

Hepatitis C virus core protein binding to lipid membranes: the role of domains 1 and 2

A. J. Pérez-Berná,¹ A. S. Veiga,² M. A. R. B. Castanho² and J. Villalaín¹ ¹Instituto de Biología Molecular y Celular, Universidad “Miguel Hernández”, Elche-Alicante, Spain; and ²Centro de Química e Bioquímica, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, Lisboa, Portugal

Received July 2007; accepted for publication October 2007

SUMMARY. We have analysed and identified different membrane-active regions of the Hepatitis C virus (HCV) core protein by observing the effect of 18-mer core-derived peptide libraries from two HCV strains on the integrity of different membrane model systems. In addition, we have studied the secondary structure of specific membrane-interacting peptides from the HCV core protein, both in aqueous solution and in the presence of model membrane systems. Our results show that the HCV core protein region comprising the C-terminus of domain 1 and the N-terminus of domain 2 seems to be the most active in membrane interaction, although a role in protein–protein interaction cannot

be excluded. Significantly, the secondary structure of nearly all the assayed peptides changes in the presence of model membranes. These sequences most probably play a relevant part in the biological action of HCV in lipid interaction. Furthermore, these membranotropic regions could be envisaged as new possible targets, as inhibition of its interaction with the membrane could potentially lead to new vaccine strategies.

Keywords: assembly, capsid, core protein, HCV, hepatitis, lipid.

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped positive single-stranded RNA virus that belongs to the genus *Hepacivirus* in the family *Flaviviridae*, and is the leading cause of acute and chronic liver disease in humans, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma [1–3]. There exists no vaccine to prevent HCV infection and current therapeutic agents have limited success against HCV [4]. The HCV genome consists of one translational open reading frame encoding a polyprotein precursor, including structural and non-structural proteins, that is cleaved by host and viral proteases (Fig. 1a). The structural proteins consist of the core protein, which forms the viral nucleocapsid, and the envelope glycoproteins E1 and E2, both of them transmem-

brane proteins. The HCV cell entry is achieved by the fusion of viral and cellular membranes, and the morphogenesis and virion budding has been suggested to take place in the endoplasmic reticulum [5]. HCV proteins are very sensitive to folding, assembly, mutations or deletions. Besides, the HCV genome is widely heterogeneous; the errors during its replication cause a high rate of mutations. Therefore, the region implicated in fusion and/or budding must interact with the membranes and should be a conservative sequence. Finding protein–membrane and protein–protein interaction inhibitors could be a good strategy against HCV infection as they might prove to be potential therapeutic agents.

The HCV core protein is highly basic and shows homology with the nucleocapsid protein of other flaviviruses. This protein is well conserved among the different HCV strains [6] and is important for HCV infection diagnosis by the detection of either specific anti-HCV core protein antibodies or circulatory viral antigens. The core protein has regulatory roles on cell functions like immune presentation, apoptosis, lipid metabolism and transcription [7–9]. Additionally, this protein has oncogenic potential playing an important role in the regulation of HCV-infected cell growth, transformation to a tumorigenic phenotype and development of hepatocellular carcinoma [10]. Recombinant cDNA expression studies of this protein have identified two major protein core species, p23 and p21 [8]. The later is the predominant species and is

Abbreviations: CF, 5-Carboxyfluorescein; Chol, cholesterol; EPC, egg 1-phosphatidylcholine; HCV, hepatitis C virus; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; N-RhB-PE, lissamineTM rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; SM, egg sphingomyelin; T_m, temperature of the gel–liquid crystalline phase transition.

Correspondence: Dr. José Villalaín, Instituto de Biología Molecular y Celular, Campus de Elche, Universidad “Miguel Hernández”, E-03202 Elche-Alicante (España-Spain). E-mail: jvillalain@umh.es

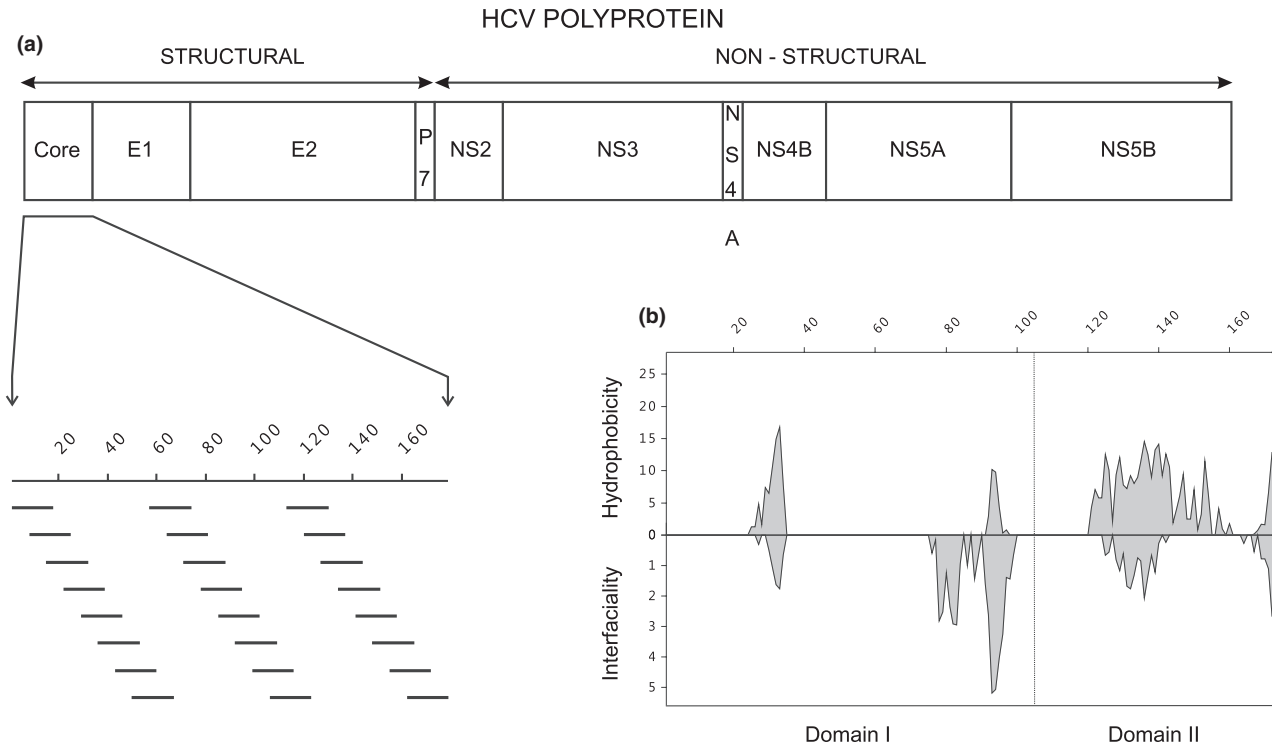


Fig. 1 (a) Scheme of the hepatitis C virus (HCV) structural and non-structural proteins according to literature consensus. The sequence and relative location of the twenty-seven 18-mer peptides derived from the HCV core protein are shown with respect to the sequence of the protein. Maximum overlap between adjacent peptides is 11 amino acids. (b) Analysis of the hydrophobicity and interfaciality distribution according to the scales of Wimley and White [22,23] using a window of 11 amino acids along the HCV core sequence without any assumption about secondary structure.

found in viral particles from infected sera, probably being the mature form of the protein. Although the three dimensional structure of this protein is unknown three distinct domains can be considered in the full-length immature protein [11,12], i.e. domain 1, comprising amino acids 1–117 and having several positive charges involved in RNA binding and including the immunodominant antigenic site, domain 2, comprising amino acids 117–171 and putatively responsible of its association with lipid droplets, and domain 3, comprising amino acids 171–191, a signal peptide upstream of the E1 glycoprotein. Significantly, the mature core protein is a dimeric helical protein exhibiting membrane protein features [11].

Recently, we have identified the membrane-active regions of the human immunodeficiency virus (HIV) gp41, severe acute respiratory syndrome coronavirus (SARS-CoV) spike and HCV E1 and E2 glycoproteins by observing the effect of glycoprotein-derived peptide libraries on model membrane integrity [13–15]. These results have permitted us to suggest the possible location of different segments in these proteins which might be implicated in protein–lipid and protein–protein interactions, helping us to understand the processes which give rise to the interaction between the protein and the membrane. Additionally, HCV membrane assembly and budding is an attractive target for anti-HCV therapy, as it

has been proposed that the core protein has an important role in viral assembly. To investigate the structural basis of the protein core interaction with the membrane and identify new targets for searching viral assembly inhibitors, we have analysed and identified different membrane-active regions of the HCV core protein by observing the effect of 18-mer core-derived peptide libraries from two HCV strains, namely, HCV 1AH77 and HCV 1B4J, on the integrity of different membrane model systems. Furthermore, we have studied the secondary structure of specific membrane-interacting peptides, both in aqueous solution and in the presence of model membrane systems, to understand the molecular mechanism of viral budding as well as making possible the future development of HCV assembly inhibitors which may lead to new vaccine strategies.

MATERIALS AND METHODS

Materials and reagents

Egg L-phosphatidylcholine (EPC), egg sphingomyelin (SM), liver lipid extract (a 2:1 chloroform:methanol extract of liver tissue) and cholesterol (Chol) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). 5-Carboxyfluorescein (CF) (>95% by high performance liquid chromatography), and

sodium dithionite were from Sigma-Aldrich (Madrid, Spain). Lissamine rhodamine B 1,2-dihexadecanoyl-snglycero-3-phosphoethanolamine (N-RhB-PE) and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD-PE) were obtained from Molecular Probes Inc. (Eugene, OR, USA). Three sets of 18-mer peptides derived from the core protein (Fig. 1a) of hepatitis C virus strains 1B4J and 1AH77 having 11-amino acid overlap between sequential peptides were obtained through the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MD, USA). All other reagents used were of analytical grade from Sigma-Aldrich. Water was deionized, twice-distilled and passed through a Milli-Q equipment (Millipore Ibérica, Madrid, Spain) to a resistivity better than 18 MΩ cm.

Sample preparation

For infrared spectroscopy, aliquots containing the appropriate amount of lipid in chloroform/methanol (2:1, v/v) were placed in a test tube containing 100 μg of dried lyophilized peptide to obtain a final lipid/peptide mole ratio of 50:1. After vortexing, the solvents were removed by evaporation under a stream of O₂-free nitrogen, and finally, traces of solvents were eliminated under vacuum in the dark for more than 3 h. The samples were hydrated in 100 μL of D₂O buffer containing 20 HEPES, 50 NaCl, 0.1 mM EDTA, pH 7.4 and incubated at 10 °C above the gel to liquid-crystalline phase transition temperature (T_m) of the phospholipid mixture with intermittent vortexing for 45 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. Finally the suspensions were centrifuged at 15 000 rpm at 25 °C for 15 min to remove the peptide possibly unbound to the membranes. The pellet was resuspended in 30 μL of D₂O buffer. The phospholipid and peptide concentrations were measured by methods described previously [16,17].

Membrane-leakage measurement

Aliquots containing the appropriate amount of lipid in chloroform/methanol (2:1 v/v) were placed in a test tube, the solvents were removed by evaporation under a stream of O₂-free nitrogen, and finally traces of solvents were eliminated under vacuum in the dark for more than 3 h. For assays of vesicle leakage at pH 7.4, buffer containing 10 Tris-HCl, 20 NaCl, 40 CF, and 0.1 mM EDTA, pH 7.4, was used [13]. To obtain MLV, 1 mL of buffer was added to the dry phospholipid mixture and vortexed at room temperature until a clear suspension was obtained. Large unilamellar vesicles (LUV) with a mean diameter of 90 nm were prepared from MLV by the extrusion method [14] using polycarbonate filters with a pore size of 0.1 μm (Nuclepore Corp.,

Cambridge, CA, USA). Breakdown of the vesicle membrane leads to content leakage. Non-encapsulated CF was separated from the vesicle suspension through a Sephadex G-75 filtration column (Pharmacia, Uppsala, Sweden) eluted with buffer containing either 10 Tris-HCl, 100 NaCl and 0.1 mM EDTA, pH 7.4. Membrane rupture (leakage) of intraliposomal CF was assayed by treating the probe-loaded liposomes (final lipid concentration, 0.125 mM) with the appropriate amounts of peptide on microtitre plates using a microplate reader (FLUOstar; BMG Labtech, Offenburg, Germany), stabilized at 25 °C with the appropriate amounts of peptide, each well containing a final volume of 170 μL. The medium in the microtitre plates was continuously stirred to allow the rapid mixing of peptide and vesicles. Leakage was assayed until no more change in fluorescence was obtained. Changes in fluorescence intensity were recorded with excitation and emission wavelengths set at 492 and 517 nm, respectively. Excitation and emission slits were set at 5 nm. One hundred per cent release was achieved by adding Triton X-100 to the microtiter plate to a final concentration of 0.5% (wt/wt). Fluorescence measurements were made initially with probe-loaded liposomes, afterwards by adding peptide solution and finally by adding Triton X-100 to obtain 100% leakage. Leakage was quantified on a percentage basis according to the equation,

$$\%L = \frac{(F_f - F_0) \times 100}{F_{100} - F_0}$$

where F_f is the equilibrium value of fluorescence after peptide addition, F_0 is the initial fluorescence of the vesicle suspension, and F_{100} is the fluorescence value after the addition of Triton X-100.

Phospholipid-mixing measurement

Peptide-induced vesicle lipid mixing (hemifusion) was measured by resonance energy transfer [18]. This assay is based on the decrease in resonance energy transfer between two probes (NBD-PE and RhB-PE) when the lipids of the probe-containing vesicles are allowed to mix with lipids from vesicles lacking the probes. The concentration of each of the fluorescent probes within the liposome membrane was 0.6 mol%. For assays of lipid mixing, 1 mL of buffer (10 HEPES, 100 mM NaCl, pH 7.4) was added to the dry phospholipid mixture (containing either 0.6 mol % NBD-PE and N-RhBPE or 0.12 mol% NBD-PE and N-RhB-PE, or no probes), and MLV were obtained by vortexing at room temperature. LUV were prepared from MLV by the extrusion method as above, using polycarbonate filters with a pore size of 0.2 μm (Nuclepore Corp). The use of 0.2 μm pore-size filters gives place to larger liposomes and henceforth greater fluorescence intensity per surface unit. Labelled and unlabelled vesicles in a proportion 1:4 were placed in a 5 mm × 5mm fluorescence cuvette at a final lipid

concentration of 100 μM in a final volume of 400 μL , stabilized at 25 °C under constant stirring. The fluorescence was measured using a Varian Cary Eclipse fluorescence spectrometer using 467 nm and 530 nm for excitation and emission, respectively. Excitation and emission slits were set at 10 nm. As labelled and unlabelled vesicles were mixed in a proportion of 1 to 4 respectively, 100% phospholipid mixing was estimated with a liposome preparation in which the membrane concentration of each probe was 0.12%. Phospholipid mixing was quantified on a percentage basis according to the equation,

$$\% \text{ PM} = \frac{(F_f - F_0) \times 100}{F_{100} - F_0},$$

F_f being the equilibrium value of fluorescence after peptide addition to a liposome mixture containing liposomes having 0.6% of each probe plus liposomes without any fluorescent probe, F_0 the initial fluorescence of the vesicles and F_{100} is the fluorescence value of the liposomes containing 0.12% of each probe.

Inner-monolayer phospholipid-mixing measurement

Peptide-induced phospholipid-mixing (fusion) of the inner monolayer was measured by a modification of the phospholipid-mixing measurement stated above [19]. LUVs were treated with sodium dithionite to completely reduce the NBD-labelled phospholipid located at the outer monolayer of the membrane. Final concentration of sodium dithionite was 100 mM (from a stock solution of 1 M dithionite in 1 M TRIS, pH 10.0) and incubated for approximately 1 h on ice in the dark. Sodium dithionite was then removed by size exclusion chromatography through a Sephadex G-75 filtration column (Pharmacia, Uppsala, Sweden) eluted with buffer containing 10 TRIS, 100 NaCl, 1 mM EDTA, pH 7.4. The proportion of labelled and unlabelled vesicles, lipid concentration and other experimental and measurement conditions were the same as indicated above for the phospholipid mixing assay.

Infrared spectroscopy

Approximately 30 μL of a pelleted sample in D_2O were placed between two CaF_2 windows separated by 56- μm thick Teflon spacers in a liquid demountable cell (Harrick, Ossining, NY, USA). The spectra were obtained in a Bruker IFS55 spectrometer (Bruker, Ettlingen, Germany) using a deuterated triglycine sulphate detector. Each spectrum was obtained by collecting 200 interferograms with a nominal resolution of 2 / cm, transformed using triangular apodization and, to average background spectra between sample spectra over the same time, a sample shuttle accessory was used to obtain sample and background spectra. The spectrometer was continuously purged with dry air at a dew point of -40 °C to remove atmospheric water vapour from the bands of interest.

All samples were equilibrated at the lowest temperature for 20 min before acquisition. An external bath circulator connected to the infrared spectrometer controlled the sample temperature. For temperature studies, samples were scanned using 20 °C intervals and a 15-min delay between each consecutive scan. Subtraction of buffer spectra taken at the same temperature as the samples was performed interactively using either GRAMS/32 or Spectra-Calc (Galactic Industries, Salem, MA, USA) as described previously [20,21]. Frequencies at the centre of gravity, when necessary were measured by taking the top 10 points of each specific band and fitted to a Gaussian band. The criterion used for buffer subtraction in the C=O and amide regions was the removal of the band near 1210 / cm, and a flat baseline between 1800 and 2100 / cm.

RESULTS

The peptide libraries used in this study and their correlation with the HCV core protein sequence are depicted in Fig. 1a, where it can be observed that the 18-mer peptide libraries include the whole HCV core protein sequence. Two and three consecutive peptides in the library have an overlap of 11 and 4 amino acids, respectively. The analysis of the hydrophobicity and interfaciality distribution along the core sequence of HCV 1B4J strain (the data obtained for the HCV 1AH77 strain is nearly identical) without any assumption about secondary structure is shown in Fig. 1b [13,22,23]. Although the three dimensional structure of the core protein is not known, these data give us a depiction of the potential surface zones that could be possibly implicated in either the modulation of membrane binding or protein-protein interaction or both. As it is observed in Fig. 1b, it is evident about the existence of different regions with large hydrophobic and interfacial values along the core sequence; these surfaces should be biologically functional in their roles as these patches of positive hydrophobicity and interfaciality along the surface of the core protein could favour the interaction with other similar patches along other core proteins, different proteins or with the surface of the membrane.

Membrane rupture, hemifusion and fusion

We have studied the effect of the 18-mer peptide libraries derived from the HCV core protein 1AH77 and 1B4J strains on membrane rupture, i.e. leakage, for different liposome compositions (Fig. 2); the lipidic composition of the model membranes have been EPC/Chol at a phospholipid molar ratio of 5:1, EPC/SM at a phospholipid molar ratio of 5:1, EPC/SM/Chol at a phospholipid molar ratio of 5:1:1, and a lipid extract of liver membranes [containing 42% EPC, 22% PE, 7% Chol, 8% phosphatidylinositol (PI), 1% lysophosphatidylcholine (LPC), and 21% neutral lipids]. The presence of both SM and Chol has been related to the occurrence of laterally segregated membrane microdomains or 'lipid rafts', and it has been found that there is

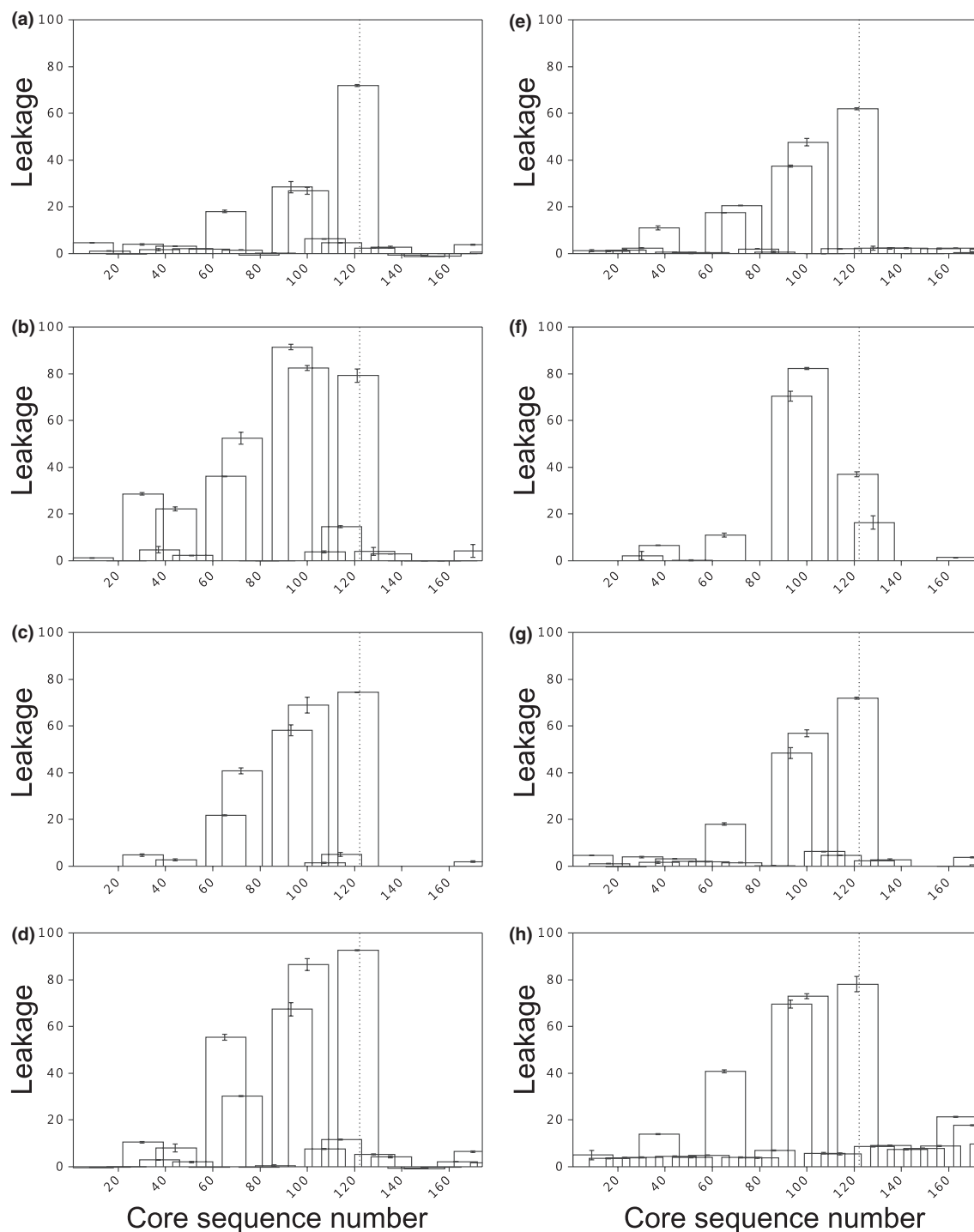


Fig. 2 Effect of the 18-mer peptides derived from the hepatitis C virus (HCV) core protein of HCV 1AH77 (a–d) and HCV 1B4J (e–h) strains on the release (membrane rupture) of large unilamellar vesicles (LUV) contents for different lipid compositions. Leakage data for LUVs composed of (a,e) egg L-phosphatidylcholine/cholesterol (EPC/Chol) at a phospholipid molar ratio of 5:1, (b,f) EPC/egg sphingomyelin (SM) at a phospholipid molar ratio of 5:1, (c,g) EPC/SM/Chol at a phospholipid molar ratio of 5:1:1, and (d,h) lipid extract of liver membranes. Vertical bars indicate SDs of the mean of triplicate samples. The dotted line divides regions corresponding to core domains I and II.

an important relationship between membrane interaction and Chol and SM membrane content for several viruses [24].

When the 18-mer peptides were assayed on EPC/Chol liposomes, some peptides exerted a significant leakage effect (Figs 2a,f). The most remarkable effects were induced

by peptide comprising residues 113–130. Other peptides which elicited important leakage values were peptides comprising residues 57–74, 85–102 and 92–109 for strain 1AH77 and 29–46, 57–74, 64–81, 85–102 and 92–109 for strain 1B4J. The leakage pattern was slightly different when liposomes composed of EPC/SM were tested (Figs 2b,f), as the most significant effect appeared now for peptides comprising residues 85–102 and 92–109. Peptide 113–130 also showed a significant leakage effect. Other significant leakage values were found for peptides 22–39, 36–53, 57–74 and 64–81 for strain 1AH77 and 64–81 and 120–137 for strain 1B4J. When liposomes composed of EPC/SM/Chol at a molar ratio of 5:1:1 were assayed (Figs 2c,g), a similar pattern to liposomes containing EPC/Chol was found. The most notable effect was induced by peptide comprising residues 113–130, whereas peptides 57–74, 64–81, 85–102 and 92–109 for strain 1AH77 and peptides 57–74, 85–102, and 92–109 for strain 1B4J elicited also significant leakage values. For liposomes composed of a lipid extract of liver membranes, the same peptide sequences displayed significant leakage values (Figs 2d,h). As it was found above, the most significant values were found for the peptide comprising residues 113–130, but peptides 85–102 and 92–109 showed also significant leakage. Peptides 57–74 and 64–81 for strain 1AH77 and peptide 57–74 for strain 1B4J displayed significant leakage values.

When the 18-mer core derived peptides from strain 1AH77 were assayed for hemifusion on both EPC/SM/Chol and liver extract liposomes, peptide comprising residues 29–46 displayed significant values (Figs 3a,b). Other peptides which elicited important hemifusion values (10%) defined a region comprising amino acids 85–130, with hemifusion values ranging from 10 to 25%. Slightly lower hemifusion values were found for core-derived peptides from strain 1B4J than for strain 1AH77 using both types of liposomes, EPC/SM/Chol and liver extract (Figs 3e,f). When the 18-mer core-derived peptides from strains 1AH77 and 1B4J were assayed for fusion on both EPC/SM/Chol and liver extract liposomes, peptide comprising residues 29–46 was the one which displayed significant fusion values (Figs 3c,d,g,h). The region comprising amino acids 85–130 (five peptides in total) was also found to have relatively high hemifusion and fusion values for both strains.

The summary of membrane leakage, membrane hemifusion and membrane fusion results obtained for both 1AH77 and 1B4J strains is presented in Fig. 4a, whereas the global average result is displayed in Fig. 4b. It is possible to detect various segments with large leakage values, which coincide with segments displaying significant hemifusion and fusion values. However, it is also possible to detect segments displaying high hemifusion and fusion values but low leakage ones. These results have permitted us to study specific peptides comprising different zones by infrared spectroscopy as shown below.

Fourier-transformed infrared spectroscopy structural assays

Peptides which displayed significant leakage, hemifusion and fusion effects (Fig. 4b), i.e. peptides corresponding to sequences 29–46, 57–74, 85–102, 106–123, and 155–172 were chosen for structural infrared assays both in aqueous solution [aqueous solutions of peptides 85–102 and 153–172 additionally contained 10% trifluoroethanol (TFE)] and in the presence of membrane model systems composed of either EPC/SM/Chol at a 5:1:1 molar ratio or a liver lipid extract (Fig. 5). Spectra were obtained at five different temperatures (5, 25, 45, 65 and 85 °C) and no any significant differences was found between them, indicating a high degree of conformational stability of the peptides. For simplicity, we will describe spectra obtained at 25 °C. The assignment of the Amide I' component bands to specific structural features has been described previously [25]. As observed in Fig. 5, there were significant changes in the Amide I' envelope band depending on the peptide and the specific lipid composition used.

The Amide I' band of the peptide derived from the core 29–46 region in aqueous solution (Fig. 5a) displays a narrow band at 1672 /cm and a shoulder with at least two broad bands at about 1645 and 1628 /cm. The band envelope of the Amide I' band of the peptide bound to raft model membranes was significantly different to that found for the peptide in aqueous solution, as maximum of the band appeared at about 1628 /cm with small bands at about 1672 and 1644 /cm (Fig. 5a). In the presence of liver lipid extract membranes, the Amide I' band was similar to the one found in the presence of raft-containing membranes, but a more intense band at about 1671 /cm. These differences in band intensities for the 29–46 peptide would imply that β -turn should be the most significant secondary structure in aqueous solution, whereas in the presence of both raft and liver extract model membranes the most significant one should be β -sheet.

The Amide I' band of the peptide derived from the core 57–74 sequence in aqueous solution displayed a broad band with a maximum at about 1672 /cm and a shoulder at about 1648 /cm (Fig. 5b). The band envelope of the Amide I' band of this peptide bound to raft model membranes was significantly different to that found for the peptide in aqueous solution, as the maximum of the band appeared at about 1637 /cm with a small shoulder at about 1648 /cm. In the presence of model membranes composed of liver lipid extract, the Amide I' band of the peptide displayed a maximum at about 1648 /cm. The intensity maxima for the core 57–74 sequence would imply that the most significant structure in aqueous solution and in the presence of liposomes composed of a liver extract should be random, but β -sheet in the presence of EPC/SM/Chol at a molar ratio of 2:1:1.

The peptides derived from the core 85–102 sequence of strains 1AH77 and 1B4J have similar sequences: the cysteine present in the 91 position of the strain 1AH77 is replaced by a

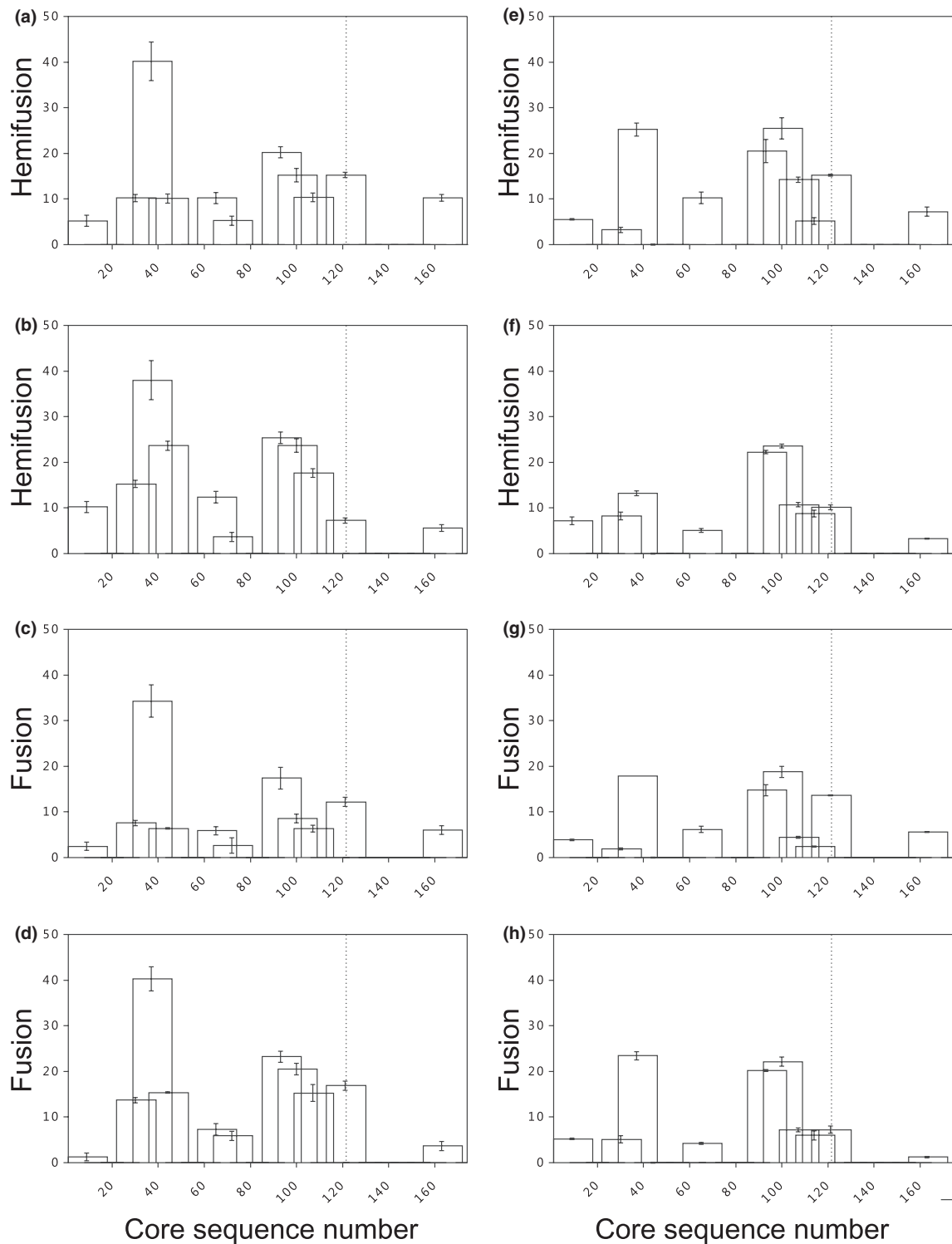


Fig. 3 Effect of the 18-mer peptides derived from the hepatitis C virus (HCV) core protein of HCV 1AH77 (a–d) and HCV 1B4J (e–h) strains on hemifusion (a,b,e,f) and fusion (c,d,g,h) for large unilamellar vesicles composed of (a,c,e,g) egg L-phosphatidylcholine/egg sphingomyelin/cholesterol at a phospholipid molar ratio of 5:1:1, and (b,d,f,h) lipid extract of liver membranes. Vertical bars indicate SDs of the mean of triplicate samples. The dotted line divides regions corresponding to core domains I and II.

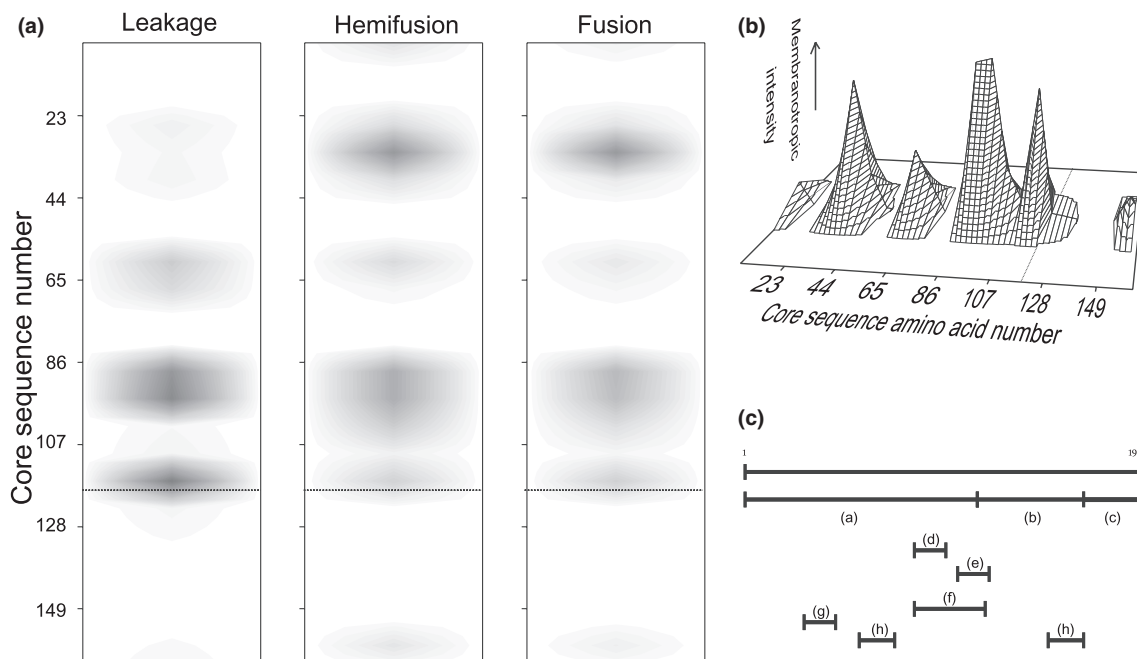


Fig. 4 (a) Summary of all experimental membrane leakage, hemifusion and fusion data presented in this work corresponding to the core-derived 18-mer peptides for both 1AH77 and 1B4J strains representing the full core sequence and for all liposome compositions studied (the darker, the greater membrane-active effect). (b) Summary of all the experimental data along the consensus scheme of the core protein highlighting the experimentally determined membrane-active regions. The dotted line divides regions corresponding to core domains I and II. (c) Pictorial survey of the most important membrane-active regions of hepatitis C virus (HCV) core protein. Membrane-active sequences found in this study (d,e,g,h) are depicted along sequences reported in the literature (a,b,c,f). (a) Domain 1: this sequence is regarded as the RNA-binding region with immunodominant antigenic properties [11,36]. (b) Domain 2: putatively responsible for the association with lipids [11,36]. (c) Domain 3: signal peptide [11,36]. (d) Membrane-leakage, hemifusion, and fusion activities with high interfacial values (see results section). (e) Membrane-leakage activity (see results section). (f) Homotypic interactions [27] and association to endoplasmic reticulum (ER) and mitochondrial membranes [35]. (g) Maximal hemifusion and fusion activities (see results section); part of the HCV major antigenic domain sequence; exposed region for protein-membrane interaction. (h) Both sequences have low but constant rupture, hemifusion and fusion properties (see results section).

leucine in the 1B4J strain. In aqueous solution, both peptides show a relatively broad Amide I' band with a maximum at approximately 1647 / cm and a shoulder at about 1672 / cm, much more intense for the peptide derived from the 1B4J strain than for the 1AH77 one (Figs 5c,d). In the presence of raft model membranes, the maximum of the Amide I' band in both cases is shifted to lower wavenumbers, displaying an Amide I' maxima at about 1638 / cm with a shoulder at about 1671 / cm. In the presence of liver lipid extract membranes, the Amide I' band of both peptides display a broad band with a maximum at about 1650 / cm. These differences in band intensities would imply that disordered structure should be the most significant secondary structure in aqueous solution for the 1AH77 derived peptide, but random and β -turn for the 1B4J peptide; however, in the presence of both raft and liver extract model membranes, the most significant one should be either disordered structure and/or β -sheet.

The Amide I' band of the peptide derived from the core 106–123 and 155–172 sequences in aqueous solution display a narrow band at about 1672 / cm and a shoulder at

about 1647 / cm (Figs 5e,f). The band envelope of the Amide I' band of both peptides bound to raft model membranes was significantly different to that found for the peptides in aqueous solution, as the maximum of the band appeared at about 1638 / cm with shoulders at about 1667 / cm. In the presence of model membranes composed of liver lipid extract, the Amide I' band of both peptides were rather similar to the ones found in the presence of raft membranes, but the band at 1665 / cm presented a higher intensity. These differences in band intensities for the core 106–123 and 155–172 sequences would imply that β -turn should be the most significant secondary structure in aqueous solution, together with random structure, whereas in the presence of both raft and liver extract model membranes, the most predominant secondary structure should be β -helix followed by β -turn.

DISCUSSION

Viral morphogenesis is one of the most important steps in the viral cycle involving lipid membranes and is not as well

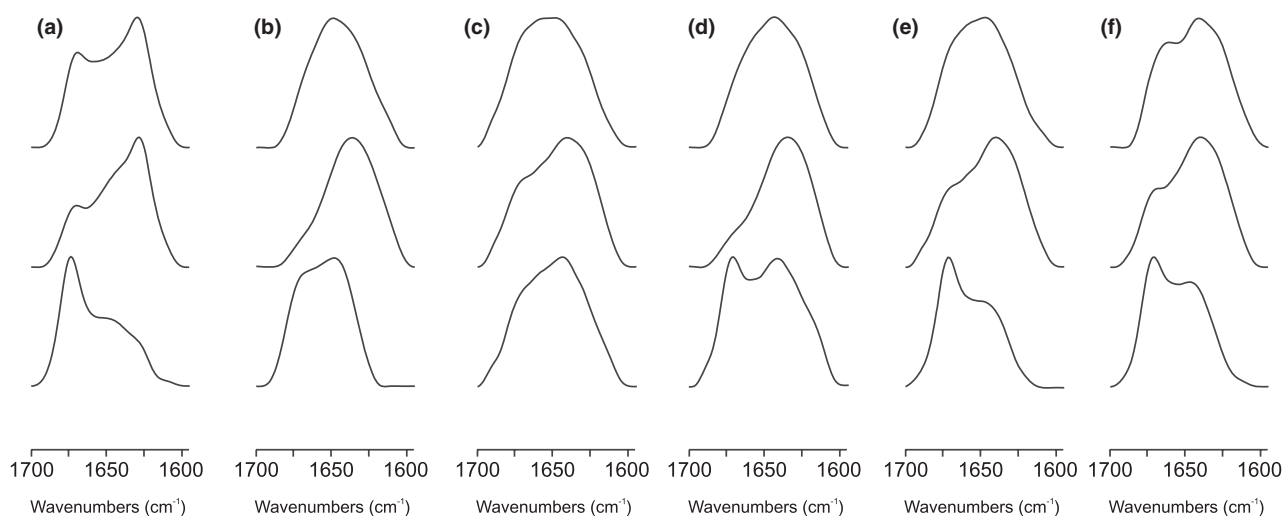


Fig. 5 Amide I' band spectra of peptides at 25 °C corresponding to core sequences (a) 29–46, (b) 57–74, (c) 85–102 (1AH77 strain), (d) 85–102 (1B4J strain), (e) 106–123 and (f) 155–172 in aqueous solution (lower spectra), in the presence of liposomes composed of egg L-phosphatidylcholine/egg sphingomyelin/ cholesterol at a 5:1:1 molar ratio (middle spectra) and in the presence of liposomes composed of a liver lipid extract (upper spectra).

characterized as the fusion event. For enveloped viruses, the assembly of the virions takes place in the host cell membrane and in the case of HCV it has been suggested that the virus particles assembly occurs in the endoplasmic reticulum membranes [26] where the core protein might play a central role in viral particle formation as well as it might drive the budding process [27]. Significantly, the HCV core protein is very well conserved among different HCV strains [6] and has important regulatory roles on different cell functions. Interestingly, the HCV core protein has been reported to be associated with raft sub-domains in the absence of other viral non-structural proteins [28]. Rafts, rigid membrane sub-domains located predominantly in the plasma membrane are described to be important in the HCV replication cycle, assembly of virions and budding from the host cell. For this reason, we have chosen membrane model systems composed not only of a liver lipid extract but also of EPC, SM and Chol [29,30], as mimetic of model membranes containing raft domains. As the biological roles of the HCV core protein can be modulated by membranes, we have identified the membrane-active regions of the HCV core protein by studying the effect of two core-derived peptide libraries from HCV on the integrity of membrane model systems. Furthermore, to obtain information on the structural basis of the interaction of the protein with model membranes, we have studied the secondary structure of specific membrane-interacting peptides both in aqueous solution and in the presence of model membrane systems. In this way, it would be possible to identify important regions which might be implicated in the budding molecular mechanism, and therefore could be used as new targets for searching viral assembly inhibitors as well as making possible the future development of HCV

assembly inhibitors which may lead to new vaccine strategies.

As it can be observed in Figs 4a,b, we have been able to discern different zones along the core sequence displaying different membranotropic properties. When leakage was assayed, and all the values were taken into account for all membrane compositions assayed, two zones displayed significant membrane rupture activity, i.e. regions defined by amino acids 85–102 and 106–123. The first region displayed a high interfacial value along its sequence; however, the second one did not show significant hydrophobic and interfacial values. In this context, it is interesting to note that the region comprising residues 85–123 has been described to be important in homotypic interactions which might be a prerequisite for assembly and budding of HCV-like viral particles [27,31]. Significantly, mutant core proteins without the 80–118 amino acid residues reduced, but did not abolish the association with lipid droplets [32,33]. Interestingly, the interaction of a region composed of amino acids 72–91 with the E1 glycoprotein [34] has been described; this region might be also important for association with the endoplasmic reticulum and the mitochondrial outer membranes [35]. Notably, region 85–102 coincides with a hydrophobic cluster located in domain 1 of the core protein and region 106–123 partially overlaps with another one located in domain 2 [36]. By infrared spectroscopy, it was possible to observe that the 85–102 region displayed different types of secondary structure, as random structure was the major one in aqueous solution but an increase in β -sheet in the presence of model membranes was observed. Although these regions present high levels of membrane rupture, hemifusion and fusion in the whole protein context, this region might be implicated in protein homodimerization but not in protein-membrane interactions.

Sequences 85–102 and 106–123, apart from being the ones which elicited the most leakage effect, displayed also significant hemifusion and fusion activities but region 29–46 displayed the highest hemifusion and fusion values together with low-leakage values (Fig. 4). As observed in Fig. 1b, this region displays high hydrophobicity and interfaciality values along its sequence. By infrared spectroscopy, it was determined that in aqueous solution, the predominant secondary structure was β -turn but β -sheet in the presence of model membranes composed of either raft or liver extract lipids. This region has two sequential glycines which might confer a high flexibility to the peptide and might act as a hinge around which the conformation could change. Moreover, this region is part of the HCV major antigenic domain sequence [37], being probably exposed and implicated in protein-membrane interactions. On the other hand, it has been demonstrated that uniformly packed virus particles are generated only by segment composed by amino acid residues 1–82, whereas region 29–46 is the only one in the N-terminal half of the core protein found to interact with lipids [38]. For this reason, this region might be an important one for the HCV assembly. Another interesting core sequences pertain to the 57–74 and 155–172 regions, as they displayed low but constant membrane rupture, hemifusion and fusion activities (Fig. 4a). It has been described that the ability of the core protein to associate with lipid droplets is lost when the 153–172 region containing the YATG motif is deleted [27]. These regions, having low but significant membrane-active activities, might be implicated in the interaction with membranes.

Additionally to its role in the viral morphogenesis and budding, the core protein is also important in the disassembly event during the viral uncoating that occurs at the target cell in a post-fusion stage. For this reason, this protein is very flexible [5]. The predicted structure of the whole protein using circular dichroism (CD) indicated that the protein has 16% of β -helix structure, 29% β -sheet structure and β -turns and 55% of random coil, whereas the C-terminal truncated protein was largely unordered [9]. As shown in this work, nearly all the peptides we have assayed display a major random coil or turn structure in solution; however, their structure changes in the presence of model membranes. This information could suggest that the membranotropic regions which we have detected along the core sequence might be involved in the interaction, not only with membranes, but also with proteins, even other core proteins and therefore responsible of their oligomerization.

Altogether, our results show that most of the HCV core protein membrane-active regions we have presented in this work are located in domain 1 (Fig. 4c), whatever interaction parameter is considered. However, the prevalent idea is that domain 1 might be the RNA-binding region whereas domain 2 of the HCV core protein would be responsible for membrane-binding (domain 3 is a signal peptide) [11,12].

Gathering related results in the literature, it is clear that a domain 1 vs domain 2 reasoning is not appropriate to unravel the foundations of HCV core-protein interaction with lipids. The region comprising the C-terminus of domain 1 and N-terminus of domain 2 seems to be the most active in membrane interaction, although a role in protein-protein interaction cannot be excluded. There is a sequence in domain 1 of maximal fusion and hemifusion (sequence f in Fig. 4c) that belongs to the major antigenic sequence of HCV. This sequence most probably plays a part in the biological action of HCV in lipid interaction whether during assembly, or fusion, or both. A more exhaustive study of these lipid-peptide interactions might help in the understanding of the HCV budding molecular mechanism as well as making possible the future development of anti-HCV drugs targeted to budding. These membranotropic regions could be envisaged as new possible targets, as inhibition of its interaction with the membrane could potentially lead to new vaccine strategies.

ACKNOWLEDGEMENTS

A. J. Pérez-Berná is a recipient of pre-doctoral fellowship from the Autonomous Government of the Valencian Community, Spain. A. S. Veiga acknowledges a grant (SFRH/BD/14336/2003) under the program POCTI to FCT-MCIES (Portugal). This study was funded in full by Ministerio de Ciencia y Tecnología, Spain, grant number BFU2005-00186-BMC (J. Villalaín). We are especially grateful to the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, for the peptides used in this work, as well as to Ana I. Gómez for expert technical assistance.

REFERENCES

- 1 Chen SL, Morgan TR. The natural history of hepatitis C virus (HCV) infection. *Int J Med Sci* 2006; 3(2): 47–52.
- 2 Penin F, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM. Structural biology of hepatitis C virus. *Hepatology* 2004; 39(1): 5–19.
- 3 Tan SL, Pause A, Shi Y, Sonenberg N. Hepatitis C therapeutics: current status and emerging strategies. *Nat Rev Drug Discov* 2002; 1(11): 867–881.
- 4 Qureshi SA. Hepatitis C virus-biology, host evasion strategies, and promising new therapies on the horizon. *Med Res Rev* 2007; 27(3): 353–373.
- 5 Vauloup-Fellous C, Pene V, Garaud-Aunis J *et al.* Signal peptide peptidase-catalyzed cleavage of hepatitis C virus core protein is dispensable for virus budding but destabilizes the viral capsid. *J Biol Chem* 2006; 281(38): 27679–27692.
- 6 Cha TA, Beall E, Irvine B *et al.* At least five related, but distinct, hepatitis C viral genotypes exist. *Proc Natl Acad Sci USA* 1992; 89(15): 7144–7148.
- 7 Lee SK, Park SO, Joe CO, Kim YS. Interaction of HCV core protein with 14-3-3epsilon protein releases Bax to activate

- apoptosis. *Biochem Biophys Res Commun* 2007; 352(3): 756–762.
- 8 Irshad M, Dhar I. Hepatitis C virus core protein: an update on its molecular biology, cellular functions and clinical implications. *Med Princ Pract* 2006; 15(6): 405–416.
 - 9 Kunkel M, Watowich SJ. Biophysical characterization of hepatitis C virus core protein: implications for interactions within the virus and host. *FEBS Lett* 2004; 3: 174–180.
 - 10 Kato N. Molecular virology of hepatitis C virus. *Acta Med Okayama* 2001; 55(3): 133–159.
 - 11 Boulant S, Vanbelle C, Ebel C, Penin F, Lavergne JP. Hepatitis C virus core protein is a dimeric alpha-helical protein exhibiting membrane protein features. *J Virol* 2005; 79(17): 11353–11365.
 - 12 McLauchlan J. Properties of the hepatitis C virus core protein: a structural protein that modulates cellular processes. *J Viral Hepat* 2000; 7(1): 2–14.
 - 13 Guillen J, Perez-Berna AJ, Moreno MR, Villalain J. 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* 2005; 79(3): 1743–1752.
 - 14 Perez-Berna AJ, Moreno MR, Guillen J, Bernabeu A, Villalain J. The membrane-active regions of the hepatitis C virus E1 and E2 envelope glycoproteins. *Biochemistry* 2006; 45(11): 3755–3768.
 - 15 Moreno MR, Giudici M, Villalain J. The membranotropic regions of the endo and ecto domains of HIV gp41 envelope glycoprotein. *Biochim Biophys Acta* 2006; 1758(1): 111–123.
 - 16 Böttcher CSF, Van Gent CM, Fries C. A rapid and sensitive sub-micro phosphorus determination. *Anal Chim Acta* 1961; 106(1): 203–204.
 - 17 Edelhoch H. Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry* 1967; 6(7): 1948–1954.
 - 18 Struck DK, Hoekstra D, Pagano RE. Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* 1981; 20(14): 4093–4099.
 - 19 Meers P, Ali S, Erukulla R, Janoff AS. Novel inner monolayer fusion assays reveal differential monolayer mixing associated with cation-dependent membrane fusion. *Biochim Biophys Acta* 2000; 1467(1): 227–243.
 - 20 Contreras LM, Aranda FJ, Gavilanes F, Gonzalez-Ros JM, Villalain J. 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* 2001; 40(10): 3196–3207.
 - 21 Pascual R, Moreno MR, Villalain J. A peptide pertaining to the loop segment of human immunodeficiency virus gp41 binds and interacts with model biomembranes: implications for the fusion mechanism. *J Virol* 2005; 79(8): 5142–5152.
 - 22 Wimley WC, White SH. Experimentally determined hydrophobicity scale for proteins at membrane interfaces. *Nat Struct Biol* 1996; 3(10): 842–848.
 - 23 White SH, Wimley WC. Membrane protein folding and stability: physical principles. *Annu Rev Biophys Biomol Struct* 1999; 28: 319–365.
 - 24 Ahn A, Gibbons DL, Kielian M. The fusion peptide of Semliki Forest virus associates with sterol-rich membrane domains. *J Virol* 2002; 76(7): 3267–3275.
 - 25 Pascual R, Contreras M, Fedorov A, Prieto M, Villalain J. Interaction of a peptide derived from the N-heptad repeat region of gp41 Env ectodomain with model membranes. Modulation of phospholipid phase behavior. *Biochemistry* 2005; 44(43): 14275–14288.
 - 26 Ait-Goughoulte M, Hourieux C, Patient R, Trassard S, Brand D, Roingard P. Core protein cleavage by signal peptide peptidase is required for hepatitis C virus-like particle assembly. *J Gen Virol* 2006; 4: 855–860.
 - 27 Hourieux C, Ait-Goughoulte M, Patient R *et al.* Core protein domains involved in hepatitis C virus-like particle assembly and budding at the endoplasmic reticulum membrane. *Cell Microbiol* 2007; 9(4): 1014–1027.
 - 28 Matto M, Rice CM, Aroeti B, Glenn JS. Hepatitis C virus core protein associates with detergent-resistant membranes distinct from classical plasma membrane rafts. *J Virol* 2004; 78(21): 12047–12053.
 - 29 de Almeida RF, Fedorov A, Prieto M. Sphingomyelin/phosphatidylcholine/cholesterol phase diagram: boundaries and composition of lipid rafts. *Biophys J* 2003; 85(4): 2406–2416.
 - 30 de Almeida RF, Loura LM, Fedorov A, Prieto M. Lipid rafts have different sizes depending on membrane composition: a time-resolved fluorescence resonance energy transfer study. *J Mol Biol* 2005; 346(4): 1109–1120.
 - 31 Nolandt O, Kern V, Muller H *et al.* Analysis of hepatitis C virus core protein interaction domains. *J Gen Virol* 1997; 6: 1331–1340.
 - 32 Hope RG, McLauchlan J. Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. *J Gen Virol* 2000; 8: 1913–1925.
 - 33 Hope RG, Murphy DJ, McLauchlan J. The domains required to direct core proteins of hepatitis C virus and GB virus-B to lipid droplets share common features with plant oleosin proteins. *J Biol Chem* 2002; 277(6): 4261–4270.
 - 34 Nakai K, Okamoto T, Kimura-Someya T *et al.* Oligomerization of hepatitis C virus core protein is crucial for interaction with the cytoplasmic domain of E1 envelope protein. *J Virol* 2006; 80(22): 11265–11273.
 - 35 Suzuki R, Sakamoto S, Tsutsumi T *et al.* Molecular determinants for subcellular localization of hepatitis C virus core protein. *J Virol* 2005; 79(2): 1271–1281.
 - 36 Boulant S, Montserret R, Hope RG *et al.* Structural determinants that target the hepatitis C virus core protein to lipid droplets. *J Biol Chem* 2006; 281(31): 22236–22247.
 - 37 Jolivet-Reynaud C, Dalbon P, Viola F *et al.* HCV core immunodominant region analysis using mouse monoclonal antibodies and human sera: characterization of major epitopes useful for antigen detection. *J Med Virol* 1998; 56(4): 300–309.
 - 38 Majeau N, Gagne V, Boivin A *et al.* The N-terminal half of the core protein of hepatitis C virus is sufficient for nucleocapsid formation. *J Gen Virol* 2004; 4: 971–81.