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The membrane-active regions of the dengue virus proteins C and E

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ABSTRACT

We have identified the membranotropic regions of proteins C and E of DENV virus by performing an exhaustive study of membrane rupture induced by two C and E-derived peptide libraries on model membranes having different phospholipid compositions as well as its ability to modulate the DEPE L_β–L_α and L_α–H_{II} phospholipid phase transitions. Protein C presents one hydrophobic leakage-prone region coincidental with a proposed membrane interacting domain, whereas protein E presents five membrane-rupture zones coincidental with different significant zones of the protein, i.e., the fusion peptide, a proline-rich sequence, a sequence containing a hydrophobic pocket as well as the stem and transmembrane domains of the protein. The identification of these membrane-active segments supports their role in viral membrane fusion, formation of the replication complex and morphogenesis and therefore attractive targets for development of new anti-viral compounds.

1. Introduction

Dengue virus (DENV) is a member of the family *Flaviviridae* in the genus *Flavivirus*. DENV is the leading cause of arboviral diseases in the tropical and subtropical regions, affecting more than 70 million people each year [1]. DENV comprises four serologically and genetically related viruses, DENV viruses 1–4, which possess 69–78% identity at the amino acid level [2]. DENV infections might be either asymptomatic or result in what is known as dengue fever; some individuals develop a severe and potentially life-threatening disease known as dengue hemorrhagic fever or dengue shock syndrome, leading to more than 25,000 deaths per year. Despite the urgent medical need and considerable efforts, no antivirals or vaccines against DENV virus are currently available, so that more than 2 billion people, mainly in poor countries, are at risk in the world [3]. DENV is a positive-sense, single-stranded RNA virus with a single open reading frame encoding a polyprotein, which is subsequently cleaved by cellular and viral proteases into three structural proteins, C, prM and E, and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [4] (Fig. 1A). Similarly to other enveloped viruses, the DENV virus enters the cells through receptor mediated endocytosis [4–7] and rearranges cell internal membranes to establish specific sites of replication [8–10]. Details about DENV replication process remain largely unclear, but most, if not all of the DENV proteins, are involved and function in a complex web of protein–protein interactions. The mature DENV virus has a capsid (C) protein core complexed with the RNA genome, surrounded by a host-derived lipid bilayer in which multiple copies of the viral envelope (E) and membrane (M) proteins are embedded.

The C proteins of *Flaviviridae* are dimeric, basic, have an overall helical fold and are responsible for genome packaging. Protein C seems also to associate with intracellular membranes through a conserved hydrophobic domain [11]. Recently, it has been found that protein C accumulates around endoplasmic reticulum (ER) derived lipid droplets [12]. Similarly to other enveloped viruses, DENV replicates its genome in a membrane-associated replication complex, and morphogenesis and virion budding has been suggested to take place in the ER or modified ER membranes. These modified membranes could provide a platform for capsid formation during viral assembly [12]. Although *Flaviviridae* C proteins are shorter than the *Hepacivirus* core proteins, their roles should be similar as well as their capacity to bind to phospholipid membranes [13–15].

The DENV E protein is a class II fusion protein, essential for attachment, membrane fusion, and assembly. The three-dimensional structure of class I and class II membrane fusion proteins is different but their function is identical, so they must share structural and functional characteristics in specific domains which interact with and disrupt biological membranes [6,16]. A series of conformational changes occurring in the DENV E protein driven by the endosomal

Abbreviations: BMP, S,R-bis(monooleoylglycero)phosphate; BPI, Bovine brain L-α-phosphadidylinositol; BPS, Bovine brain L-α-phosphatidylserine; CF, 5-Carboxyfluorescein; CHOL, Cholesterol; CL, 1′,3′-bis[1,2-dimyristoyl-*sn*-glycero-3-phospho]-*sn*-glycerol; DENV, Dengue virus; DEPE, 1,2-Dielaidoyl-sn-glycero-3-phosphatidylethanolamine; DSC, Differential Scanning Calorimetry; EPA, Egg L-α-phosphatidic acid; EPC, Egg L-α-phosphatidylcholine; ER, Endoplasmic reticulum; ESM, Egg sphingomyelin; LUV, Large unilamellar vesicles; MLV, Multilamellar vesicles; NS, Non-structural protein; TFE, Trifluoroethanol; T_m, Temperature of the gel-to–liquid crystalline phase transition; TM, Transmembrane domain; TPE, Egg trans-esterified L-α-phosphatidylethanolamine

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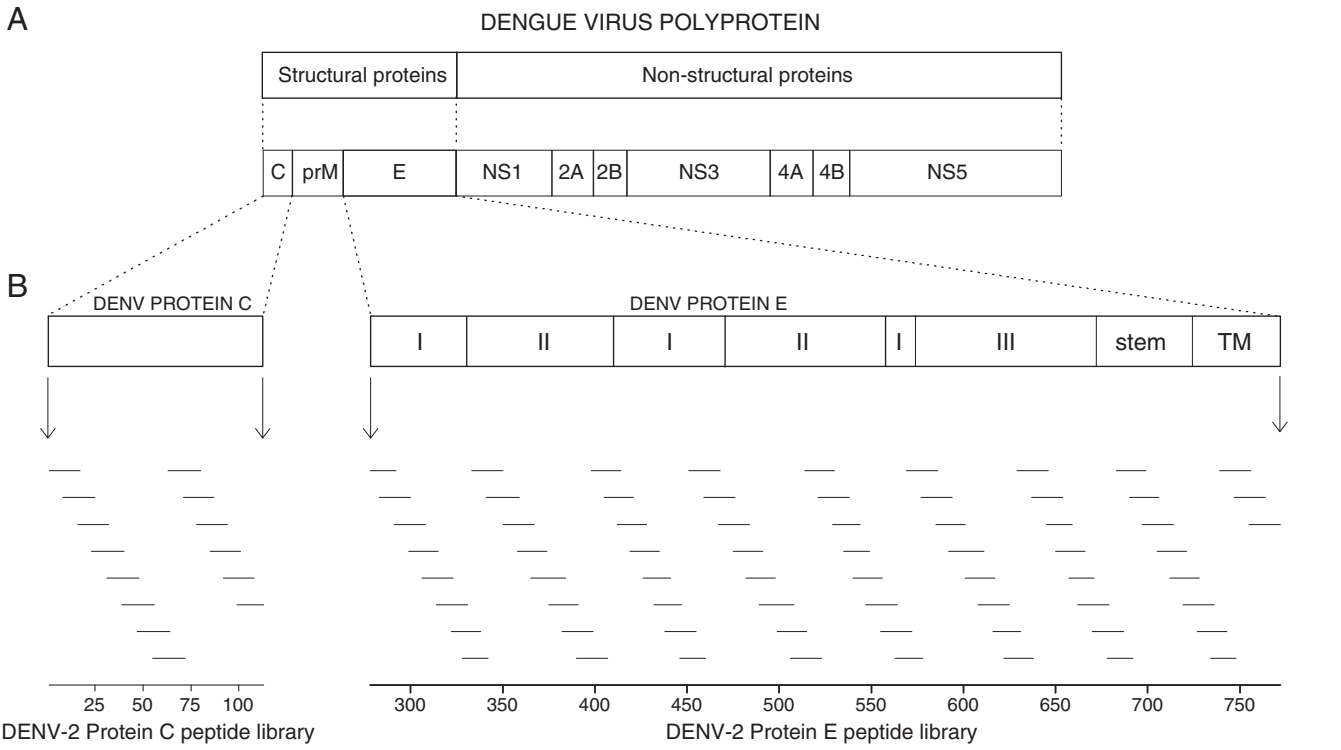

Fig. 1. (A) Scheme of the structure of the DENV virus structural (C, prM and E) and non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), according to literature consensus. The approximate segments of domains I, II, III, the stem and the transmembrane anchor of E protein are depicted (see text for details). (B) The sequence and relative location of the peptide libraries derived from the Dengue Virus Type 2 NGC C (14 peptides) and E proteins (67 peptides) are shown with respect to the full sequence of both proteins. Peptide line length is related to the number of amino acids in the peptide. Maximum overlap between adjacent peptides is 11 amino acids.

low-pH gives place to the fusion of the viral and endosomal membranes [4,7]. The DENV E protein, being the first point of contact between the virus and the host cell, is a determinant of tropism and virulence, as well as the major target of DENV neutralizing and enhancing antibodies [7]. The N-terminal ectodomain of the protein presents three domains consisting predominantly of β-strands (amino acids 1 to 395, DENV2 numbering) [5,7]. The fusion loop of the E protein is located between amino acids 98 and 112 [17]. Two α-helices that link the soluble ectodomain and the two transmembrane domains of the E protein form a stem (amino acids 396 to 447) which contribute to the flexibility required for the conformational change [18]. Based on tick-borne encephalitis virus E protein, DENV E protein would have two transmembrane (TM) domains (amino acids 448 to 491) and both of them are required for assembly of E protein into particles; similarly, the two α-helices are implicated either in homo and/or in hetero protein–protein interaction or membrane interaction or both [19,20]. Interestingly, E protein might interact with other proteins through a conserved Pro-rich motif [21]. Significantly, the stem region of the E protein has been proposed to be engaged in the fusion process but the critical regions of the stem region involved are not known with certainty [4–6,22].

We have recently identified the membrane-active regions of a number of viral proteins by observing the effect of glycoprotein-derived peptide libraries on model membrane integrity [15,23–26]. These results allowed us to propose the location of different segments in these proteins that are implicated in either protein–lipid or protein–protein interactions and helped us to understand the mechanisms underlying the interaction between viral proteins and membranes. There are still many questions to be answered regarding the C and E mode of action in membrane fusion, assembly, replication and/or release during the DENV viral cycle. Segments of both C and E proteins have been used as vaccine candidates for DENV [27]. For example, domain III of the E protein can block the entry of the virus as well as peptides derived from the fusion loop can interfere with infectivity [28]. Additionally, DENV membrane interaction is an attractive target for anti-DENV therapy. To investigate the structural basis of the interaction of proteins C and D from DENV virus and identify new targets for searching new DENV inhibitors, we have carried out the analysis of the different regions of DENV C and E proteins which might interact with phospholipid membranes using a similar approach to that used before [26,29,30]. By monitoring the effect of these peptide libraries on membrane integrity we have identified different regions on DENV C and E proteins with membrane-interacting capabilities, suggesting the location of different segments implicated in oligomerization (protein–protein binding) and membrane interaction and destabilization. These results should help in our understanding of the molecular mechanism of viral fusion and morphogenesis as well as making possible the future development of DENV entry inhibitors which may lead to new vaccine strategies.

2. Materials and methods

2.1. Materials and reagents

Two sets of 14 (Table 1) and 67 (Table 2) peptides derived from Dengue Virus Type 2 NGC C and E proteins were obtained through BEI Resources, National Institute of Allergy and Infectious Diseases, Manassas, VA, USA. Peptides were solubilized in water/TFE at 70:30 ratios (v/v). Bovine brain phosphatidylserine (BPS), S,R-bis(monooleoylglycero)phosphate ammonium salt (BMP), bovine liver L-α-phosphatidylinositol (BPI), cholesterol (CHOL), egg phosphatidic acid (EPA), egg L-α-phosphatidylcholine (EPC), egg sphingomyelin (ESM), egg trans-esterified L-α-phosphatidylethanolamine (TPE), 1′,3′-bis[1,2-dimyristoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (cardiolipin, CL), dielaidoyl-sn-glycero-3-