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Properties and Functions of the Dengue Virus Capsid Protein

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Abstract

Dengue virus affects hundreds of millions of people each year around the world, causing a tremendous social and economic impact on affected countries. The aim of this review is to summarize our current knowledge of the functions, structure, and interactions of the viral capsid protein. The primary role of capsid is to package the viral genome. There are two processes linked to this function: the recruitment of the viral RNA during assembly and the release of the genome during infection. Although particle assembly takes place on endoplasmic reticulum membranes, capsid localizes in nucleoli and lipid droplets. Why capsid accumulates in these locations during infection remains unknown. In this review, we describe available data and discuss new ideas on dengue virus capsid functions and interactions. We believe that a deeper understanding of how the capsid protein works during infection will create opportunities for novel antiviral strategies, which are urgently needed to control dengue virus infections.

Keywords

RNA virus; flavivirus; arbovirus; dengue virus; capsid protein; viral encapsidation; viral assembly; lipid droplets

DENGUE VIRUS

Dengue virus (DENV) is the most significant arthropod-borne viral pathogen in humans. The geographical spread and incidence of DENV infections have increased dramatically in recent years. DENV is estimated to cause around 390 million infections per year, placing over 3 billion people at risk of infection (1). In addition to the heavy burden placed on public health, DENV epidemics have a huge economic impact on affected countries.

DENV is a member of the *Flavivirus* genus of the *Flaviviridae* family (2). The *Flavivirus* genus includes other important emerging and reemerging human pathogens such as Zika virus (ZIKV), West Nile virus (WNV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), and Saint Louis encephalitis virus (SLEV) (3). Most flaviviruses are arthropod-borne; however, vertebrate-and invertebrate-specific viruses are also members of the group (for a recent review see 4). DENV cycles in nature between *Aedes* mosquito vectors (mainly

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Aedes albopictus and *Aedes aegypti*) and humans. Four DENV serotypes (DENV1, DENV2, DENV3, and DENV4) circulate in tropical and subtropical regions of the globe (5). They differ from one another by 25–40% at the amino acid level and are further separated into genotypes. Clinical outcomes for all serotypes can be unapparent or result in a spectrum of diseases ranging from self-limited dengue fever to severe dengue, a potentially lethal hemorrhagic illness. The incidence of dengue disease is growing as the mosquito vector spreads owing to urbanization, population growth, international travel, insufficient mosquito control efforts, and global warming.

Although vaccines and antivirals are still unavailable to control DENV infections, a great effort is being made in this direction, and solutions will likely be accessible in the near future.

OVERVIEW OF THE VIRAL LIFE CYCLE

The structure of the DENV particle was solved through a combination of cryo–electron microscopy, imaging reconstruction, and X-ray crystallography (6–8). The particle comprises an electron-dense core surrounded by a lipid bilayer, in which two transmembrane viral proteins are inserted to form a glycoprotein shell. This shell is well defined and consists of 180 copies of the envelope (E) and membrane (prM/M) proteins. The core contains the nucleocapsid (NC), formed by one copy of the single-stranded capped RNA genome in complex with multiple copies of the capsid protein.

DENV enters host cells by receptor-mediated endocytosis, which involves attachment and receptor binding by the viral E protein. Different mammalian and mosquito host proteins have been shown to interact with E at the cell surface, but a bona fide receptor for virus entry has not yet been identified (for review, see 9). The virus enters primarily through clathrin-mediated endocytosis (10–15). Upon internalization and acidification of the endosome, fusion of viral and vesicular membranes mediated by conformational changes in the E protein allows NC release into the cytoplasm (16, 17; for review, see 18, 19). Genome uncoating involves dissociation of capsid, which frees the RNA that is directly used for viral translation. Viral protein synthesis takes place in the rough endoplasmic reticulum (RER) and renders a large polyprotein with a complex topology on endoplasmic reticulum (ER) membranes (20–22). The viral polyprotein is cleaved cotranslationally and posttranslationally into at least ten mature proteins: three structural proteins (capsid, prM, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (2). Most of the NS proteins are multifunctional. They provide enzymatic activities and render the proper environment for viral RNA replication, including remodeling cellular membranes and suppressing host antiviral responses. Infection induces hypertrophy of intracellular membranes, which provides structures in which the genome is amplified (23–27). RNA synthesis is catalyzed by the viral polymerase NS5 in a process that requires specific promoter recognition and genome cyclization (28, 29). The newly synthesized genome associates with capsid to form the NC, and this ribonucleoprotein complex buds into the ER lumen, acquiring the lipid bilayer, together with the viral E and prM proteins. The immature viral particles travel through the secretory pathway. Furin-mediated proteolysis of

prM in the *trans* Golgi network triggers rearrangement, homodimerization of E, and formation of mature viral particles, which are subsequently secreted (30).

CAPSID PROTEIN STRUCTURE

Mature DENV capsid is a highly basic protein of 12 kDa that forms homodimers in solution with affinity for both nucleic acids and lipid membranes (31, 32). The 100-residue monomer contains 26 basic amino acids and only 3 acidic residues. The tridimensional structures of the DENV and WNV capsid proteins were solved by nuclear magnetic resonance and crystallography, respectively (33, 34). The dimer shows asymmetric charge distribution, with basic residues accumulating on one face of the molecule and a concave apolar surface on the opposite side. Structural studies showed that the monomer has four α-helices (α1 to α4) (Figure 1*a*), in agreement with previous analysis (32). The N-terminal domain is unstructured in solution and has a high density of positive charges (8 lysines or arginines in the first 22 residues). Purification of recombinant DENV and YFV capsid proteins was associated with truncations of the N-terminal region, supporting the idea that this segment is flexible or structurally disordered. Helices α2 and α4 of one monomer are antiparallel to helices α2 and α4 of the neighboring monomer, respectively, and the two interfaces contribute the majority of the dimer contact surface. Structure-based mutagenesis indicates that α4–α4′ helix interaction is crucial for dimer formation, protein stability, and infectious particle production (35). The first three helices (α1 to α3) form a right-handed bundle that composes the monomer core. The different orientation of α1 in WNV and DENV suggests that this helix is more flexible. The longest helix, α4, extends away from the monomer core and has basic residues on the solvent-accessible surface (33). On the opposite side of the molecule, the surface contributed by α2–α2′ and α1–α1′ is largely uncharged (33, 36). Accordingly, pioneering studies by Markoff and colleagues (37) described a conserved internal hydrophobic region spanning residues 45 to 65 of DENV4. These authors showed that the mature capsid protein remains associated to ER membranes via this hydrophobic region, which is conserved in a wide range of mosquito- and tick-borne flaviviruses.

On the basis of structural studies, it has been proposed that the α4–α4′ region, which is rich in basic residues, interacts with the viral RNA, whereas the hydrophobic cleft, including the apolar α2–α2′ region, interacts with membranes (33). Functional analysis has provided evidence that basic residues at the N-terminal region of DENV capsid also contribute to RNA binding and viral particle formation (38). A model of RNA and lipid membrane binding of capsid is presented in Figure 1*b*.

RNA-BINDING AND CHAPERONE ACTIVITY OF CAPSID

Capsid–RNA interaction studies have been hampered by the fact that capsid aggregates upon nucleic acid binding. In particular, positive charge neutralization by RNA interaction might drive aggregation through the hydrophobic region; however, experimental analyses of the aggregation process are still needed. RNA binding to the N- and C-terminal domains of WNV capsid has been observed in vitro (39). WNV capsid phosphorylation has also been proposed to regulate RNA binding (40). For DENV, the capsid protein binds RNA with high affinity and low specificity, with dissociation constants of approximately 20 nM (41).

The capsid proteins of DENV and WNV were shown to assist nucleic acid rearrangements, acting as RNA chaperones in vitro (42, 43). RNA chaperones promote folding of RNA molecules either by preventing their misfolding or by resolving misfolded RNA species without ATP consumption (44, 45). The RNA chaperone activity of WNV capsid was mapped to the C-terminal RNA-binding region of the protein (43) and was proposed to facilitate long-range interactions in the viral genome (46). A hallmark of active RNA chaperone domains is a high content of basic residues that are structurally flexible, a property shared among all flavivirus capsid proteins; thus, capsid proteins from other members of the genus are expected to display the same RNA remodeling capacity.

CAPSID PROTEIN MATURATION

Capsid is the first protein encoded in the viral genome, followed by prM (Figure 2). These two proteins are connected by an internal hydrophobic signal peptide, known as anchor, which spans the ER membrane and is responsible for the translocation of prM into the ER lumen. A model of a coordinated two-step proteolytic processing, at the N-and C-termini of anchor in the capsid–prM junction, has been proposed for different flaviviruses (47–50). Matured capsid is released by proteolytic processing of the capsid–anchor junction by the viral NS3 protease, which requires the viral NS2B cofactor (NS2B-3). In the ER lumen, the host signal peptidase cleaves the anchor–prM junction.

It has been demonstrated that the anchor peptide is not efficiently recognized by the signal peptidase in the ER and that, for certain flaviviruses, cleavage at the cytoplasmic side by the NS2B-3 protease allows peptide accommodation for efficient cleavage of anchor–prM. Mutagenesis within anchor that increased peptidase processing uncoupled the sequential order of the two cleavages but impaired viral particle formation (51). For Murray Valley encephalitis virus (MVEV), a premature cleavage of anchor–prM was responsible for an increased release of empty particles, which contained E and prM but lacked NCs (52). Thus, the accepted model suggests that capsid protein maturation triggers prM maturation and particle assembly in a timely and spatially coordinated process.

NUCLEOCAPSID ASSEMBLY

Capsid is crucial for NC formation, which is the first step during DENV assembly. NCs contain a single molecule of the viral genome and multiple copies of capsid but lack a defined symmetry (6, 7, 53). This RNA–capsid complex has never been isolated from DENV-infected cells, suggesting a coordinated process between genome recruitment by capsid and budding of the NC into the ER. Also, RNA encapsidation signals in DENV and other flavivirus genomes have not been identified. Thus, the specific manner in which capsid directs NC formation remains unclear. Capsid–RNA binding has been proposed to be nonspecific and mainly driven by electrostatic interactions.

Viral RNA synthesis occurs in replication complexes that contain membranous structures formed by ER invaginations, known as vesicle packets (VPs). It has been shown that these vesicles have necks open to the cytoplasm, through which the newly synthesized RNA exits (24). Interestingly, replication complexes have been observed by using transmission electron

microscopy and electron tomography of DENV-infected cells as physically linked to capsid-containing ER membranes. A model has been proposed wherein the viral genome is transported directly to sites of NC assembly at the ER membranes, and the budding particles in the ER lumen acquire the lipid bilayer, E, and prM (23, 24, 26) (Figure 3). NC incorporation into the budding particle is not driven by interactions between the NC and the cytoplasmic domains of E or prM inserted into the viral membrane. Structural studies using DENV particles described a low-density gap between the density contributed by the NC and that contributed by the lipid bilayer without evidence of a contact between the NC and E or M (6, 7). Budding of viral particles into the ER is NC independent, because empty flavivirus particles (lacking capsid and viral RNA) can be produced by overexpressing only prM and E proteins (54). Coupling between RNA replication and RNA encapsidation has also been reported for flaviviruses (55, 56). In this regard, it has been shown that viral RNAs are not encapsidated if they were not actively synthesized in replication complexes (55).

The sequential order of cleavages at the C–prM junction possibly enhances NC uptake into budding membranes (49). In this respect, the presence of the NS2B-3 active protease (required for capsid maturation) may play additional roles in coordinating genome recruitment (Figure 3). NS3 also contains RNA helicase and RNA annealing activities and interacts with the viral genome; thus, this protein may be the missing link between the viral genome and capsid for NC formation. Interestingly, a function of NS3 during viral assembly, independent of the enzymatic activities for genome replication, has been observed for YFV (57) and DENV (58). A genetic link between NS2A and NS3 for viral particle formation has been described, suggesting that particle assembly uses a complex system that includes different host and viral components (59). Moreover, a recent report showed that viruses with specific mutations within NS1, which were still competent for replication, release up to 100-fold fewer infectious DENV particles than the parental virus, providing novel evidence for a function of NS1 in viral particle assembly (60). Although a great deal of information on DENV NC assembly and/or particle production has become available, the interplay between the identified components and the mechanism by which they function remains largely unknown. Further studies are necessary to understand the complex network of proteins involved in genome recruitment and to elaborate more comprehensive models of viral NC assembly.

OVERLAPPING RNA SIGNALS IN THE CAPSID-CODING SEQUENCE

The coding sequence for capsid contains a number of RNA structures necessary for viral genome replication, limiting the genetic manipulation of protein residues. Incorporation of mutations within capsid must take into account potential effects on viral RNA synthesis. A systematic analysis including structure prediction and biochemical probing of the complete capsid-coding RNA was recently reported (61). This study indicates that the first 160 of the 300 nucleotides encoding DENV capsid are involved in conserved RNA structures. Well-characterized functional *cis*-acting RNA elements present in DENV capsid include the following: (*a*) the RNA cyclization signal known as 5′CS, which is essential for RNA synthesis (for review, see 62); (*b*) a stable hairpin known as cHP, which is involved in RNA synthesis (63); and (*c*) a pseudoknot structure included in a region known as C1, which contains a sequence complementary to a region present at the viral 3′ end that enhances

genome cyclization and RNA synthesis (61, 64). Therefore, functional studies of DENV capsid require uncoupling these *cis*-acting RNA elements for RNA synthesis from the capsid-coding sequence. Recently reported strategies include duplication of specific RNA structures or duplication of the complete capsid-coding sequence (38, 61). These genetic tools have been used to study capsid determinants for DENV particle formation without affecting viral RNA replication.

FUNCTION AND STRUCTURAL FLEXIBILITY OF CAPSID

Alignments of capsid-coding sequences from different flaviviruses show less than 40% sequence identity. In particular, comparisons between DENV and YFV capsid proteins (which bear the least capsid sequence identity among flaviviruses) indicate approximately 22% sequence identity (32) (Figure 4). Although capsid is the least conserved of the flavivirus proteins, the structural properties are very similar and the charge distribution is well conserved.

Mutational studies were performed using reporter DENV systems to uncouple *cis*-acting RNA structures from the capsid-coding sequence (38, 41). A highly conserved sequence at the N-terminus of capsid, 13-NML-17 (present in all DENV serotypes), was important at the RNA level for RNA synthesis but not for capsid function (38). In contrast, deletion of the basic-rich N-terminal sequence of capsid impaired DENV particle formation. In this sequence, two clusters of basic residues (R5K6K7R9 and K17R18R20R22) were defined as essential for DENV propagation. Interestingly, differential requirements were noticed for infections in mosquito and human cells (38). A systematic mutational analysis indicated that at least two positive charges in each of the two clusters were necessary for viral particle assembly in human cells. On the basis of these studies, an accumulation of positive charges rather than of residues in specific positions was proposed to be crucial for DENV particle formation.

Mutations in other regions of DENV capsid were also reported. Although mutations within α2 impaired protein function, substitutions of uncharged residues in α1 or in the connecting loop between α1 and α2 did not affect DENV propagation (41). The importance of an internal hydrophobic region, including the α2 helix, was originally described using DENV4, and further studies reported that this region was also important for efficient propagation of different flaviviruses (37, 65–67). A study using WNV reported that deletions within the most hydrophobic section of the α2 helix (LALLAFF) impaired viral propagation (68). However, pseudo-revertants with extended deletions of capsid from amino acid 40 to 76 were recovered in culture. These results indicated that a large deletion of approximately 36 amino acids was better tolerated than a small deletion of 4–7 amino acids in the hydrophobic region, suggesting that a short version of capsid could form NCs by an alternative mechanism.

Remarkable flexibility in capsid protein function has been observed among various flaviviruses. In tick-borne encephalitis virus (TBEV), infectious viruses were still recovered even after deletion of up to 16 residues in the central region of capsid (between positions 28 and 48) (66). Also, TBEV with deletions ranging from 19 to 30 residues long in a

hydrophobic region resulted in viruses with second-site mutations that increased the hydrophobicity of the protein (69). Interestingly, viral mutants lacking 16 amino acids of capsid were found to be attenuated but very immunogenic in adult mice (66). Studies using a YFV replicon *trans*-packaging system demonstrated that large deletions in the N- and C-terminal regions of capsid were also tolerated for particle formation (65). This observation provides evidence that one of the two proposed RNA-binding regions is sufficient for NC assembly of this virus, in contrast with observations on DENV (38).

In conclusion, capsid proteins from different flaviviruses tolerate extensive deletions and mutations, suggesting that they do not require a defined 3D structure for their function but rather rely on basic residues to recruit the viral RNA. Nevertheless, distinct flavivirus capsid proteins show different degrees of tolerance for structural changes; DENV capsid is one of the least tolerant proteins in this regard.

SUBCELLULAR DISTRIBUTION OF CAPSID

Although DENV particle assembly occurs in the cytoplasm, capsid has been detected in both the cytoplasm and the nucleus of infected cells (70–75) (Summary Figure). Inside the nucleus, capsid accumulates in nucleoli, whereas in the cytoplasm it is distributed between ER membranes and the surface of lipid droplets (LDs) (41). This distribution has been observed early after DENV infection, suggesting that it is not a consequence of cell damage during viral replication (74).

Studies using WNV and DENV proposed that the ER-associated protein is in close proximity to RNA exit sites of VPs and near viral budding particles, suggesting a role of capsid in this location (23–27, 76, 77). However, the functional significance of the fraction of capsid associated with the nucleus and LDs is still unclear. Capsid subcellular distribution could be temporally and spatially controlled during DENV infection. In this regard, mutations in capsid that lead to protein mislocalization during infection greatly inhibit viral RNA synthesis (41). This observation is puzzling because, although deletion of the complete capsid-coding sequence does not affect viral RNA synthesis, point mutations that alter its localization do. Removal of mature capsid from ER sites, near RNA replication, could be important to avoid premature capsid interaction with the viral RNA; thus, sequestration of the protein in the nucleus or LDs could be a mechanism to regulate protein availability during the viral life cycle. If proteolytic maturation of capsid–anchor is associated with NC assembly and particle budding, it is possible that ER-associated capsid is used for NC formation and capsid localized in nucleoli or LDs plays auxiliary functions during infection. We next summarize the available data on DENV capsid subcellular localization.

Nuclear Localization

Transport of capsid into the nucleus has been proposed to be mediated by nuclear localization signals (NLSs). Three putative NLSs in DENV2 capsid protein have been predicted: ^{6}KKAR$^{9;}$ ^{73}KKSK^{76}; and the third motif, with a bipartite structure, ^{85}RKEIGRMLNILNRRRR^{100} (70, 72). Using protein overexpression, it was originally proposed that capsid nuclear localization was predominantly due to the bipartite sequence (72); however, studies using DENV2-infected cells with mutations in the three

putative NLSs of capsid showed a reduction of nuclear localization for mutants in each of the three sites (75). This study suggested a lack of correlation between capsid nuclear accumulation and viral propagation in cell culture. Thus, the functional significance of the nuclear-associated capsid during viral infection remains unclear.

Nuclear localization of capsid from other flaviviruses has also been observed. Using JEV as a model, capsid accumulation in the nucleus was dependent on amino acids G42 and P43, both in mammalian and infected insect cells. Mutations of these residues resulted in a reduction of JEV pathogenesis in mice and lower titers in cell culture (78). These residues are conserved among different flaviviruses (Figure 4), but whether they are also involved in the nuclear accumulation of capsid from other members of the genus remains to be seen. WNV capsid was also found in nucleoli and nucleoplasm of infected cells (79), and the nuclear transport of WNV capsid was shown to be mediated by the importin-α/β complex. The capsid residues involved in translocation were the consensus sequence of a bipartite NLS (located between residues 85 and 101) and amino acids 42 and 43. These authors also reported that capsid interaction with importin-α/β appeared to be relevant for efficient virus production and that WNV capsid phosphorylation influenced nuclear trafficking by modulating capsid–importin-α binding (80, 81).

It is important to bear in mind that the predicted NLSs are patches of basic residues in the capsid protein that are also involved in RNA binding. Therefore, further analysis of mutant viruses is necessary to dissociate DENV capsid requirements for nuclear localization and NC assembly.

Lipid Droplet Accumulation

DENV capsid progressively accumulates around LDs in mosquito and infected human cells (41). Mutagenesis analysis using infectious DENV clones indicated that hydrophobic residues in the α2 helix of capsid are involved in LD association. In particular, single substitutions of residues L50 or L54 in the hydrophobic cleft were sufficient to abrogate capsid accumulation on LDs and to reduce viral particle formation. In vitro studies using atomic force microscopy provided evidence that a peptide corresponding to the disordered N-terminal region of capsid interacts with negatively charged LDs, suggesting that this region also facilitates LD binding (82). Further studies indicated that the N-terminal peptide inhibits, in a dose-dependent manner, in vitro binding of capsid to LDs (83). It has also been proposed that capsid binding to LDs depends on high concentrations of potassium and that this binding could be mediated by the LD-associated protein TIP47 (84). Studies to define the significance of capsid on LDs during viral infection are complicated by the fact that hydrophobic residues involved in LD accumulation may also be important for ER membrane association. Thus, the lack of viral particle formation for mutant viruses with substitutions in the hydrophilic region may be due to defects in viral morphogenesis.

Capsid accumulation on LDs could be a mechanism to store or sequester mature capsid during early stages of viral replication. A biological role of LDs as transient deposits of proteins that are in temporal excess has been demonstrated for histones and other cellular proteins (85). Also, capsid association to LDs could be important to modulate lipid metabolism during infection and/or regulate the host antiviral response. LDs are dynamic

organelles involved in lipid metabolism that regulate storage and turnover of neutral lipids. They contain mostly triacylglycerols and sterol esters surrounded by a phospholipid monolayer, coated by different proteins (86–88). Also, LDs participate in the generation of prostaglandins and leukotrienes, which are important inflammation mediators in immune responses (for review, see 89)

A link between DENV infection and LD abundance has been well documented. Studies using leukocytes have demonstrated augmented numbers of LDs per cell in samples from patients with severe dengue, compared with samples from healthy volunteers (90). Also, an increased number of LDs was reported in different DENV-infected cells (41, 90–92). The increase in LD abundance can be associated with a redistribution of fatty acid synthase (FASN) to the ER observed during DENV infection. In this regard, binding of FASN to the viral protein NS3 increases cellular fatty acid synthesis (93), and pharmacological inhibition of FASN activity blocks induction of LDs during DENV infection, reducing the production of infectious viral particles (41). A link between autophagy-mediated LD degradation and DENV infection has also been observed (94). Lipid degradation has been suggested as necessary during DENV RNA synthesis; however, inhibition of autophagy appears to have a modest effect on viral RNA synthesis but a profound effect on viral particle infectivity (95). Also, Rab18, which participates in lipid exchange between ER and LD compartments, was shown to be necessary for efficient DENV replication and to have a positive role in LD induction (92). Given these findings, both lipid biosynthesis and degradation are likely necessary during different stages of the viral life cycle, and capsid on LDs could play a role regulating this lipid metabolism.

The association of different pathogens, including viruses, bacteria, and parasites, with LDs is a common theme (89, 96, 97). Regarding viruses, the best-studied case is hepatitis C virus (HCV). During HCV infection, the core protein, as well as other viral proteins, accumulates on the surface of LDs (98–100). This localization of the core protein has been proposed to play an important role in HCV encapsidation (100–108).

Hundreds of cellular proteins are present on the surface of LDs (88); however, the mechanism by which the proteins are delivered to these organelles is still unclear. A function of components of the vesicle trafficking systems, coat proteins II and I (COPII and COPI), has been reported for the transport of different cellular proteins to LDs (109–112). Regarding DENV capsid, an active GBF1/Arf/COPI pathway was found to be necessary for capsid transport from the ER to LDs in infected human cells (74). This process was demonstrated to be independent of COPII components and did not require Golgi functions. Normally, COPII vesicles mediate transport of proteins and lipids from the ER to the Golgi, whereas COPI mainly participates in the retrieval of proteins from the Golgi back to the ER. Thus, the involvement of COPI in DENV capsid transport suggests a noncanonical function of this transport system.

Even though it has been shown that DENV capsid accumulates on LDs in infected cells, and different lines of evidence link DENV infection to LD metabolism, the functional significance of capsid on LDs deserves further studies.

CAPSID ASSOCIATION WITH HOST PROTEINS

DENV capsid has been reported to interact with a number of nuclear and cytoplasmic proteins. Different methodologies have been used to identify capsid interactions; these include affinity purification of overexpressed capsid followed by mass spectrometry and yeast two-hybrid screens. In the nucleus, DENV capsid has been found to interact with different cellular proteins, including DAXX, core histones (H2A, H2B, H3, and H4), hnRNP-K, and nucleolin (NCL) (73, 113–115). Capsid binding to DAXX was suggested to induce Fas-dependent apoptosis, whereas capsid binding to histones was suggested to regulate transcription by nucleosome disruption. In this regard, DENV infection was associated with increased levels of histones and increased phosphorylation of H2A (113). The interaction of capsid with NCL was disrupted by an NCL-binding aptamer, and both silencing of NCL and the addition of an aptamer to cells reduced DENV titers, suggesting a functional interaction (115).

The link between DENV capsid and apoptosis is complex because, although the interaction with DAXX appears to induce apoptosis, capsid was also suggested to inhibit apoptosis through interactions with the calcium-modulating cyclophilin-binding ligand (116). Proapoptotic and antiapoptotic functions have been reported for the capsid proteins of different flaviviruses. In the case of WNV, transient expression of capsid in different human cell lines resulted in induction of apoptosis with loss of mitochondrial membrane potential and activation of caspase-9 and caspase-3 (117). WNV capsid also interacts with HDM2, which could be proapoptotic by upregulation of Bax via p53 (118). Expression of WNV capsid in the brain cells of mice was also reported to cause local inflammation and cell death (119).

Other studies using yeast two-hybrid assays with the viral proteins as baits identified different cellular proteins that interact with the DENV and WNV capsid proteins, including the human Sec 3 protein (hSec3p) (120, 121). The Jab1 protein was also identified in a yeast two-hybrid screen to interact with WNV capsid and was proposed to facilitate nuclear export of the viral protein (122). It has also been reported that WNV capsid expression upregulates phosphatase 2A (PP2A) activity (123) and induces redistribution of host DDX56 from the nucleus to the cytoplasm, a process that appears to be specific for WNV, because DENV infection does not change DDX56 localization (124, 125). Recent in vitro studies have reported that DENV capsid interacts with very low-density lipoproteins (VLDL) (126). A link between flavivirus capsid and the formation of stress granules has been also suggested using JEV (127). Proteomic studies overexpressing a tagged JEV capsid identified caprine-1 as a specific binder and proposed a function of capsid in suppressing stress granule accumulation (127). Recent reports provide an interesting association of both DENV capsid and WNV capsid with peroxisome biogenesis. Flavivirus infection produces a significant loss of peroxisomes; this process was associated with the ability of overexpressed capsid to form a stable complex with the peroxisome biogenesis factor Pex19 (128).

The capsid proteins of DENV and other flavivirus appear to be very promiscuous in their interaction with host proteins, membranes, and nucleic acids. Thus, the functional

significance of the identified capsid interactions must be validated in the context of viral infections.

CAPSID PROTEIN AND ANTIVIRAL STRATEGIES

Despite strong efforts and growing public interest to develop antiviral therapies against DENV, no approved vaccines or therapeutics are available. Historically, the focus on antiviral drug development has been centered on viral enzymes; however, the idea that multifunctional viral proteins may be even more vulnerable to interference is starting to gain interest. Thus, capsid proteins of enveloped viruses are emerging as promising targets for a new generation of antiviral agents. Capsid proteins have to undergo different conformational changes and engage in distinct types of macromolecular interactions during the viral life cycle. In fact, the processes of viral genome release and encapsidation, which depend on controlled NC assembly and disassembly, have been proved to be extremely sensitive to even subtle molecular disturbances (129).

A DENV capsid inhibitor based on a peptide mimic of the N-terminal region of the viral protein has been proposed (82, 83). This peptide has been suggested to compete with different capsid functions, such as LD association. A recent high-throughput screen identified a small molecule (ST-148) that potently inhibits replication of all four DENV serotypes in vitro by targeting the capsid protein (130). ST-148 has been proposed to enhance capsid self-interaction, likely perturbing viral assembly and disassembly (91). Interestingly, despite the presence of viral variants with resistance to ST-148 in the population, selection for resistant viruses was not observed (131). According to the concept of genetic dominance of defective subunits, coexpression of both resistant and susceptible capsid units in infected cells would generate nonfunctional complexes, delaying emergence of resistant viruses (131). This observation makes capsid an attractive target for DENV inhibition.

PERSPECTIVES

Great advances in our understanding of key steps in the DENV life cycle have been made in recent years. We have learned about the structure of the DENV capsid protein, its subcellular distribution during infection, and its interaction with host components. Nevertheless, a number of important questions remain unanswered. We still need to understand how capsid recruits the viral genome to assemble NCs and how viral and host proteins contribute to this process. Also, the functional significance and dynamics of capsid accumulation on LDs and nucleoli need further investigation. The role of this association could be the sequestration of capsid to prevent premature binding to viral RNA; however, the viral protein likely has auxiliary functions in those locations. These functions require further study. Another important topic is the interaction of capsid with nucleic acids. This interaction has been challenging to study because the protein aggregates upon RNA binding and NC intermediates have not been detected in DENV-infected cells. Thus, biochemical settings are necessary to define structural properties of capsid involved in RNA and lipid membrane binding. Moreover, how capsid releases the viral genome during uncoating remains an understudied viral process that requires further attention. Answers to these questions will

hopefully facilitate development of antiviral strategies, which are necessary to control dengue and other emerging and reemerging flaviviruses.

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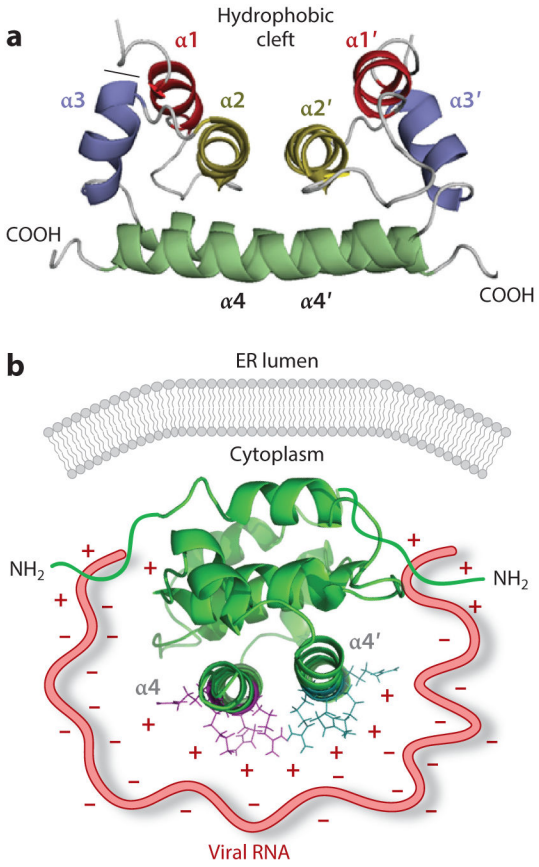


Figure 1.

Structure of the dengue virus (DENV) capsid protein. (*a*) The homodimer is represented and the four α-helices are indicated: helices α1 (*red*), α2 (*yellow*), α3 (*blue*), and α4 (*green*). Labels for one of the two dimer subunits are designated with prime symbols (helices α1′, α2′, α3′, and α4′) (33). The hydrophobic cleft is indicated. (*b*) Model of capsid protein interaction with endoplasmic reticulum (ER) membranes and the viral genome. The unstructured N-terminal region of the capsid protein is represented and shown interacting with the viral RNA together with the large α4–α4′ region.

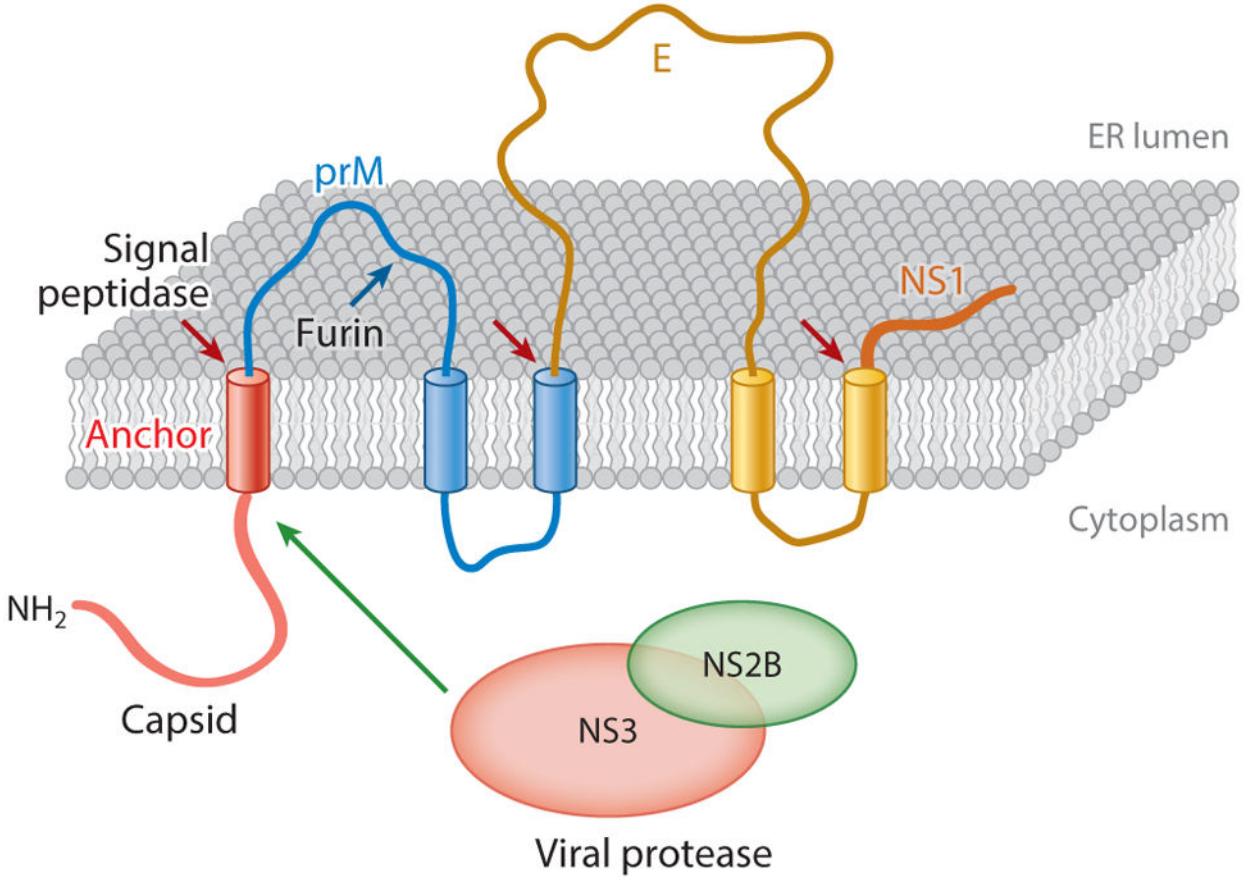


Figure 2.

Membrane topology of dengue virus structural proteins. The orientation of the structural proteins across the ER membrane is shown. Transmembrane helices are indicated by cylinders, and the sites of posttranslational cleavage by signal peptidase are indicated by red arrows. The cleavage site of the viral NS2B-3 protease is indicated by a green arrow and the furin cleavage site by a blue arrow. Abbreviations: E, envelope protein; ER, endoplasmic reticulum; NS, nonstructural protein; prM, pre–membrane protein.

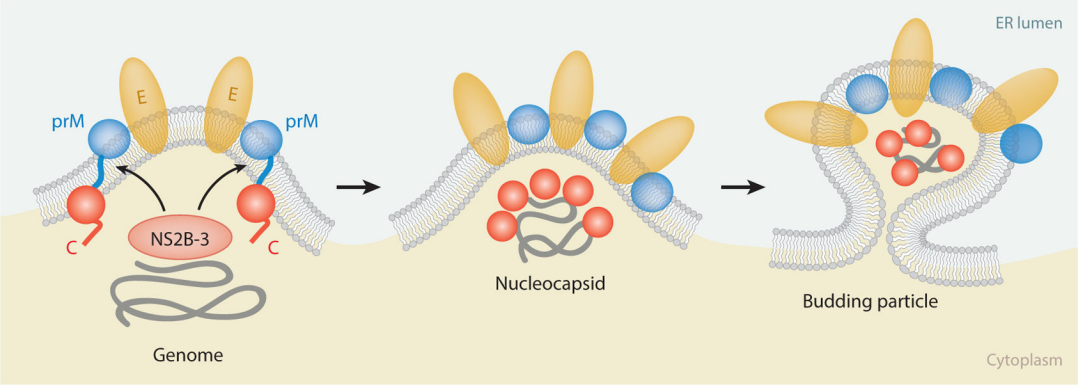


Figure 3.

Model of dengue virus assembly. Capsid maturation by NS2B-3 on the ER membrane is shown. The budding viral particle, containing the nucleocapsid, the viral proteins E and prM, and lipid membranes, is shown within the ER. Abbreviations: C, capsid protein; E, envelope protein; ER, endoplasmic reticulum; NS, nonstructural protein; prM, pre–membrane protein.

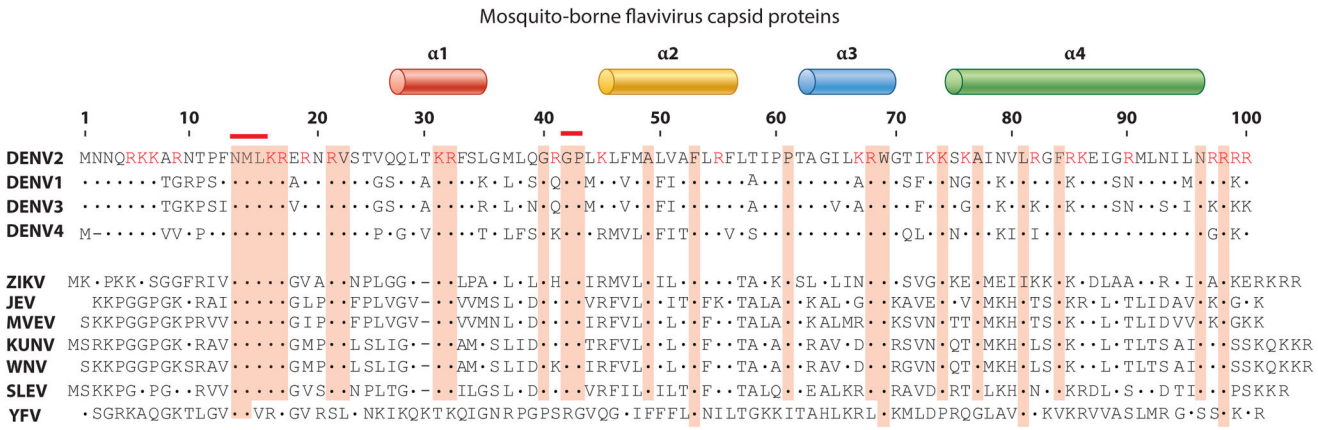
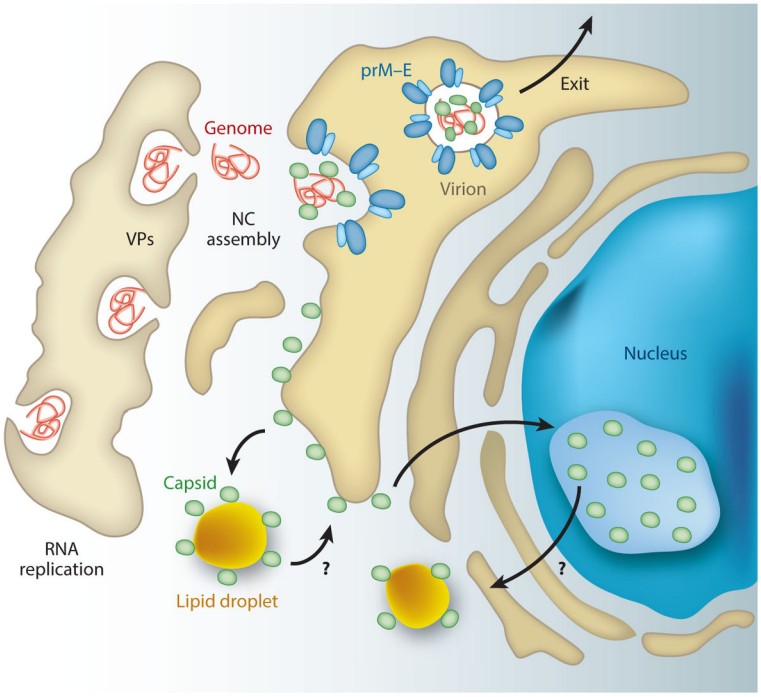


Figure 4.

A multiple-sequence alignment of mosquito-borne flavivirus capsid proteins indicates low sequence conservation. Dots indicate conserved residues, and dashes indicate deletions. The red text indicates basic residues in the capsid protein of DENV2. Pink boxes highlight conserved residues along the different mosquito-borne flaviviruses. The red lines indicate the highly conserved sequence MNL and the putative residues involved in nuclear localization of JEV (G42 and P43). The locations of the four helices (α1, α2, α3, and α4) are indicated by barrels at the top. Available GeneBank sequences were aligned to obtain consensus sequences for each of the viruses shown (DENV1, DENV2, DENV3, DENV4, ZIKV, JEV, MVEV, KUNV, WNV, SLEV, and YFV). The figure shows the alignment of these consensus sequences, performed using Geneious 3.6.1. Abbreviations: DENV, dengue virus; JEV, Japanese encephalitis virus; KUNV, Kunjin virus; MVEV, Murray Valley encephalitis virus; SLEV, Saint Louis encephalitis virus; WNV, West Nile virus; YFV, yellow fever virus; ZIKV, Zika virus.



Summary Figure.

Dengue virus particle assembly, capsid protein interactions, and subcellular distribution in an infected cell. Viral RNA is amplified in replication complexes inside membranous structures called VPs. The newly synthesized viral genome exits the VPs and is recruited by the capsid protein (*green*) to form the NC, which buds into the ER and acquires lipid membranes and the structural viral proteins E and prM. The capsid protein is also distributed in different cellular compartments, ER membranes, nucleoli, and lipid droplets. Abbreviations: E, envelope protein; ER, endoplasmic reticulum; NC, nucleocapsid; prM, pre–membrane protein; VP, vesicle packet.