International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 11 (2014) pp. 1025-1039**
http://www.ijcmas.com

Review Article

Review on Dengue viral Replication, assembly and entry into the host cells

R.Aruna*

Research and Development Centre, Bharathiar University, Coimbatore, India

**Corresponding author*

K e y w o r d s

Dengue fever, viral Replication, and assembly

A B S T R A C T

Dengue fever is a mosquito-borne virus disease of humans. It is estimated that more than 3 billion humans live in dengue endemic regions of the world, and currently, more than 50 million infections occur annually with at least 500,000 individuals requiring hospitalization. In this review we will discuss the replication of dengue virus, involvement of the structural proteins in this process. NS3 and NS5 methyl transferase 's predictive function in the entry of hostcells

Introduction

Dengue fever is a mosquito-borne virus disease of humans. In terms of numbers of individuals infected, it is by far the most devastating of all the recognised arthropod-transmitted virus diseases. Dengue has become a global problem since the Second World War and is endemic in more than 110 countries. It is estimated that more than 3 billion humans live in dengue endemic regions of the world, and currently, more than 50 million infections occur annually with at least 500,000 individuals requiring hospitalisation [1]. Of these, tens of thousands have a high risk of developing haemorrhagic disease, potentially with fatal consequences depending to a large extent on the quality of the available medical services. The dengue viruses are positive stranded RNA viruses in the genus *Flavivirus*, family *Flaviviridae* [2].

There are four distinct dengue virus (DENV) serotypes that share antigenic relationships (DENV-1, DENV-2, DENV-3, and DENV-4), and although infection with one serotype confers lifelong protection against that serotype, it does not necessarily protect against a secondary infection with a heterologous serotype. Indeed, nonprotective but cross-reactive antibodies may enhance disease severity [3]. Currently, there are no effective vaccines or antiviral drugs against these viruses. This problem is being addressed as a matter of urgency as failure to develop effective DENV control strategies will inevitably result in a further increase in the number of infected humans, as predicted more than a decade ago [4]. This problem is also exacerbated by the continuing dispersal of these viruses to new geographic regions.

In this review, we will discuss on advances in structural studies on DENV and its proteins. We will focuses the relevance of the functional mechanisms of the viral proteins, their interactions with host proteins, and their role in the viral life cycle.

DENV is a member of the Flaviviridae family and is grouped within the flavivirus genus together with other pathogenic viruses including West Nile virus (WNV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV) and yellow fever virus (YFV) [5]. The viral genome consists of a positive-sense RNA of ~11kb. This RNA encodes 3 structural proteins (C, prM and E) that form the components of the virion, and 7 non-structural proteins (NS1, NS2A/B, NS3, NS4A/B, NS5) involved in viral RNA replication (Figure 1).

Replication and assembly of dengue virus particles

A schematic representation of the DENV genomic RNA and the translation of the viral proteins are depicted in Fig. 3. After virus cell entry and uncoating of the nucleocapsid, the RNA molecule is translated as a single polyprotein [28].

During this process, the signal- and stop-transfer sequences of the polyprotein direct its back-and-forth translocation across the endoplasmic reticulum (ER) membrane. The polyprotein is processed co- and posttranslationally by cellular and virus-derived proteases into three structural proteins (C, prM, and E) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The E protein is glycosylated at amino acid residue Asn67 and Asn153 to assure proper folding of the protein [23, 29]. Other potential N-linked glycosylation sites are located in prM at position 7, 31, and 52 and within NS1 at position 130 and 207 [30, 31]. Upon protein translation and folding of the individual proteins, the NS proteins initiate replication of the viral genome [28]. The newly synthesized RNA is subsequently packaged by the C protein to form a nucleocapsid. The prM and E proteins form heterodimers that are oriented into the lumen of the ER. Then, the prM/E heterodimers associate into trimers and these oligomeric interactions are believed to induce a curved surface lattice, which guides virion budding [25, 26]. It is unclear how this is synchronized with the engulfment of the nucleocapsid since no specific interactions between C and prM/E proteins have been identified yet [32, 33]. Interestingly, encapsulation of the nucleocapsid during virus assembly is not crucial as the formation and release of capsidless subviral particles has been often documented [34–37]. Structural analysis of newly assembled immature virions revealed that a single virion contains 180 prM/E heterodimers that project vertically outward from the viral surface as 60 trimeric spikes [32, 33, 38]. The immature particles formed in the ER mature as they travel through the secretory pathway. The slightly acidic pH (*5.8–6.0) of the trans-Golgi network (TGN) triggers dissociation of the prM/E heterodimers, which leads to the formation of 90 dimers that lie flat on the surface of the particle, with prM capping the fusion peptide of the E protein. This global structural reorganization of the glycoproteins enables the cellular endoprotease furin to cleave prM [39–41]. Furin cleavage occurs at a Arg-X-(Lys/Arg)-Arg (where X is any amino acid) recognition sequence and leads to the generation of membrane-associated M and a ‘‘pr’’ peptide. A recent study has shown that the pr peptide remains associated with the virion until the virus is secreted to the extracellular milieu [40]. Both the prM protein as well as the pr peptide are believed to act as

chaperones stabilizing the E protein during transit through the secretory pathway, thereby preventing premature conformational changes of the E protein that would lead to membrane fusion. Upon dissociation of the pr peptide, mature virions are formed that are able to infect new cells.

Virus assembly and maturation

The structure of DENV was previously solved through a combination of cryo-electron microscopy and X-ray crystallography[6,7,8,9]. Recently, the structures of intermediates in the assembly process have also been obtained and these provide insight into the assembly and maturation process of DENV [16,17]. In supernatants of infected cells, the virus is found either as a mature or immature particle with a diameter of about 50 nm and 60 nm, respectively. Both particles consist of an outer glycoprotein shell and an internal host derived lipid bilayer. Within this bilayer is an RNA-protein core consisting of genome RNA and capsid proteins (C). The glycoprotein shell is well defined and consists of 180 copies each of an envelope (E) and membrane protein (prM/M). These two proteins have different conformations in the immature and mature DENV particles and therefore, confer unique structural features to both forms of particles. In the immature virion, prM and E form 90 heterodimers that extend as 60 trimeric spikes from the particle surface (figure 3A). In the mature virion, E is found as 90 homodimers that lie flat against the viral surface forming a 'smooth' protein shell (figure 3D). The 'pr' peptide is cleaved from prM during maturation and M remains in the mature particle as a transmembrane protein beneath the E protein shell. The structural transitions from immature ('spiky') to mature ('smooth') morphology occur while in transit through the Trans-Golgi Network (TGN) and are driven predominantly by conformational changes in the E protein [8,9,16]. These conformational changes in E are triggered by low pH (~5.8–6.0) and occur prior to the maturation cleavage of prM by a host encoded furin protease (figure 3B) [17]. It was demonstrated that these conformational changes were reversible (based on pH). This suggested that the immature particle could exist reversibly in either 'spiky' or 'smooth' forms depending on the pH of the cellular environment. This is a remarkable observation considering that such reversible conformational changes require the E protein to toggle between forming heterodimeric interactions with prM in spikes that projects vertically outwards from the particle to forming homodimeric structures that lie flat against the viral surface. The molecular reorganization required for these changes are extensive, the details of which still remain illusive. Following maturation however, the mature particle cannot revert back to its immature morphology. Once cleaved, the pr peptide remains associated with E (figure 3C) until the mature particle is released into the neutral pH of the extracellular environment. Therefore, it was proposed that the pr peptide functioned as a cap-like structure that protected the fusion peptide on E from undergoing premature fusion prior to virus release [19].

Recently, the atomic structures of prM were solved at pH 5.5 (2.2Å) and 7.0 (2.6Å) [13]. Both structures were similar indicating that pH did not affect the tertiary structure of prM. The construct consisted of a prM-E fusion protein where the prM protein was fused to the ectodomain of E. The transmembrane domain of prM was replaced by a linker. The furin cleavage site was also mutated. The resulting structure of prM has a unique fold and consists of 7 antiparallel β-strands, stabilized with three S-S bonds.

The glycosylation modification at asparagine 69 was also observed. The structure of E in this construct was similar to its prefusion dimeric conformation [17]. As predicted previously, the pr peptide was positioned as a 'cap' on E protecting the hydrophobic fusion loop [18]. These recent studies on the immature virions and proteins have provided new details of DENV assembly and maturation. They have also opened new avenues that can be explored toward understanding the molecular mechanism behind E protein dynamics and the role of the immature particles in the virus life cycle. The E protein also provides the first point of contact between the virus and the host cell. Several cellular proteins and carbohydrate molecules that function as attachment factors mediating viral entry have been identified, and these molecules have been shown to interact with the E protein [18–24]. These attachment factors assist in concentrating the virus on the cell surface increasing its access to specific cellular receptor/s. Structural insight into the interaction of E with one of these attachment factors, dendritic-cell-specific ICAM3-grabbing non-integrin (DC-SIGN) [26] has been obtained. Unfortunately, a specific cellular receptor for dengue has not yet been identified. It is possible that currently identified attachment molecules could function as specific cellular receptors for dengue. However, it would be necessary to demonstrate that they mediate clathrin-mediated endocyotsis of the particle and this has not been shown for any of these putative receptors to date.

NS3 protease-helicase

NS3 is a multifunctional protein of 618 amino acids that functions both as a chymotrypsin like serine protease as well as an RNA helicase and RTPase/NTPase. The protease domain is N terminal in NS3 (residues 1–180) and cleaves the viral polyprotein at several sites as depicted in figure 1. The enzyme consists of 6 β-strands that form two β-barrels with the catalytic triad (His-51, Asp-75 and Ser-135) sandwiched between them. Activity of the protease is critically dependent upon the presence of its co-factor, NS2B which is conserved among the flaviviruses [28,32,33]. Recently, the structure of the full-length NS3 of DENV-4 was solved to 3.15Å resolution (figure 4) [34]. This structure suggests that NS3 is an extended molecule with the protease domain spatially oriented on top of subdomains I and II of the helicase. The NS3 protein included residues 49–66 from the NS2B which were linked to the N-terminus of fulllength NS3 by a Gly-Ser linker. However, its protease domain was inactive but retains the same active site conformation and overall fold as an active form of the DENV-2 protease domain that included an extended region of NS2B (residues 40–80) [35].

Analysis of these two structures as well as the structure of the substrate-bound form of the WNV protease domain [36] indicated that residues 67–80 of NS2B are critically important for protease activity of NS3. This is due to NS2B wrapping around the protease domain as a 'belt-like' structure and forming an integral part of the protease active site. While the central region of NS2B (residues 67–80) interacts with the protease, flanking hydrophobic regions of NS2B are predicted to anchor the NS2B-NS3 complex in the ER membrane [37]. This geometry places the protease active site close to predicted transmembrane domains that must be cleaved within the polyprotein. The consensus cleavage site of NS3 requires a dibasic (Arg/Lys)-Arg motif at the P2 and P1 positions respectively and a small amino acid (Gly) at the P1′ position. The substrate specificity as well as the cis/trans activity of

many of the flavivirus proteases have also recently been characterized [38,39]. These studies provided evidence of a required cis-cleavage of the NS3 helicase domain at a site hidden from exposure to the protease domain. This indicated that the NS3 is structurally dynamic and may indeed require an extended conformation for its function. Such a conformation of NS3 could explain the ability of the NS2B-NS3 protease to function on sites not readily accessible to it. These same studies have also suggested that the cis-trans activity of the protease may play an important role in controlling the dynamics of viral protein translation versus RNA replication by controlling the availability of viral proteins. The helicase domain of NS3 (residues 180–618) has seven structural motifs reminiscent of superfamily 2 helicases [40]. It has three subdomains with significant sequence identity and structural similarity to other flavivirus helicases [34,41–43]. Subdomains I and II are also structurally similar to the corresponding domains in hepatitis C virus suggesting a common functional mechanism [44,45], however, the fold of subdomain III is unique to the flaviviruses, and may be a site for protein binding. Both subdomains I (residues 181–326) and II (residues 327–481) are composed of a central six-stranded parallel β-sheet, which is flanked by 4 α-helices. Subdomain III (residues 482–618) has 4 approximately parallel α-helices surrounded by three shorter α-helices and two solvent-exposed antiparallel β-strands. In the recent structure of full-length NS3 [34], the helicase domain adopts a similar structure to the isolated DENV helicase [41], with the exception that several regions previously disordered in the isolated domain structure are now visible. However, the helicase activity of the full-length NS3 protein is ~30-fold higher than the isolated domain [41] indicating that the protease domain may influence the enzymatic activities of the helicase. The protease and helicase are linked by an interdomain linker that shares little conservation between the flaviviruses, but plays an important role in the association between the two domains in NS3 (the buried surface area between the domains is ~1380Å2 and ~380Å2 with and without the linker, respectively). The helicase domain of NS3 is also implicated in interacting with the polymerase, NS5 [46] and in WNV implicated in virulence [47]. Recently, a residue in the NS3 helicase domain (W349) was shown to be involved in virus assembly suggesting that NS3 plays an additional role in the life cycle following viral RNA replication [48]. The NS3 protein has also been implicated in inducing apoptosis in infected cells [50]. Overall, the full-length structure of NS3 represents a significant milestone in flavivirus biology as crystallization of full-length viral proteins has long been a challenge.

NS5 methyltransferase-polymerase

The largest (900 residues, 104kDa) and the most conserved protein in DENV is NS5 (67% sequence identity among DENV serotypes 1–4). It is also a bifunctional enzyme with a methyltransferase domain (MTase; residues 1–296) at its N-terminal end and a RNA-dependent RNA polymerase (RdRp; residues 320–900) at its C-terminal end. The structure of the MTase domain was previously solved for DENV [50] and recently solved for WNV [51]. Both structures have an S-adenosyl-methionine-dependent MTase core structure that folds into a α/ β/β sandwich cradled between N- and C-terminal subdomains (figure 4). Overall, the two MTase structures are very similar and this domain has recently been shown to sequentially catalyze both guanine N-7 and ribose 2′-O-methylation [50,52]. The primary difference between the two structures is observed in the SAM-binding

site and the cap-binding site, with the former having a more open conformation in DENV versus WNV, and the latter being more open in WNV [50,51]. It is suggested that the differences in the SAM-binding site may reflect two distinct states of the enzyme, with the closed conformation (in WNV) representing tight SAM-binding, and the open state representing the release of the by-product of the methylation reaction, S-adenosyl-homocysteine (SAH). Both enzymes have a highly positively charged surface at the GTP and SAM binding sites to accommodate capped RNA substrates. The crystal structures for both DENV [53] and WNV [54]polymerases have been solved. The polymerase domain of NS5 assumes a structure similar to other RdRp molecules, and is composed of a canonical right hand conformation with palm, fingers, thumb subdomains [55]. It also shares a common catalytic mechanism for the incorporation of nucleotides utilizing two metal ions coordinated by structurally conserved aspartic acid residues (also known as the GDD motif). The RdRps differ from DNA-dependent RNA polymerases by the existence of the 'finger tips' that connect the fingers and thumb subdomains to create a fully encircled active site. The DENV RdRp displays a more flexible fingers subdomain and therefore through a rotation of ~8° away from the thumb subdomain, forms a more 'open' conformation compared to WNV [53,54]. Interestingly, the flavivirus RdRps have a nuclear localization signal between residues 320–405. This NLS region in NS5 was previously thought to be a flexible interdomain linker, but is now known to be a well-defined structural component of the RdRp [53- 57]. Specifically, residues 320–368 are strictly conserved among the flaviviruses and bind β-importin. These residues are also implicated in interacting with NS3 [46,56]. In DENV infections, the NS5 protein is primarily localized within the nucleus. However, not all flavivirus RdRps localize to the nucleus. The rationalization for a viral RdRp localizing to the nucleus when its actual enzymatic functions in the virus life cycle are required in the cytoplasm is currently unknown but is actively being investigated [60]. However, these observations do suggest that apart from its enzymatic functions, NS5 may also engage in virus-host interactions and actively interact with the host environment.

Other viral non-structural proteins

Unfortunately, there is no structural information available for viral proteins NS1, NS2A and NS4A/4B. NS1 is a 45kDa glycoprotein that is translocated into the lumen of the ER and secreted from the cell [59–61]. It is implicated in functions within the viral RNA replication complex [62–64] as well as in viral defense through inhibition of complement activation [65]. Although the protein forms stable oligomers (dimers and hexamers) in solution, structural analyses of this protein and has been quite challenging [66,67]. This is also true for the hydrophobic proteins NS2A and NS4A/4B. NS2A is a ~22kDa protein that is implicated to form part of the replication complex [67]. As a result of an internal NS2B-NS3 cleavage, two forms are observed, NS2A and NS2Aα Both forms are important for virus production [69]. NS4A (16kDa) and NS4B (27kDa) are integral membrane proteins. NS4A is proposed to induce membrane alterations important for virus replication [31,70]. NS4B is implicated in assisting viral RNA replication through its direct interaction with NS3 [71]. It is also suggested to block IFN α/β-induced signal transduction [32,69]. Structural analayses of these three proteins have been unsuccessful since they all possess multiple transmembrane hydrophobic segments. However, the membrane topology of NS4A

and NS4B has been predicted through biochemical analyses (figure 2B) [31,71].

Receptor interaction and viral entry

During natural infection, cells of the mononuclear phagocyte lineage [monocytes (MO), macrophages (MØ), and dendritic cells (DCs)], including the skin-resident Langerhans cells, are primary targets for DENV infection [42, 43]. In insects, DENV was found to initially infect the midgut from where it spreads and replicates in many body compartments and organs [44–47]. Also, DENV has been shown to infect numerous cell lines, including human (K562, U937, THP-1, HepG2, HUVEC, ECV304, Raji, HSB-2, Jurkat, LoVo, KU812), mosquito (C6/36), monkey (Vero, BS-C-1, CV-1, LLC-MK2), hamster (BHK), as well as murine MØ (Raw, P388D1, J774) cell lineages [39, 48–61]. The wide range of DENV-permissive cells indicates that the virus must bind to an ubiquitous cell-surface molecule, or exploit multiple receptors to mediate infection. Over the last decade, several candidate receptors and/ or attachment factors have been identified, which suggests that DENV is capable of utilizing multiple molecules to enter the cell. In mosquito cells, DENV has been shown to interact with heat-shock protein 70 (Hsp70) [62], R80, R67 [63], and a 45-kDa protein [64]. Heparan sulfate [65–67], Hsp90 [62], CD14 [68], GRP78/BiP [69], and a 37/67-kDa high-affinity laminin receptor [70] have been identified as receptors on mammalian cells. C-type lectin receptors (CLR) are involved in the interaction of DENV particles with human myeloid cells [71]. These include DC-specific intracellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN, CD209) [72–74], mannose receptor (MR) [75] and C-type lectin domain family 5, member A (CLEC5, MDL-1) [52]. DENV [76–78] as well as other flaviviruses [79, 80] use clathrin-mediated endocytosis for cell entry. Using a single- particle tracking approach, we have revealed that DENV-2 strain S1 particles land on the cell surface and migrate in a diffusive manner toward a pre-existing clathrin- coated pit [77]. This suggests that DENV particles move along the cell surface by rolling over different receptors, or migrate as virus–receptor complexes. Upon internalization, the particles are delivered to Rab5-positive early endosomes, which mature into Rab7-positive late endosomes, where membrane fusion primarily occurs [77]. A recent report also demonstrated that DENV, depending on the serotype and/or the target cell type used, is able to utilize an alternative internalization pathway, independent of clathrin, caveolae, and lipid rafts [81]. The subcellular organelle from which membrane fusion occurs is most likely dependent on the pH-dependent membrane fusion properties of the virus and may therefore vary between individual DENV strains [77, 78]. Numerous functional and structural studies have been undertaken to unravel the molecular mechanisms involved in the membrane fusion process of the virus [27, 82–85]. It is postulated that the acidic pH in endosomes triggers dissociation of E homodimers, which then leads to the outward projection of domain II and exposure of the hydrophobic fusion peptide to the target membrane [86]. Subsequently, the hydrophobic residues in the fusion loop would insert into the outer leaflet of the target membrane, triggering the assembly of E trimers. Next, domain III is assumed to shift and fold back toward the fusion peptide into a hairpin-like conformation. This folding-back mechanism would force the target membrane and the viral membrane to bend towards each other and eventually to fuse, releasing the nucleocapsid into the cell cytosol.

Fig.1 Life cycle of Dengue virus. Adapted from H. M. van der Schaar

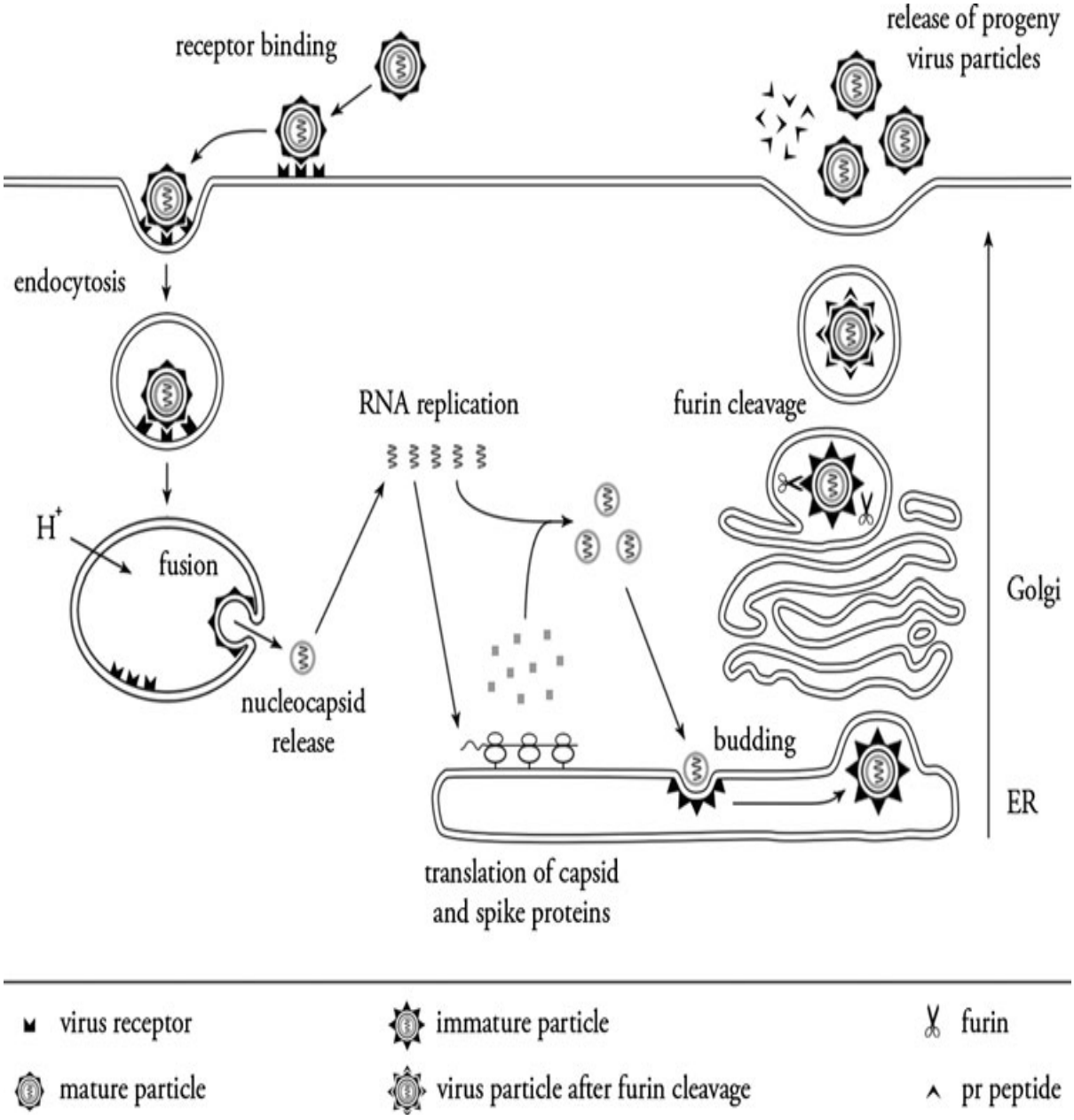
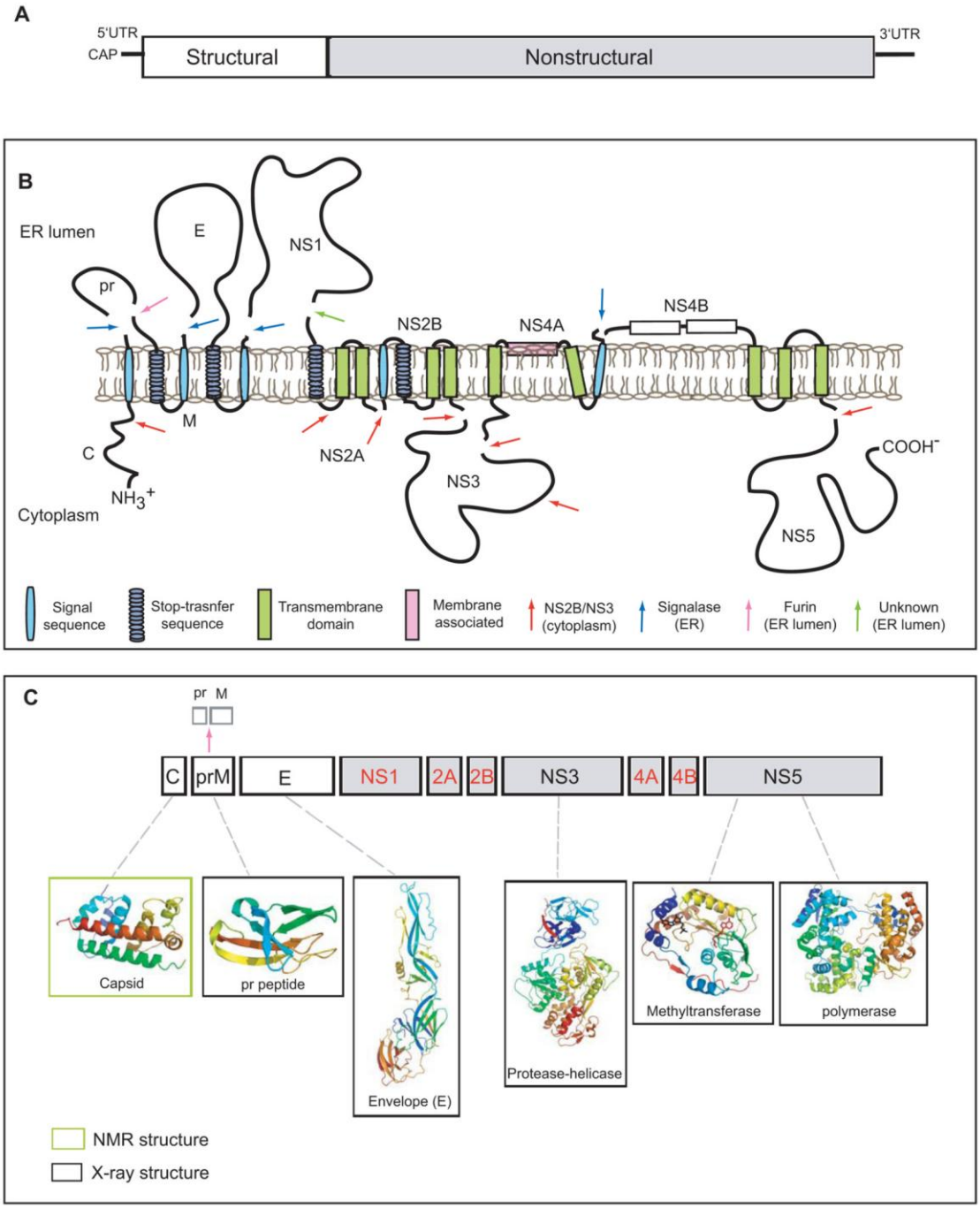


Fig.2 Schematic diagram of the dengue virus genome and polyprotein



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