Chapter 15
Flaviviruses: Introduction to Dengue Viruses

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Core Message Flaviviruses are a major concern of the World Health Organization, and dengue virus is now the most widespread arthropod-borne viral disease infecting humans. Currently no vaccine or specific antiviral drugs are available to combat dengue fever. Thus, investigations and research to better understand this virus and its relation with its host are of great importance.

1 Flaviviruses

1.1 Etiologic Agent and Natural History

1.1.1 Definition

Flaviviruses *sensu lato* are all the members of the viral family *Flaviviridae*, which includes the four genera *Flavivirus*, *Hepacivirus*, *Pegivirus*, and *Pestivirus*. Members of these different genera are distantly related but their genomes share a similar gene order and conserved nonstructural protein motifs [1]. Important and well-known members of the family are bovine viral diarrhea virus, classical swine fever virus, dengue viruses, hepatitis C virus, and yellow fever virus.

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1.1.2 Etiology and Evolution

This chapter addresses the members of the genus *Flavivirus* (flaviviruses *sensu stricto*). The first bona fide flavivirus characterized was yellow fever virus (YFV), the causative agent of yellow fever (named after the prominent icterus of infected patients). Together with YFV, more than 70 viruses are currently classified in the genus, which also is the taxonomic home of other important human and animal pathogens such as dengue viruses (DENV1-4), Japanese encephalitis virus, Murray Valley encephalitis, St. Louis encephalitis virus, tick-borne encephalitis virus (TBEV), and West Nile virus (WNV) [1]. Flaviviruses can be categorized into three groups based on phylogenetic analysis of genomic sequences, and these groups correlate largely with their respective insect vector: the mosquito-borne viruses, the tick-borne viruses, and viruses for which no vector has been identified and that may spread without a vector. Table 15.1 summarizes the major characteristics of some well-known flaviviruses. Dengue fever, transmitted by mosquitoes, is currently the most widespread arthropod-borne viral disease of humans. Due to this prevalence, we here use DENV as an example to discuss, introduce, and describe flaviviruses.

1.1.3 Geographic Distribution and Economic Effects of Dengue Virus Infection

In the past 50 years, dengue viruses have become a major global human health threat. The World Health Organization estimates that about 100 million human infections occur annually, whereas a recent study estimated this number to actually be closer to 300 million [2]. Dengue fever is predominantly found in tropical and subtropical regions. In 2013, more than half of the world's human population lived in areas at risk of infection [3, 4]. Currently, there is no specific treatment for or vaccine against these viruses. Control of the primary DENV vector, the *Aedes aegypti* mosquito, is currently the principal measure available to prevent and control DENV transmission.

1.2 Pathogenesis and Clinical Features

1.2.1 Pathogenesis and Immunology

Four antigenically and phylogenetically distinct dengue viruses are known to be established in humans although sylvatic viruses that lie outside of these four serotypes can also infect humans. Immunoglobulin M (IgM) can be detected at the end of the febrile phase of dengue fever, followed by a moderate IgG response that is thought to confer lifelong protection against that DENV serotype. During subsequent infection with a heterologous serotype of dengue virus, IgG against both serotypes arise rapidly, but antibody titers are higher against the first virus serotype than against the second virus serotype [5].

Table 15.1 Characteristics of clinically important flaviviruses

Viruses	Vertebrate host	Clinical signs	Prevalence/epidemiology	Ref.
Dengue virus 1-4 (DENV1-4)	Humans	See text	See text	See text
West Nile virus (WNV)	Birds; humans and horses are accidental hosts	• Approximately 20 % develop influenza-like signs for 3–6 days • Less than 1 % develop severe (meningitis, encephalitis, myelitis) or lethal disease, especially in elderly and immunodeficient patients	• US 2012 outbreak: total of 5,387 cases (243 deaths) in humans • Enzootic and endemic in Africa and Asia. More sporadic in America and Europe	[98–100]
Japanese encephalitis virus (JEV)	Birds, pigs, and humans	• Incubation period: 5–10 days. • Most cases are asymptomatic or mild • About 1/300 infections induce disease ranging from febrile illness to severe meningoencephalitis (lethal in 25 %) and long-term neurological defects	• 30,000–50,000 total cases/year • 10,000–15,000 deaths/year in Asia and Australasia	[9, 101–103]
Yellow fever virus (YFV)	Humans, nonhuman primates	• Fever, chills, malaise, headache, back pain, nausea, dizziness, jaundice and hemorrhage • ≈15 % of cases develop severe disease with hemorrhage • ≈20–50 % of patients develop fatal liver and kidney failure	• ≈200,000 worldwide cases/year, causing 30,000 deaths • Increase observed over the last 10 years • Endemic in tropical areas of Africa and South America	[9, 104]
Tick-borne encephalitis virus (TBEV)	Humans, small mammals, birds, and livestock	• Influenza-like illness with severe headache, nausea, vomiting, and severe back pain; often focal epilepsy, flaccid paralysis (e.g., shoulder girdle) • ≈30 % of cases develop a second phase with high fever, CNS signs, (meningoencephalitis, paralysis), with possible sequelae or death • The case-fatality rate: ≈20 % with Asian strains, 1–5 % European strains	• 10,000–12,000 cases/year • Endemic in forested areas of Europe, northern China, Mongolia, and Russia	[9, 105]

Clinical signs of dengue fever appear 3–14 days after a bite by a DENV-infected female mosquito. These signs vary from unapparent, mild febrile illness to severe dengue infection that can result in complications and death. About 1–70 % of the infections lead to influenza-like disease characterized by high fever, rash, joint and muscle pain, and mild hemorrhagic manifestations, such as petechiae, purpura, ecchymoses, and nose bleeds [3, 6–8]. Dengue fever can progress to a painful and debilitating disease commonly referred to as "break bone fever." Severe dengue, formerly referred to as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), is characterized by a significant increase in vascular permeability, plasma leakage, microvascular bleeding, and reduced functioning of the coagulation cascade. Hemorrhages occur in multiple organs, frequently including the gastrointestinal tract, and fluids may pool within body cavities. Severe dengue is characterized by a sudden drop in blood pressure due to vascular leakage that leads to hypovolemia and collapse of the vascular system. Since there is currently no specific antiviral treatment for DENV infection, supportive care that maintains the patient's body fluid volume is critical [9]. Under the supervision of experienced medical personnel, supportive care can reduce fatality rates for severe dengue from more than 20 % to less than 1 %.

Despite much study, the determinants of disease severity in dengue infection are complex, and the absence of a fully immunocompetent animal model that faithfully reproduces all aspects of human dengue virus infection has delayed scientific progress in this area. Considerable evidence exists that disease is correlated with greater viral burden [7], although some patients experiencing secondary infection with a heterologous serotype of DENV exhibit high viremia in the absence of severe disease [3]. Prior infection with a heterologous serotype of DENV has been identified as a major risk factor for severe dengue fever. Enhancement of secondary dengue fever is thought to be due to non-neutralizing antibodies that promote uptake and productive entry of virions (reviewed in ref. 10). The antibody-dependent enhancement (ADE) of viral infection is a phenomenon that has been described for several flaviviruses [11–13]. In particular, antibodies that recognize the DENV structural precursor membrane protein (prM) are thought to promote ADE by increasing uptake of immature virions. Since the presence of infection-enhancing antibodies is not always correlated with disease severity [14], other variables including viral [13, 14] and host genetics [11, 12] and kinetics (i.e., time between infections) are also likely to be important.

1.3 Flavivirus Characteristics

1.3.1 Flavivirions

Flavivirions are spherical particles of approximately 50 nm in diameter. Each virion contains a single copy of the positive-sense RNA genome surrounded by multiple copies of the viral core protein to form the viral nucleocapsid. This nucleocapsid is

protected by a lipid bilayer bearing 180 copies of the viral envelope protein (E) arranged as a well-ordered lattice of 90 homodimers organized in a herringbone pattern on the virion surface [15]. A glycoprotein, E, functions in viral entry by mediating the virion's interactions with entry factors and receptors and undergoing conformational changes. These conformational changes catalyze fusion of viral and cellular membranes following exposure to acidic pH in the endosome (see below). On immature virions, the E glycoprotein forms trimers in which each E monomer is protected by a copy of the chaperone premembrane protein, prM [16]; each trimer of E-prM heterodimers forms a "spike" on the virion surface [17] (schematic representation in Fig. 15.1). This association of E with prM prevents premature fusion of immature virions with cellular membranes in the acidic environment of the Golgi [18, 19]. The prM protein is processed to pr and M by furin, and pr is released by the virion once in the neutral pH of the extracellular space. This release produces the smooth-surfaced, mature virion observed in cryo-electron microscopy reconstructions [20, 21]. Studies using neutralizing antibodies indicate that E protein on the virion surface undergoes dynamic structural changes at physiological temperatures, and these changes have a significant effect on the accessibility of epitopes and neutralization of virus [22].

1.3.2 Flavivirus Genomes

The DENV genome is a single-stranded linear RNA of positive polarity ((+)ssRNA)) of approximately 11 kb (Fig. 15.2). Like that of other flaviviruses, the genome encodes a single open reading frame (ORF) flanked by highly structured 5′ and 3′ untranslated regions (UTRs) of about 100 and 400 nucleotides, respectively. The 5′ end bears a type I cap structure (m^7GpppAmG) for cap-dependent translation of a single polyprotein. The 3′ UTR terminus lacks a polyadenylated tail but ends with a highly conserved stem loop structure (3′SL). A high number of *cis*-acting RNA elements, located in the coding and noncoding regions of the genome, act as promoters, enhancers, and circularization signals that are required for efficient RNA replication or translation (for reviews see refs. [23–25]). A RNA hairpin structure in the capsid-coding region (cHP) directs start codon selection and is also required for viral replication [26]. The highly conserved 3′SL is required for viral RNA replication [27–29] but also promotes translation by facilitating binding of the RNA to polysomes [30].

1.3.3 Flavivirus Proteins

The single polyprotein encoded by flavivirus genomes is cleaved into ten individual proteins by a combination of viral and cellular proteases and peptidases. For DENV, these cleavages are catalyzed by the viral NS2B-NS3 protease, host signal peptidase (a protease of the *trans*-Golgi network), and by furin [31, 32]. Collectively, these cleavage events lead to the production of ten mature viral proteins: three structural

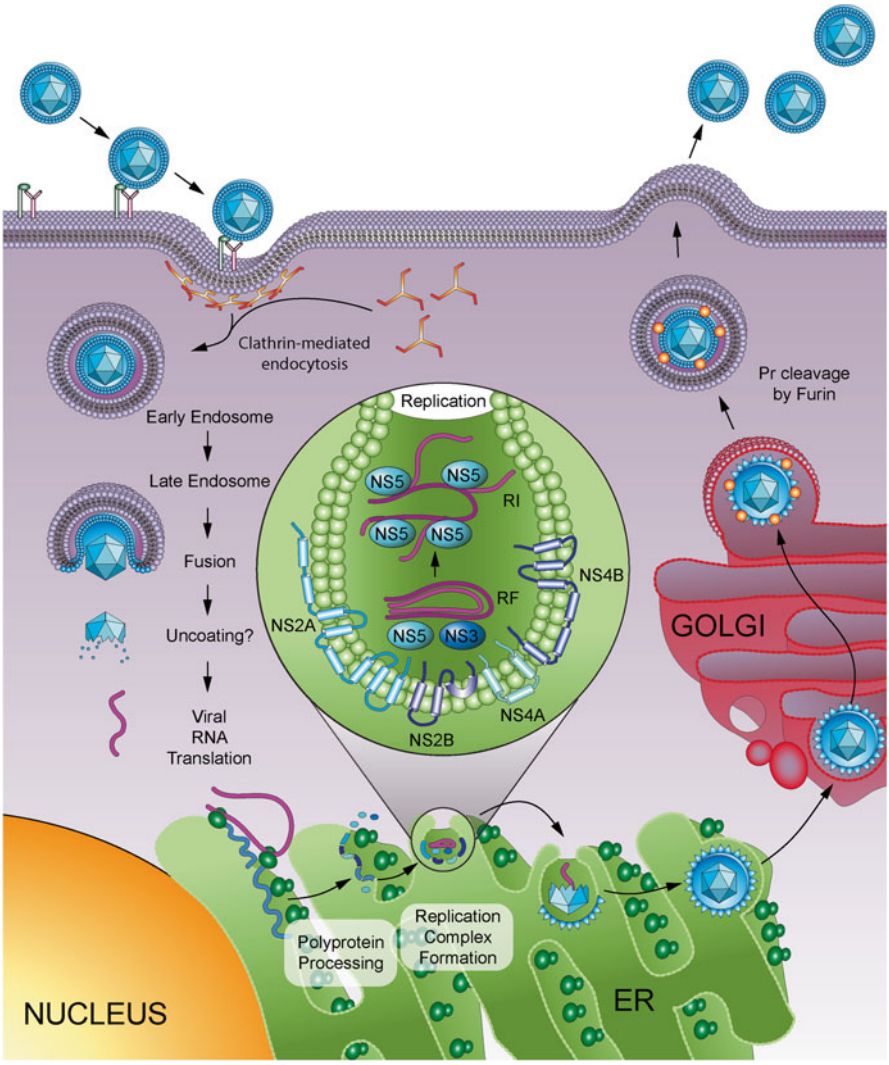

Fig. 15.1 Dengue cycle. Mature virus particles bind to host cell receptors and coreceptors and are internalized by clathrin-mediated endocytosis. In the late endosome, acidic pH triggers structural rearrangements of E that catalyze fusion of the viral and endosomal membranes. This fusion process is also affected by the presence of negatively charged lipids in the target endosomal membrane. The decapsidation process is not well known, but the released viral genomic RNA (*light pink*) is directly translated by the cellular ribosomes (*green*) to produce viral polyproteins (*blue*). The polyprotein is cotranslationally and posttranslationally processed by viral and host proteases. The nonstructural (NS) proteins produced from the polyprotein induce membrane rearrangement (invagination in the endoplasmic reticulum) to form the specialized compartment in which the replication complex is assembled and genome replication occurs. The viral genomic (+)ssRNA (light pink) is a template for the synthesis of a negative-sense RNA by NS5 and leads to the formation of double-stranded RNA (dsRNA) intermediates called the replication form (RF). The newly synthesized (−)ssRNA (dark pink) is used in turn as a template for the synthesis of multiple (+)ssRNA via a replication intermediate (RI). Newly synthesized (+)ssRNA can either serve as a template for translation or replication or undergo encapsidation. During encapsidation, RNA interacts with the capsid protein and buds into the lumen of the endoplasmic reticulum, thereby acquiring lipid bilayer and the precursor membrane (prM) and envelope (E) viral proteins.

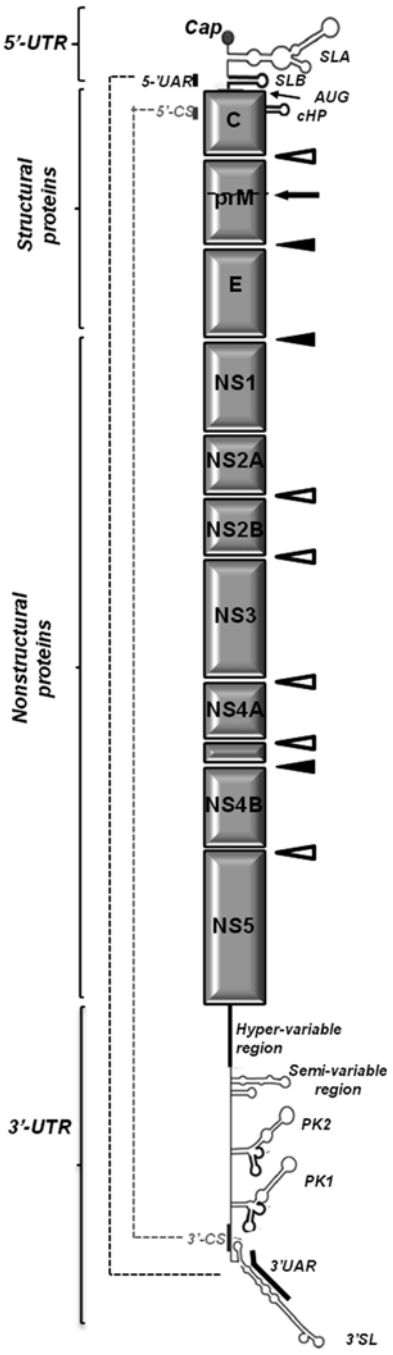

Fig. 15.2 Flavivirus genomic organization. The genome of Dengue viruses is a positive, 5′-capped, single-stranded RNA. The genomic RNA consists of a single ORF that encodes a polyprotein (*dark grey*) flanked by 5′ and 3′ untranslated regions (UTRs). The polyprotein is cleaved during and after translation by the viral NS2B-NS3 proteases (*open triangles*) or host proteases, such as furin (*arrow*), signal peptidase (*closed triangles*), or unknown factors. Abbreviations: *AUG* translation initiation codon, *C* capsid, *cHP* capsid hairpin, *E* envelope, *NS* nonstructural, *PK* pseudoknot, *prM* precursor membrane, *SL* stem loop, *UAR* upstream of the AUG region

Fig. 15.1 These immature viral particles, recognized by the spikes of prM-E heterodimers on the particle surface, are transported along the secretory pathway to the Golgi. Proteolytic processing of prM by the host furin protease (*orange*) produces a pr peptide that remains associated with the viral particle until it reaches the neutral pH of the extracellular environment, where it is released from mature viral particles (smooth surface with E homodimers)

proteins (core/capsid (C), pre-membrane (prM), and envelope (E)) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). An overview of the characteristics of these structural and nonstructural proteins is provided in Tables 15.2 and 15.3, respectively.

1.4 Flavivirus Replication Cycle

1.4.1 Flavivirus Cell Entry

Flavivirion entry is complex, and the exact mechanisms involved are still not fully elucidated. The general events leading to productive flavivirus entry include attachment of the virion to the cell surface, particle endocytosis, fusion with the endosomal membrane, movement of the nucleocapsid into the cytoplasm, and delivery of the viral genome to the site of translation (Fig. 15.1).

The E glycoprotein mediates DENV entry and functions at several steps during this process, beginning with attachment of the viral particle to the plasma membrane of the target cell. This attachment to the cell surface is mediated by the putative receptor binding domain located in domain III of the E glycoprotein [33]. Domain II, which contains the hydrophobic fusion loop, is protected between domains I and III in mature virions. Exposure to acidic pH leads to significant structural changes including insertion of the fusion peptide into the target endosomal membrane and refolding of E as a post-fusion trimer. While conserved histidine residues in the E protein of TBEV function as a "switch" triggering membrane fusion upon protonation [34], this protonation is not required for WNV entry [35]. Likewise, fusion catalyzed by the DENV E protein appears to be regulated by networks of residues located (1) proximal to the fusion loop, (2) in the "latch" between E and M, and (3) in the hinge regions between domains I–II and domains I–III. Collectively, these residues stabilize the pre-fusion dimer with the fusion peptide protected at neutral pH and promote refolding of E into its post-fusion trimeric conformation upon exposure to acidic pH [36]. In vitro, an acidic pH is sufficient to trigger fusion and nucleocapsid release of DENV and other flaviviruses [37, 38]. In vivo, DENV fusion is thought to occur within a small endosomal vesicle in the late endosome via a process that requires the presence of negatively charged lipids [39]. Anionic lipids act downstream of the low-pH-dependent step and promote the steps of fusion from the earliest hemi-fusion intermediates to opening of the fusion pore [39].

Although DENV has broad cell tropisms, the target cells in humans are primarily dendritic cells (DC), monocytes, macrophages, and hepatocytes. Many different cellular proteins facilitate entry of DENVs including, heparan sulfate expressed at the surface of most cell types [40, 41], dendritic-cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) expressed by immature DCs [42], heat-shock proteins 70 and 90 [43], glucose-regulated protein 78 [44], laminin receptor [45], mannose receptor [46], and the T-cell immunoglobulin and mucin domain (TIM) and tyrosine 3-, AXL-, and MER-tyrosine kinase (TAM) family of phosphatidylserine receptors [47, 48]. The prevailing model today suggests a

Table 15.2 Overview of dengue virus structural proteins

Protein	Genomic sequence, protein properties and domains	Function	Ref.
Capsid (C)	• nt 97–396/438 • Immature C: 114 aa • Mature C: – 100 aa/11 kDa – Central hydrophobic region • 3 NLS • N- and C-terminal charged residues	• Forms nucleocapsid with viral genomic RNA • Immature C: anchored to ER membrane by 14 aa in C-terminal, cleaved by NS2B-3 to form mature C • Mature C: associates with ER membrane through internal hydrophobic region • 18 basic N-terminal aa required for viral encapsidation and possibly interaction with RNA • Binding to lipid droplets required for viral particle formation • Interacts with nucleolin during virion morphogenesis. Inhibition of nucleolin decreases viral titer but not RNA synthesis • Interacts with hSec3 to delay DENV translation and replication possibly through sequestration of eEF1α • 3 NLS interact with DAXX and induces apoptosis	[80, 81, 106–111]
Precursor membrane (prM)	• nt 439–936 • 166 aa/34 kDa • pr: 91 aa/26 kDa • M: 75 aa/8 kDa	• Interacts with claudin-1 for efficient virus entry • Forms heterodimer with E protein to prevent premature fusion of immature virion with host membrane • His residue at M39 in M protein influences virus assembly • Host furin in post-Golgi vesicles cleaves the prM into "pr" and "M" for virus maturation • prM and vacuolar-ATPase interaction influences efficient virion egress	[16, 112–114]
Envelope (E)	• nt 937–2421 • 495 aa/50 kDa • 3 domains (I–III)	• Class II fusion protein • Mediates attachment to cell surface entry factors • Undergoes structural changes at low pH that are coupled to fusion of viral and endosomal membranes during viral entry • Interacts with ER resident chaperones for folding and virus assembly • Among flaviviruses, Asn 67 (in DENV) associated with hemorrhagic fever • Major immunogenic protein; interacts with NKp44, a NK surface receptor, and activate NK cells	[16, 115–118]

Abbreviations: *Aa* amino acids, kDa kiloDalton, *DAXX* death domain-associated protein, *EF1α* elongation factor 1 alpha, *ER* endoplasmic reticulum, *His* histidine, *NLS* nuclear localization signal, *NS* nonstructural, *NK* natural killer, *nt* nucleotide

Table 15.3 Overview of dengue virus nonstructural proteins

Protein	Genomic sequence, protein properties and domains	Function	Ref.
NS1	• nt 2422–3477 • 352 aa/46–55 kDa • Glycosylated	• Cell-membrane-bound form: intracellular vesicular compartments, cell surface • Soluble form: secreted as hexameric lipoparticle • Implicated in disease pathogenesis and protection • Circulates in sera of DENV-infected patients • Important biomarker for early diagnosis • Interacts with NS4A, co-localizes with dsRNA • Involved in RNA replication but exact role undetermined	[71, 72, 122, 123]
NS2A	• nt 3478–4131 • 218 aa/22 kDa	• Transmembrane protein, associates with the ER membrane • Component of the viral replication complex • Functions in virion assembly • Along with NS4A and NS4B, inhibits type 1 IFN signaling upstream of STAT1 activation	[119, 120]
NS2B	• nt 4132–4521 • 130 aa/14 kDa	• Transmembrane protein, associates with the ER membrane • Component of the viral replication complex • Hydrophobic loop of NS2B necessary for NS2B-NS3 serine protease activity • NS2B-NS3 cleaves human adaptor molecule STING and inhibits type I IFN production • NS2B-NS3 interacts with IKKε to block IFN induction pathway	[121–123]
NS3	• nt 4522–6375 • 618 aa/70 kDa • C terminus domain: – NTPase/RNA helicase • N-terminal domain: – Serine protease – RNA 5' triphosphatase	• RNA helicase and NTPase important for replication of the viral RNA genome • Activates fatty acid synthase and induces fatty acid production at the RC • NS2B-NS3 impairs type 1 IFN production • Interacts with La protein	[69, 124, 125]
NS4A	• nt 6376–6756 • 127 aa/16 kDa • C-terminal: 2 K fragment	• Associates with ER membrane • Component of the viral RC; may serve as a scaffold for formation of RC • Up-regulates autophagy in epithelial cells • Induces intracellular membrane rearrangements • Along with NS2A and NS4B, inhibits type 1 IFN signaling upstream of STAT1 activation	[67, 119, 126]
2 K	• nt 6757–6825 • 23 aa	• Regulates induction of intracellular membrane rearrangements by NS4A	[67]

(continued)

Table 15.3 (continued)

Protein	Genomic sequence, protein properties and domains	Function	Ref.
NS4B	• nt 6826–7569 • 248 aa/27 kDa	• Transmembrane protein • Component of the viral RC • Colocalizes with NS3, dissociates NS3 from ssRNA, and enhances the helicase activity of NS3 in vitro • Along with NS2A and NS4A, inhibits type 1 IFN signaling upstream of activation of STAT1	[119, 127]
NS5	• nt 7570–10269 • 900 aa/103 kDa • 2 NLS • 1 NES • RNA polymerase • MTase	• Mostly in nucleus (possesses 2 NLS), only hypophosphorylated NS5 in cytoplasm • RNA-dependent RNA polymerase, synthesizes positive and negative sense viral RNA • Caps 5′ end of viral genomic RNA through 2′O methyltransferase (MTase) activity • Interacts with and activates NS3 • Inhibits IFNα signaling via binding and inhibition of phosphorylation STAT2 • Interacts with La protein • CRM1-mediated nuclear export of NS5 modulates IL-8 induction and virus production	[73–76, 125, 128]

Abbreviations: *Aa* amino acids, *ds* double stranded, kDa kiloDalton *ER* endoplasmic reticulum, *IL* interleukin, *IFN* interferon, *NES* nuclear export signal, *NLS* nuclear localization signal, *NS* nonstructural, *nt* nucleotide, *RC* replication complex, *ss* single stranded, *STAT* signal transducer and activator of transcription, *STING* stimulator of interferon genes

multistep process in which E protein interacts sequentially with at least two cellular entry factors. Which host factor serves as the bona fide receptor for cell entry remains unclear. The initial interaction on the plasma membrane concentrates virions on the cell surface and thereby facilitates interaction of the virion with a high affinity, second receptor that mediates virion internalization through receptor-mediated endocytosis [49, 50]. The phenomenon of ADE, in which non-neutralizing antibodies increase infectivity by concentrating virus on the plasma membrane, is consistent with this model.

Following receptor binding, uptake of virions is a clathrin-dependent process [51, 52], and trafficking from early to late endosomes requires actin and microtubules [53–56]. Receptor binding of DENV on endothelial cells activates the RHO-family GTPases Rac1 and cell division control protein 42 (CDC42), which induce actin reorganization and formation of filopodia required for efficient virus entry [57, 58]. Following membrane fusion and creation of the fusion pore, the viral nucleocapsid traffics to the cytosol and is disassembled, and the RNA genome is delivered to the site on the endoplasmic reticulum (ER) membrane for translation. Although these events almost certainly are regulated by host factors, the specific molecular mechanisms remain poorly understood.

1.4.2 Flavivirus Translation

Flaviviruses, like most viruses, utilize the host cell machinery for translation of protein-coding ORFs. Flaviviruses have a capped, (+)ssRNA genome that is directly translated as a single polyprotein. DENV translation occurs mostly via cap-dependent initiation [59] although noncanonical initiation of translation has been described under conditions that inhibit cap-dependent translation in an internal ribosomal entry site (IRES)-independent manner [60]. During cap-dependent translation, initiation occurs when the eukaryotic initiation factor 4E (eIF4E) recognizes and binds the DENV genomic 5′ cap. Recruitment of the 43S preinitiation complex (composed of the 40S ribosomal subunit, eIF1A, eIF3, Met-tRNA-GTP) by eIF4F (composed of eIF4E, eIF4G, eIF4B, and the helicase eIF4A) leads to formation of the 48S complex. The 48S complex scans the viral 5′UTR, unwinding secondary structures until it reaches a start codon. Interestingly, the poly(A)-binding protein (PABP) can bind to the DENV genome 3′UTR (especially 3′SL) despite the absence of a poly(A) tail [61]. This finding suggests that circularization of the genome by the interaction of PABP with eIF4G is important for efficient translation. DENV and other mosquito-borne flaviviruses initiate translation of the C protein from a start AUG codon in a suboptimal context, and multiple additional in-frame AUGs are downstream from the start codon [26]. An RNA hairpin structure in the capsid coding region (cHP) (Fig. 15.2) directs translation start site selection [26]. The optimal distance from the start codon to the cHP is about 15 nucleotides [25], which corresponds to the footprint of a ribosome paused over a start codon [62]. Thus, the scanning initiation machinery is thought to pause at the structural cHP to unwind the cHP and, in this poor initiation context, the scanning initiation complex stalls momentarily over the first AUG [25, 26]. Association of the 60S subunit with eIF3 at the initiation codon forms the 80S ribosomal complex that is needed for translation elongation to proceed. The 3′SL present at the very end of the 3′UTR (Fig. 15.2) facilitates mRNA binding to polysomes and promotes efficient DENV mRNA translation, notably during the first round of translation in the absence of synthesized viral proteins [30].

Efficient translation of incoming genomes early in infection is an essential step in the flavivirus virus replication cycle. Synthesis of nonstructural proteins is a requirement for flavivirus replication since the cells do not possess an RNA-dependent-RNA polymerase capable of replicating flaviviral RNA. Deficiencies in translation can therefore significantly reduce viral replication and production of infectious particles [63].

1.4.3 Flavivirus Replication

The viral (+)ssRNA genome (light pink in Fig. 15.1) initially serves as the template for translation of the viral polyprotein, but eventually serves as a template for RNA replication. The transition between these two processes is not fully understood. The incoming (+)ssRNA is a template for the synthesis of a negative-stranded RNA that leads to the formation of double-stranded RNA (dsRNA) intermediates called

the replicative form (RF) [25]. The newly synthesized (−)ssRNA (dark pink in Fig. 15.1) is used in turn as a template for the synthesis of multiple (+)ssRNA via a replication intermediate (RI). Approximately five nascent (+)ssRNAs are present on an RI and 12–15 min are necessary to synthesize each strand [64]. This process leads to the production of ≈10–100-fold more (+)ssRNA than (−)ssRNA [29]. Thereafter, the (+)ssRNAs serve as substrates for a new round of translation, templates for production of (−)ssRNA, or are encapsidated for assembly into new virions. Replication of the flaviviral genome does not occur freely in the cytoplasm, probably to limit recognition of the dsRNA intermediates by intracellular cytosolic innate immune sensors such as retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated protein 5 (MDA5). Concentration of the necessary substrates and catalysts within these specialized membrane-bound compartments also serves to increase the efficiency of RNA replication.

Extensive intracellular membrane rearrangement takes place in flavivirus-infected cells [65], and electron tomography, immuno-electron microscopy, and transmission electron microscopy have been used to characterize the architecture of DENV-induced membrane alterations [66]. DENV replication occurs inside replication complexes (RC) formed by membranous invaginated vesicles (≈90 nm in diameter) that are derived from the ER and are associated with most of the DENV NS proteins (schematic representation of the RC in Fig. 15.1). NS4A, along with additional viral and host factors, induces the membrane curvatures and rearrangements for invaginated vesicle formation [67, 68].

During DENV infection, fatty acid synthase re-localizes to the RC and is activated by interaction with NS3. The newly produced lipids are incorporated into RC, as these lipids co-purify with viral RNA in biochemical fractionation experiments. [69]. This incorporation may facilitate the extension of membranes and the formation of the RC. Virus-induced autophagy also leads to liberation of fatty acids from lipid droplets, and these fatty acids undergo beta-oxidation, producing ATP that fuel the energy demands of replication [70]. The hydrophobic NS2A, NS4A, and NS4B proteins are thought to anchor the RC to the ER membrane [67, 71, 72] although the molecular details of these interactions are still subject to investigation. Likewise, although the NS1 protein is known to interact with NS4B and to be essential for viral RNA replication, its function in this process remains obscure [71, 72].

NS5, a bifunctional protein with N-terminal methyl transferase and C-terminal RdRp-dependent RNA polymerase (RdRp), possesses two nuclear localization signals (NLS) [73] and a nuclear export signal (NES) [74]. NS5 is mostly located in the nucleus as a phospho-protein and only the hypophosphorylated form is located in the cytoplasm. Within the cytoplasm, NS5 replicates viral RNA and also interacts and modulates the enzymatic activity of NS3 [74–76]. NS3 is a multifunctional protein required for polyprotein processing and RNA replication. Besides its N-terminal protease activity, NS3 has RNA-stimulated nucleoside triphosphatase (NTPase), RNA helicase, and RNA triphosphatase (RTPase) activities that are absolutely required for viral RNA replication. RTPase activity is believed to be responsible for the dephosphorylation of the 5′ end of the genomic RNA before cap addition by NS5 [77]. NS3 binds to the 3′SL and NS5 enhances NS3's NTPase activity [78].

A number of *cis*-acting RNA elements, located in the coding and noncoding regions of flavivirus genomes, act as promoters, enhancers, and circularization signals necessary for efficient RNA replication [23–25]. The two inverted pairs of complementary sequences (5′-3′ CS and 5′-3′ upstream of AUG region [UAR], Fig. 15.2) at both extremities of the genome are required for genome circularization. The replication process begins with 5′-3′ UAR and 5′-3′ CS hybridization, which triggers circularization of the genome. NS5 binds specifically to the 5′ stem-loop A (SLA), and through this long range RNA-RNA interaction the 5′ promoter and the 3′ end of the genome are brought together. This enables transfer of NS5 to the site of initiation at the 3' end of the genome and the initiation of RNA synthesis. Circularization of the viral genome may also play an important role during ORF translation. Although both circular and linear forms of DENV RNA are necessary for virion production, viral RNA replication is highly vulnerable to changes that alter the balance between circular and linear forms of the RNA [79].

1.4.4 Flavivirion Egress

The last steps of the flavivirus replication cycle are the encapsidation of genomic progeny RNA, envelopment of the viral nucleocapsid, maturation of the virion surface proteins, and egress of infectious particles. Encapsidation of flavivirus genomes is thought to be directly linked to genome replication, as only nascent (+)ssRNAs from the RC are encapsidated [71]. Indeed, the presence of budding vesicles that may correspond to the formation of viral particles at the ER membrane is directly apposed to the RC [66]. Two clusters of basic amino acids that confer a high density of positive charges at the N-terminus of the C protein are essential for genome encapsidation in human cells [80]. Since encapsidation in mosquito cells still occurs when these residues are deleted, albeit in a less efficient fashion, interaction of the N-terminus of the C protein with a host factor likely differs between humans and mosquitoes.

The DENV C protein accumulates on the surface of lipid droplets (LD), and loss of LD targeting results in abrogation of particle formation [81]. This observation indicates a role of LD in virion assembly or release. In contrast to hepatitis C virus, no evidence of recruitment of lipid droplets to the RC or DENV RNA to the lipid droplets has been reported [66, 82]. An alternate explanation for the sequestration of C protein by LD is that this sequestration prevents the binding of C to newly synthesized viral RNA and thus averts premature encapsidation of the viral genome and the inhibitory effect this would have on replication of the viral genome [82]. Interestingly, DENV induces autophagy and degradation of the LD in autophagosomes to liberate fatty acid and produce energy [70, 82]. Although the kinetics and the sequence of these events have not yet been described in detail, coupling of virus-induced autophagy to the release of C protein is hypothesized to provide a mechanism for delaying viral assembly until sufficient genome replication has occurred.

Virus assembly occurs at the surface of the ER. The DENV C protein associates with newly synthesized RNA genomes liberated from the RC through a pore-like structure [66]. Newly formed immature virions contain a genomic RNA within an icosahedral capsid. Budding of this capsid into the lumen of the ER in close proximity

to the RC results in its envelopment within a lipid bilayer, yielding immature virions in which trimers of E and prM heterodimers appear as spikes on the particle surface. Individual virions travel toward distal sites of the ER lumen and are thought to be collected in dilated ER cisternae [66]. Maturation of these immature particles occurs as they traffic through the host secretory system. Processing of the N-linked glycan on the E protein by host enzymes in the ER and Golgi is required for efficient secretion of infectious virions in mammalian but not insect cells [83]. The prM portion of the trimer with E is cleaved by furin. The pr protein, corresponding to the N-terminal 91 residues of prM, dissociates from the virion upon exposure to neutral pH in the extracellular space while the M protein remains in the virion [16]. Processing of the dengue virion is inefficient, as a large proportion of secreted virions have unprocessed prM [84, 85].

1.5 Vaccines and Antiviral Agents

Vector control has been the most widely used strategy to prevent dengue virus infection. No FDA-approved vaccine is currently available to prevent dengue fever. The risk that an ineffective vaccine might exacerbate infection through ADE has provided an additional challenge to efforts to develop a vaccine providing pan-serotype protection. Several candidate antivirals show promise in vitro and in animal models of DENV infection (reviewed in ref. 86, 87). These include agents that act against viral targets, such as ST-148, which targets the C protein, and NITD-008, a nucleoside that inhibits the NS5 RNA-dependent RNA polymerase. NITD-451 and other benzomorphans are specific inhibitors of DENV translation. Some antisense morpholino-oligomers that directly or indirectly affect translation of DENV [88, 89] and other flaviviruses [90–92] are potent inhibitors of these viruses in cell culture.

Compounds that inhibit DENV translation and replication are currently under investigation as potential antivirals [93, 94]. Although some of these agents (e.g., NITD-008, NITD-451) have demonstrated antiviral activity in a mouse model of DENV infection, their therapeutic window is limited [87, 94]. In addition, fenretinide, a synthetic retinoid, has been shown to inhibit the replication of DENV and other flaviviruses in cell culture and in mouse models through effects on genome replication [95, 95b]. Celgosivir, which targets the host alpha-glucosidase, is thought to inhibit DENV pathogenesis and replication by causing misfolding of E, prM, and NS1 proteins [96, 97]. Now being tested in a phase Ib trial for treatment of dengue fever, celgosivir is currently the most advanced clinical candidate.

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