Biochimica et Biophysica Acta 1818 (2012) 2818–2830

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

NS4A and NS4B proteins from dengue virus: Membranotropic regions

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ARTICLE INFO

Article history:
Received 9 May 2012
Received in revised form 26 June 2012
Accepted 27 June 2012
Available online 5 July 2012

Keywords:
DENV replication
DENV
Lipid–peptide interaction
Membranous web
Replication complex

ABSTRACT

Proteins NS4A and NS4B from Dengue Virus (DENV) are highly hydrophobic transmembrane proteins which are responsible, at least in part, for the membrane arrangements leading to the formation of the viral replication complex, essential for the viral life cycle. In this work we have identified the membranotropic regions of DENV NS4A and NS4B proteins by performing an exhaustive study of membrane rupture induced by NS4A and NS4B peptide libraries on simple and complex model membranes as well as their ability to modulate the phospholipid phase transitions $P_{\beta'}$–L_α of DMPC and L_β–L_α/L_α–H_{II} of DEPE. Protein NS4A presents three membrane active regions coincident with putative transmembrane segments, whereas NS4B presented up to nine membrane active regions, four of them presumably putative transmembrane segments. These data recognize the existence of different membrane-active segments on these proteins and support their role in the formation of the replication complex and therefore directly implicated in the DENV life cycle.

1. Introduction

The *Flaviviridae* family contains three genera, *Flavivirus, Hepacivirus and Pestivirus.* Dengue virus (DENV), a member of the genus *Flavivirus*, is the leading cause of arboviral diseases in the tropical and subtropical regions, affecting 70 to 100 million people every year of dengue fever and dengue hemorrhagic fever [1,2]. DENV comprises four serologically and genetically related viruses which possess 69–78% identity at the amino acid level [3]. Despite the urgent medical need and considerable efforts to treat DENV derived infections, 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 [4]. Furthermore, due to the increasing world global temperature as well as travelling, there is a real risk of mosquito vector spreading to previously unaffected zones.

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 and seven non-structural (NS) proteins [5]. Similarly to other enveloped viruses, DENV enters the cells through receptor mediated endocytosis [5–8] and rearranges cell internal membranes to establish specific sites of replication [9–11]. DENV replicates its genome in a membrane-associated replication complex, and morphogenesis and virion budding have been suggested to take place in the endoplasmic reticulum (ER) or modified ER membranes. These modified membranes could provide a platform for capsid formation during viral assembly [12]. 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 [5,8]. The majority of the NS proteins are thought to be responsible for both polyprotein processing and viral RNA replication, the latter taking place in the membrane-associated replication complexes (RC) of the virus [13]. The exact function of each of the NS proteins is far from explained, yet some studies have unveiled some information. It has been reported that NS1, in association with NS4A, might be determinant in the early steps of viral RNA replication and mutations in NS1 affected the start of the minus RNA strand synthesis [14,15]. NS3 is a multifunctional protein with RNA helicase, 5′-terminal RNA triphosphatase and serine protease functions [16]. NS5, the most conserved protein in DENV has RNA-dependent RNA polymerase activity at its C-terminal domain and methyltransferase activity at its N-terminal domains, essential functions for capping of the mRNA [17].

As for the remaining small hydrophobic DENV proteins, i.e., NS2A, NS4A and NS4B, little is known hitherto about their function in the viral cycle of dengue virus and remain the most poorly characterized proteins [18]. NS4A, a highly hydrophobic protein, contains an initial sequence (residues 1 to 49) that apparently does not interact with membranes and appears to function as a cofactor of NS3 [19], three hydrophobic regions (residues 50 to 73, residues 76 to 89, and residues 101 to 127) which are tightly associated to membranes, a small loop

Abbreviations: BMP, S,R-bis(monooleoylglycero)phosphate; BPI, bovine liver L-α-phosphadidylinositol; BPS, bovine brain L-α-phosphatidylserine; CF, 5-carboxyfluorescein; CHOL, cholesterol; CL, bovine heart cardiolipin; DENV, dengue virus; DEPE, 1,2-Dielaidoyl-sn-glycero-3-phosphatidylethanolamine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry; EPA, egg L-α-phosphatidic acid; EPC, egg L-α-phosphatidylcholine; EPG, egg L-α-phosphatidylglycerol; ER, endoplasmic reticulum; ESM, egg sphingomyelin; HCV, hepatitis C virus; LEM, late endosome membrane; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; NS, non-structural protein; TFE, 2,2,2-Trifluoroethanol; T_m, temperature of the gel-to-liquid crystalline phase transition; TM, transmembrane domain; TPE, egg transphosphatidylated L-α-phosphatidylethanolamine

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doi:10.1016/j.bbamem.2012.06.022

that exposes the NS4A-2k cleavage site (residues 123 to 130) and a C-terminal fragment called 2k that acts as the signal sequence for translocation of the NS4B protein into the ER lumen [9]. NS4A, in concert with other viral and host proteins, promotes the membrane rearrangements essential for viral replication [9,20,21]. Another evidence of the role of NS4A in viral RNA replication of DENV is given by the fact that this protein was found in reticular structures and cytoplasmic foci (derived from or associated to the ER) [9,18,22,23]. Interestingly, it has been recently shown that NS4A induces autophagy in epithelial cells, protecting the host cell against death [21]. NS4B is another highly hydrophobic membrane protein which appears to have two hydrophobic segments (residues 1 to 56 and residues 56–93) which are probably associated to the ER lumen side of the membrane and supposedly three C-terminal TM segments (residues 93 to 146, residues 146 to 190 and residues 190 to 248) [18]. NS4B is capable of interfering with phosphorylation of STAT1 blocking the IFN-α/β induced signal transduction cascade [24]. NS4B is also a negative modulator of the NS3 helicase function, being this modulation dependent on the conformation of NS4B. This model is supported, among other evidences, by the fact that a single point mutation disrupts the interaction between NS3 and NS4B; furthermore, NS3, NS4B and NS5 might form a complex that holds the separated strands apart as the helicase moves along the duplex [18,25]. Lastly, NS4A and NS4B might function cooperatively in viral replication and the anti-host response [9,26].

We have recently identified the membrane-active regions of a number of viral proteins by observing the effect of protein-derived peptide libraries on model membrane integrity [27–32]. These results allowed us to propose the location of different protein segments implicated in either protein–lipid or protein–protein interactions and help us to understand the mechanisms underlying the interaction between viral proteins and membranes. Motivated by the need to understand the interaction between the highly hydrophobic DENV NS4A and NS4B proteins with membranes, considering that they are fundamental in the viral RNA replication process, and additionally, that DENV protein/membrane interaction is an attractive target for antiviral drug development, we have characterized the membranotropic regions of DENV proteins NS4A and NS4B. By using peptide libraries encompassing the full length of both proteins, by observing their effect on membrane integrity as well as their effect on model biomembranes, we have identified different regions on DENV proteins NS4A and NS4B with membrane-interacting capabilities. These data will help us to understand 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

The peptide library, consisting of 66 peptides (Table 1), was derived from Dengue Virus Type 2 NGC NS4A, 2k and NS4B proteins and was obtained from BEI Resources, National Institute of Allergy and Infectious Diseases, Manassas, VA, USA. The peptides had a purity of about 80%. Peptides were solubilized in water/TFE/DMSO at 50:20:30 ratios (v/v/v). Bovine brain phosphatidylserine (BPS), bovine liver L-α-phosphatidylinositol (BPI), cholesterol (Chol), egg L-α-phosphatidic acid (EPA), egg L-α-phosphatidylcholine (EPC), egg sphingomyelin (ESM), egg transphosphatidylated L-α-phosphatidylethanolamine (TPE), bovine heart cardiolipin (CL), 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC), 1,2-dielaidoyl-sn-glycero-3-phosphatidylethanolamine (DEPE) and liver lipid extract were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The lipid composition of the synthetic endoplasmic reticulum was EPC/CL/BPI/

Table 1
Sequence and residue position of all peptides contained in the DENV NS4A/2k/NS4B libraries. The amino acid position in the protein sequence is relative to each protein. Residues in cursive constitute the 2k fragment.

Protein	Peptide number	Amino acid sequence	Amino acid position	Protein	Peptide number	Amino acid sequence	Amino acid position
NS4A	1	SLTLNLITEMGRLPTFM	1–17	NS4B	9	YAVATTFVTPMLRHSIE	40–57
NS4A	2	ITEMGRLPTFMTQKARD	7–23	NS4B	10	FVTPMLRHSIENSSVNV	46–63
NS4A	3	LPTFMTQKARDALDNLA	13–29	NS4B	11	RHSIENSSVNVSLTAIA	52–69
NS4A	4	TQKARDALDNLAVLHTA	18–34	NS4B	12	SSVNVSLTAIANQATVL	58–75
NS4A	5	ALDNLAVLHTAEAGGRA	24–40	NS4B	13	LTAIANQATVLMGLGKG	64–81
NS4A	6	VLHTAEAGGRAYNHAL	30–45	NS4B	14	NQATVLMGLGKGWPLSK	69–86
NS4A	7	AEAGGRAYNHALSELPE	34–50	NS4B	15	MGLGKGWPLSKMDIGV	75–91
NS4A	8	AYNHALSELPETLETLL	40–56	NS4B	16	GWPLSKMDIGVPLLAIG	80–97
NS4A	9	SELPETLETLLLLTLLA	46–62	NS4B	17	MDIGVPLLAIGCYSQVN	86–103
NS4A	10	LETLLLLTLLATVTGGI	52–68	NS4B	18	LLAIGCYSQVNPITLTA	92–109
NS4A	11	LTLLATVTGGIFLFLM	58–73	NS4B	19	YSQVNPITLTAALFLLV	98–115
NS4A	12	TVTGGIFLFLMSGRGIG	63–79	NS4B	20	ITLTAALFLLVAHYAII	104–121
NS4A	13	FLFLMSGRGIGKMTLGM	69–85	NS4B	21	LFLLVAHYAIIGPGLQA	110–127
NS4A	14	GRGIGKMTLGMCCIITA	75–91	NS4B	22	HYAIIGPGLQAKATREA	116–133
NS4A	15	MTLGMCCIITASILLWY	81–97	NS4B	23	PGLQAKATREAQKR	122–136
NS4A	16	CIITASILLWYAQIQPH	87–103	NS4B	24	AAAGIMKNPTVDGITVI	136–153
NS4A	17	ILLWYAQIQPHWIAASI	93–109	NS4B	25	KNPTVDGITVIDLDPI	142–158
NS4A	18	AQIQPHWIAASIILEFF	98–114	NS4B	26	DGITVIDLDPIPYDPKF	147–164
NS4A	19	WIAASIILEFFLIVLLI	104–120	NS4B	27	DLDPIPYDPKFEKQLGQ	153–170
NS4A	20	ILEFFLIVLLIPEPEKQ	110–126	NS4B	28	YDPKFEKQLGQVMLLVL	159–176
NS4A	21	IVLLIPEPEKQRTPQDN	116–132	NS4B	29	KQLGQVMLLVLCVTQVL	165–182
NS4A/2k	22	PEPEKQR*TPQDNQLTYV*	121–137	NS4B	30	MLLVLCVTQVLMMRTTW	171–188
NS4A/2k	23	R*TPQDNQLTYVVIAILT*	127–143	NS4B	31	VTQVLMMRTTWALCEAL	177–194
2k	24	*NQLTYVVIAILTVVAAT*	132–148	NS4B	32	MRTTWALCEALTLATG	183–199
2k/NS4B	25	*VIAILTVVAATMA*NEMG	138–150	NS4B	33	ALCEALTLATGPISTLW	188–205
2k/NS4B	1	VVAATMANEMGFLEKTK	−7–10	NS4B	34	TLATGPISTLWEGNPGR	194–211
2k/NS4B	2	ANEMGFLEKTKKDLGLG	−1–16	NS4B	35	ISTLWEGNPGRFWNTTI	200–217
NS4B	3	LEKTKKDLGLGSITTQQ	5–22	NS4B	36	GNPGRFWNTTIAVSMAN	206–223
NS4B	4	DLGLGSITTQQPESNIL	11–28	NS4B	37	WNTTIAVSMANIFRGSY	212–229
NS4B	5	ITTQQPESNILDIDLR	17–33	NS4B	38	VSMANIFRGSYLAGAGL	218–235
NS4B	6	PESNILDIDLRPASAWT	22–39	NS4B	39	FRGSYLAGAGLLFSIMK	224–241
NS4B	7	DIDLRPASAWTLYAVAT	28–45	NS4B	40	AGAGLLFSIMKNTTNTR	230–247
NS4B	8	ASAWTLYAVATTFVTPM	34–51	NS4B	41	FSIMKNTTNTRR	236–248