Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1758 (2006) 755–763
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Membrane-perturbing properties of three peptides corresponding to the ectodomain of hepatitis C virus E2 envelope protein

Beatriz Pacheco [a], Julián Gómez-Gutiérrez [a], Belén Yélamos [a], Carmen Delgado [a], Fernando Roncal [b], Juan P. Albar [b], Darrell Peterson [c], Francisco Gavilanes [a,*]

[a] *Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas, Universidad Complutense, 28040 Madrid, Spain*
[b] *Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología, CSIC, 28049 Madrid, Spain*
[c] *Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298, USA*

Received 13 January 2006; received in revised form 31 March 2006; accepted 24 April 2006
Available online 10 May 2006

Abstract

Based on the predicted capacity to interact with membranes at the interface, we have found three regions in the ectodomain of the hepatitis C virus envelope glycoprotein E2 (430–449, 543–560 and 603–624) with the ability to destabilize membranes. Three peptides corresponding to the sequence of these regions have been synthesized and their interaction with liposomes have been characterized. The three peptides were able to insert deeply into the hydrophobic core of negatively charged phospholipids as stated by fluorescence depolarization of the probe 1,6-diphenyl-1,3,5-hexatriene. Peptides $E2_{430–449}$ and $E2_{603–624}$ were able to induce aggregation of phosphatidylglycerol vesicles in a concentration-dependent manner both at neutral and acidic pH while peptide $E2_{543–560}$ did not induce any increase of optical density at 360 nm in the concentration range studied. The three peptides induced lipid mixing and the release of the internal contents in a dose-dependent manner when acidic phospholipids were used. Fourier transformed infrared spectroscopy indicated that the peptides adopted mainly a β-sheet conformation which is not modified by the presence of acidic phospholipids. Taken together, our results point out to the involvement of these three regions in the fusion mechanism of HCV at the plasma membrane level.

1. Introduction

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B viral hepatitis throughout the world [1]. HCV is a positive-stranded RNA virus which belongs to the Flaviviridae family [2]. The HCV genomic RNA contains a long open reading frame (ORF) that encodes a polyprotein that is proteolytically cleaved by viral and cellular proteases to generate the structural (C, E1, E2 and p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins [3]. Recently, additional proteins, synthesized by ribosomal frameshift from a coding sequence that overlaps the core protein reading frame, have been reported [4].

The mechanism of HCV attachment and entry into target cells is not known. Although some cell lines exhibit limited replication of HCV [5,6], studies of the infection mechanisms of HCV have been hampered by the lack of an efficient, reliable cell culture system that support the full replication of HCV.

HCV envelope proteins E1 and E2 (residues 192–383 and 384–746 of the polyprotein, respectively) are type I transmembrane proteins, highly glycosylated, consisting of a large N-terminal ectodomain and a C-terminal hydrophobic anchor that retains them in the endoplasmic reticulum [7]. The extracellular domains of E1 and E2 are thought to play an important role in the interaction between the virus and its receptor as well as in the fusion of the viral and cellular membranes. To date, the identification of the cellular receptor for HCV remains elusive and controversial. Among others, human CD81, a member of the tetraspanin superfamily [8,9], the low-density lipoprotein receptor (LDL-r) [10], the human scavenger receptor class B type I [11] and the C-type lectins DC-SIGN/DC-SIGNR [12] have been reported as receptors for the HCV.

Entry of an envelope virus in a host cell requires not only its binding to one or more receptors in the cell surface but also the

* Corresponding author. Tel.: +34 913944266; fax: +34 913944159.
E-mail address: pacog@bbm1.ucm.es (F. Gavilanes).

0005-2736/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2006.04.024

viral–cell membrane fusion. The fusion step mainly occurs either at the cell plasma membrane, mediated by the interaction of the viral envelope proteins and their receptors in a pH-independent manner, or in the endosomal compartment, at acidic pH, after uptake of the virus by receptor-mediated endocytosis [13].

The majority of the viral fusion proteins that have been studied can be divided into two classes. Class I includes the fusion proteins of ortomyxo-, retro-, paramyxo-, and filoviruses, all of which are activated by proteolytic cleavage [14]. In these proteins, the fusion peptide is just C-terminal to the cleavage point in the precursor and becomes – or is near to – the N terminus of the mature fusion protein. Class II includes the fusion proteins of flaviviruses and alphaviruses and possesses internal fusion peptides that are not proteolytically cleaved during maturation but are associated with a second protein whose cleavage is essential for activation of fusion activity [15].

Available data concerning the fusion step of HCV are scarce. However, some studies suggest that the entry of HCV into the host cell takes place through the endosome at acidic pH. Thus, the cell fusion activity of HCV envelope proteins have been studied using a CHO cell line constitutively expressing HCV envelope proteins E1 and E2 on the cell surface [16]. This study demonstrated the requirement for both chimeric E1 and E2 proteins and the increase of the fusion activity of HCV envelope proteins by the low pH treatment. On the other hand, the entry of pseudotyped vesicular stomatitis virus (VSV) possessing E1 or E2 into the cell is inhibited by lysosomotropic bases or inhibitors of vacuolar H^+ ATPases [17]. However, up to date, no sequence of the HCV envelope proteins has been reported to be implicated in the fusion process.

In the present work, we show the existence of three internal sequences in the ectodomain of E2, based on the hydrophobicity scale of Wimley and White [18], which have destabilizing effects in membrane model systems and, hence, could participate in the fusion of the viral and cellular membranes.

2. Methods

2.1. Reagents

N-(7-nitro-2,1,3-benzoxadiazol-4-yl)dimyristoylphosphatidylethanolamine (NBD-PE), *N*-(lissamine rhodamine B sulfonyl)-diacylphosphatidylethanolamine (Rh-PE), egg phosphatidylcholine (PC), egg phosphatidylglycerol (PG), and dimyristoylphosphatidylglycerol (DMPG) were provided by Avanti Polar Lipids. 8-Aminonaphtalene-1,3,6-trisulfonic acid (ANTS), *p*-xylenebis(pyridinium) bromide (DPX) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Molecular Probes. Triton X-100 was purchased from Boehringer Mannheim. All other reagents were obtained from Merck and Sigma. All solvents were of HPLC grade.

2.2. Peptide synthesis

Peptides were synthesized as the C-terminal amide on an automated multiple peptide synthesizer (AMS 422, Abimed) using previously described procedures [19] by the solid-phase procedure and standard Fmoc chemistry in a base of 25 μmol. Briefly, the synthesis was carried out on a *N*-α-Fmoc-DMP resin [4-(2′,4′-dimethoxyphenil-Fmoc-aminomethyl)phenoxy resin (Novabiochem)] with Fmoc-protected amino acids activated in situ with PyBOC (benzotriazole-1-yl-oxy-*tris*-pyrrolidinophosphonium hexafluorophosphate) in the presence of *N*-methylmorpholine and 20% piperidine/dimethylformamide for deprotection. The protecting side chains were as follows: Asn, Gln and His (Trt), Arg (pmc), Asp (OtBu), Cys (Acm), Lys (Boc), Ser, Thr and Tyr (tBu). Peptides were purified by reverse-phase HPLC using an Ultrasphere-ODS C_{18} column (10 × 150 mm) with a linear gradient (0–35%) of acetonitrile in 0.1% trifluoroacetic acid. Due to the low solubility of the peptides in aqueous buffers, a 10 mg/ml stock solution in dimethylsulfoxide (DMSO) was kept perfectly sealed at −20 °C and employed in the various assays described in this work. The final organic solvent concentration was always kept under 2% (v/v) and had no measurable effect on vesicle stability. The peptide concentration of the stock solutions was determined by amino acid analysis which was performed on a Beckman 6300 amino acid analyzer.

2.3. Vesicle preparation

In all cases, a lipid film was obtained by drying a cloroform:methanol (2:1) solution of the lipid under a current of nitrogen and this film was further kept under vacuum for 4–5 h to completely remove the organic solvent. The phospholipids were resuspended at a concentration of 1 mg/ml in medium buffer (100 mM NaCl, 5 mM MES, 5 mM sodium citrate, 5 mM Tris, 1 mM EDTA) at the appropriate pH value and incubated for 1 h at 37 °C and eventually vigorously vortexed. This suspension was sonicated in a bath sonicator (Branson 1200) and was subsequently subjected to fifteen cycles of extrusion in a Liposo Fast-Basic extruder apparatus (Avestin, Inc.) with 100-nm polycarbonate filters (Costar). When encapsulation was required, an additional step of five freeze–thawing cycles was included after the sonication process.

2.4. Vesicle aggregation

The increase in the optical density at 360 nm (ΔOD_{360}) produced by addition of the peptide from a concentrated stock solution in DMSO to a phospholipid vesicle suspension, in medium buffer at the appropriated pH, was measured on a Beckman DU-7 spectrophotometer after incubation for 1 h at 37 °C. Values of control samples containing corresponding amounts of peptide dissolved in DMSO were subtracted at each peptide concentration. The final phospholipid concentration was 0.13 mM.

2.5. Release of aqueous contents

Leakage was determined by the ANTS/DPX assay [20], which is based on the dequenching of ANTS fluorescence caused by its dilution upon release of the aqueous contents of one vesicle preparation containing both ANTS and DPX. It was performed by coencapsulating 12.5 mM ANTS and 45 mM DPX in 10 mM Tris, 20 mM NaCl, pH 7.2, in phospholipid vesicles. The lipid film was hydrated as described previously, and the vesicles were sonicated 30 min. Afterwards, the vesicles were subjected to five cycles of freeze–thawing in liquid nitrogen an passed 15 times through an extruder apparatus (Avestin, Inc.) with 100-nm polycarbonate filters (Costar). After the vesicles with the coencapsulated probe and quencher were formed, the whole sample was passed through a Sephadex G-75 column (Pharmacia) to separate the vesicles from the non encapsulated material using medium buffer for elution [21]. The final phospholipid concentration in the assay was 0.13 mM and medium buffer at the appropriated pH value was used in all cases. After adding the peptide from a DMSO stock solution, the samples were incubated for 1 h at 37 °C and measured in a SLM Aminco 8000 C spectrofluorimeter. The organic solvent had no measurable effect on vesicle stability. The excitation wavelength was set at 385 nm, and the ANTS emission was monitored at 520 nm. Both the excitation and emission slits were set at 4 mm. The excitation and emission polarizers were kept constant at 90° and 0°, respectively, to minimize interference due to dispersion. The fluorescence scale was set to 100% by addition of 0.5% Triton X-100, and 0% leakage was obtained measuring the fluorescence of control vesicles without peptide.

2.6. Lipid mixing assay

Lipid mixing was monitored by using the classical fluorescent probe dilution assay [22], in which the decrease in the efficiency of the fluorescence energy transfer between NBD-PE (energy donor) and Rh-PE (energy acceptor) incorporated into liposomes, as a consequence of lipid mixing, is monitored. Liposomes, in medium buffer at the appropriated pH, labeled with 1 mol% NBD-PE and 1 mol

% Rh-PE were mixed with unlabeled liposomes in a 1:9 molar ratio. Lipid mixing was initiated by addition of the peptide from a stock solution in DMSO. The samples were incubated for 1 h at 37 °C, and the emission spectra were recorded with the excitation wavelength set at 450 nm. Both the excitation and emission slits were set at 4 mm. The excitation polarizer was kept constant at 90°, and the emission polarizer was kept constant at 0° to minimize dispersive interference. The efficiency of the energy transfer was calculated from the ratio of the emission intensities at 530 and 590 nm and the appropriated calibration curve. The final phospholipid concentration was 0.13 mM. The organic solvent itself had no effect on the efficiency of the energy transfer.

2.7. Fluorescence polarization

Fluorescence polarization measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH) were taken by using 10 mm Glan-Thompson polarizers. DMPG vesicles (0.13 mM) were prepared as indicated above containing the probe DPH at a weight ratio of 1:500. The peptide was added from a stock concentrated solution in DMSO. Peptide-vesicle mixtures were incubated for 1 h at 37 °C and then cooled. The excitation was set at 365 nm and emission was measured at 425 nm, after equilibration of the samples at the indicated temperature. The temperature in the cuvette was maintained with a circulating water bath.

2.8. Fourier transformed infrared spectroscopy

Lyophilized aliquots of peptides (300 μg) were dissolved in 50 μl of deuterated medium buffer, pD 5.4, to avoid the interference of H_2O infrared absorbance (1645 cm^{-1}) on the protein amide I band [23]. Infrared spectra were acquired on an IFS 66/S instrument (Bruker) equipped with a DTGS detector. Usually, 600 scans/sample were taken, averaged, apodized with a Happ-Genzel function and Fourier transformed to give a nominal resolution of 2 cm^{-1}. Fourier self-deconvolution was performed using a Lorenztian bandwidth of 18 cm^{-1} and a resolution enhancement factor of 2 [24,25]. Peptide secondary structure was quantified by band deconvolution of the amide I band. The number and position (wavenumber) of the bands were taken from the deconvoluted spectra. The iterative curve-fitting process was performed in CURVEFIT running under SpectraCalc (Galactic Industries Corp.). The number, position, and band shape were fixed during the first 200 iterations. The fitting was further refined by allowing the band positions to vary for 50 additional iterations. The goodness of the fit was assessed from the χ^2 values (4×10^{-5} to 6.5×10^{-5}). The area of the fitted band was used to calculate the percentage of secondary structure [24–26].

3. Results

3.1. Putative fusion peptides of HCV

Based on the water-to-membrane interface transfer free energies for each amino acid, Wimley and White have elaborated a whole-residue hydrophobicity scale compiling the energetic components that dictate initial partitioning of unfolded peptide sequences into membranes [18]. This scale has been applied to the detection of membrane-partitioning regions within the ectodomains of several viral fusion proteins [27,28]. Fig. 1 shows a combined representation of the hydrophobicity plots of Wimley–White and Kyte–Doolittle [29] of E1 and E2 proteins. Both plots identified a stretch in the C-terminal region of both E1 and E2 with a high hydrophobic index that corresponds to the proposed transmembrane domains of the envelope proteins. In the ectodomain of E1, both profiles identified a broad highly hydrophobic region (residues 265–295) which could also constitute a transmembrane region. The interfacial hydrophobicity plot revealed three regions in the ectodomain of E2 (residues 430–449, 543–560 and 603–624) with capacity to destabilize membranes, due to its high tendency to interact with membranes at the interface.

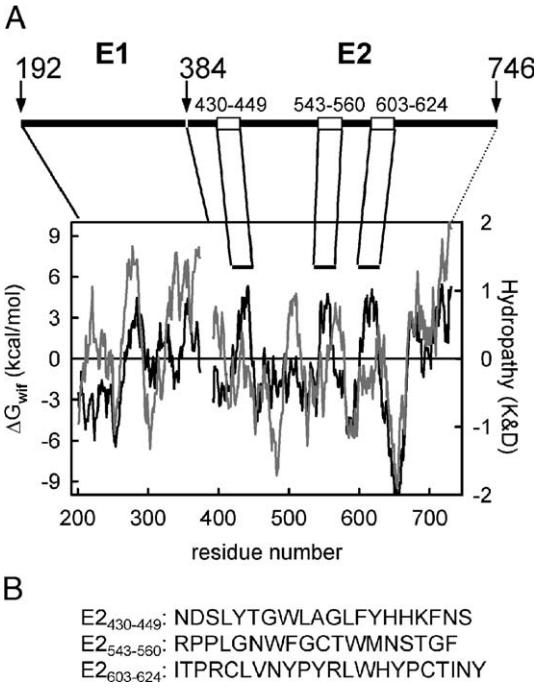

Fig. 1. (A) Hydrophobicity plot of E1 and E2 proteins. The scales of Wimley and White (black line) and Kyte and Doolittle (grey line) were used. The sequences of E1 and E2 correspond to residues 192–383 and 384–746 of the polyprotein, respectively. A 19-amino-acid window was employed and the average value was assigned to residue 10th. (B) Amino acid sequence of the three E2 peptides.

Moreover, the regions 543–560 and 603–624 are immediately followed by stretches identified as hydrophobic in the scale of Kyte–Doolittle and, thus, can interact with internal regions in the lipid bilayer. It has been described that a sequence with the ability to interact with the lipid interface immediately followed by a transmembrane region can constitute a new structural motif implicated in viral fusion [27,28]. Finally, the Kyte–Doolittle plot revealed two hydrophobic regions in E2 (N-terminal and residues 497–515) not detected in the Wimley–White plot. These regions are not predicted to interact with lipid bilayers at the interface and thus to destabilize membranes, although their interaction with the internal region of the bilayer is not discarded. Hence, the three interfacial regions detected in the ectodomain of E2 are the best candidates to act as fusion peptides of HCV. Thus, peptides corresponding to these regions were chemically synthesized. Their amino acid sequences are shown in Fig. 1B. All three peptides were purified by HPLC, yielding preparations with purity higher than 95%. Their amino acid composition correlates perfectly with that expected from their amino acid sequence. MALDI-TOF mass spectrometry yielded the following results: peptide $E2_{430–449}$, *m/z* 2370.3 (calculated 2370.6); peptide $E2_{543–560}$, *m/z* 2071.0 (calculated 2071.3); peptide $E2_{603–624}$, *m/z* 2786.3 (calculated 2786.2).

3.2. Vesicle aggregation

The ability of the peptides to induce vesicle aggregation was monitored by measuring the increment in the optical density at

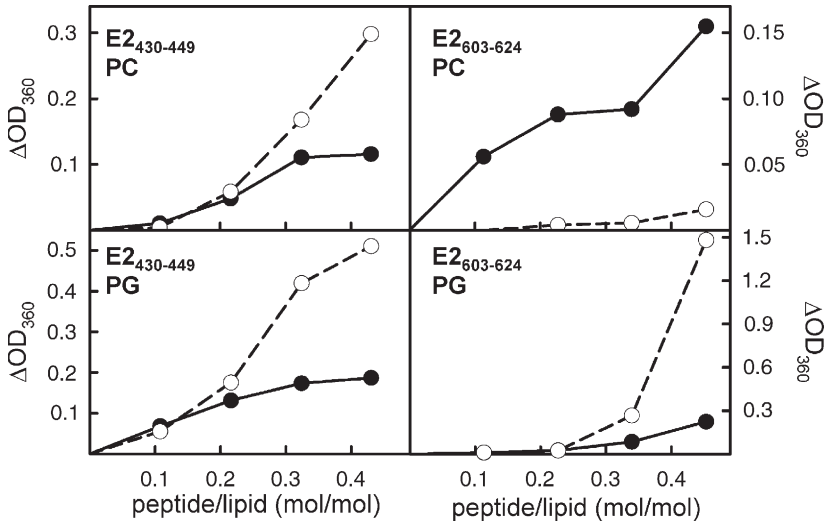

Fig. 2. Aggregation of PC and PG phospholipids vesicles induced by E2 peptides. The optical density at 360 nm (ΔOD_{360}) was measured after incubation of vesicles in medium buffer at pH 7.5 (●) and 5.0 (○) with aliquots of peptide from a stock solution in DMSO. The concentration of DMSO was kept below 1%. The results shown are representative of those obtained for at least three different experiments.

360 nm (ΔOD_{360}) of a liposome suspension as a result of the increase in vesicle size. As shown in Fig. 2, peptides $E2_{430–449}$ and $E2_{603–624}$ were able to induce aggregation of neutral and acidic phospholipid vesicles in a concentration-dependent manner both at neutral and acidic pHs. The OD_{360} values reached with $E2_{430–449}$ peptide and either PC or PG are around 3 times higher at pH 5.0 than at pH 7.5. This was also the case for peptide $E2_{603–624}$ which induced an increase in the OD_{360} of PG vesicles 5 times higher at pH 5.0 than at pH 7.5. Moreover, the increase in OD_{360} induced by the peptide $E2_{603–624}$ at pH 5.0 was 3 times higher than that observed with the peptide $E2_{430–449}$. When neutral phospholipids were employed $E2_{603–624}$ almost did not induce any vesicle aggregation at pH 5.0 (Fig. 2). On the other hand, peptide $E2_{543–560}$ did not induce any increase of the optical density at 360 nm of either neutral or acidic phospholipids in the concentration range studied (data not shown).

3.3. Release of aqueous contents

The ability of these peptides to destabilize the lipid bilayer was assessed by measuring the release of aqueous contents of phospholipid vesicles. Liposome leakage was followed by measuring the increase in ANTS fluorescence at 520 nm [20]. Fig. 3 shows the leakage induced by the synthetic peptides added to PG vesicles. Either peptide was able to induce the release of the internal contents of the liposomes in a dose-dependent manner. The peptide concentrations needed to attain the maximum effect were substantially lower than those needed to induce vesicle aggregation (Fig. 2).

Peptide $E2_{430–449}$ induced release of aqueous contents at acidic pH reaching 100% at a peptide/lipid molar ratio of 0.05 (6.5 μM peptide), while at the same ratio and neutral pH leakage was very low, 10%, being necessary a peptide concentration 4-fold higher to attain 100% of leakage. Although peptide $E2_{543–560}$ did not induce vesicle aggregation, it was able to destabilize the membrane leading to the release of the aqueous content at relatively low

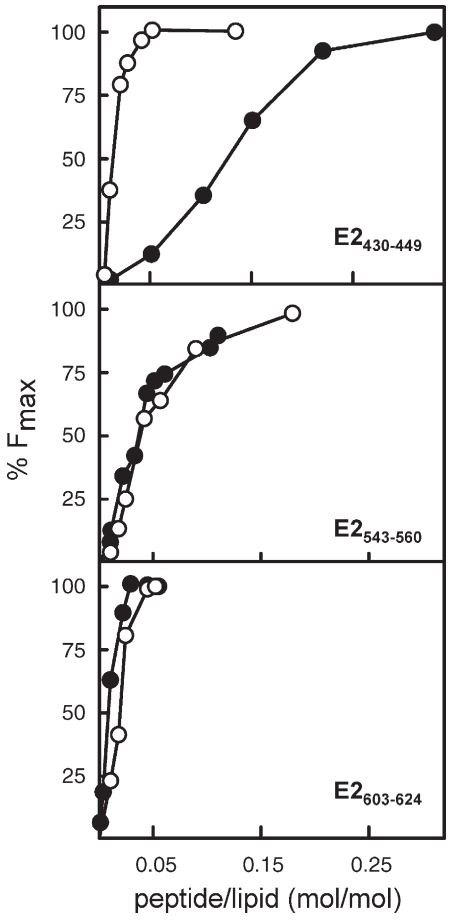

Fig. 3. Leakage of ANTS/DPX from PG vesicles induced by E2 peptides. Increasing concentrations of E2 peptides were added to vesicles loaded with ANTS and DPX in medium buffer at pH 7.5 (●) and 5.0 (○).The concentration of DMSO was kept below 1% and had no measurable effect on vesicle stability. The results shown are representative of those obtained for at least three different experiments.

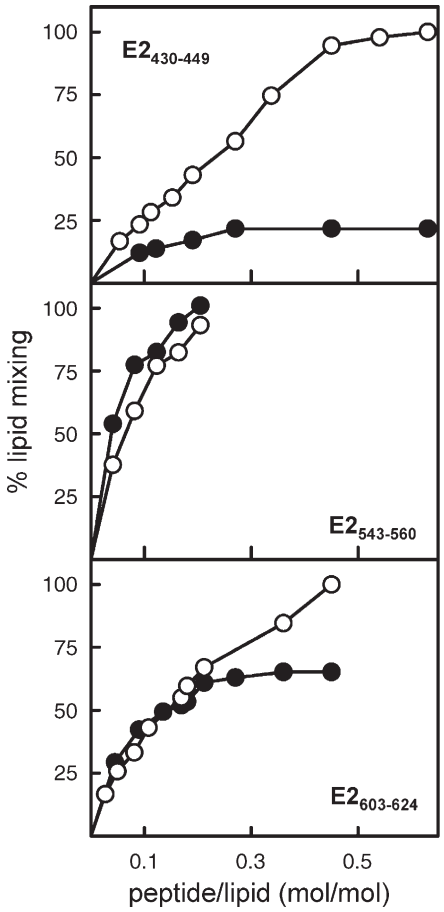

Fig. 4. Lipid mixing induced by E2 peptides. Increasing concentrations of the E2 peptides were added to a 1:9 mixture of labeled (NBD-PE 1% and Rh-PE 1%) and unlabeled PG vesicles hydrated in medium buffer at pH 7.5 (●) and 5.0 (○). The concentration of DMSO was kept below 1%. The results shown are representative of those obtained for three different experiments.

peptide/lipid ratios. In this case, there was not dependence with pH, and 90% leakage was reached at a peptide/lipid molar ratio of 0.1 (13 μM peptide) at both pHs. Finally, $E2_{603–624}$ peptide reached 100% leakage at a peptide/lipid molar ratio as low as 0.04 (about 5 μM peptide concentration), at which no vesicle aggregation was observed (Fig. 2). The effect of pH on breaking the integrity of the vesicles by peptide $E2_{603–624}$ was low, showing slightly higher values at pH 7.5 than at pH 5.0.

Neither peptide destabilized the bilayer of phospholipid vesicles composed of pure PC (data not shown).

3.4. Lipid mixing

Mixing of membrane lipids was followed by the resonance energy transfer (RET) assay between the fluorescence probes NBD-PE and Rh-PE [22]. In this assay, mixing of phospholipids from labeled and unlabeled liposomes results in an increase in the distance between the donor (NBD) and the acceptor (Rh), with the concomitant decrease in energy transfer which reflects accurately the degree of fusion [30].

Either peptide was able to induce lipid mixing of PG vesicles, showing a similar dependence with pH to that observed for leakage, although the peptide/lipid molar ratios needed to reach maximum values were higher (Fig. 4).

$E2_{430–449}$ peptide induced 100% lipid mixing of PG vesicles at pH 5.0 at a peptide/lipid molar ratio of about 0.45 (58 μM peptide), and at pH 7.5 and the same ratio this percentage was much lower attaining only a 20% of energy transfer. $E2_{543–560}$ peptide was able to induce lipid mixing in a virtually pH-independent manner, reaching a complete lipid mixing at a peptide/lipid molar ratio of 0.2 (26 μM peptide). Finally, lipid mixing induced by peptide $E2_{603–624}$ was independent of pH up to peptide/lipid molar ratios of 0.2. Above this ratio, the percentage

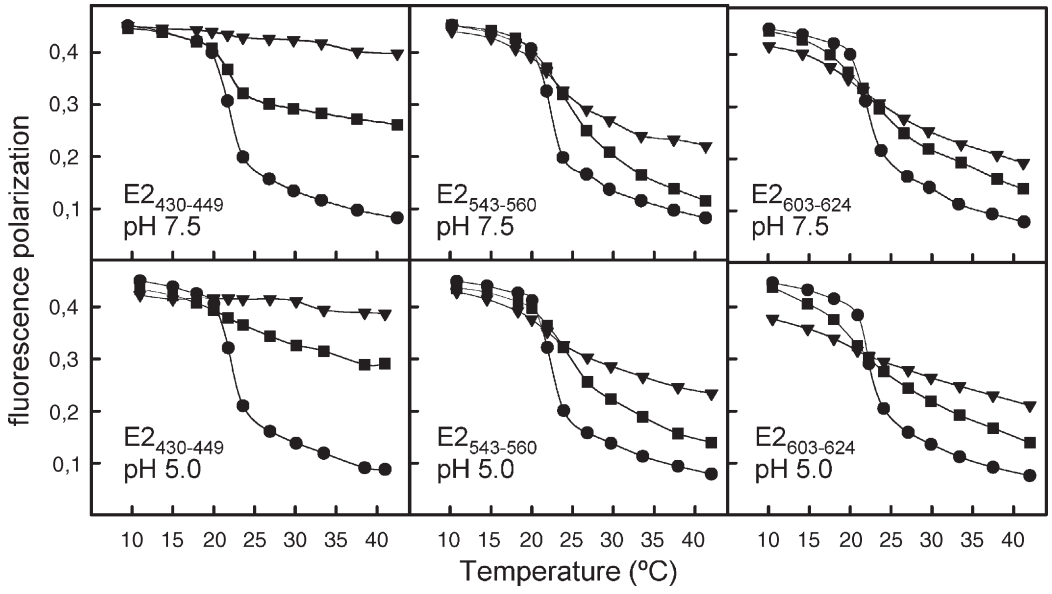

Fig. 5. Temperature dependence of fluorescence polarization of DPH-labeled DMPG liposomes. The peptide/lipid molar ratios employed were (●) 0.0, (■) 0.206 ($E2_{430–449}$), 0.175 ($E2_{543–560}$) and 0.203 ($E2_{603–624}$) and (▼) 0.515 ($E2_{430–449}$), 0.433 ($E2_{543–560}$) and 0.506 ($E2_{603–624}$). The results shown are representative of those obtained for three different experiments.

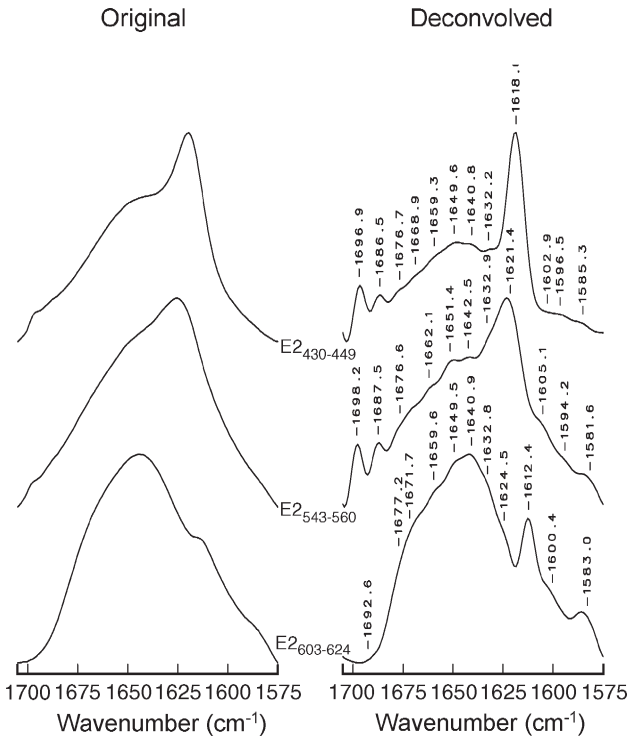

Fig. 6. Amide I infrared band of the original and deconvolved spectra of the E2 peptides. Aliquots of peptides (300 μg) were dissolved in 50 μl of deuterated medium buffer, pD 5.4.

of lipid mixing at pH 7.5 was stabilized at about 60%, while at pH 5.0, it continued increasing, attaining 100% at a peptide/lipid molar ratio of 0.45.

As it occurs with the release of aqueous contents neither peptide induced mixing of lipids of vesicles composed of pure PC (data not shown).

3.5. Peptide insertion into phospholipid bilayers

The effect of these peptides on the thermotropic behavior of dimyristoylphosphatidylglycerol (DMPG) vesicles has been studied. We have measured the fluorescence polarization of DMPG vesicles labeled in the hydrophobic core of the bilayer with the fluorescent probe DPH at increasing temperatures. The results obtained in the absence and the presence of peptides at different peptide/phospholipid molar ratios at pH 7.5 and 5.0 are depicted in Fig. 5. The addition of the peptides to DMPG vesicles induced a decrease in the amplitude of the transition in a concentration-dependent manner. At a peptide/lipid molar ratio of around 0.5 (65 μM peptide), either peptide almost completely abolished the phase transition, affecting mainly the polarization values above the transition temperature, indicating that the peptides affect mainly the acyl chains in the liquid–crystal phase, inducing a higher order in the chain packing. The interaction of peptide $E2_{543–560}$ with phospholipids caused a slight increase in the transition temperature from 22.5 °C to 24.8 °C. Peptide $E2_{603–624}$ reduced the enthalpy of the phase transition as a consequence of the decrease of the fluorescence polarization in the gel phase as well as an increase in the fluorescence polarization in the liquid crystal phase. The results were similar at both pH 7.5 and 5.0.

3.6. FTIR spectroscopy

The conformation adopted by the peptides both in the absence and in the presence of lipids was monitored by Fourier transformed infrared spectroscopy (FTIR). Fig. 6 shows the conformation sensitive amide I region of the infrared spectrum of the peptides alone in D_2O medium buffer at pD 5.4. Similar results were obtained at pD 7.5. The spectrum of $E2_{430–449}$ and $E2_{543–560}$ peptides exhibited a maximum centered around 1620 cm^{-1} while the spectrum of the peptide $E2_{603–624}$ was different with a broad maximum centered at 1640 cm^{-1}. Band-narrowing deconvolution of the amide I band (Fig. 6) shows that $E2_{430–449}$ and $E2_{543–560}$ possess a maximum at 1618–1621 cm^{-1}, characteristic of aggregates with a β-sheet conformation, and that $E2_{603–624}$ possesses, besides a maximum at 1612 cm^{-1}, two maxima centered at around 1641 cm^{-1} and 1649 cm^{-1} that can be assigned to non-ordered structures and α-helix, respectively. Fig. 6 shows that the amide I region exhibited other maxima that can be assigned to vibration of the carbonyl groups in peptide bonds within different secondary structural motifs. The components between 1618 cm^{-1} and 1633 cm^{-1} are assigned to β-structures, those around 1648–1652 cm^{-1} to α-helix, those between 1659 and 1690 cm^{-1} to β-turns and the components in the region around 1580 and 1605 cm^{-1} are assigned to side chains [31].

The original infrared spectra can be fitted with the bands obtained in the deconvolution of the spectra, which allows the amide band I to be adjusted to the different components (data not shown). The percentages of secondary structures calculated from the area of the fitted bands are represented in Table 1. As reflected in the table, the three peptides adopted mainly an extended β-structure, although the percentage of this structure was higher for peptides $E2_{430–449}$ and $E2_{543–560}$ than for $E2_{603–624}$ peptide which exhibited a slightly higher helical content.

The interaction with acidic phospholipids did not induce important changes in the shape of spectra nor in the position of the maxima, and thus, in the conformation of the peptides (data not shown).

4. Discussion

The knowledge of the HCV viral life cycle is scarce, and the sequence(s) involved in the fusion step have not yet been identified.

Table 1
Secondary structure of E2 peptides in medium buffer

% [a]	$E2_{430–449}$	$E2_{543–560}$	$E2_{603–624}$
Helix	16	10	18
Sheet	48	53	38
Turn	29	29	32
Non-ordered	7	8	12

[a] The percentages were determined by deconvolution of the amide I band. The number and position (wavenumber) of the bands were taken from the deconvolved spectra. The area of the fitted band was used to calculate the percentage of secondary structure.

E1 was proposed as the fusion protein based upon the existence in its sequence of a putative fusion peptide [32]. However, and based on homology studies, E2 has also been proposed as the protein responsible of the fusion step [33]. In the present study, we have scanned the sequence of the HCV envelope proteins searching for regions able to interact with lipid bilayers and thus to participate in the fusion process. The use of the interfacial hydrophobicity scale of Wimley and White has allowed us to identify three regions within the ectodomain of E2 with the ability to interact with membranes at the interface. In spite of the limitations of the use of peptides to study the fusion step between the viral and cellular membranes, the research on the fusogenic properties of synthetic peptides representing a protein stretch able to interact and destabilize a lipid bilayer has allowed the characterization of the initial steps of several viral infection processes [34].

The results obtained in this work show that synthetic peptides corresponding to these three interfacial regions identified in the ectodomain of E2 may interact with lipids, destabilizing phospholipids vesicles and promoting processes of aggregation, fusion and leakage both at neutral and acidic pHs. Fluorescence depolarization studies show that the three E2 peptides reduce the DMPG transition amplitude without affecting the transition temperature. This effect is characteristic of integral membrane proteins [35], suggesting that these peptides are able to insert into the membrane interacting hydrophobically with the hydrocarbon core and restricting the mobility of the acyl chains. The fact that lipid mixing and release of contents take place only when negatively charged phospholipids were used could be explained if the ionic interaction between the peptides and the polar head group of the phospholipid promotes the subsequent insertion into the hydrophobic core. This later step would be responsible of the destabilization which gives rise to fusion. This dependence with the polar head group of the phospholipid has been observed in several fusion proteins and peptides of other viruses. Thus, the fusion peptides of influenza and Sendai viruses interact preferably with PC vesicles while the fusion peptide of VSV shows preference for PS vesicles [21] and the fusion peptide of Ebola virus require the presence of PI in the target membrane [36]. Also, studies of the fusion of HIV and SIV with model membranes show a preference for negatively charged liposomes demonstrating higher fusogenic activity at acidic pH, although viral entry into the host cell is pH independent [37]. However, this dependence with the pH and lipid composition observed in viral fusion peptides or even with whole viruses does not necessarily have a physiological significance.

The pH dependence observed in the different assays when acidic phospholipids were employed could be due to a conformational change induced by acidic pH. However, FTIR showed that all the peptides maintain their conformation independently of the pH. The pH dependence may also be explained in terms of the net charge that the peptides have. Thus, $E2_{430–449}$ has charge 0 at pH 7.5 while the charge at pH 5.0 is +2. This increase would give this peptide the highest pH dependence as it is observed in virtually all the assays. The effect of pH on the behavior of $E2_{543–560}$ and $E2_{603–624}$ peptides would be smaller, since $E2_{543–560}$ has a +1 charge at both pHs and $E2_{603–624}$ increase the positive charge from +2 to +3 when decreasing the pH from 7.5 to 5.0. Indeed, leakage and lipid mixing results are similar for both peptides at both pHs. However, the fact that decreasing the pH resulted in the highest increase in OD_{360} observed for $E2_{603–624}$ would indicate that the peptide sequence, besides the positive charge, also play an essential role in determining the destabilization effects. On the other hand, $E2_{543–560}$ peptide did not induce any increase of the optical density at 360 nm although this peptide is as effective as the other two in terms of promoting lipid mixing and release of contents. The absence of aggregation could be due to the fragmentation of previously aggregated and fused vesicles as it has been demonstrated for other fusion peptides [19].

The three putative fusion peptides would occupy an exposed location on the three dimensional model which has been proposed for HCV E2 protein based on fold recognition methods [33]. The exposed character is also indicated by the fact that amino acids 430–449 constitute the epitope that binds monoclonal antibody mAb 7/16b, amino acids 544–551 take part in the binding of monoclonal antibody mAb 6/53 and residues 623–625 contain the sequence N-X-T which can be glycosylated in the native structure [33]. Moreover, the three peptides have features characteristic of fusogenic peptides. Thus, they are short (between 16 and 26 residues) and hydrophobic sequences [38], $E2_{430–449}$ and $E2_{543–560}$ are rich in Gly, Ala and Phe residues [38], $E2_{430–449}$ contain the tripeptide GLF which is highly conserved among the fusion peptides of class I proteins [39]. The peptide $E2_{603–624}$ is rich in aromatic residues, a pattern reminiscent of pre-transmembrane regions in retroviruses and filoviruses.

Another feature of many internal fusion peptides is a proline at or near their centers which could mediate the loop structure which is thought to insert into the lipid bilayer [40–42]. On the other hand, several evidences suggest that the internal fusion peptide of ASLV exists as a loop that is stabilized by a disulfide bond at its base and that this stabilized loop serves an important function during virus–cell fusion [43]. Also, the structure of the tick-borne encephalitis virus envelope protein (TBE-E) solved by X-ray crystallography shows that in its native prefusogenic state the fusion peptide conforms a β-strand-turn-β-strand motif stabilized by disulfide bonds, although in this case there is not any proline residue [44]. From the FTIR studies, it can be concluded that the E2 peptides in the absence of vesicles form predominantly aggregates with an extended β-structure. Moreover, the fact that infrared spectra were hardly modified by the presence of PG vesicles suggests that E2 putative fusion peptides interact with lipids mostly in an extended conformation, compatible with a β-strand-turn-β-strand motif. Peptide $E2_{603–624}$ also contains two cysteine residues near its ends and a central proline suggesting that in the E2 protein this region could indeed adopt a loop structure. Furthermore, this region is highly conserved between the different variants of HCV. The involvement of $E2_{430–449}$ in the fusion process would be indicated by the fact that monoclonal antibodies against amino acids 412–447 of E2 neutralize the infectivity of HIV pseudotypes bearing native hepatitis C virus glycoproteins [45]. Thus, all these observations taken together

would point to the direct participation of these regions in the fusion between the viral and cellular membranes. It is difficult to think of the fusion process between the viral and cellular membranes with only a small region of the envelope protein carrying the membrane destabilization, since this is a complex process that requires the approach between two membranes and involves several biochemical and biophysical interactions. In fact, the existence of two fusion peptides (one N-terminal and one internal) in the envelope proteins of measles, Sendai and HIV-1 viruses has been described [28,46,47].

In conclusion, the three peptides of E2 studied in this work induce, although to a different extent, and both at neutral and acidic pH, close apposition of membranes and destabilization of acidic phospholipids vesicles, the essential steps required for fusion [48]. Taken together, our results point out to the involvement of these three regions in the fusion mechanism of HCV at the plasma membrane level. The effect of point mutations introduced in these regions in the membrane destabilizing properties of the complete E2 protein could offer a better understanding of the early steps in the infective cycle of HCV.

Acknowledgments

This work was supported by a Grant from the Dirección General de Investigación of the Ministerio de Educación y Ciencia (Spain) (BMC2003-06631).

References

[1] Q.-L. Choo, G. Kuo, A.J. Weiner, L.R. Overby, D.W. Bradley, M. Houghton, Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome, Science 244 (1989) 359–362.
[2] R.I.B. Franki, C.M. Fauquet, D.L. Knudson, F. Brown, Classification and nomenclature of viruses: fifth report of the International Committee on Taxonomy of Viruses, Arch. Virol., Suppl. 2 (1991) 223.
[3] K.E. Reed, C.M. Rice, Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties, Curr. Top. Microbiol. Immunol. 242 (2000) 55–84.
[4] A.D. Branch, D.D. Stump, J.A. Gutierrez, F. Eng, J.L. Walewski, The hepatitis C virus alternate reading frame (ARF) and its family of novel products: the alternate reading frame protein/F-protein, the double-frameshift protein, and others, Semin. Liver Dis. 25 (2005) 105–117.
[5] R. Bartenschlager, V. Lohmann, Replication of hepatitis C virus, J. Gen. Virol. 81 (2000) 1631–1648.
[6] T. Heller, S. Saito, J. Auerbach, T. Williams, T.R. Moreen, A. Jazwinski, B. Cruz, N. Jeurkar, R. Sapp, G. Luo, T.J. Liang, An in vitro model of hepatitis C virion production, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 2579–2583.
[7] C.M. Rice, Flaviviridae: the viruses and their replication, in: B.N. Fields, D.M. Knipe, P.M. Howley (Eds.), Fields Virology, 3rd ed., Lippincott-Raven Publishers, Philadelphia, 1996, pp. 931–959.
[8] P. Pileri, Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A.J. Weiner, M. Houghton, D. Rosa, G. Grandi, S. Abrignani, Binding of hepatitis C virus to CD81, Science 282 (1998) 938–941.
[9] J.A. McKeating, L.Q. Zhang, C. Logvinoff, M. Flint, J. Zhang, J. Yu, D. Butera, D.D. Ho, L.B. Dustin, C.M. Rice, P. Balfe, Diverse hepatitis C virus glycoproteins mediate viral infection in a CD81-dependent manner, J. Virol. 78 (2004) 8496–8505.
[10] V. Agnello, G. Ábel, M. Elfahal, G.B. Knight, Q.-X. Zhang, Hepatitis C virus and other Flaviviridae viruses enter cells via low density lipoprotein receptor, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 12766–12771.
[11] E. Scarselli, H. Ansuini, R. Cerino, R.M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, A. Vitelli, The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus, EMBO J. 21 (2002) 5017–5025.
[12] S. Pöhlmann, J. Zhang, F. Baribaud, Z. Chen, G.J. Leslie, G. Lin, A. Granelli-Piperno, R.W. Doms, C.M. Rice, J.A. McKeating, Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR, J. Virol. 77 (2003) 4070–4080.
[13] L.D. Hernandez, L.R. Hoffman, T.G. Wolfsberg, J.M. White, Virus–cell and cell–cell fusion, Annu. Rev. Cell Dev. Biol. 12 (1996) 627–661.
[14] H.D. Klenk, W. Garten, Host cell proteases controlling virus pathogenicity, Trends Microbiol. 2 (1994) 39–43.
[15] F.X. Heinz, S.L. Allison, Structures and mechanisms in flavivirus fusion, Adv. Virus Res. 55 (2000) 231–269.
[16] S. Takikawa, K. Ishii, H. Aizaki, T. Suzuki, H. Asakura, Y. Matsuura, T. Miyamura, Cell fusion activity of hepatitis C virus envelope proteins, J. Virol. 74 (2000) 5066–5074.
[17] K. Meyer, A. Basu, R. Ray, Functional features of hepatitis C virus glycoproteins for pseudotyped virus entry into mammalian cells, Virology 276 (2000) 214–226.
[18] W.C. Wimley, S.H. White, Experimentally determined hydrophobicity scale for proteins at membrane interfaces, Nat. Struct. Biol. 3 (1996) 842–848.
[19] I. Rodríguez-Crespo, E. Núñez, B. Yélamos, J. Gómez-Gutiérrez, J.P. Albar, D.L. Peterson, F. Gavilanes, Fusogenic activity of hepadnavirus peptides corresponding to sequences downstream of the putative cleavage site, Virology 261 (1999) 133–142.
[20] H. Ellens, J. Bentz, F.C. Szoka, pH-induced destabilization of phosphatidylethanolamine-containing liposomes: role of bilayer contact, Biochemistry 23 (1984) 1532–1538.
[21] N. Düzgünes, S.A. Shavnin, Membrane destabilization by N-terminal peptides of viral envelope proteins, J. Membr. Biol. 128 (1992) 71–80.
[22] D.K. Struck, D. Hoekstra, R.E. Pagano, Use of resonance energy transfer to monitor membrane fusion, Biochemistry 20 (1981) 4093–4099.
[23] R. Mendelsohn, H.H. Mantsch, Fourier transform infrared studies of lipid–protein interaction, in: A. Watts, A. DePond (Eds.), Progress in Protein-Lipid Interactions, Elsevier Science Publisher, New York, 1986, pp. 103–146.
[24] D.J. Moffatt, H.H. Mantsch, Fourier resolution enhancement of infrared spectral data, Methods Enzymol. 210 (1992) 192–200.
[25] W.K. Surewicz, H.H. Mantsch, D. Chapman, Determination of protein secondary structure by Fourier transform infrared spectroscopy: a critical assessment, Biochemistry 32 (1993) 389–394.
[26] I. Echabe, J.A. Encinar, J.L.R. Arrondo, Removal of spectral noise in the quantitation of protein structure through infrared band decomposition, Biospectroscopy 3 (1997) 469–475.
[27] J.L. Nieva, T. Suárez, Hydrophobic-at-interface regions in viral fusion protein ectodomains, Biosci. Rep. 20 (2000) 519–533.
[28] T. Suárez, W.R. Gallaher, A. Aguirre, F.M. Goñi, J.L. Nieva, Membrane interface-interacting sequences within the ectodomain of the human immunodeficiency virus type 1 envelope glycoprotein: putative role during viral fusion, J. Virol. 74 (2000) 8038–8047.
[29] J. Kyte, R.F. Doolittle, A simple method for displaying the hydropathic character of a protein, J. Mol. Biol. 157 (1982) 105–132.
[30] N. Düzgünes, T.M. Allen, J. Fedor, D. Papahadjopoulos, Lipid mixing during membrane aggregation and fusion: why fusion assays disagree, Biochemistry 26 (1987) 8435–8442.
[31] J.L. Arrondo, A. Muga, J. Castresana, F.M. Goñi, Quantitative studies of the structure of proteins in solution by Fourier-transform infrared spectroscopy, Prog. Biophys. Mol. Biol. 59 (1993) 23–56.
[32] M. Flint, J.M. Thomas, C.M. Maidens, C. Shotton, S. Levy, W.S. Barclay, J.A. McKeating, Functional analysis of cell surface-expressed hepatitis C virus E2 glycoprotein, J. Virol. 73 (1999) 6782–6790.
[33] A.T. Yagnik, A. Lahm, A. Meola, R.M. Roccasecca, B.B. Ercole, A. Nicosia, A. Tramontano, A model for the hepatitic C virus envelope glycoprotein E2, Proteins 40 (2000) 355–366.
[34] E.I. Pécheur, J. Sainte-Marie, A. Bienvenüe, D. Hoekstra, Peptides and membrane fusion: towards an understanding of the molecular mechanism of protein-induced fusion, J. Membr. Biol. 167 (1999) 1–17.
[35] D. Papahadjopoulos, M. Moscarello, E.H. Eylar, T. Isac, Effects of proteins on thermotropic phase transitions of phospholipids membranes, Biochim. Biophys. Acta 401 (1975) 317–335.

[36] M.B. Ruiz-Argüello, F.M. Goñi, F.B. Pereira, J.L. Nieva, Phosphatidylinositol-dependent membrane fusion induced by a putative fusogenic sequence of ebola virus, J. Virol. 72 (1998) 1775–1781.
[37] C.E. Larsen, S. Nir, D.R. Alford, M. Jennings, K.-D. Lee, N. Düzgünes, Human immunodeficiency virus type 1 (HIV-1) fusion with model membranes: kinetic analysis and the role of lipid composition, pH and divalent cations, Biochim. Biophys. Acta 1147 (1993) 223–236.
[38] M.L. Bosch, P.L. Earl, K. Fargnoli, S. Picciafuoco, F. Giombini, F. Wong-Staal, G. Franchini, Identification of the fusion peptide of primate immunodeficiency viruses, Science 244 (1989) 694–697.
[39] M. Pritsker, J. Rucker, T.L. Hoffman, R.W. Doms, Y. Shai, Effect of nonpolar substitutions of the conserved Phe11 in the fusion peptide of HIV-1 gp41 on its function, structure, and organization in membranes, Biochemistry 38 (1999) 11359–11371.
[40] J.M. White, Membrane fusion, Science 258 (1992) 917–924.
[41] L. Zhang, H.P. Ghosh, Characterization of the putative fusogenic domain in vesicular stomatitis virus glycoprotein G, J. Virol. 68 (1994) 2186–2193.
[42] S.E. Delos, J.M. Gilbert, J.M. White, The central proline of an internal viral fusion peptide serves two important roles, J. Virol. 74 (2000) 1686–1693.
[43] S.E. Delos, J.M. White, Critical role for the cysteines flankining the internal fusion peptide of avian sarcoma/leukosis virus envelope glycoprotein, J. Virol. 74 (2000) 9738–9741.
[44] F.A. Rey, F.X. Heinz, C. Mandl, C. Kunz, S.C. Harrison, The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution, Nature 375 (1995) 291–298.
[45] M. Hsu, J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C.M. Rice, J.A. Mckeating, Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 7271–7276.
[46] O. Samuel, Y. Shai, Participation of two fusion peptides in measles virus-induced membrane fusion: emerging similarity with other paramyxoviruses, Biochemistry 40 (2001) 1340–1349.
[47] S.G. Peisajovich, O. Samuel, Y. Shai, Paramyxovirus F1 protein has two fusion peptides: implications for the mechanism of membrane fusion, J. Mol. Biol. 296 (2000) 1353–1365.
[48] J. Bentz, N. Düzgünes, S. Nir, Kinetics of divalent cation induced fusion of phosphatidylserine vesicles. Correlation between fusogenic properties and binding affinities, Biochemistry 22 (1983) 3320–3330.