The Membrane-Active Regions of the Hepatitis C Virus E1 and E2 Envelope Glycoproteins†

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ABSTRACT: We have identified the membrane-active regions of the full sequences of the HCV E1 and E2 envelope glycoproteins by performing an exhaustive study of membrane leakage, hemifusion, and fusion induced by 18-mer peptide libraries on model membranes having different phospholipid compositions. The data and their comparison have led us to identify different E1 and E2 membrane-active segments which might be implicated in viral membrane fusion, membrane interaction, and/or protein−protein binding. Moreover, it has permitted us to suggest that the fusion peptide might be located in the E1 glycoprotein and, more specifically, the segment comprised by amino acid residues 265−296. The identification of these membrane-active segments from the E1 and E2 envelope glycoproteins, as well as their membranotropic propensity, supports their direct role in HCV-mediated membrane fusion, sustains the notion that different segments provide the driving force for the merging of the viral and target cell membranes, and defines those segments as attractive targets for further development of new antiviral compounds.

Hepatitis C virus (HCV)[1] is a small, enveloped RNA virus that belongs to the genus *Hepacivirus* in the family Flaviviridae (which also comprises the genera *Flavivirus* and *Pestivirus*) and is the leading cause of acute and chronic liver disease in humans, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (*1*, *2*). There exists no vaccine to prevent HCV infection, and current therapeutic agents such as pegylated interferon and ribavirin, apart from being associated to different adverse effects, have limited success against HCV (*3*). Therefore, the development of effective treatments is mandatory to control HCV infection.

The HCV genome is a 9.6 kb long single-stranded RNA molecule of positive polarity that is translated into a polyprotein of approximately 3010 amino acids (*4*), flanked by 5′ and 3′ untranslated regions (Figure 1). The structural proteins include the core, which forms the viral nucleocapsid, and the envelope glycoproteins E1 (gp35) and E2 (gp70), both of them transmembrane proteins. The structural proteins are separated from the nonstructural proteins by the short membrane peptide p7, thought to be a viroporin. The HCV genome shows a remarkable sequence variation since more than 90 genotypes distributed into six main types and subtypes have been identified (*5*, *6*). The relationship between HCV sequence variability and liver disease status or resistance to current antivirals is unclear and likely is multifactorial (*7*). HCV is thought to adopt a classical icosahedral scaffold in which its two envelope glycoproteins, E1 and E2, are anchored to the host-cell-derived double-layer lipid envelope. E1 and E2 are thought to play pivotal roles at different steps of the HCV replicative cycle. There is now strong evidence that they are essential for host-cell entry, binding to receptor(s), and inducing fusion with the host-cell membrane as well as in viral particle assembly (*8*). These distinct functions imply that the envelope proteins adopt markedly different conformations and that the latter must be controlled tightly to occur at the appropriate phases of the replicative cycle. E1 and E2 are type I transmembrane (TM) glycoproteins, with N-terminal ectodomains and a short C-terminal TM domain. These proteins interact with each other and assemble as noncovalent heterodimers, and their TM play a major role in the E1−E2 heterodimer formation, membrane anchoring, and endoplasmic reticulum retention (*9*−*12*). Interestingly, the E2 glycoprotein has two hypervariable regions (HVR1 and HVR2) which could function as a decoy to help the virus escape the immune system (*1*). Like other viral envelope proteins involved in host-cell entry, HCV envelope proteins are thought to induce fusion between the viral envelope and a host-cell membrane. The HCV envelope glycoproteins E1 and E2 are thought to be class II fusion proteins because the putative fusion peptide is supposedly localized in an internal sequence linked by antiparallel β-sheets; moreover, proteomic computational analyses suggest that HCV envelope glycoprotein E1 and

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[1] Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; CF, 5-carboxyfluorescein; Chol, cholesterol; DPX, *p*-xylenebispyridinium bromide; E1, gp35 HCV envelope glycoprotein; E2, gp70 HCV envelope glycoprotein; HCV, hepatitis C virus; HVR, hypervariable region; LUV, large unilamellar vesicles; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; N-RhB-PE, lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; PA, egg L-α-phosphatidic acid; PC, egg L-α-phosphatidylcholine; PE, egg trans-sterified L-α-phosphatidylethanolamine; PI, bovine brain L-α-phosphatidylinositol; PS, bovine brain L-α-phosphatidylserine; SM, egg sphingomyelin; TM, transmembrane domain.

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pestivirus envelope glycoprotein E2 are truncated class II fusion proteins (*13*).

Although much information has been gathered in recent years, we do not know the processes which give place to membrane fusion. The mechanism by which proteins facilitate the formation of fusion intermediates is a complex process involving several segments of fusion proteins (*14*, *15*). These regions, either directly or indirectly, might interact with biological membranes, contributing to the viral envelope and cell membrane merging. The existence of a quasi-species population appears to hamper the development of vaccines against HCV and favor the perpetuation of the virus in the organism. It is well-known that highly variable infectious agents can induce persistent infections, probably through generation of escape mutants (*16*), so that HCV infection evolves to chronicity in the majority of infected patients (*17*). The inability of the immune system to eliminate this virus has been ascribed to lack of immune recognition, insufficient $CD4^+$ T cell help, induction of unresponsiveness in HCV-specific T cells, immune deviation, and activation of incomplete immune cell functions (*18*, *19*). There are still many questions to be answered regarding the E1 and E2 mode of action in accelerating membrane fusion, and moreover, HCV membrane entry is an attractive target for anti-HCV therapy. To investigate the structural basis of HCV membrane fusion and identify new targets for searching new fusion inhibitors, we have carried the analysis of the different regions of HCV E1 and E2 envelope glycoproteins which might interact with phospholipid membranes using an approach similar to that used for studying HIV gp41 and SARS-CoV S proteins (*20*, *21*), i.e., the identification of membrane-active regions of HCV E1 and E2 envelope glycoproteins by determining the effect on membrane integrity, hemifusion, and fusion of 18-mer E1 and E2 glycoprotein-derived peptide libraries. By monitoring the effect of these peptide libraries on membrane integrity, we have identified different regions on the HCV E1 and E2 envelope glycoproteins with membrane-interacting capabilities, suggest the possible location of the fusion domain plus other zones which might be implicated in oligomerization (protein−protein binding), and therefore might help in the understanding of the molecular mechanism of membrane merging as well as making possible the future development of HCV entry inhibitors which may lead to new vaccine strategies.

MATERIALS AND METHODS

Materials and Reagents. Egg L-α-phosphatidylcholine (PC), egg sphingomyelin (SM), egg trans-sterified L-α-phosphatidylethanolamine (PE), egg L-α-phosphatidic acid (PA), bovine brain L-α-phosphatidylserine (PS), bovine brain L-α-phosphatidylinositol (PI), liver lipid extract (a 2:1 chloroform:methanol extract of liver tissue), and cholesterol (Chol) were obtained from Avanti Polar Lipids (Alabaster, AL). 5-Carboxyfluorescein (CF) (>95% by HPLC), calcein, and sodium dithionite were from Sigma-Aldrich (Madrid, Spain). Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (N-RhB-PE), *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE), 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), and *p*-xylenebispyridinium bromide (DPX) were obtained from Molecular Probes Inc. (Eugene, OR). Three sets of 18-mer peptides derived from the E1 envelope glycoprotein (26 peptides) and from the E2 envelope glycoprotein (52 peptides) from 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). All other reagents used were of analytical grade from Merck (Darmstadt, Germany). Water was deonized, twice distilled, and passed through Milli-Q equipment (Millipore Ibérica, Madrid, Spain) to a resistivity better than 18 MΩ cm.

Sample Preparation. 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 O_2-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 mM Tris-HCl, 20 mM NaCl, 40 mM CF, and 0.1 mM EDTA, pH 7.4, was used (*20*), whereas for assays of vesicle leakage at pH 5.4, buffer containing 20 mM citrate, 20 mM NaCl, and 40 mM calcein, pH 5.4, was used (*22*). To obtain multilamellar vesicles, 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 multilamellar vesicles by the extrusion method (*20*) using polycarbonate filters with a pore size of 0.1 μm (Nuclepore Corp., Cambridge, CA). Breakdown of the vesicle membrane leads to content leakage, i.e., CF or calcein fluorescence. Nonencapsulated CF or calcein was separated from the vesicle suspension through a Sephadex G-75 filtration column (Pharmacia, Uppsala, Sweden) eluted with buffer containing either 10 mM Tris-HCl, 100 mM NaCl, and 0.1 mM EDTA, pH 7.4, or 20 mM citrate, 100 mM NaCl, and 0.1 mM EDTA, pH 5.4.

For assays of lipid mixing, 1 mL of buffer (10 mM Hepes, 100 mM NaCl, pH 7.4) was added to the dry phospholipid mixture (containing either 0.6 mol % NBD-PE and N-RhB-PE or 0.12 mol % NBD-PE and N-RhB-PE, or no probes), and multilamellar vesicles were obtained by vortexing at room temperature. Large unilamellar vesicles (LUV) were prepared from multilamellar vesicles by the extrusion method as above, using polycarbonate filters with a pore size of 0.2 μm (Nuclepore Corp., Cambridge, CA). The use of 0.2 μm pore-size filters gives place to larger liposomes and henceforth greater fluorescence intensity per surface unit. For assays of inner monolayer lipid mixing, the sample preparation was similar to lipid mixing, but sodium dithionite was added to preformed LUVs labeled with NBD-PE and N-RhB-PE at a final concentration of 100 mM. After 1 h incubation in ice, the dithionite was removed from the liposomes by size exclusion chromatography (*23*). For assays of vesicle fusion using the ANTS/DPX method (*21*), 1 mL of buffer (5 mM Hepes, pH 7.4, and either 40 mM NaCl and 25 mM ANTS or 40 mM NaCl and 90 mM DPX or 20 mM NaCl plus 12.5 mM ANTS and 45 mM DPX) was added to the dry phospholipid mixture, and multilamellar vesicles were obtained by vortexing at room temperature. Large unilamellar vesicles (LUV) were prepared from multilamellar vesicles by the extrusion method using polycarbonate filters with a pore size of 0.1 μm (Nuclepore Corp., Cambridge, CA). Nonencapsulated ANTS and DPX were separated from the

vesicle suspension through a Sephadex G-75 filtration column (Pharmacia, Uppsala, Sweden) eluted with buffer containing 5 mM Hepes and 100 mM NaCl, pH 7.4.

All peptides were dissolved in buffer, but only those peptides which presented solubility problems were dissolved in 5% DMSO. In all cases, no aggregation was visible after DMSO addition. The phospholipid concentration was measured by methods described previously (*24*).

Membrane Leakage Measurement. Leakage of intraliposomal CF or calcein was assayed by treating the probe-loaded liposomes (final lipid concentration, 0.125 mM) with the appropriate amounts of peptide on microtiter plates stabilized at 25 °C using a microplate reader (FLUOstar, BMG Labtech, Germany), each well containing a final volume of 170 μL. The medium in the microtiter plates was continuously stirred to allow the rapid mixing of peptide and vesicles. Leakage was measured at an approximate peptide-to-lipid molar ratio of 1:15. Changes in fluorescence intensity were recorded with excitation and emission wavelengths set at 492 and 517 nm, respectively. One hundred percent release was achieved by adding Triton X-100 to a final concentration of 0.5% (w/w) to the microtiter plates. Fluorescence measurements were made initially with probe-loaded liposomes and afterward by adding peptide solution and finally adding Triton X-100 to obtain 100% leakage. Leakage was quantified on a percentage basis according to the equation

$$\% \text{ release} = \frac{F_f - F_0}{F_{100} - F_0} \times 100$$

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

Hemifusion (Outer Monolayer Phospholipid-Mixing) Measurement. Peptide-induced vesicle lipid mixing (hemifusion) was measured by resonance energy transfer (*25*). This assay is based on the decrease in resonance energy transfer between two probes (NBD-PE and N-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 %. Liposomes were prepared as described above. Labeled and unlabeled vesicles in a proportion 1:4 were placed in a 5 mm × 5 mm fluorescence cuvette at a final lipid concentration of 0.1 mM in a final volume of 400 μL, stabilized at 25 °C under constant stirring. The fluorescence was measured using a Varian Cary Eclipse spectrofluorometer, using 467 and 530 nm for excitation and emission, respectively. Excitation and emission slits were set at 10 nm. Since labeled and unlabeled 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

$$\% \text{ phospholipid mixing} = \frac{F_f - F_0}{F_{100} - F_0} \times 100$$

F_f being the value of fluorescence obtained 15 min 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.

Membrane Fusion Measurements. Fusion of membrane vesicles was assayed using two methods, the ANTS/DPX fusion assay (*21*) and the dithionite fusion assay (*23*). For the ANTS/DPX fusion assay, LUVs containing 25 mM ANTS were mixed with LUVs containing 90 mM DPX in a 1:1 (mol/mol) portion. LUVs were placed in a 10 mm × 10 mm fluorescence cuvette at a final lipid concentration of 0.1 mM in a final volume of 1 mL, stabilized at 25 °C under constant stirring. The fluorescence was measured using a Varian Cary Eclipse spectrofluorometer, using 353 and 520 nm for excitation and emission, respectively. Excitation and emission slits were set at 10 nm. Membrane fusion was quantified on a percentage basis according to the equation

$$\% \text{ fusion} = 100 - \left(\frac{F_f - F_0}{F_{100} - F_0} \times 100\right)$$

F_f being the value of fluorescence obtained 15 min after peptide addition to a mixture of LUVs containing 25 mM ANTS plus LUVs containing 90 mM DPX, F_0 the initial fluorescence of the vesicles, and F_{100} is the fluorescence value of an independent LUV preparation in which the internal probe concentration was 12.5 mM ANTS and 45 mM DPX. Peptide-induced phospholipid mixing of the inner monolayer was measured by a modification of the hemifusion assay (*23*). LUVs were treated with sodium dithionite to completely reduce the NBD-labeled phospholipid located at the outer monolayer of the membrane. The final concentration of sodium dithionite was 100 mM (from a stock solution of 1 M dithionite in 1 M Tris-HCl, pH 10.0), and the mixture was 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 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA, pH 7.4. The proportion of labeled and unlabeled vesicles, lipid concentration, and other experimental and measurement conditions were the same as indicated above.

Hydrophobic Moments, Hydrophobicity, and Interfaciality. The scale for calculating hydrophobic moments was taken from Engelman et al. (*26*). Hydrophobicity and interfacial values, i.e., whole residue scales for the transfer of an amino acid of an unfolded chain into the membrane hydrocarbon palisade and the membrane interface, respectively, have been obtained from http://blanco.biomol.uci.edu/hydrophobicity_scales.html (*27*, *28*). To detect membrane partitioning and/or membrane interacting surfaces along the whole E1 and E2 envelope glycoproteins, two-dimensional plots of the hydrophobic moments, hydrophobicity, and interfaciality have been obtained, taking into consideration the arrangement of the amino acids in the space and assuming an α-helical structure (for a detailed explanation of how to obtain the two-dimensional plots, see ref *20*). Positive values represent positive bilayer-to-water transfer free energy values, and therefore, the higher the value, the greater the probability to interact with the membrane surface and/or hydrophobic core.

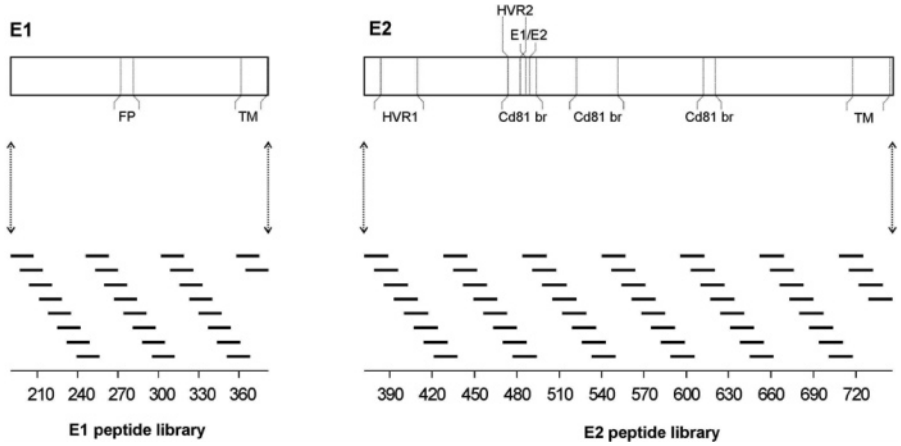

FIGURE 1: Scheme of the structure of the HCV E1 and E2 envelope glycoproteins according to literature consensus. The important supposed functional regions are highlighted, i.e., the putative fusion domain (FP), the transmembrane domain (TM), the hypervariable regions (HVR), the CD81 binding regions (CD81br), and the E1/E2 interaction domain (E1/E2). The sequence and relative location of the 18-mer peptides derived from HCV envelope glycoproteins E1 (26 peptides) and E2 (52 peptides) are shown with respect to the sequence of both proteins. Maximum overlap between adjacent peptides is 11 amino acids.

RESULTS

The peptide libraries we have used in this study and their correlation with the HCV E1 and E2 envelope glycoprotein sequences are shown in Figure 1, where it can be observed that the 18-mer peptide libraries include the whole sequence of both glycoproteins. Since two and three consecutive peptides in the library have an overlap of 11 and 4 amino acids, respectively, it seems reasonable to think on peptide-defined regions as we will present below.

Membranotropic Surfaces in E1 and E2. The E1 envelope glycoprotein is thought to be responsible for the membrane fusion process whereas the E2 envelope glycoprotein is thought to mediate the binding to the host cell, although other roles could not be ruled out (*29*, *30*). Several hydrophobic patches have been identified in both E1 and E2 proteins which might be important, not only for modulating membrane binding and interaction but also for protein−protein interaction (*29*, *31*, *32*). To detect surfaces along the E1 and E2 envelope glycoprotein sequences which might be identified as membrane-partitioning and/or membrane-interacting zones which might be related to either tertiary or quaternary structures or both, we have plotted the average surface hydrophobic moment, hydrophobicity, and interfaciality versus the amino acid sequence of the E1 and E2 glycoprotein sequences of HCV_1B4J (the data obtained for the HCV_1AH77 strain are nearly identical), supposing it adopts an α-helical structure along the whole sequence (Figure 2; see ref *20*). Although the E1 and E2 HCV glycoproteins are supposed to be class II membrane fusion proteins and therefore their α-helix content should not be as high as that of class I membrane fusion proteins, it gives us a depiction of the potential surface zones that could possibly be implicated in membranotropic action (*20*). As observed in Figure 2, the existence of different regions with large hydrophobic moment values along both HCV E1 and E2 envelope glycoproteins is readily evident. These sequences should show comparable capability to partition and/or interact with membranes and should be biologically functional in their roles. In addition, there are other regions which show patches of large hydrophobic moment, hydrophobicity, and/or interfaciality values. These patches of positive hydrophobicity and interfaciality along the surface of the protein could favor the interaction with other similar patches along these or other proteins as well as with the surface of the membrane. By observing these data, it would be possible to detect three and six distinct highly positive regions in E1 and E2, respectively (see Figure 2). In the case of E1, these regions would be comprised by amino acid residues 260−294, 316−336, and 344−382, whereas for E2 these regions would be comprised by amino acid residues 385−404, 436−446, 485−509, 548−572, 667−691, and 702−745. These data would suggest that these regions could show a tendency to partition into membranes and/or interact with the membrane surface; however, it should not be ruled out that some areas corresponding to the HCV E1 and E2 envelope glycoproteins could also be responsible for the interaction with other proteins or even between both of them as has been suggested previously (*32*). The generation of hydrophobic-rich surfaces along the structure of the HCV E1 and E2 envelope glycoproteins emphasizes that the actual distribution of hydrophobicity and interfaciality, i.e., structure-related factors, along E1 and E2 would affect the biological function of these sequences.

Membrane Rupture. In the first instance, we have studied the effect of the 18-mer peptide libraries derived from the HCV_1B4J E1 and E2 envelope glycoproteins and two different pH values, 7.4 and 5.4, on membrane rupture, i.e., leakage, for six different liposome compositions (Figures 3 and 4); the lipidic composition of the model membranes has been PC/Chol at a molar ratio of 5:1, PC/SM at a molar ratio of 5:1, PC/SM/Chol at a molar ratio of 5:1:1, PC/SM/Chol at a molar ratio of 26:9:15, a synthetic lipid mixture resembling the hepatocyte plasma membrane consisting of PC/PS/PI/SM/Chol at a molar ratio of 51:2.4:5.3:7.48:33.42 (*33*), and a lipid extract of liver membranes (containing 42% PC, 22% PE, 7% Chol, 8% PI, 1% LPC, and 21% neutral lipids).

When the 18-mer peptides from the E1 library were assayed on PC/Chol liposomes at pH 7.4, some peptides exerted an important leakage effect (Figure 3A, upper panel). The most remarkable effects were observed for peptides 14 and 18, which produced leakage values of about 20−25%. At pH 5.4 (Figure 3A, lower panel), only peptides 18 and

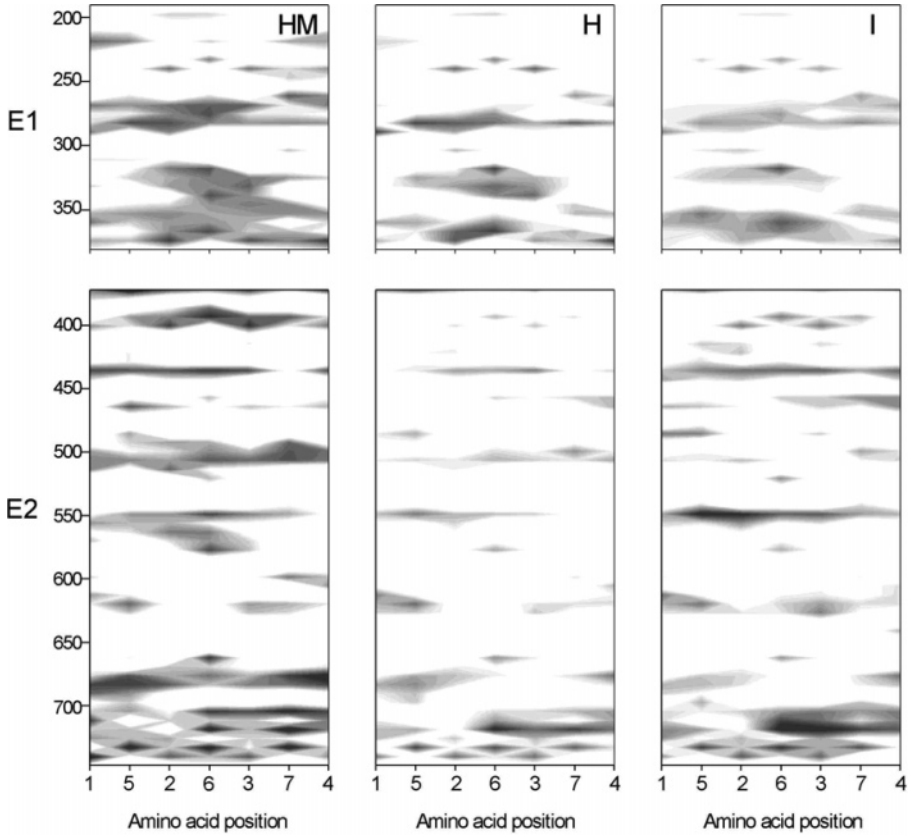

FIGURE 2: Hydrophobic moment, hydrophobicity, and interfaciality distribution of HCV E1 and E2 envelope glycoproteins assuming they form an α-helical wheel. The hydrophobic moment, hydrophobicity, and interfaciality plots show only positive bilayer-to-water transfer free energy values. The residue numbers are indicated at the left, whereas column numbers define amino acid positions (see ref *28*).

24 showed relevant leakage values (about 15−23%). When liposomes composed of PC/SM at pH 7.4 were tested, the leakage pattern was different (Figure 3B, upper panel), since peptide 13 was the only one which showed a significant leakage effect (about 60%). When these liposomes were tested at pH 5.4 (Figure 3B, lower panel), peptides 18 and 19 were the ones that showed major leakage values (about 25−30%). The same peptides which, at pH 7.4, showed major effects on PC/Chol liposomes had a similar effect on PC/SM/Chol liposomes at a molar ratio of 5:1:1, but their leakage values were in general slightly higher (Figure 3C, upper panel). These were peptides 2, 14, 18, and 24 (leakage values of about 25−30%). At pH 5.4, peptides 14, 18 (about 60−65% leakage), and 19 (about 45% leakage) presented major values compared to the other ones (Figure 3C, lower panel). When liposomes composed of PC/SM/Chol at a molar ratio of 26:9:15 at pH 7.4 were assayed (Figure 3D, upper panel), peptides 14, 15, 21, 25, and 26 presented leakage values of about 10−15%. At pH 5.4 a similar pattern was found (Figure 3D, lower panel), since peptide 18 elicited a leakage value of about 20%, whereas peptides 2, 14, 19, 20, 24, and 25 showed leakage values of about 10−15%. When liposomes resembling the hepatocyte plasma membrane were assayed at pH 7.4, a completely different pattern was obtained (Figure 3E, upper panel), since peptide 25 was the only one which showed a major leakage value (about 20%). At pH 5.4 (Figure 3E, lower panel) peptides 2, 8, and 25 showed relatively higher leakage values (about 10−13%), but there was an ample region ranging from peptide 1 to peptide 11 which showed leakage values of about 5−7%. For liposomes composed of a lipid extract of liver membranes at pH 7.4 (Figure 3F, upper panel), two peptides, 14 and 18, showed significant leakage values (about 65−70%), but peptides 19, 20, and 24−26 also showed major leakage values (about 15−30%). At pH 5.4 (Figure 3F, lower panel) peptides 18, 20, and 24 showed leakage values of about 30−40% whereas peptides 11, 14, 25, and 26 showed leakage values of about 20−22%.

Similarly to what was found above for E1, when the peptide library from E2 was assayed on PC/Chol liposomes at pH 7.4, some peptides induced a major effect on leakage but others did not (Figure 4A, upper panel). In this case, peptides 10, 35, and 36 showed major leakage values (about 60−80%), whereas peptides 5, 16, 17, 34, 36, 37, and 49 induced lower but important leakage values (about 15−30%). At pH 5.4, lower absolute leakage values were found, but the pattern was similar to what was found at pH 7.4 (Figure 4A, lower panel). When liposomes composed of PC/SM at pH 7.4 were used, the pattern was very similar to that found for PC/Chol liposomes (Figure 3B, upper panel), since peptides 10 and 35 were the ones which produced the major leakage values (about 40−50%). Peptides 5, 36, and 49 induced lower leakage values (about 20−25%) but significant when compared to the other ones (Figure 3B, upper panel). At pH 5.4, the absolute leakage values were higher than those found at pH 7.4, but the leakage pattern was also very similar (Figure 4B, lower panel). When leakage was assayed on PC/SM/Chol liposomes at molar ratios of 5:1:1 and 26:9:15 and pH 7.4, it was interesting to find a pattern similar to what was described above (see Figure 4C,D, upper panels). In this case, peptides 10 and 35 were again the ones which induced the higher leakage values, whereas peptides 5, 16, 17, 25,

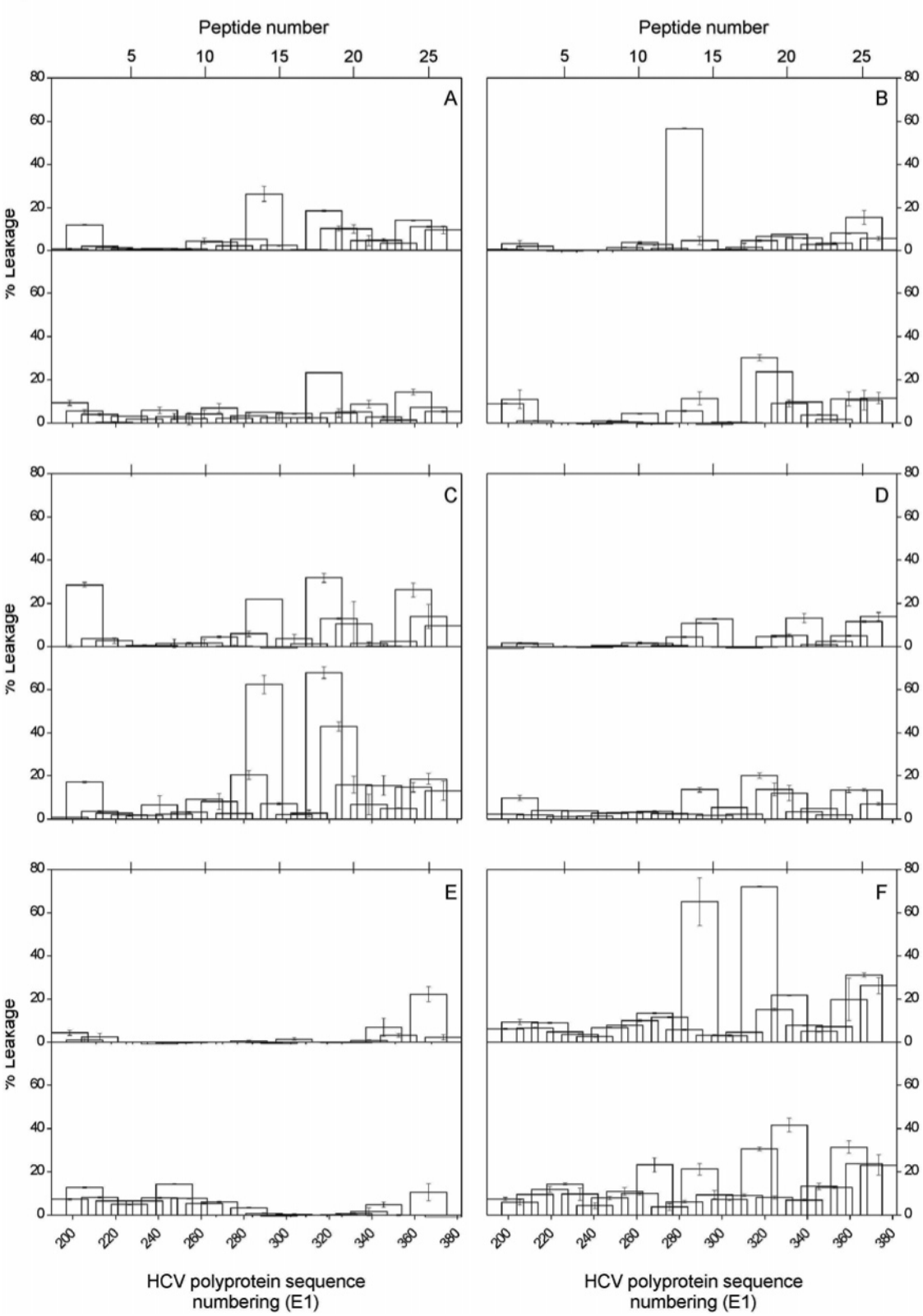

FIGURE 3: Effect of the 18-mer peptides derived from the E1 envelope glycoprotein of HCV_1B4J on the release of LUV contents for different lipid compositions and two different pH values, 7.4 and 5.4 (upper and lower panels, respectively). Leakage data for LUV composed of (A) PC/Chol at a phospholipid molar ratio of 5:1, (B) PC/SM at a phospholipid molar ratio of 5:1, (C) PC/SM/Chol at a phospholipid molar ratio of 5:1:1, (D) PC/SM/Chol at a phospholipid molar ratio of 26:9:15, (E) synthetic hepatocyte plasma membrane, and (F) lipid extract of liver membranes. Vertical bars indicate standard deviations of the mean of triplicate samples.

34, and 35 induced lower but important leakage values. It should be noted that peptide 49 induced a major leakage effect on liposomes composed of PC/SM/Chol at a molar ratio of 26:9:15 whereas it did not show any effect on liposomes composed of PC/SM/Chol at a molar ratio of 5:1:1 (Figure 4C,D, upper panels). At pH 5.4, a similar pattern was again found, since peptides 10 and 35 were the ones which exerted the higher leakage effect, although peptides

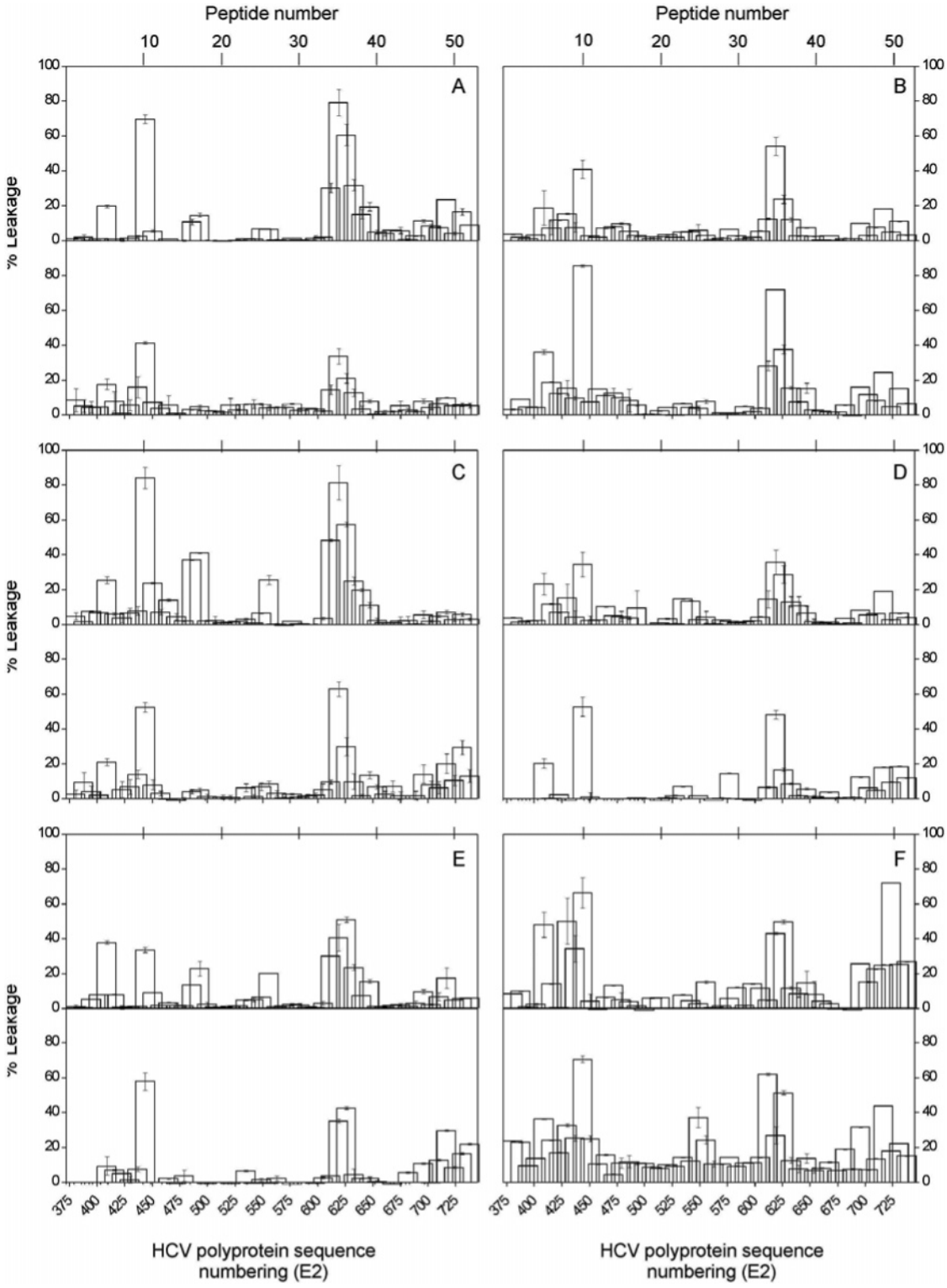

FIGURE 4: Effect of the 18-mer peptides derived from the E2 envelope glycoprotein of HCV_1B4J on the release of LUV contents for different lipid compositions and two different pH values, 7.4 and 5.4 (upper and lower panels, respectively). Leakage data for LUV composed of (A) PC/Chol at a phospholipid molar ratio of 5:1, (B) PC/SM at a phospholipid molar ratio of 5:1, (C) PC/SM/Chol at a phospholipid molar ratio of 5:1:1, (D) PC/SM/Chol at a phospholipid molar ratio of 26:9:15, (E) synthetic hepatocyte plasma membrane, and (F) liver extract of liver membranes. Vertical bars indicate standard deviations of the mean of triplicate samples.

5, 36, and 49−51 induced also relatively larger leakage values (Figure 4C,D, lower panels). It is interesting to note that peptides 16 and 17 did not show any effect at pH 5.4 but a major one at pH 7.4. When liposomes resembling the hepatocyte plasma membrane composition were assayed at pH 7.4, peptides 5, 10, 35, and 36 were the ones which induced a major leakage value (Figure 4E, upper panel). Other peptides which presented relevant leakage values were peptides 16, 17, 25, and 49. At pH 5.4 a similar pattern was found, except that no leakage activity was found between peptides 10 and 35 (Figure 4E, lower panel). The pattern which was obtained for liposomes composed of a lipid extract of liver membranes at pH 7.4 and 5.4 was indeed similar to those described above, since peptide groups 5−10, 34−36,

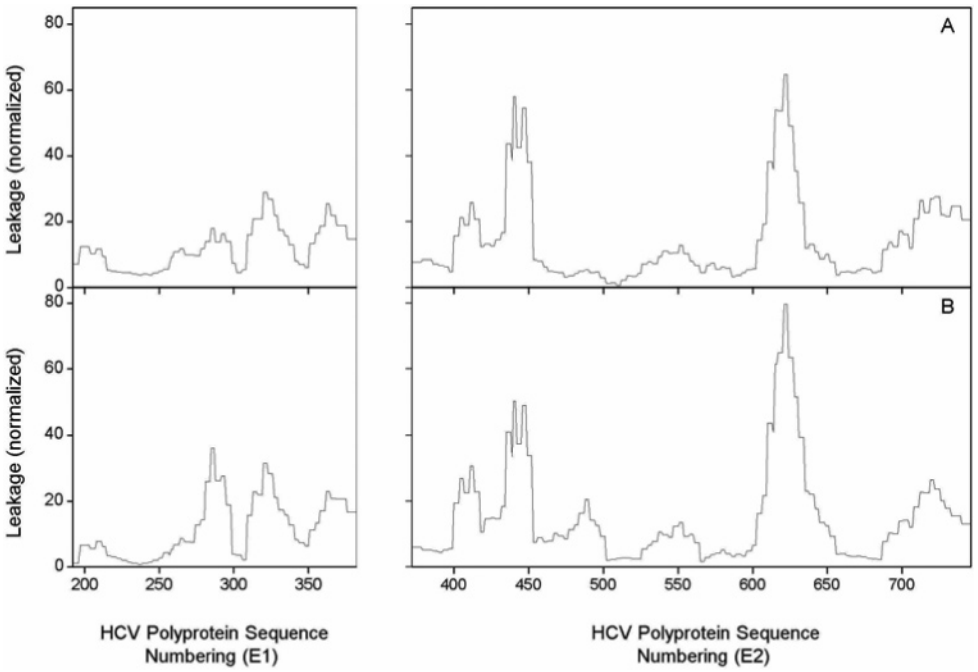

FIGURE 5: Summary of the normalized (percentage) experimental membrane rupture data (leakage) corresponding to the 18-mer peptide libraries derived from the HCV_1B4J envelope glycoproteins E1 and E2 at (A) pH 5.4 and (B) pH 7.4 for all model membranes studied.

49, and 50 were the ones which induced major leakage values (Figure 4F, upper and lower panels).

As was noted above, it seems reasonable to think on the combined effect of peptide groups or segments rather than on the effect of isolated peptides. In this context, leakage values shown in Figures 3 and 4 would define different protein segments with high relative leakage activity for both envelope glycoproteins E1 and E2. In Figure 5, we show the normalized experimental leakage data (the summation of all of the percentages of leakage for all model membranes) corresponding to the E1 and E2 derived 18-mer peptide libraries; as shown in the figure, defined leakage zones are clearly observed. For E1 and pH values 7.4 and 5.4, there are three zones having consistent relatively similar high leakage values which would approximately correspond to amino acid segments 274−298, 309−343, and 351−382. In a similar way, five zones are observed for E2 and pH 7.4, corresponding approximately to amino acid segments 394−452, 457−502, 531−565, 604−656, and 688−739. As was commented above, it is interesting to note that all of these segments show leakage at both pH values except segment 457−502 (Figure 5).

Hemifusion and Fusion. Perturbation of membranes is not sufficient to complete the process of viral and cellular membrane fusion, since it is also necessary for the merge of the monolayers and the stalk formation, according to the stalk model for membrane fusion (*34*). However, there is not a clear quantitative criterion to characterize fusion peptides using membrane destabilization (*14*). Therefore, we have also studied the effect of the peptide libraries derived from the E1 and E2 envelope glycoproteins on both membrane hemifusion and fusion using liposomes of different compositions (Figures 6 and 7). As has been shown previously (*35*), the energetic barrier for hemifusion and fusion is larger than for leakage (pore formation), so that peptides which might show hemifusion, and/or fusion effects in general induce leakage but peptides which induce leakage might not provoke hemifusion and/or fusion. At the same time, leakage values, as measured here, are usually higher than hemifusion and fusion ones. Because of that, we have used two E1 and E2 envelope glycoprotein 18-mer derived libraries from two different strains of HCV, namely, HCV_1B4J and HCV_1AH77, to define more precisely the membrane-active regions of both E1 and E2 envelope glycoproteins which elicit hemifusion and fusion effects. Moreover, these studies have been made at pH 7.4 since there were no significant differences on leakage at the two pHs studied (see above).

The hemifusion pattern which was found when the E1 libraries were assayed on PC/SM/Chol liposomes defined clearly the effect induced by peptide 13 (hemifusion values of about 15−20%) and to a lesser extent peptide 14 (Figure 6A). When liposomes composed of PC/PS/Chol were used, a dramatic effect on hemifusion was observed for peptide 14, since 100% hemifusion was attained for the HCV_1AH77 strain whereas about 55% was observed for the HCV_1B4J one (Figure 6B). Similarly high hemifusion values were found when PC/PI/Chol liposomes were used (Figure 6C), since about 80% hemifusion values were found for peptides 13 (strain HCV_1AH77) and 14 (strain HCV_1B4J). Smaller but significant hemifusion values were found for peptides 18/20 and 25/26 (about 15−20%). Similar results were also found when model membranes composed of a lipid extract of liver membranes were used (Figure 6D). In the case of the HCV_1AH77 strain, peptides 14 and 19 were the ones which elicited significant hemifusion values (about 50−70%), whereas peptide 13 was the one which induced high hemifusion values (about 65%) for the HCV_1B4J strain. When fusion was assayed on liposomes composed of PC/SM/Chol and a lipid extract of liver membranes using both HCV_1AH77 and HCV_1B4J E1 libraries, similar results to those found above were obtained, since peptides 13 and 14 were the ones which educed the higher fusion values (Figure 6E−H).

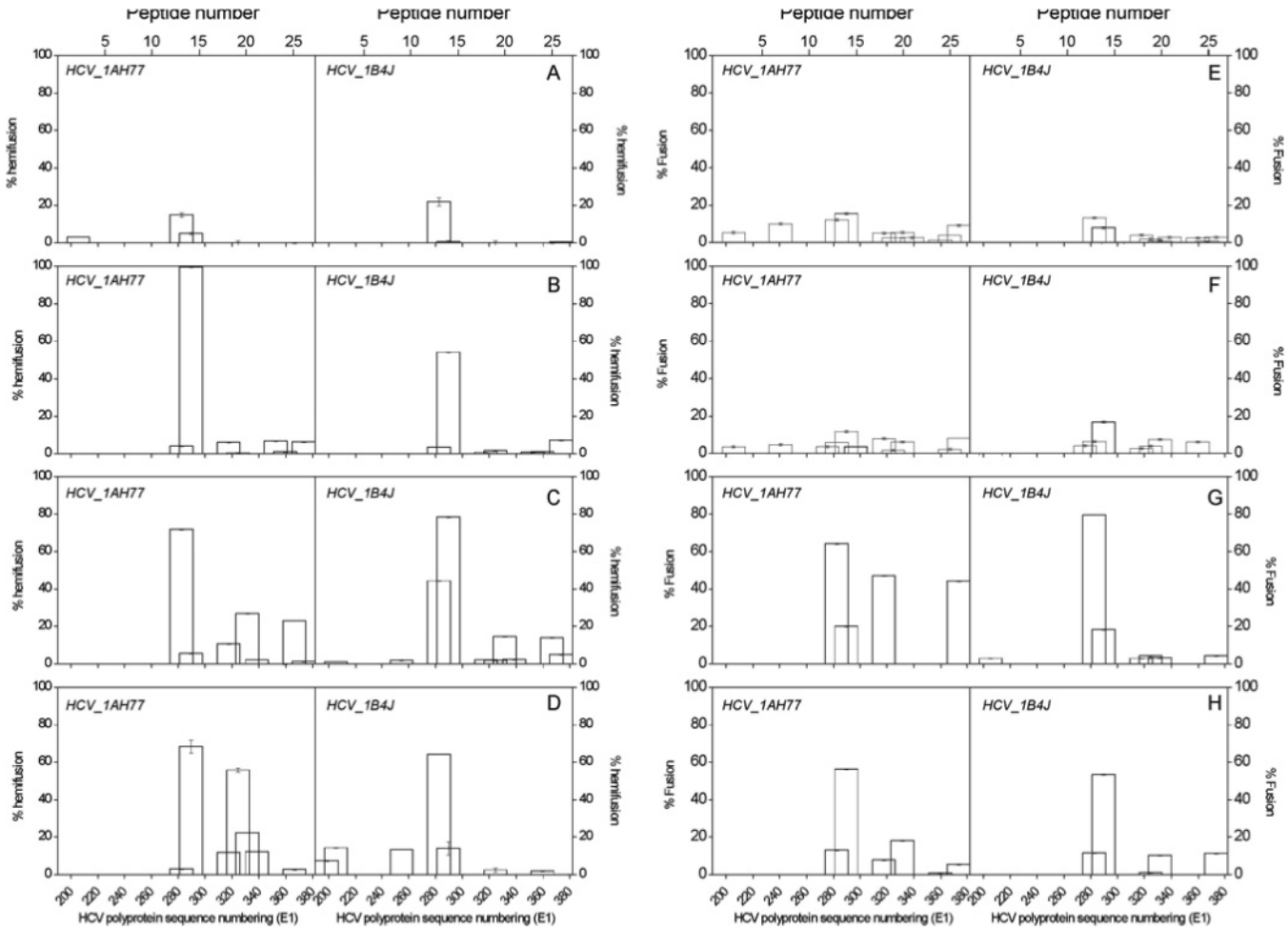

FIGURE 6: Effect of the 18-mer peptides derived from the HCV E1 envelope glycoprotein strains 1AH77 and 1B4J (left and right panels, respectively) on hemifusion (A−D) and fusion (E−H) for LUVs composed of (A, E, G) PC/SM/Chol at a phospholipid molar ratio of 5:1:1, (B) PC/PS/Chol at a phospholipid molar ratio of 5:4:1, (C) PC/PI/Chol at a phospholipid molar ratio of 5:4:1, and (D, F, H) lipid extract of liver membranes. Aqueous content fusion is shown in (E) and (F), whereas inner monolayer fusion is shown in (G) and (H). Note the different vertical scales. Vertical bars indicate standard deviations of the mean of triplicate samples.

Similarly to what was found above, when the envelope glycoprotein E2 derived libraries were assayed on PC/SM/Chol liposomes, a small number of peptides gave place to hemifusion (Figure 7A). In the case of the HCV_1AH77 library, peptide 9 was the one which induced the higher hemifusion value (about 7%) whereas from the HCV_1B4J library it was peptide 52 which induced the highest hemifusion (about 20%). When liposomes composed of PC/PS/Chol were used, peptide 36 from both HCV strains induced a relatively high hemifusion effect (Figure 7B), although peptide 10 from HCV_1B4J also elicited a major hemifusion value (about 30%). Peptide 10 from both strains was also the one which showed an important hemifusion effect (about 45−50%) when liposomes composed of PC/PI/Chol were used (Figure 7C); peptide 35 from HCV_1AH77 elicited also a major hemifusion value (about 30%). The number of peptides which induced hemifusion effects was augmented when liposomes were composed of a lipid extract of liver membranes (Figure 7D). In this case, peptides 6 and 9 (about 35−40%) from the HCV_1AH77 library and peptides 51 and 52 (about 15%) from the HCV_1B4J library were the ones which induced the higher hemifusion effects. When fusion was assayed on liposomes composed of PC/SM/Chol and a lipid extract of liver membranes, there were two main peptide groups which induced the higher relative hemifusion values (Figure 7E−H). These groups were formed by peptides 6−10 and 35−63, whose fusion values ranged from 10% to 40%.

Following the same reasoning which was followed above, hemifusion and fusion values presented in Figures 6 and 7 would define different protein segments with high relative hemifusion and fusion activities for both envelope glycoproteins E1 and E2. To compare and relate the experimental hemifusion and fusion data with the different membranotropic segments we found by studying the hydrophobicity and interfaciality along the full E1 and E2 sequences (Figure 2), we show in Figure 8A the normalized experimental hemifusion and fusion data corresponding to the E1 and E2 derived 18-mer peptide libraries. It is possible to observe defined hemifusion and fusion zones, similarly to what was found previously for the leakage experiments, matching the segments we found previously. For E1 three zones with high hemifusion and fusion values are found, corresponding to amino acid segments 268−298, 317−339, and 360−382 (Figure 8A), which also match the ones which were detected previously by assaying membrane rupture (see Figure 5). Similarly, five zones are found for E2, which approximately correspond to amino acid segments 419−453, 453−467, 541−558, 611−628, and 713−737 (Figure 8A).

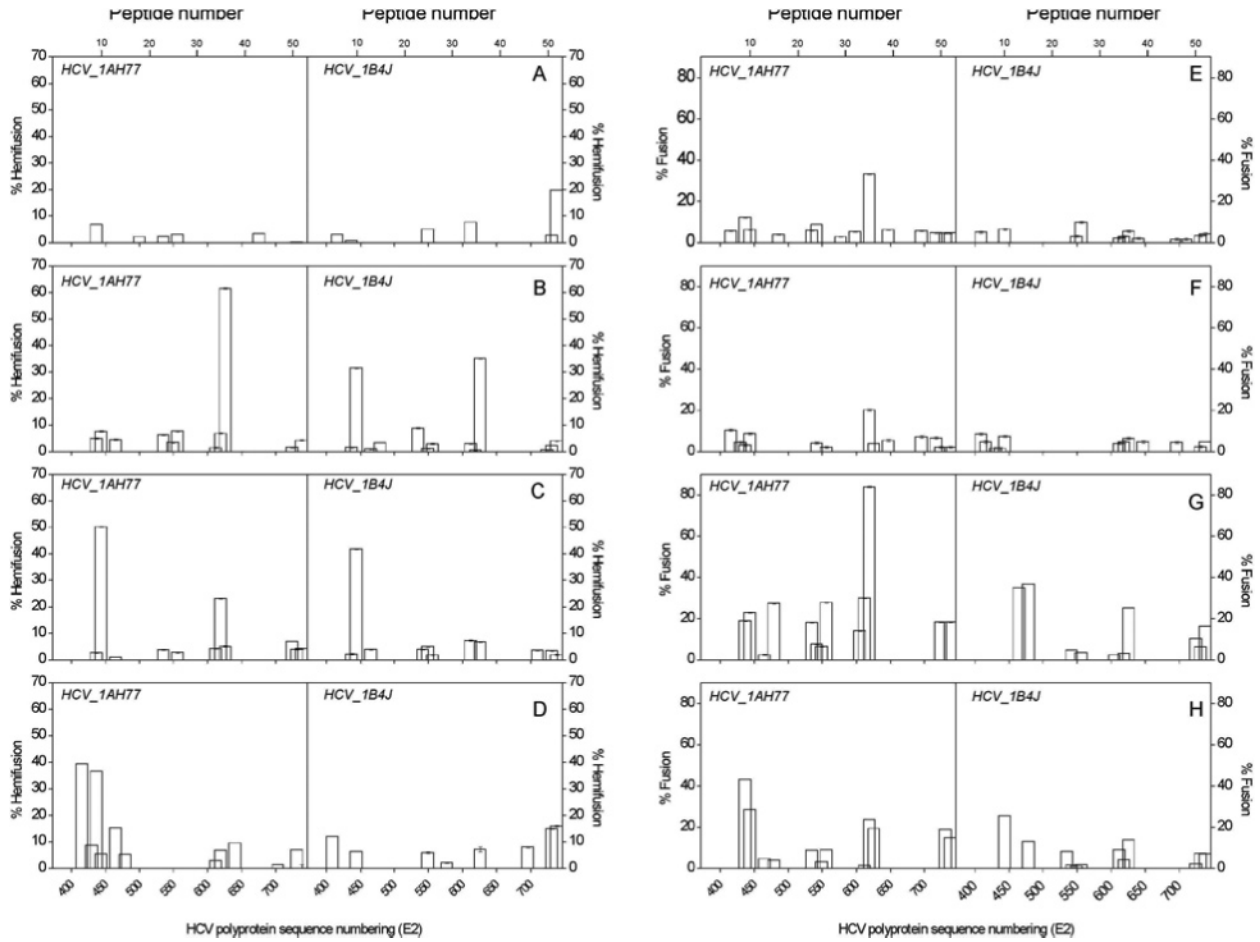

FIGURE 7: Effect of the 18-mer peptides derived from the HCV E2 envelope glycoprotein strains 1AH77 and 1B4J (left and right panels, respectively) on hemifusion (A−D) and fusion (E−H) for LUVs composed of (A, E, G) PC/SM/Chol at a phospholipid molar ratio of 5:1:1, (B) PC/PS/Chol at a phospholipid molar ratio of 5:4:1, (C) PC/PI/Chol at a phospholipid molar ratio of 5:4:1, and (D, F, H) liver extract of liver membranes. Aqueous content fusion is shown in (E) and (F), whereas inner monolayer fusion is shown in (G) and (H). Note the different vertical scales. Vertical bars indicate standard deviations of the mean of triplicate samples.

DISCUSSION

The fusion of viral and cellular membranes, the critical early events in viral infection, are mediated by class I and class II envelope fusion glycoproteins located on the outer surface of the viral membranes (*14*, *36*, *37*). Whereas class I membrane fusion proteins possess a fusion peptide at or near the amino terminus, a pair of extended α-helices, and, generally, a cluster of aromatic amino acids proximal to a hydrophobic transmembrane domain, class II fusion proteins possess an internal fusion peptide stabilized by cysteine linkages and different domains comprised mostly of antiparallel *β*-sheets (*38*). Although their three-dimensional structure is different, their function is identical, i.e., fusion between two opposing lipid bilayers. Accumulated evidence suggests that the mechanism of membrane fusion induced by class I and class II membrane fusion proteins is similar and, in addition, requires the concerted action of different membranotropic segments of the implicated proteins (*13*, *38*, *39*). It is known that both HCV E1 and E2 envelope glycoproteins are essential for receptor binding, host-cell entry, and membrane fusion, but their specific role(s) in the different processes of the viral life cycle remain(s) uncharacterized (*8*, *29*). The E1/E2 heterodimer is thought to be the functional unit of the HCV spike and low pH would induce its dissociation leading to homooligomerization of the active form of the fusion protein (*40*). However, there is some controversy about the identity of the HCV fusion protein, either E1 or E2 or both. It was first proposed that E1 might be a good candidate, because the ectodomain of E1 might contain a putative fusion peptide (residues 265−287) (*13*, *41*); however, structural homology with other fusion proteins suggested that E2 could be the fusion protein (*29*, *32*).

Both E1 and E2 envelope glycoproteins possess different segments with significant properties. For example, it has been proposed that E1 could be capable of adopting a polytopic form, since, apart from containing the C-terminal membrane-spanning domain (residues 331−381), it contains a potential transmembrane one (residues 270−291) (*42*, *43*). This segment has also been suggested to be the fusion peptide (*13*, *41*). E2 comprises a receptor-binding domain which is connected to the transmembrane hydrophobic domain (residues 718−746) via a highly conserved sequence (residues 675−699), which seems to be necessary for heterodimerization with the E1 glycoprotein. E2 carries also regions of extreme hypervariability; while the most variable region, HVR-1, is located within the N-terminal part of E2 (residues 384−411), the HVR-2 region resides in the 476−480 segment (*44*). Another important E2 segment which determines the correct folding and subunit aggregation is comprised within residues 525−660 (*30*); different parts of this segment have also been implicated in CD81 binding (*32*).

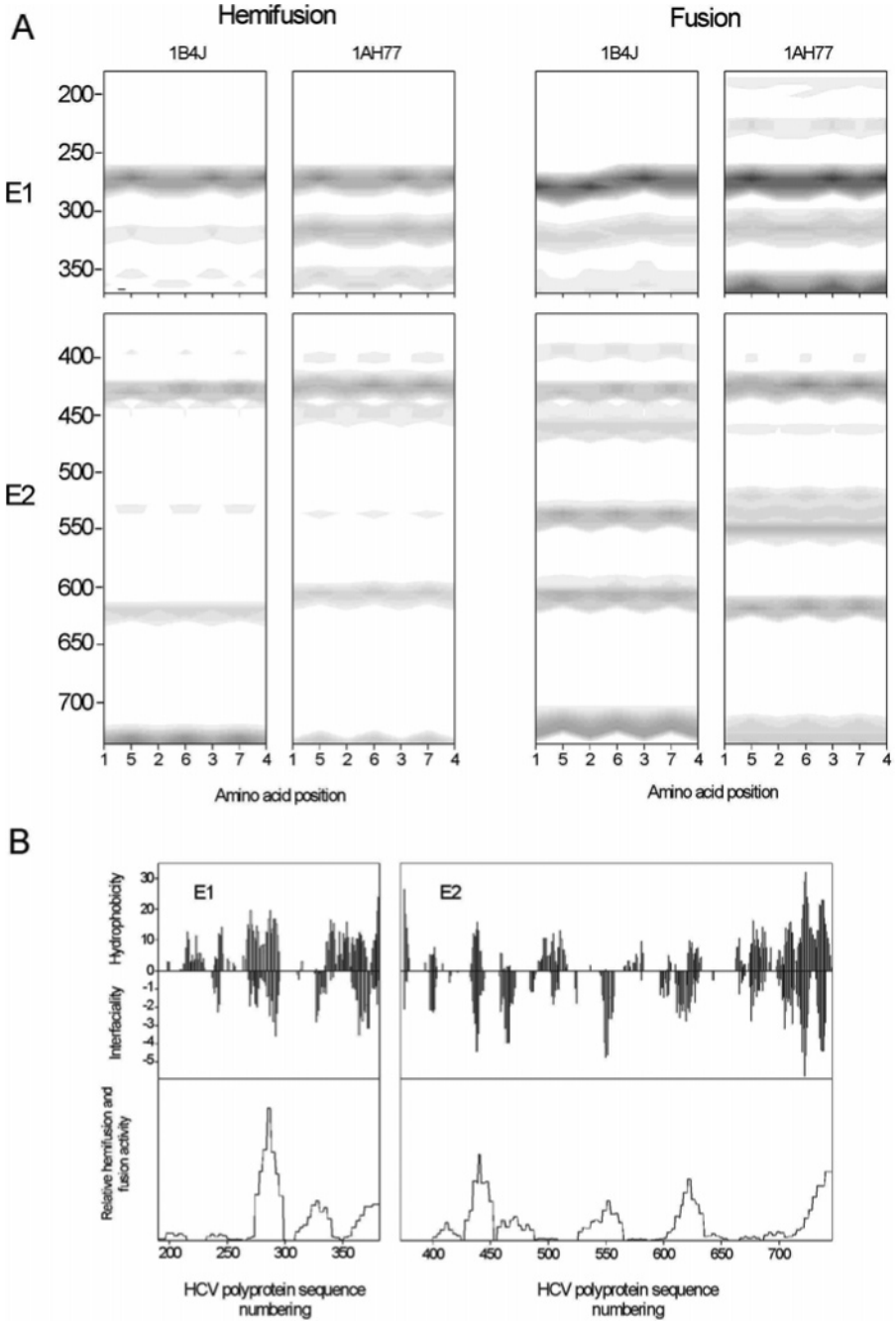

FIGURE 8: (A) Summary of the normalized (percentage) experimental membrane rupture data corresponding to the 18-mer peptide libraries derived from the envelope glycoproteins E1 and E2 corresponding to the HCV_1B4J and HCV_1AH77 strains for all model membranes studied (the darker, the higher the value). The residue numbers are indicated at the left, whereas column numbers define amino acid positions (see Figure 2). (B) Analysis of the interfacial and hydrophobic distribution for the full sequence of the envelope glycoproteins E1 and E2 using a window of 15 amino acids according to the scales of Winley and White (see text) along with the normalized hemifusion and fusion experimental data shown in (A), highlighting the relationship between the theoretical and experimentally determined membrane-active regions.

Understanding the factors that may determine the specificity and stability of the metastable native and the stable fusogenic conformations of both class I and class II membrane fusion proteins, being located on the ectodomain or endodomain or both, is required to know the mechanism of viral membrane fusion and consequent viral entry into cells.

We have previously shown the existence of different segments with membrane-interacting capabilities in the HIV gp41 and SARS S1 and S2 glycoproteins (*20*, *21*). In this work we have made an exhaustive study of the effect on membrane integrity of a peptide scan corresponding to two peptide libraries from the HCV E1 and E2 envelope glycoproteins of two different HCV virus strains, as well as studied their effect not only on membrane rupture but also on membrane-specific approaches such as hemifusion and fusion. Although the use of peptide fragments might not mimic the properties of the intact protein, our results give us an indication of the relative propensity of the different domains to bind, interact, and affect membranes in relation to each other, i.e., help us to classify the different regions and segments of the HCV E1 and E2 envelope glycoproteins according to their effect in an ample representation of membrane systems. As apparent from the results described above, there are different peptides corresponding to different

regions which, depending on liposome composition, sequence, and origin, have different effects on the three membrane-related items studied. It is also true that some peptides which are very active in one model membrane system are much less active in other ones, and vice versa. However, it should be noted that membrane systems having different lipid compositions have diverse properties which make them behave in a significant different way (*20*). For example, 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 an important relationship between membrane fusion and Chol and SM membrane content for several viruses (*45*, *46*). In this context, it is interesting to note that it has recently been described that the presence of Chol facilitates the fusion of HCV pseudoparticles with the target membrane (*47*). Taking into account all of this information, we have gathered the membrane-interacting propensity of all its domains and therefore have an indication to the possible function of each segment of the HCV E1 and E2 envelope glycoproteins in each step of the fusion process. This information could provide us with an outline of the possible mechanism of membrane fusion as driven by this membrane fusion protein.

As we have shown above, different segments of both E1 and E2 present high positive values of hydrophobic moment, hydrophobicity, and interfaciality (Figure 2). It is very interesting to note that these segments match exceedingly well with segments showing major leakage and fusion values for all liposome types and conditions (Figures 5 and 8). As is already known, the fusion peptide is an essential factor in viral fusion proteins; however, there exists some controversy about its exact identification. The E1 envelope glycoprotein segment comprising residues 265−296 has a significant theoretical and experimental membranotropic effect as shown here and, therefore, should be the candidate to contain the fusion peptide domain. This sequence possesses a high degree of hydrophobicity and interfaciality, essential properties for membrane-interacting segments in proteins (Figure 8B). The other two membrane-active regions of E1, though showing less membranotropic activity than the first one, possess also a significant effect. Since the C-terminal transmembrane domain is comprised by residues 331−381, the segment comprising residues 310−331 could be involved in promotion of membrane destabilization, as well as pore formation and enlargement, in a manner similar to that of the pretransmembrane and/or loop domains of class I fusion proteins (*15*, *48*−*51*). Similarly to E1, there are different segments on E2, which show major positive values of hydrophobic moments, hydrophobicity, and interfaciality, that also show major leakage and fusion values. From the theoretical point of view, six zones could be identified (Figure 2) but five from experimental data (Figures 5 and 8). One of the most membranotropic regions of E2 is comprised by residues 715−746, which corresponds to the C-terminal transmembrane domain of the protein (Figure 8B). Consecutively to this one, we found two membranotropic regions comprised by residues 603−635 and 525−565, which have been implicated in folding and receptor binding. Following these, we found the segment comprised by residues 455−489 which includes the hypervariable HVR-2 region. Adjacent to this one we find the most membranotropic region of E2, that comprising residues 423−453. While this region is significantly more membranotropic than the other E2 regions, it is not as membrane active as region 265−296 from E1 (Figure 8B). If the fusion peptide were located in E2, this region should be the candidate to contain it. Similarly to what was noted above, the different membrane-active segments found in E2 could be involved in promotion of membrane destabilization, pore formation, and/or enlargement, probably in combination with other E1 regions. It is interesting to note that two significant regions of E2, the highly conserved sequence necessary for heterodimerization with E1 (residues 675−699) and the hypervariable HVR-1 region (residues 384−411), have no or very limited membranotropic activity (Figures 5 and 8A,B). This is in contrast with hydropathy analysis that revealed the highly hydrophobic domain of HVR-1 (*44*).

For membrane fusion to occur, fusion proteins must pull the viral and cellular membranes, create membrane defects, induce hemifusion and fusion, and, last but not least, induce pore formation, stabilization, and enlargement (*52*). The fusion domain is mainly responsible for the first steps of membrane fusion, but in the last instance other HCV E1 and E2 envelope glycoprotein segments, in combination with the previous one, drive and accomplish membrane fusion. The binding to the surface and the modulation of the phospholipid biophysical properties which take place when the HCV E1 and E2 envelope glycoprotein domains bind to the membrane could be related to the conformational changes which occur upon binding of the envelope glycoprotein to its receptors. The change in conformation and the possible formation of oligomeric forms in the presence of membranes could indicate the propensity of the protein to self-assemble and suggest that these changes might be part of the structural transition that transform HCV E1 and E2 envelope glycoproteins from the inactive to the active state, being most probably the dominant form during membrane fusion and, probably, driven by the interaction of the different segments indicated in this work. The destabilization of the phospholipid bilayer as a result of the interaction of the HCV E1 and E2 envelope glycoprotein segments would provide a way to lower the energy barrier necessary for fusion. The change in free energy associated with the structural changes taking place should be sufficient to cause phospholipid mixing and membrane fusion. In addition, the knowledge of all membrane-active regions of HCV E1 and E2 envelope glycoproteins could aid in the search for molecules which could revert or reduce the action of those specific membrane-active peptides; i.e., those molecules which inhibit peptide-induced membrane leakage, hemifusion, or fusion could be potential candidates as inhibitors of membrane fusion, as has been done successfully for class I fusion inhibitors.

CONCLUDING REMARKS

The HCV entry process is considered to be an attractive target for chemotherapeutic intervention, as blocking HCV entry into its target cell leads to suppression of viral infectivity, replication, and the cytotoxicity induced by virus-cell contacts. Therefore, the inhibition of membrane fusion by direct action on either E1 or E2 or both is increasing in importance as an additional approach either to combat directly against HCV infection or to prevent its spread. An understanding of the structural features of the fusogenic intermediates is essential because they are attractive drug

targets. The results reported in this work sustain the notion that different membranotropic segments of both HCV E1 and E2 provide the driving force for the merging of the viral and target cell membranes, as well as they could help us to develop inhibitors of HCV infection in a similar way as HIV peptide inhibitors have been generated.

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