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

Henrique Nemésio, Francis Palomares-Jerez, José Villalaín *

Instituto de Biología Molecular y Celular, Universidad "Miguel Hernández", E-03202 Elche-Alicante, Spain

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

* Corresponding author. Tel.: +34 966 658 762; fax: +34 966 658 758.
E-mail address: jvillalain@umh.es (J. Villalaín).

0005-2736/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
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

TPE/BPS/EPA/ESM/Chol at a molar ratio of 59:0.37:7.7:18:3.1:1.2:3.4:7.8 [33,34]. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Molecular Probes (Eugene, OR). 5-Carboxyfluorescein (CF, >95% by HPLC), Triton X-100, EDTA and HEPES were purchased from Sigma-Aldrich (Madrid, ES). All other chemicals were commercial samples of the highest purity available (Sigma-Aldrich, Madrid, ES). Water was deionized, twice-distilled and passed through a Milli-Q equipment (Millipore Ibérica, Madrid, ES) to a resistivity higher than 18 MΩ cm.

2.2. Vesicle preparation

Aliquots containing the appropriate amount of lipid in chloroform-methanol (2:1 vol/vol) were placed in a test tube, the solvents were removed by evaporation under a stream of O_2-free nitrogen, and finally, traces of solvents were eliminated under vacuum in the dark for >3 h. The lipid films were resuspended in an appropriate buffer and incubated either at 25 °C or 10 °C above the phase transition temperature (T_m) with intermittent vortexing for 30 min to hydrate the samples and obtain multilamellar vesicles (MLV). The samples were frozen and thawed five times to ensure complete homogenization and maximization of peptide/lipid contacts with occasional vortexing. Large unilamellar vesicles (LUV) with a mean diameter of 0.1 μm were prepared from MLV by the extrusion method [35] using polycarbonate filters with a pore size of 0.1 μm (Nuclepore Corp., Cambridge, CA, USA). Breakdown of the vesicle membrane leads to contents leakage, i.e., CF fluorescence. Non-encapsulated CF was separated from the vesicle suspension through a Sephadex G-75 filtration column (Pharmacia, Uppsala, SW, EU) eluted with buffer containing either 10 mM Tris, 100 mM NaCl, 0.1 mM EDTA, pH 7.4. Phospholipid and peptide concentration were measured by methods described previously [36,37].

2.3. Membrane leakage measurement

Leakage of intraliposomal CF was assayed by treating the probe-loaded liposomes (final lipid concentration, 0.125 mM) with the appropriate amounts of peptides on microtiter plates stabilized at 25 °C using a microplate reader (FLUOstar, BMG Labtech, GER, EU), each well containing a final volume of 170 μl. The medium in the microtiter plates was continuously stirred to allow the rapid mixing of peptide and vesicles. Leakage was measured at an approximate peptide-to-lipid molar ratio of 1:25. Changes in fluorescence intensity were recorded with excitation and emission wavelengths set at 492 and 517 nm, respectively. One hundred percent release was achieved by adding Triton X-100 to a final concentration of 0.5% (w/w) to the microtiter plates. Fluorescence measurements were made initially with probe-loaded liposomes, afterwards by adding peptide solution and finally adding Triton X-100 to obtain 100% leakage. Leakage was quantified on a percentage basis according to the equation, % Release = $[(F_f - F_0)/(F_{100} - F_0)] * 100$, F_f being the equilibrium value of fluorescence after peptide addition, F_0 the initial fluorescence of the vesicle suspension and F_{100} the fluorescence value after addition of Triton X-100. For details see refs. [38,39].

2.4. Differential scanning calorimetry

MLVs were formed as stated above in 20 mM HEPES, 100 mM NaCl, 0.1 mM EDTA, pH 7.4. The peptides were added to obtain a peptide/lipid molar ratio of 1:15 and incubated 10 °C above the T_m of DEPE for 30 min with occasional vortexing and then centrifuged. Samples containing 1.5 mg of total phospholipid were transferred to 50 μl DSC aluminum and hermetically sealed pans and subjected to DSC analysis in a differential scanning calorimeter Pyris 6 DSC (Perkin-Elmer Instruments, Shelton, U.S.A.) under a constant external pressure of 30 psi in order to avoid bubble formation. Thermograms were recorded at a constant rate of 4 °C/min. After data acquisition, the pans were opened and the phospholipid content was determined. To avoid artefacts due to the thermal history of the sample, the first scan was never considered; second and further scans were carried out until a reproducible and reversible pattern was obtained. Data acquisition was performed using the Pyris Software for Thermal Analysis, version 4.0 (Perkin-Elmer Instruments LLC) and Microcal Origin software (Microcal Software Inc., Northampton, MA, U.S.A.) was used for data analysis. The thermograms were defined by the onset and completion temperatures of the transition peaks obtained from heating scans. The phase transition temperature was defined as the temperature at the peak maximum.

2.5. Steady-state fluorescence anisotropy

MLVs were formed in a buffer composed of 100 mM NaCl, 0.1 mM EDTA, 20 mM HEPES at pH 7.4 (at 25 °C). Aliquots of DPH in N,N′-dimethylformamide (0.2 mM) were directly added to the lipid suspension to obtain a probe/lipid molar ratio of 1:500. DPH, a popular membrane fluorescent probe for monitoring the organization and dynamics of membranes, is known to partition mainly into the hydrophobic core of the membrane [40]. Samples were incubated for 60 min at 10 °C above the gel to liquid-crystalline phase transition temperature T_m of the phospholipid mixture. Afterwards, the peptides were added to obtain a peptide/lipid molar ratio of 1:15 and incubated 10 °C above the T_m of each lipid for 1 h, with occasional vortexing. All fluorescence studies were carried using 5 mm x 5 mm quartz cuvettes in a final volume of 400 μl (315 μM lipid concentration). The steady state fluorescence anisotropy was measured with an automated polarization accessory using a Varian Cary Eclipse fluorescence spectrometer, coupled to a Peltier for automatic temperature change. The vertically and horizontally polarized emission intensities, elicited by vertically polarized excitation, were corrected for background scattering by subtracting the corresponding polarized intensities of a phospholipid preparation lacking probes. The G-factor, accounting for differential polarization sensitivity, was determined by measuring the polarized components of the fluorescence of the probe with horizontally polarized excitation ($G = I_{HV}/I_{HH}$). Samples were excited at 360 nm and emission was recorded at 430 nm, with excitation and emission slits of 5 nm. Anisotropy values were calculated using the formula $\langle r \rangle = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively.

3. Results

In a similar way to other enveloped viruses, DENV virus modifies cell internal membranes to establish specific sites of replication described as the membranous web or replication complex (RC), fundamental for the viral life cycle [9,10]. The small, hydrophobic and poorly characterized DENV NS4A and NS4B proteins are responsible for the membrane rearrangements essential for viral replication and they are engaged in a plethora of other functions [9,18,20,21,25,26]. In order to detect surfaces along the NS4A and NS4B proteins which might be identified as membrane partitioning and/or membrane interacting zones, two-dimensional plots of hydrophobicity, obtained taking into consideration the arrangement of the amino acids in the space assuming they adopt an α-helical structure along the whole sequence [28,41], are shown in Fig. 1. It is readily evident the existence of different regions with large hydrophobic moment values along the NS4A and NS4B proteins. As it has been noted before, using these two-dimensional plots, it would be possible to distinguish two types of patches, those which do not comprise the perimeter of the helix and those which embrace the full perimeter [41]. The first type could favor the interaction with other similar patches along the same or other proteins as well as with the membrane surface. The second one would encompass patches containing more than 15 amino acids which could represent TM

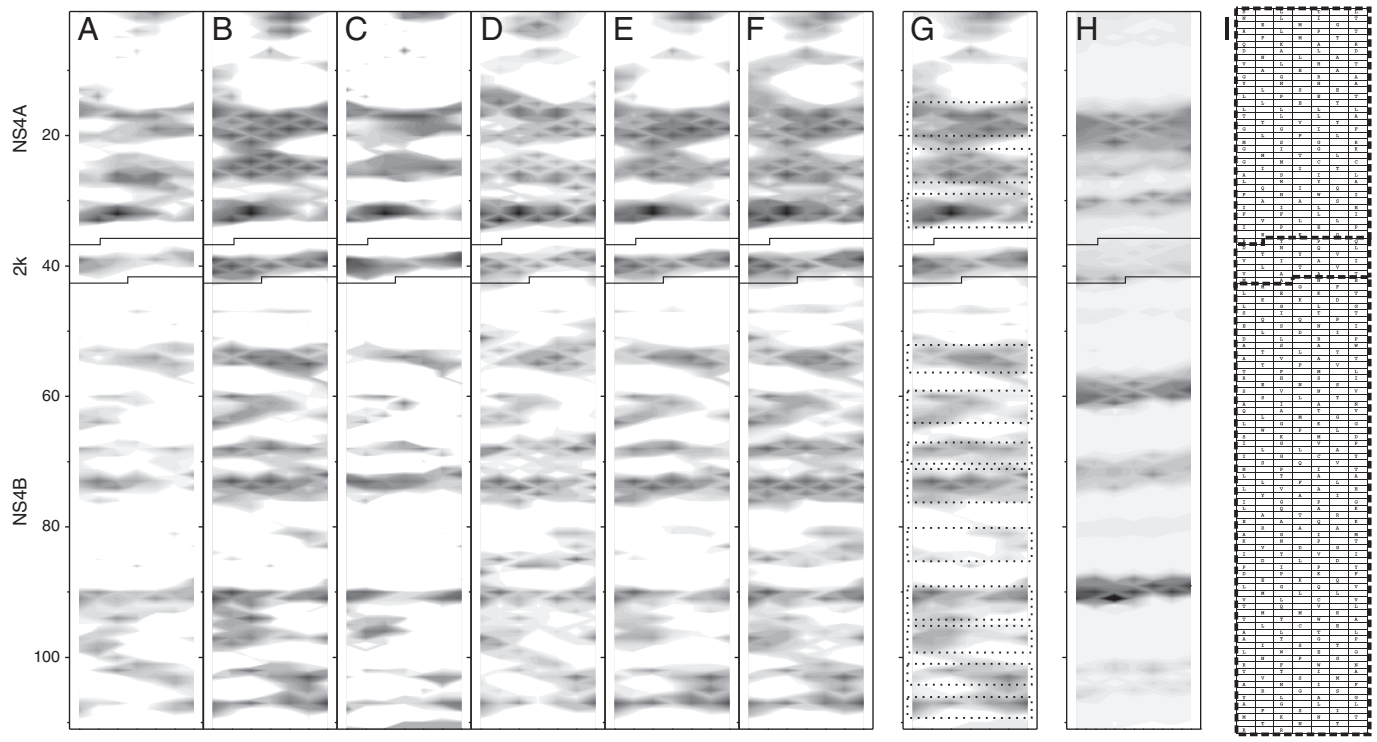


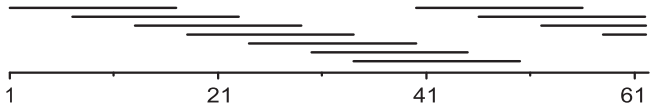
Fig. 1. Two-dimensional plots of the values of the water–membrane transfer free energy scales in kcal/mol for DENV NS4A, 2k and NS4B proteins according to (A) Wimley and White [50], (B) Engelman et al. [51], (C) Hessa et al. [52], (D) Moon and Fleming [53], (E) Meiler et al. [54] and (F) Eisenberg et al. [55]. Positive values represent positive bilayer-to-water transfer free energy values and therefore the higher (darker) the value, the greater the probability to interact with the membrane surface and/or hydrophobic core. The average of scales (A)–(F) is shown in (G), the average experimental leakage values are shown in (H) and the sequences of the NS4A, 2k and NS4B proteins in relation to the two-dimensional plots are shown in (I). The hydrophobic regions discussed in the text are boxed in (G).

domains and patches containing 15 or less amino acids which could represent membrane interacting domains [27,29,41,42].

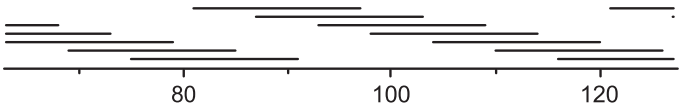
By observing the NS4A protein data, it is possible to detect three localized highly positive hydrophobic regions covering the full horizontal length of the plot. These regions would be comprised approximately from amino acid residues 51 to 72, residues 78 to 98 and residues 103 to 120 (Fig. 1). These results are in accordance with former data, since it was previously proposed the presence of highly hydrophobic patches from residues 50 to 73, residues 76 to 89, and residues 101 to 127 [9]. These patches contain about 22, 21 and 18 amino acids so that they could match (and represent) TM domains [27,29,41,42]. Two patches located along limited zones of the protein surface can be described from residues 2 to 11 and from 21 to 31 approximately. Whereas the previous three hydrophobic regions supposedly traverse the palisade structure of the membrane, these last two regions could show a tendency to interact with the membrane surface; however, it should not be ruled out that these areas could also be responsible for the interaction with other proteins [43]. The 2k fragment, which functions as a signal sequence [9], presents as expected a highly positive hydrophobic region (from residues 7 to 20, 2k numbering, Fig. 1). The two-dimensional plot corresponding to protein NS4B, shown in Fig. 1, displays different highly positive hydrophobic moment zones presenting diverse characteristics along the full sequence of the protein. Nine patches can be described for NS4B, i.e., from residues 35 to 52 (21 amino acids in length), from residues 60 to 78 (19 amino acids), from residues 89 to 100 (12 amino acids), from residues 103 to 122 (20 amino acids), from residues 137 to 155 (19 amino acids), from residues 168 to 188 (21 amino acids), from residues 190 to 205 (16 amino acids), from residues 212 to 225 (14 amino acids), and from residues 229 to 240 (12 amino acids). As it was commented above, there is not a clear separation between patches corresponding to either membrane interacting or TM domains. However, it is very clear from Fig. 1 that NS4B is a highly hydrophobic protein with the capacity of traversing the membrane several times [18]. It is interesting to note that this description of NS4B hydrophobic-rich surfaces fits very well with some of the previously described regions of the protein, highlighting the specific roles they might have for the proper biological functioning of the protein. In this way, the distribution of hydrophobicity and interfaciality, i.e., structure-related factors, along NS4A and NS4B proteins would affect their biological function.

The peptide libraries we have used in this study, composed by a total of 66 peptides, and their correlation with the NS4A, 2k, and NS4B protein sequences are shown in Fig. 2; it can be observed in the figure that the peptide libraries include the whole sequence of the proteins. Since two and three consecutive peptides in the library have an overlap of approximately 11 and 4 amino acids respectively, it seems reasonably to think on the combined effect of peptide groups or segments rather than on the effect of isolated peptides, so that leakage data elicited by each peptide would define protein segments or zones as commented below. It is also observed in Fig. 2 that the sequences of the four DENV strains are very well conserved, so that the information gathered studying DENV strain 2 should be essentially similar to the other DENV strains. We have studied the effect of these peptide libraries on membrane rupture by monitoring leakage from different liposome compositions and the results are presented in Figs. 3 and 4 [42]. We have tested different lipid compositions, from simple to complex (Fig. 3). The set of simple compositions contained EPC/Chol at a phospholipid molar ratio of 5:1 and EPC/SM/Chol at a phospholipid molar ratio of 5:2:1. Two complex lipid compositions were used, a lipid extract of liver membranes (42%, PC, 22% PE, 7% Chol, 8% PI, 1% LPC, and 21% neutral lipids as stated by the manufacturer) and a synthetic lipid mixture resembling the ER membrane (EPC/CL/BPI/TPE/BPS/EPA/ESM/Chol at a molar ratio of 59:0.37:7.7:18:3.1:1.2:3.4:7.8 [33,34]). It should be recalled that DENV virus is associated with membranes of the ER or an ER-derived modified compartment. In order to check the effect of each lipid in

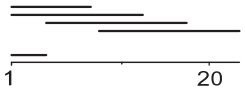
Strain		NS4A (residues 1-62)
Nauru/West Pac/1974	(DENV-1)	SVSGDLILEIGKLPQHLTQRAQNALDNLVMLHNSEQGGKAYRHAMEELPDTIETLMLLALIA
Thailand/NGS-C/1944	(DENV-2)	SLTLNLITEMGRLPTFMTQKARDALDNLAVLHTAEAGGRAYNHALSELPETLETLLLLTLLA
Philippines/H87/1956	(DENV-3)	SIALDLVTEIGRVPSHLAHRTRNALDNLVMLHTSEHGGRAYRHAVEELPETMETLLLLGLMI
Thailand/0476/1997	(DENV-4)	SITLDILTEIASLPTYLSSRAKLALDNIVMLHTTERGGRAYQHALNELPESLETLMLVALLG
Alignment (Clustalw)		*:: ::: *:. :* .:: ::: ****:.:**.:* **:**.**:.***:::***:*: *:



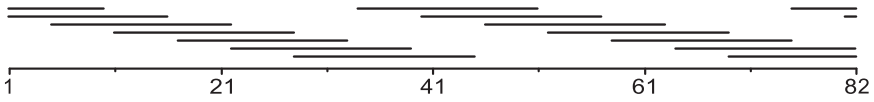
Strain		NS4A (residues 63 -127)
Nauru/West Pac/1974	(DENV-1)	VLTGGVTLFFLSGRGLGKTSIGLLCVIASSALLWMASVEPHWIAASIILEFFLMVLLIPEPDRQR
Thailand/NGS-C/1944	(DENV-2)	TVTGGIFLFLMSGRGIGKMTLGMCCIITASILLWYAQIQPHWIAASIILEFFLIVLLIPEPEKQR
Philippines/H87/1956	(DENV-3)	LLTGGAMLFLISGKGIGKTSIGLICVIASSGMLWMADVPLQWIASAIVLEFFMMVLLIPEPEKQR
Thailand/0476/1997	(DENV-4)	AMTAGIFLFFMQGKGIGKLSVGLIAIAVASGLLWVAEIQPQWIAASIILEFFLMVLLIPEPEKQR
Alignment (Clustalw)		:*.* **::.*:*:** ::*: .: .:* :** *.: :***::*:****::*******::**



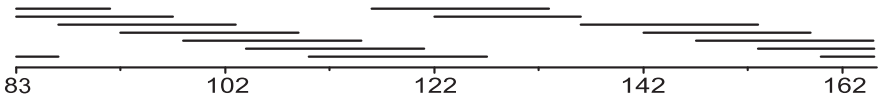
Strain		2k fragment (1-23)
Nauru/West Pac/1974	(DENV-1)	TPQDNQLAYVVIGLLFMILTAAA
Thailand/NGS-C/1944	(DENV-2)	TPQDNQLAYVVIGLLFMILTAAA
Philippines/H87/1956	(DENV-3)	TPQDNQLAYVVIGILTLAAIVAA
Thailand/0476/1997	(DENV-4)	TPQDNQLIYVILAILTIIGLVAA
Alignment (Clustalw)		******* **:::.:* : .**



Strain		NS4B (residues 1 – 82)
Nauru/West Pac/1974	(DENV-1)	NEMGLLETTKKDLGIGHAAAENHHHAAMLDVDLHPASAWTLYAVATTIITPMMRHTIENTTANISLTAIANQAAILMGLDKG
Thailand/NGS-C/1944	(DENV-2)	NEMGFLEKTKKDLGLG-SITTQQPESNILDIDLRPASAWTLYAVATTFVTPMLRHSIENSSVNVSLTAIANQATVLMGLGKG
Philippines/H87/1956	(DENV-3)	NEMGLLETTKRDLGMSKEPGVVSP-TSYLDVDLHPASAWTLYAVATTVITPMLRHTIENSTANVSLAAIANQAVVLMGLDKG
Thailand/0476/1997	(DENV-4)	NEMGLIEKTKADFGFY-QVKT---ETTILDVDLRPASAWTLYAVATTILTPMLRHTIENTSANLSLAAIANQAAVLMGLGKG
Alignment (Clustalw)		*: :**:******:******** ** *::.:*:.********************:************ .***:*: **.**



Strain		NS4B (residues 83 – 165)
Nauru/West Pac/1974	(DENV-1)	WPISKMDIGVPLLALGCYSQVNPLTLTAAVFMLVAHYAIIGPGLQAKATREAQKRTAAGIMKNPTVDGIVAIDLDPVVYDAKF
Thailand/NGS-C/1944	(DENV-2)	WPLSKMDIGVPLLAIGCYSQVNPITLTAALFLLVAHYAIIGPGLQAKATREAQKRAAAGIMKNPTVDGITVIDLDPIPYDPKF
Philippines/H87/1956	(DENV-3)	WPISKMDLGVPLLALGCYSQVNPLTLIAAVLLLVTHYAIIGPGLQAKATREAQKRTAAGIMKNPTVDGIMTIDLDPVIYDSKF
Thailand/0476/1997	(DENV-4)	WPLHRMDLGVPLLAMGCYSQVNPTTLTASLVMLLVHYAIIGPGLQAKATREAQKRTAAGIMKNPTVDGITVIDLEPISYDPKF
Alignment (Clustalw)		**: :**:******:******** ** *::.:*:.********************:************ .***:*: **.**



Strain		NS4B (residues 166 – 249)
Nauru/West Pac/1974	(DENV-1)	EKQLGQIMLLILCTSQILLMRTTWALCESITLATGPLTTLWEGSPGKFWNTTIAVSMANIFRGSYLAGAGLAFSLMKSLGGGRR
Thailand/NGS-C/1944	(DENV-2)	EKQLGQVMLLVLCVTQVLMMRTTWALCEALTLATGPISTLWEGNPGRFWNTTIAVSMANIFRGSYLAGAGLLFSIMKNTTNTRR
Philippines/H87/1956	(DENV-3)	EKQLGQVMLLVLCAVQLLLMRTSWALCEVLTLATGPITTLWEGSPGKFWNTTIAVSMANIFRGSYLAGAGLALSIMKSVGTGKR
Thailand/0476/1997	(DENV-4)	EKQLGQVMLLVLCAGQLLLMRTTWAFCEVLTLATGPVLTLWEGNPGRFWNTTIAVSTANIFRGSYLAGAGLAFSLIKNAQTPRR
Alignment (Clustalw)		******:***:**. *:*:***:**:** :******: *****.**:********* ********** :*::*. :*

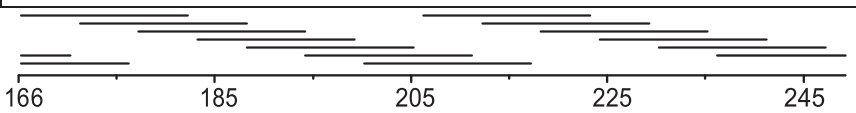


Fig. 2. Sequences of the NS4A (first and second tables from the top), 2k (third table from the top) and NS4B (fourth, fifth and sixth tables from the top) proteins for the four different dengue virus serotypes according to literature. The sequences were split for better visualization. For each protein, a global alignment was computed using Clustalw. Below each table there is a graphic showing the relative location of each peptide in the peptide library. Peptide line length is related to the number of amino acids in the peptide. Maximum overlap between adjacent peptides is 11 amino acids. It should be noted that the 2k fragment is composed of peptides included in the NS4A peptide library.

this complex composition we have designed an ER synthetic membrane composed of EPC/CL/BPI/TPE/BPS/EPA/ESM/CHOL at a molar ratio of 58:6:6:6.6:6:6:6 ($ER^{58:6}$) and tested this mixture as well as seven different lipid mixtures lacking one and only one of the lipids in the mixture (except EPC) (Fig. 4). These lipid compositions could be very useful to study the effect of each lipid component on the interaction of each peptide of the peptide library with the membrane.

The leakage data corresponding to the NS4A protein derived peptide library (Fig. 3) show that some peptides exerted a significant leakage effect. A quick bird's eye view of the leakage data shows the presence

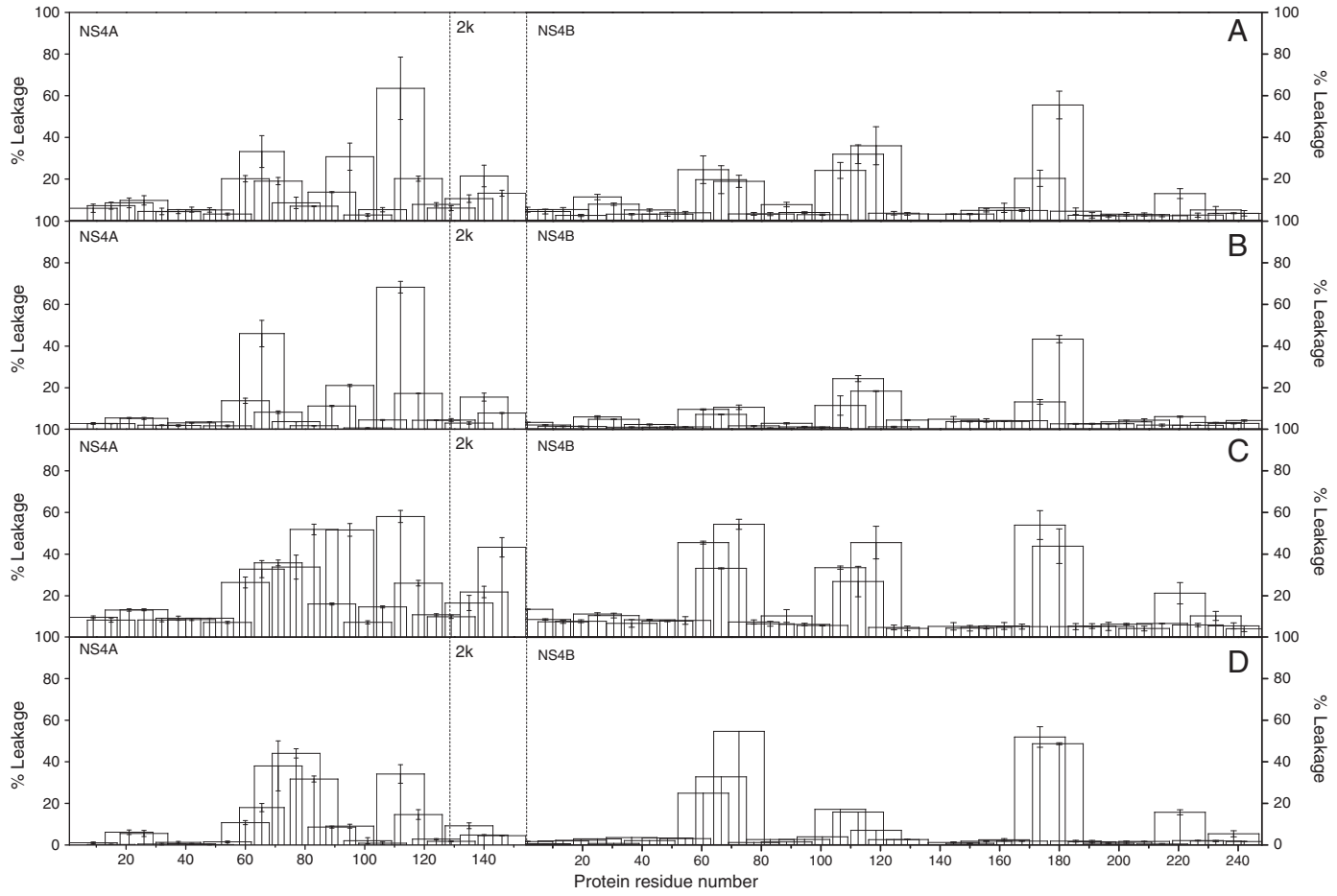


Fig. 3. Effect of the peptide library derived from the NS4A, 2k, and NS4B proteins on the release of LUV contents for different lipid compositions. Leakage data (membrane rupture) for LUVs composed of (A) EPC/Chol at a molar proportion of 5:1, (B) EPC/ESM/Chol at a molar proportion of 5:2:1, (C) lipid extract of liver membranes, and (D) ER complex synthetic lipid mixture (EPC/CL/BPI/TPE/BPS/EPA/ESM/Chol at a molar proportion of 59:0.37:7.7:18:3.1:1.2:3.4:7.8). Vertical bars indicate standard deviations of the mean of quadruplicate samples.

of two broad segments from residues 52 to 90 and residues 90 to 125, approximately. Although there were some differences depending on liposome composition, they were not significant to infer any specific relationship between lipid composition and leakage. Interestingly, leakage values were significant, since for segment 52–90 it oscillated between 30 and 50% whereas for segment 90–125 it fluctuated between 40 and 60%. For liposomes containing the $ER^{58:6}$ complex mixture and its variations (Fig. 4), results were similar; however, although the segment comprised by residues 55 to 90 elicited significant leakage values, the segment comprising residues 90 to 127 induced smaller leakage values. The average leakage of all liposome compositions tested for the NS4A protein, presented in Fig. 1H as a two-dimensional plot, shows the relationship between membrane leakage and hydrophobicity. As displayed in the figure, these two leakage zones, i.e., 52–90 and 90–125, fit perfectly well with the hydrophobic regions detected in the two dimensional plots, i.e., 51–72, 78–98 and 103–120 (Fig. 1A–F).

The leakage data corresponding to the NS4B protein derived peptide library (Fig. 3) defined in this case four broad segments, i.e., from residues 50 to 80, from residues 94 to 127, from residues 163 to 190 and from residues 210 to 240, approximately. Similarly to NS4A, differences on leakage were not significant to infer any specific relationship between leakage and lipid composition. There were also variations in the extent of leakage for the different segments, since leakage values between 20–50%, 10–40%, 40–50% and 30–50% approximately were observed for those four segments commented above. When liposomes containing the $ER^{58:6}$ complex mixture and its variations were tested, similar results were obtained (Fig. 4). The same four segments commented above, i.e., segments comprising residues 50 to 80, 94 to 127, 163 to 190 and 210 to 240, produced a significant leakage effect, i.e., 20–30%, 10–15%, 30–40% and 10–15% for all membranes tested (Fig. 4). The total average leakage, presented in Fig. 1H, shows the relationship between membrane leakage and hydrophobicity for the NS4B protein. In this representation the four leakage regions are well defined. As observed in the figure, leakage zones from residues 50 to 80, from residues 94 to 127, from residues 163 to 190 and from residues 210 to 240, fit with the hydrophobic regions detected in the two dimensional plots, i.e., from residues 60 to 78, from residues 89 to 100 and 103 to 122, from residues 168 to 188 and from residues 212 to 225 and 229 to 240 (Fig. 1A–F). The first leakage zone comprises the proposed hydrophobic segment from residues 56 to 93, whereas the other three leakage zones comprise the proposed TM segments from residues 93 to 146, from residues 146 to 190 and from residues 190 to 248. In summary, peptides from both NS4A and NS4B, capable of inducing membrane leakage, did not have any specific interaction with any specific lipid, as they elicited membrane rupture on all types of membrane model systems independently of phospholipid head group, charge and structure. The coincidental results obtained through both the theoretical and experimental data, would point out that these sequences should be important regions of this protein and would be engaged in membrane interaction.

Membrane lipids undergo a cooperative melting reaction, linked to the loss of conformational order of the lipid chains and influenced by

many types of molecules including peptides and proteins. To examine the interaction of the NS4A and NS4B peptide libraries on the phase transitions of DMPC and DEPE as a function of temperature, we have used the steady-state fluorescence anisotropy of the fluorescent probe DPH incorporated into model membranes composed of DMPC (Figs. 5 and 6 for the NS4A and NS4B peptide libraries, respectively) and differential scanning calorimetry (DSC) for model membranes composed of DEPE (Fig. 7). When DMPC was studied in the presence of each one of the peptides corresponding to the NS4A derived peptide library, some of them elicited a significant effect both on the $P_{\beta'}$–L_α transition temperature and the anisotropy (Fig. 5). Peptides 1, 2, 12 and 14 significantly changed the T_m of DMPC whereas peptides 10, 13-20, 23 and 25 increased the anisotropy above but not below the T_m of the phospholipid. When the peptides corresponding to the NS4B derived peptide library were studied, some of them elicited a significant effect both on the transition temperature and on the anisotropy (Fig. 6). Peptides 1, 8–11, 13–16, 18–21 and 41 significantly changed the T_m of DMPC whereas peptides 1, 7, 9–11, 13–23, 28–32, and 34–35 changed the anisotropy either above, below or both the T_m of the phospholipid. Therefore, some peptides pertaining to the NS4A and NS4B derived libraries were capable of affecting the thermal transition T_m of DMPC, hence the conclusion that their effect should be primarily due to their location at the lipid–water interface influencing the fluidity of the phospholipids [44].

Aqueous dispersions of pure DEPE undergo a gel to liquid-crystalline phase transition (L_β–L_α) T_m in the lamellar phase at about 38 °C and in addition a lamellar liquid-crystalline to hexagonal-H_{II} (L_α–H_{II}) phase transition at about 63 °C [45]. As observed in Fig. 7, both gel to liquid-crystalline and lamellar liquid-crystalline to hexagonal-H_{II} transitions were present in all samples, independently of the peptide tested. No peptide induced any significant change on the main transition but some of them did change both the transition temperature and the enthalpy of the lamellar liquid-crystalline to hexagonal-H_{II} transition of DEPE, since this transition is much more sensitive than the lamellar one to molecular interactions [45]. When the peptides corresponding to the NS4A derived peptide library were mixed with DEPE, peptide 18 was the only one that elicited a significant effect, since the L_α–H_{II} transition decreased about 5 °C and the L_β–L_α transition about 0.2 °C (Fig. 7). When the NS4B derived peptides were assayed, the ones which elicited some effect on the L_α–H_{II} phase transition were peptides 5, 18, 26, 29, 35 and 41 (Fig. 7). However, the differences were not significant ($\pm$0.2 °C for the L_β–L_α transition and $\pm$1°C for the L_α–H_{II} transition). The coincidence of these relatively high-effect peptides with high leakage zones demonstrates their specific interaction with the membrane as well as their modulatory effect.

4. Discussion

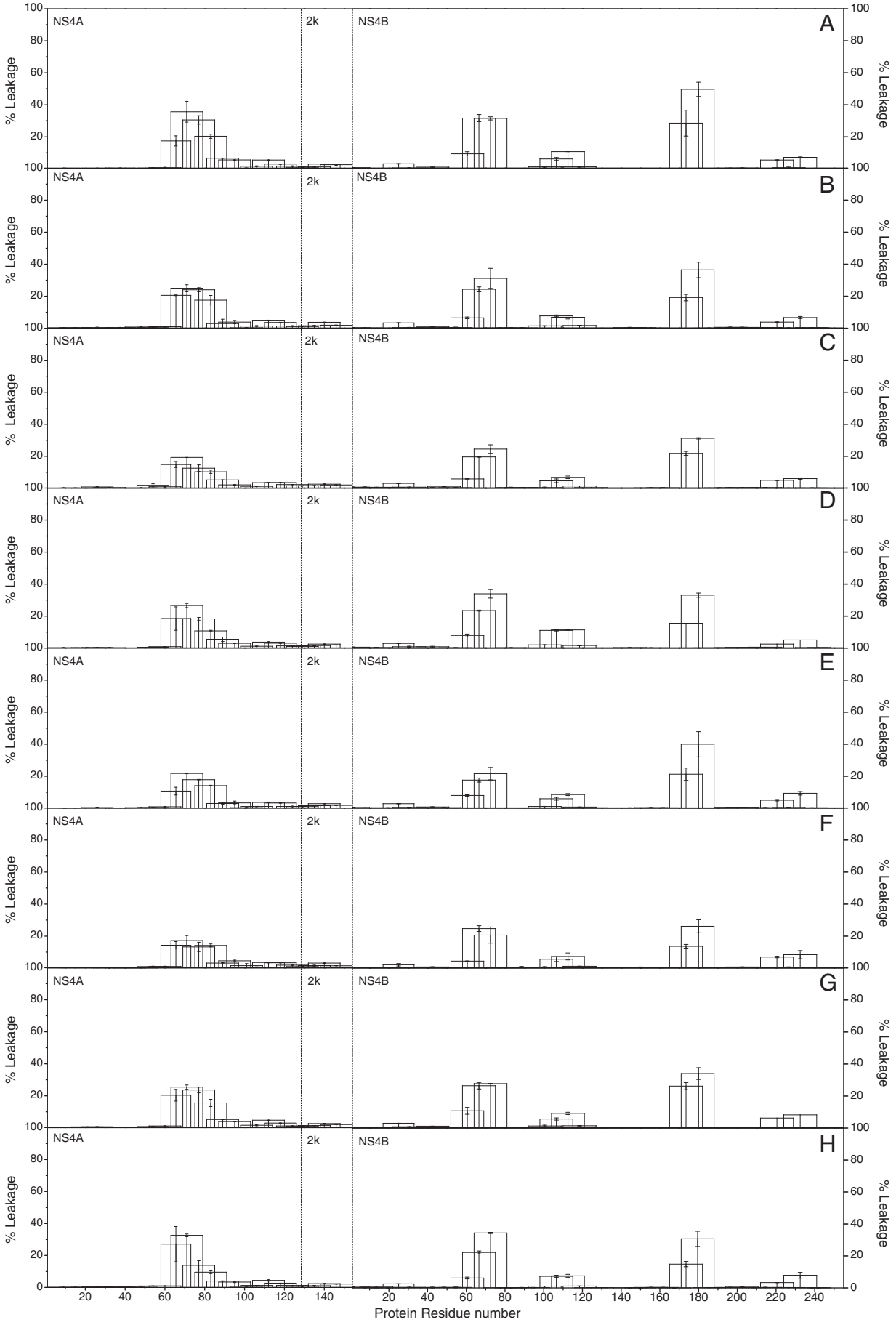
The virus family Flaviviridae includes Dengue virus (DENV) as well as other viruses such as Japanese encephalitis, Yellow fever, West Nile and tick-borne encephalitis viruses. Similarly to other enveloped viruses, they enter the cell through receptor mediated endocytosis and rearrange internal cell membranes to form the RC, an essential step for viral replication [5–11]. DENV NS4A and NS4B proteins are responsible of the membrane rearrangements which lead to the formation of the RC but its high hydrophobicity precludes the gathering of useful information in a straightforward manner. There are still many questions to be answered regarding the effect both NS4A and NS4B have on membranes. Adding to that and considering the stated previously on this text, they are highly attractive targets for anti-DENV therapy. Therefore, we have carried an exhaustive analysis of the different regions of DENV NS4A and NS4B proteins which might interact with phospholipid membranes using a similar approach to that used before [31,32] and have identified different membranotropic regions on these proteins with the capacity to interact and disrupt membranes.

For NS4A, in accordance with previously obtained data [9], three localized highly positive hydrophobic regions covering the full horizontal length of the two-dimensional plot were observed, segments 51–72, 78–98 and 103–120. These three segments would have therefore the possibility to transverse the membrane as a TM segment. Additionally, in the N-terminal region of the protein we localized two patches along limited zones of the protein surface, i.e., which are characterized by having one polar and one hydrophobic side, segments 2–11 and 21–31, which would represent membrane- and/or protein-interacting zones [43]. In the case of protein NS4B, a total of nine hydrophobic zones were observed, i.e., 35–52, 60–78, 89–100, 103–122, 137–55, 168–188, 190–205, 212–225, and 229–240. Of these, zone 35–52 has 21 amino acids in length, zone 60–78 19 amino acids, zone 103–122 20 amino acids, zone 137–155 19 amino acids and zone 168–188 21 amino acids, so that these five zones could be candidates to transverse the membrane, i.e., constitute TM domains. The other shorter zones could represent membrane- and/or protein-interacting zones. From this picture, it is clear that the distribution of hydrophobicity and interfaciality along the surface of these proteins determine its biological function and membrane interaction.

In this work, we have made an exhaustive study of the effect on membrane integrity of DENV NS4A and NS4B peptide libraries by monitoring leakage from simple and complex liposome compositions. We are aware that a) the use of peptides might not mimic the properties of the intact protein and b) it is not obvious that peptide–membrane interaction is directly related to membrane rupture [31,41,46]. However, two and three consecutive peptides in the library have an overlap of approximately 11 and 4 amino acids respectively, so it seems reasonably to think on the combined effect of peptide groups, i.e., segments, rather than on the effect of isolated peptides. These segments would define therefore membrane interacting domains.

For NS4A, the three hydrophobic regions detected in the two dimensional plots, i.e., 51–72, 78–98 and 103–120, coincided pretty well with the observed leakage zones, i.e., 52–90 and 90–125, though no specificity on liposome composition was observed. Interestingly, peptide no. 18 pertaining to the NS4A peptide library and comprising amino acids 98 to 114 affected significantly the polymorphic phase of DEPE, so that the NS4A region this peptide belongs to could be engaged in changing the membrane phase of lipids, which would lead to the formation of the RC complex by rearrangement of membranes. The highly hydrophobic character of NS4A is remarkable, implying that this protein should be the most important one to be engaged in membrane interaction and structure modulation. The NS4A topology model described previously by Bartensschlager et al. suggests the existence of two transmembrane domains and a tightly membrane associated one [9], coincidental with the three hydrophobic regions commented above. It is also known that NS4A, in concert with other viral and host proteins, promotes significant intracellular membrane changes essential for viral replication [9,20,21]. It would be possible that the tightly membrane associated domain described by Bartensschlager et al. could behave similarly to other comparable domains of other non-structural viral proteins, such as the AH2 domain of NS4B protein from hepatitis C virus [47,48]. These domains would have the potential to traverse the phospholipid bilayer as a transmembrane segment, would be engaged in oligomerization, and have the capability of modifying the membrane phase. For NS4B, leakage data defined four segments, 50–80, 94–127, 163–190 and 210–240, which coincided with four hydrophobic regions detected

Fig. 4. Effect of the peptide library derived from the NS4A, 2k, and NS4B proteins on the release of LUV contents for different lipid compositions. Leakage data (membrane rupture) for LUVs composed of (A) ER59:6 complex lipid mixture (EPC/CL/BPI/TPE/BPS/EPA/ESM/CHOL at a molar proportion of 59:6:6:6.6:6:6:6), (B) ER59:6 minus BPI, (C) ER59:6 minus CHOL, (D) ER59:6 minus EPA, (E) ER59:6 minus ESM, (F) ER59:6 minus TPE, (G) ER59:6 minus BPS and (H) ER59:6 minus CL. Vertical bars indicate standard deviations of the mean of quadruplicate samples.


NS4A
2k
NS4B
A
B
C
D
E
F
G
H
% Leakage
Protein Residue number

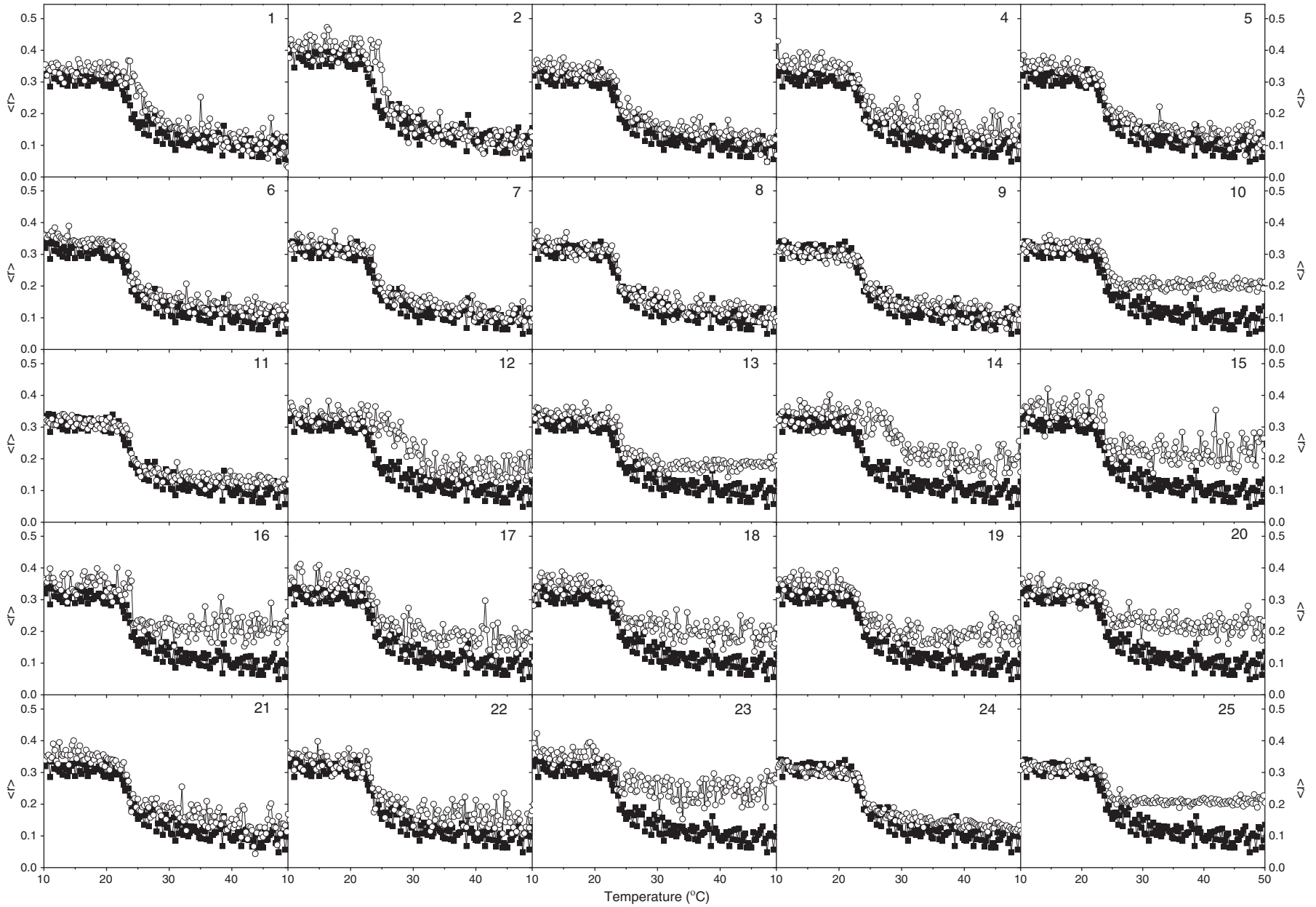


Fig. 5. Steady-state anisotropy, <r>, of DPH incorporated into DMPC model membranes as a function of temperature in the presence of the peptide library corresponding to NS4A/2k. Each peptide is identified by its corresponding number. Data correspond to vesicles containing pure phospholipids (●) and phospholipids plus peptides (○).The peptide to phospholipid molar ratio was 1:15.

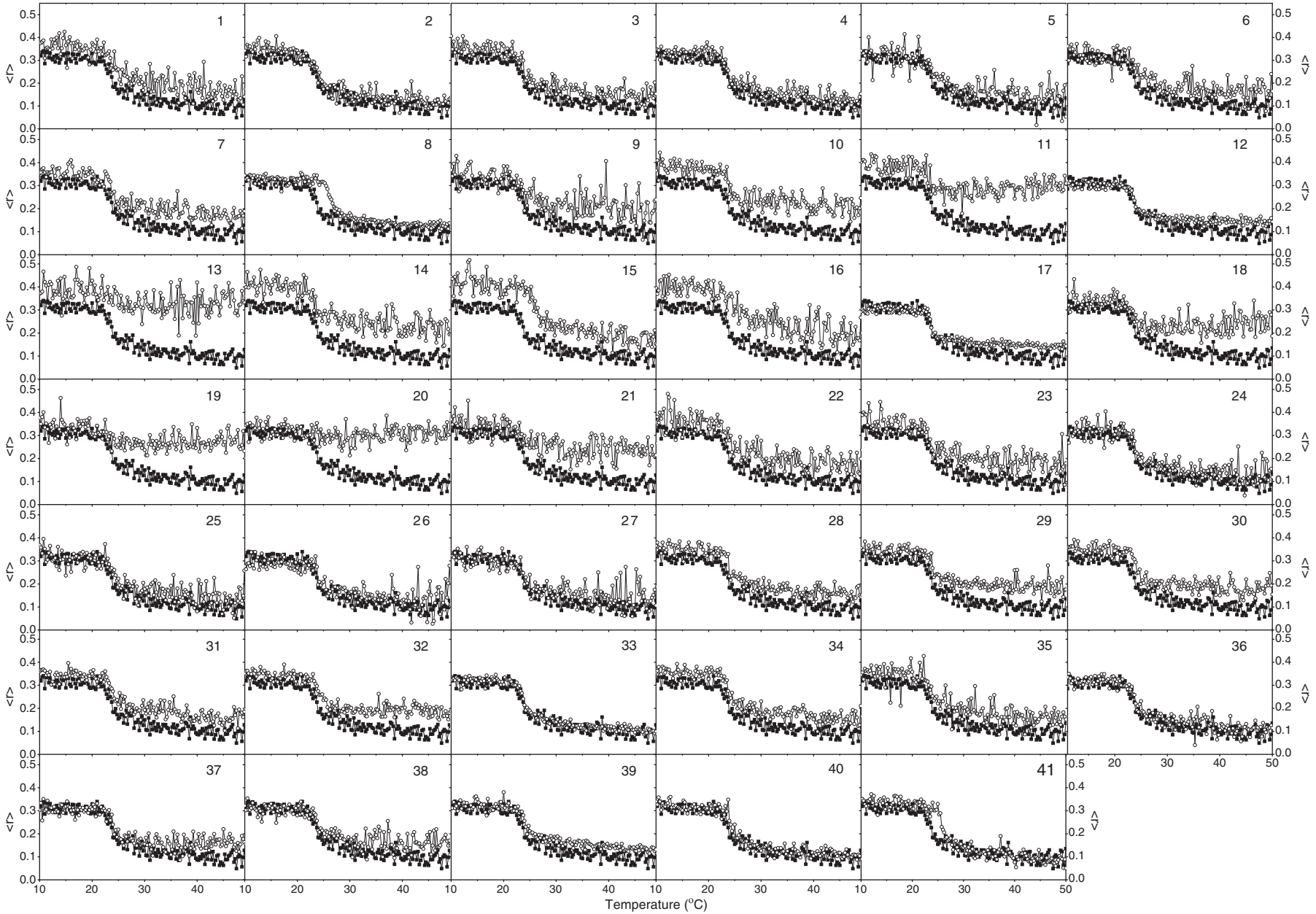


Fig. 6. Steady-state anisotropy, <r>, of DPH incorporated into DMPC model membranes as a function of temperature in the presence of the peptide library corresponding to NS4B. Each peptide is identified by its corresponding number. Data correspond to vesicles containing pure phospholipids (●) and phospholipids plus peptides (○).The peptide to phospholipid molar ratio was 1:15.

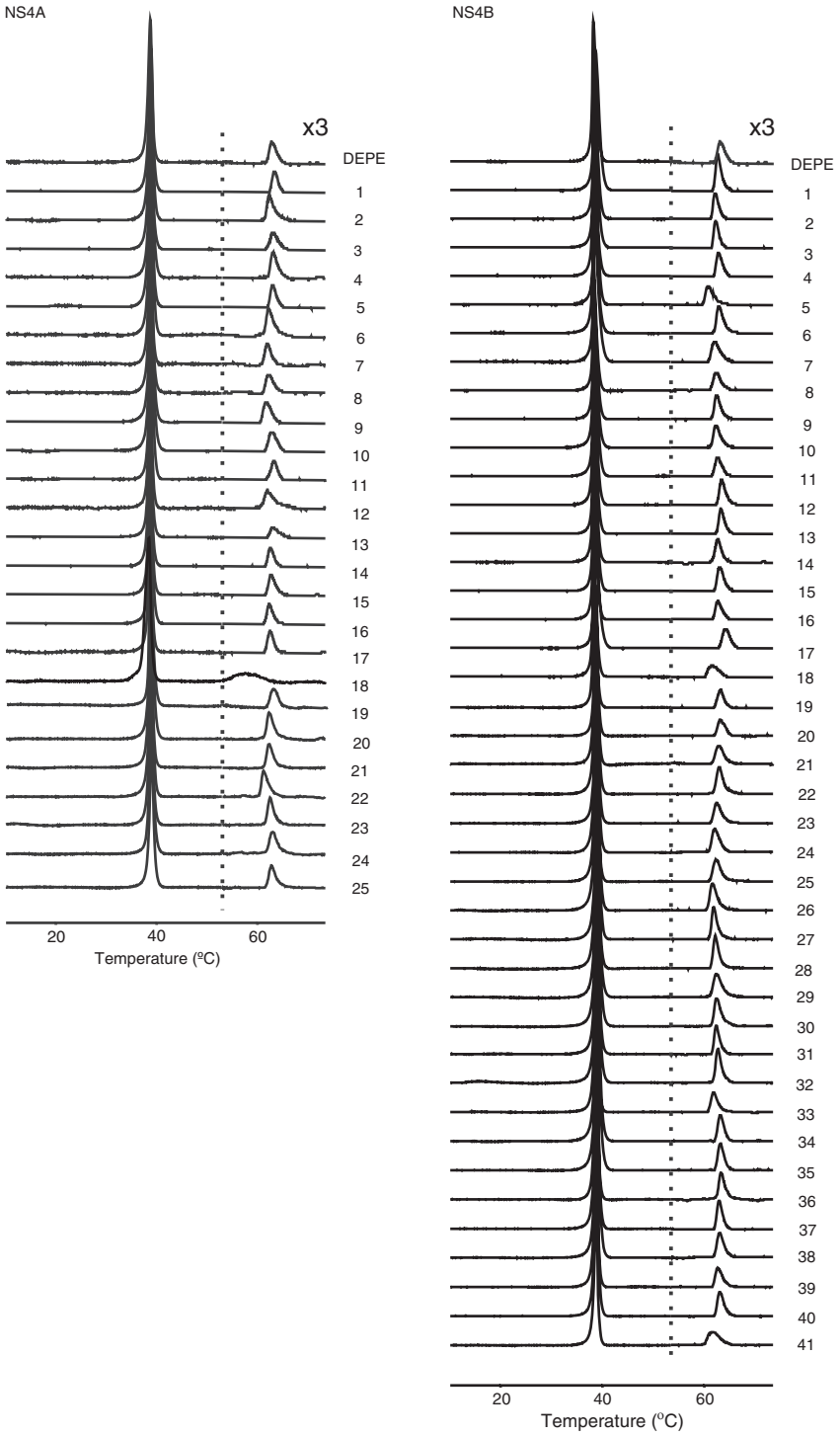


Fig. 7. Differential scanning calorimetry heating-scan thermograms of DEPE in the presence of peptides from NS4A (left) and NS4B (right) DENV protein libraries at a phospholipid/peptide molar ratio of 15:1. The thermogram of pure DEPE is shown on top of each column for comparison. Numbers represent the peptide number in the library. All the thermograms were normalized to the same amount of lipid.

in the two dimensional plots. Again, no specificity on liposome composition was observed. These data would imply that these regions could represent membrane-traversing regions, whereas the other NS4B detected regions could represent membrane surface interacting regions. The identified membrane-traversing regions would include the membrane spanning domains, essential for NS4B function [18] and the membrane interacting regions would include a region needed for IFN antagonism, essential for the viral life cycle [24]. These characteristics would imply that NS4B should be engaged in both membrane arrangement and protein/protein and/or lipid/protein interactions. These proteins would have the ability to fluctuate between different conformational states and that would be one of the main differences with fusion proteins; for example, DENV E protein, a class II fusion protein, possesses several membrane interacting domains apart from two proposed transmembrane ones [32]. DENV E protein would have the capability to change from a pre-fusion metastable conformation to a fusion stable one, but not to fluctuate between them [49].

The existence of a large number of hydrophobic and interfacial regions in both DENV NS4A and NS4B proteins would suggest that they could oscillate between metastable and stable conformations defining therefore the mechanism of formation of the replication complex. As it has been commented above and similarly to other enveloped viruses, DENV rearranges cell internal membranes to establish specific sites of replication, critical for the virus life cycle [9–11]. DENV NS4A and NS4B proteins are the main proteins, if solely, engaged in this task. Their function, and therefore their properties, should be similar to other non–structural proteins from other enveloped viruses engaged in similar functions, such as NS4B from hepatitis C virus [41,42]. These proteins might be multifunctional but at least they should have the capacity of disrupting the bilayer structure by modulating the biophysical properties of the membrane phospholipids as it has been shown here. This perturbation should be strong enough to make possible the special rearrangement of the host membranes that gives place to the formation of the replication complex. The required structure inter-conversions might be part of the structural transitions that transform them from the inactive to the active state; they are probably driven by the interaction of different membranotropic segments, such as those described in this work. The inhibition of membrane interaction by direct action on both proteins would be an additional approach to fight against DENV infection. An understanding of the structural features of these processes, directly related to membrane interaction, is essential because they are attractive drug targets. In summary, membrane inducing leakage peptides from both NS4A and NS4B did not have any specific interaction with any specific lipid, and peptides eliciting leakage rupture all types of membrane model systems independently of phospholipid head group, charge and structure. The coincidental results obtained through both the theoretical and experimental data, would point out that these sequences should be important regions of this protein and would be engaged in membrane interaction.

Acknowledgements

This work was partially supported by grant BFU2008-02617-BMC (Ministerio de Ciencia y Tecnología, Spain) to J.V. We are especially grateful to BEI Resources, National Institute of Allergy and Infectious Diseases, Manassas, VA, USA, for the peptides used in this work. H.N. is supported by a “Santiago Grisolía” fellowship from Generalitat Valenciana.

References

[1] M.G. Guzman, G. Kouri, Dengue: an update, Lancet Infect. Dis. 2 (2002) 33–42.
[2] S.B. Halstead, Dengue, Lancet 370 (2007) 1644–1652.
[3] S. Urcuqui-Inchima, C. Patino, S. Torres, A.L. Haenni, F.J. Diaz, Recent developments in understanding dengue virus replication, Adv. Virus Res. 77 (2010) 1–39.
[4] B. Pastorino, A. Nougairede, N. Wurtz, E. Gould, X. de Lamballerie, Role of host cell factors in flavivirus infection: implications for pathogenesis and development of antiviral drugs, Antiviral. Res. 87 (2010) 281–294.
[5] R. Perera, R.J. Kuhn, Structural proteomics of dengue virus, Curr. Opin. Microbiol. 11 (2008) 369–377.
[6] S. Bressanelli, K. Stiasny, S.L. Allison, E.A. Stura, S. Duquerroy, J. Lescar, F.X. Heinz, F.A. Rey, Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation, EMBO J. 23 (2004) 728–738.
[7] M. Kielian, F.A. Rey, Virus membrane-fusion proteins: more than one way to make a hairpin, Nat. Rev. Microbiol. 4 (2006) 67–76.
[8] S. Mukhopadhyay, R.J. Kuhn, M.G. Rossmann, A structural perspective of the flavivirus life cycle, Nat. Rev. Microbiol. 3 (2005) 13–22.
[9] S. Miller, S. Kastner, J. Krijnse-Locker, S. Buhler, R. Bartenschlager, The non-structural protein 4A of dengue virus is an integral membrane protein inducing membrane alterations in a 2K-regulated manner, J. Biol. Chem. 282 (2007) 8873–8882.
[10] S. Miller, J. Krijnse-Locker, Modification of intracellular membrane structures for virus replication, Nat. Rev. Microbiol. 6 (2008) 363–374.
[11] S. Welsch, S. Miller, I. Romero-Brey, A. Merz, C.K. Bleck, P. Walther, S.D. Fuller, C. Antony, J. Krijnse-Locker, R. Bartenschlager, Composition and three-dimensional architecture of the dengue virus replication and assembly sites, Cell Host Microbe 5 (2009) 365–375.
[12] M.M. Samsa, J.A. Mondotte, N.G. Iglesias, I. Assuncao-Miranda, G. Barbosa-Lima, A.T. Da Poian, P.T. Bozza, A.V. Gamarnik, Dengue virus capsid protein usurps lipid droplets for viral particle formation, PLoS Pathog. 5 (2009) e1000632.
[13] J.M. Mackenzie, A.A. Khromykh, M.K. Jones, E.G. Westaway, Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A, Virology 245 (1998) 203–215.
[14] B.D. Lindenbach, C.M. Rice, Trans-complementation of yellow fever virus NS1 reveals a role in early RNA replication, J. Virol. 71 (1997) 9608–9617.
[15] B.D. Lindenbach, C.M. Rice, Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function, J. Virol. 73 (1999) 4611–4621.
[16] A.E. Gorbalenya, A.P. Donchenko, E.V. Koonin, V.M. Blinov, N-terminal domains of putative helicases of flavi- and pestiviruses may be serine proteases, Nucleic Acids Res. 17 (1989) 3889–3897.
[17] E.V. Koonin, Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and lambda 2 protein of reovirus, J. Gen. Virol. 74 (Pt 4) (1993) 733–740.
[18] S. Miller, S. Sparacio, R. Bartenschlager, Subcellular localization and membrane topology of the dengue virus type 2 non-structural protein 4B, J. Biol. Chem. 281 (2006) 8854–8863.
[19] S.A. Shiryaev, A.V. Chernov, A.E. Aleshin, T.N. Shiryaeva, A.Y. Strongin, NS4A regulates the ATPase activity of the NS3 helicase: a novel cofactor role of the non-structural protein NS4A from West Nile virus, J. Gen. Virol. 90 (2009) 2081–2085.
[20] J. Roosendaal, E.G. Westaway, A. Khromykh, J.M. Mackenzie, Regulated cleavages at the West Nile virus NS4A-2K-NS4B junctions play a major role in rearranging cytoplasmic membranes and Golgi trafficking of the NS4A protein, J. Virol. 80 (2006) 4623–4632.
[21] J.E. McLean, A. Wudzinska, E. Datan, D. Quaglino, Z. Zakeri, Flavivirus NS4A-induced autophagy protects cells against death and enhances virus replication, J. Biol. Chem. 286 (2011) 22147–22159.
[22] M.L. Ng, S.S. Hong, Flavivirus infection: essential ultrastructural changes and association of Kunjin virus NS3 protein with microtubules, Arch. Virol. 106 (1989) 103–120.
[23] E.G. Westaway, J.M. Mackenzie, M.T. Kenney, M.K. Jones, A.A. Khromykh, Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures, J. Virol. 71 (1997) 6650–6661.
[24] J.L. Munoz-Jordan, G.G. Sanchez-Burgos, M. Laurent-Rolle, A. Garcia-Sastre, Inhibition of interferon signaling by dengue virus, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 14333–14338.
[25] I. Umareddy, A. Chao, A. Sampath, F. Gu, S.G. Vasudevan, Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA, J. Gen. Virol. 87 (2006) 2605–2614.
[26] S. Tajima, T. Takasaki, I. Kurane, Restoration of replication-defective dengue type 1 virus bearing mutations in the N-terminal cytoplasmic portion of NS4A by additional mutations in NS4B, Arch. Virol. 156 (2011) 63–69.
[27] A.J. Perez-Berna, A.S. Veiga, M.A. Castanho, J. Villalain, Hepatitis C virus core protein binding to lipid membranes: the role of domains 1 and 2, J. Viral Hepat. 15 (2008) 346–356.
[28] J. Guillen, A.J. Perez-Berna, M.R. Moreno, J. Villalain, Identification of the membrane-active regions of the severe acute respiratory syndrome coronavirus spike membrane glycoprotein using a 16/18-mer peptide scan: implications for the viral fusion mechanism, J. Virol. 79 (2005) 1743–1752.
[29] A.J. Perez-Berna, M.R. Moreno, J. Guillen, A. Bernabeu, J. Villalain, The membrane-active regions of the hepatitis C virus E1 and E2 envelope glycoproteins, Biochemistry 45 (2006) 3755–3768.
[30] M.R. Moreno, M. Giudici, J. Villalain, The membranotropic regions of the endo and ecto domains of HIV gp41 envelope glycoprotein, Biochim. Biophys. Acta 1758 (2006) 111–123.
[31] A.J. Perez-Berna, J. Guillen, M.R. Moreno, A. Bernabeu, G. Pabst, P. Laggner, J. Villalain, Identification of the membrane-active regions of hepatitis C virus p7 protein: biophysical characterization of the loop region, J. Biol. Chem. 283 (2008) 8089–8101.
[32] H. Nemesio, F. Palomares-Jerez, J. Villalain, The membrane-active regions of the dengue virus proteins C and E, Biochim. Biophys. Acta 1808 (2011) 2390–2402.
[33] T.W. Keenan, D.J. Morre, Phospholipid class and fatty acid composition of Golgi apparatus isolated from rat liver and comparison with other cell fractions, Biochemistry 9 (1970) 19–25.
[34] A.G. Krainev, D.A. Ferrington, T.D. Williams, T.C. Squier, D.J. Bigelow, Adaptive changes in lipid composition of skeletal sarcoplasmic reticulum membranes associated with aging, Biochim. Biophys. Acta 1235 (1995) 406–418.
[35] L.D. Mayer, M.J. Hope, P.R. Cullis, Vesicles of variable sizes produced by a rapid extrusion procedure, Biochim. Biophys. Acta 858 (1986) 161–168.
[36] C.S.F. Böttcher, C.M. Van Gent, C. Fries, A rapid and sensitive sub-micro phosphorus determination, Anal. Chim. Acta 1061 (1961) 203–204.
[37] H. Edelhoch, Spectroscopic determination of tryptophan and tyrosine in proteins, Biochemistry 6 (1967) 1948–1954.
[38] A. Bernabeu, J. Guillen, A.J. Perez-Berna, M.R. Moreno, J. Villalain, Structure of the C-terminal domain of the pro-apoptotic protein Hrk and its interaction with model membranes, Biochim. Biophys. Acta 1768 (2007) 1659–1670.
[39] M.R. Moreno, J. Guillen, A.J. Perez-Berna, D. Amoros, A.I. Gomez, A. Bernabeu, J. Villalain, Characterization of the interaction of two peptides from the N terminus of the NHR domain of HIV-1 gp41 with phospholipid membranes, Biochemistry 46 (2007) 10572–10584.
[40] B.R. Lentz, Use of fluorescent probes to monitor molecular order and motions within liposome bilayers, Chem. Phys. Lipids 64 (1993) 99–116.

[41] J. Guillen, A. Gonzalez-Alvarez, J. Villalain, A membranotropic region in the C-terminal domain of hepatitis C virus protein NS4B Interaction with membranes, Biochim. Biophys. Acta 1798 (2010) 327–337.
[42] M.F. Palomares-Jerez, J. Villalain, Membrane interaction of segment H1 (NS4B(H1)) from hepatitis C virus non-structural protein 4B, Biochim. Biophys. Acta 1808 (2011) 1219–1229.
[43] R.A. Gadkari, N. Srinivasan, Prediction of protein–protein interactions in dengue virus coat proteins guided by low resolution cryoEM structures, BMC Struct. Biol. 10 (2010) 17.
[44] L.M. Contreras, F.J. Aranda, F. Gavilanes, J.M. Gonzalez-Ros, J. Villalain, Structure and interaction with membrane model systems of a peptide derived from the major epitope region of HIV protein gp41: implications on viral fusion mechanism, Biochemistry 40 (2001) 3196–3207.
[45] R.M. Epand, Lipid polymorphism and protein–lipid interactions, Biochim. Biophys. Acta 1376 (1998) 353–368.
[46] A.J. Perez-Berna, G. Pabst, P. Laggner, J. Villalain, Biophysical characterization of the fusogenic region of HCV envelope glycoprotein E1, Biochim. Biophys. Acta 1788 (2009) 2183–2193.
[47] J. Gouttenoire, V. Castet, R. Montserret, N. Arora, V. Raussens, J.M. Ruysschaert, E. Diesis, H.E. Blum, F. Penin, D. Moradpour, Identification of a novel determinant for membrane association in hepatitis C virus nonstructural protein 4B, J. Virol. 83 (2009) 6257–6268.
[48] J. Gouttenoire, P. Roingeard, F. Penin, D. Moradpour, Amphipathic alpha-helix AH2 is a major determinant for the oligomerization of hepatitis C virus nonstructural protein 4B, J. Virol. 84 (2010) 12529–12537.
[49] J.M. Smit, B. Moesker, I. Rodenhuis-Zybert, J. Wilschut, Flavivirus cell entry and membrane fusion, Viruses 3 (2011) 160–171.
[50] S.H. White, W.C. Wimley, Membrane protein folding and stability: physical principles, Annu. Rev. Biophys. Biomol. Struct. 28 (1999) 319–365.
[51] D.M. Engelman, T.A. Steitz, A. Goldman, Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins, Annu. Rev. Biophys. Biophys. Chem. 15 (1986) 321–353.
[52] T. Hessa, N.M. Meindl-Beinker, A. Bernsel, H. Kim, Y. Sato, M. Lerch-Bader, I. Nilsson, S.H. White, G. von Heijne, Molecular code for transmembrane-helix recognition by the Sec61 translocon, Nature 450 (2007) 1026–1030.
[53] C.P. Moon, K.G. Fleming, Side-chain hydrophobicity scale derived from transmembrane protein folding into lipid bilayers, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 10174–10177.
[54] J. Koehler, N. Woetzel, R. Staritzbichler, C.R. Sanders, J. Meiler, A unified hydrophobicity scale for multispan membrane proteins, Proteins 76 (2009) 13–29.
[55] D. Eisenberg, R.M. Weiss, T.C. Terwilliger, The helical hydrophobic moment: a measure of the amphiphilicity of a helix, Nature 299 (1982) 371–374.