus entry. Studies of mice exposed to WNV showed that treatment with E16 five days postexposure to WNV resulted in a 90% survival rate, with complete clearance of the virus in the brains of 68% of the treated mice (Oliphant et al., 2005). Interestingly, the interaction of E16 with WNV seems to be quite different from the interaction the 1A1D2 Fab with DENV (Lok et al., 2008).

In DENV infections, the monoclonal antibody 1A1D2 strongly neutralizes DENV serotypes 1–3 by inhibiting attachment of virus to host cells, but it does not bind to serotype 4 (Roehrig et al., 1998). A pseudo-atomic structure of the Fab fragment of 1A1D2 in complex with the virus has recently been determined and suggests a mechanism for virus neutralization (Lok et al., 2008). In this structure, it was observed that the Fab bound to an epitope on DIII of the E protein that was normally occluded in the mature virus. Fab binding required higher temperatures, suggesting that "breathing" of the virus was a prerequisite for antibody recognition. Based on these observations, structural transitions that might require breathing of the viral proteins could be trapped through the use of antibodies. It has already been demonstrated that such trapped complexes are incapable of efficient infection (as demonstrated by the neutralization efficacy of 1A1D2). This structure also provides insight into cryptic epitopes in E that could be targeted by antivirals. As discussed previously, this phenomenon has been observed for nodavirus (Bothner et al., 1998) and rhinovirus (Lewis et al., 1998). For instance, the breathing of rhinovirus exposed internal regions of the VP4 structural protein making this protein sensitive to proteolysis. These breathing motions were inhibited however, in the presence of antiviral compounds that bound in a cavity within VP1 that stabilized the virion (Lewis et al., 1998; Reisdorph et al., 2003; Smith et al., 1986; Badger et al., 1988; Kim et al., 1993). Further evidence that hidden epitopes might function as immunogenic sites and potential targets for antivirals stems from a recent report that immunoglobulins raised against linear epitopes of all three domains of the E protein protected mice against lethal WNV challenge (Ledizet et al., 2007).

An alternate scenario is possible for MAbs that bind DI and DII epitopes of the E protein. As mentioned previously, ADE is a phenomenon with devastating consequences, caused by a weakly neutralizing immune response to a prior DENV infection and is a serious concern for flavivirus vaccine development (Halstead, 1979). Several MAbs directed against DI and DII of the E proteins demonstrated more enhancing effects (characteristic of ADE) than neutralization properties during virus challenge, in contrast to antibodies against DIII, which were strongly neutralizing (Oliphant et al., 2006). The authors therefore suggested that productive neutralization of DENV would require redirecting the antibody response from the enhancing effects of the DI and DII epitopes towards the more

protective DIII epitopes. Structure-based antiviral design could allow this by eliminating or inhibiting the availability of epitopes of DI and DII to the immune response, preventing their ADE effects, and prove to be a potential strategy to prevent flavivirus infection.

5. Concluding remarks

Given all of these possible strategies, it is remarkable that few successful antivirals have been developed against the flaviviruses, but the challenges ahead are clear. New structural insights into the flavivirus life cycle and viral interactions with cellular molecules and antibodies provide great opportunities for identifying new classes of inhibitors. The ability to obtain high-resolution structures of viral components and inhibitory compounds suggests that powerful structure-based approaches could rapidly focus the development of highly efficacious compounds. The same techniques could be used to design compounds that evade virus resistance and demonstrate broad anti-flaviviral activity. However, it is important to recognize that rapid and precise diagnosis will be essential to the effectiveness of anti-dengue drugs. Therefore, improvement in diagnostic tests for dengue must proceed in parallel with new therapies. The severity and duration of dengue fever might be effectively controlled by antivirals upon early diagnosis, however, these compounds may not be as effective if the infection has progressed to DHF. In this form of the disease, due to complications caused by an active immune response, immunomodulatory compounds and strategies may have greater impact on disease intervention.

Acknowledgements

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