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The membranotropic regions of the endo and ecto domains of HIV gp41 envelope glycoprotein

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Abstract

We have identified the membranotropic regions of the full sequence of the HIV gp41 envelope glycoprotein by performing an exhaustive study of membrane rupture, phospholipid-mixing and fusion induced by two 15-mer gp41-derived peptide libraries from HIV strains HIV_MN and HIV_consensus_B on model membranes having different phospholipid compositions. The data obtained for the two strains and its comparison have led us to identify different gp41 membranotropic segments in both ecto- and endodomains which might be implicated in viral membrane fusion and/or membrane interaction. The membranotropic segments corresponding to the gp41 ectodomain were the fusion domain, a stretch located on the N-heptad repeat region adjacent to the fusion domain, part of the immunodominant loop, the pre-transmembrane domain and the transmembrane domain. The membranotropic segments corresponding to the gp41 endodomain were mainly located at some specific parts of the previously described lentivirus lytic sequences. Significantly, the C-heptad repeat region and the Kennedy sequence located in the ectodomain and in the endodomain, respectively, presented no membranotropic activity in any model membrane assayed. The identification of these gp41 segments as well as their membranotropic propensity sustain the notion that different segments of gp41 provide the driving force for the merging of the viral and target cell membranes as well as they help us to define those segments as attractive targets for further development of new anti-viral compounds.

Keywords: Membrane fusion; gp41; HIV

1. Introduction

The attachment of enveloped viruses, such as human immunodeficiency (HIV)[1] virus, to cell membranes and the consequent membrane fusion, the critical early events in viral infection, are mediated by envelope glycoproteins located on the outer surface of the viral membrane [1–4]. In the case of HIV, the surface glycoprotein, initially synthesized as a highly glycosilated precursor, gp160, is endoproteolytically cleaved into the surface protein, gp120, that determines the viral tropism through the cellular surface receptors, and the transmembrane protein, gp41, which is responsible for the membrane fusion process. Both proteins gp120 and gp41 remain non-covalently associated forming trimers in their native prefusogenic state [1,2]. The native state of the HIV gp41 envelope protein is thought to be metastable by interacting with gp120 [4]. To accomplish the fusion and mixing of the viral and cellular contents, gp41 must undergo a complex series of conformational changes, apparently triggered by the attachment of gp120 to the CD4 primary receptor and the CCR5 or CXCR4 coreceptors of the target cell [5]. These conformational changes result in exposure of the fusion peptide and its insertion into the host membrane, followed by juxtaposition of viral and host cell membranes, and finally leading to activation of its membrane fusion properties [1,2,4,6].

Abbreviations: CF, 5-Carboxyfluorescein; Chol, Cholesterol; CHR, C-Terminal heptad repeat region; FP, Fusion peptide; HIV, Human immunodeficiency virus; LLP, Lentivirus lytic peptide; LUV, Large unilamellar vesicles; NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; NHR, N-Terminal heptad repeat region; 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-phosphadidylinositol; PS, Bovine brain L-α-phosphatidylserine; PTM, Pre-transmembrane; SM, Egg sphingomyelin; TM, Transmembrane

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The highly conserved gp41 sequence has been conventionally divided in three domains: the ectodomain, which contains the N-terminal fusion sequence, the C-terminal endodomain and the transmembrane spanning domain (TM) which connects both of them. The gp41 endodomain has been generally assumed to be entirely inside the virion, but it has been suggested recently that it is more complex than generally accepted and that part of its sequence could be exposed to the outer surface of the membrane [7,8]. The gp41 ectodomain contains different functional regions that are critical for membrane fusion (Fig. 1). At the N terminus of gp41, a stretch of about 15 hydrophobic residues, named the fusion peptide (FP), is believed to insert into and destabilize the membrane, thus facilitating viral and cell membrane fusion. Consecutive to the fusion peptide, two heptad repeat regions (NHR and CHR, respectively) have been identified. In the absence of lipid membranes, three NHR molecules fold into a central parallel triple-stranded α-helical coiled-coil, and wrapped anti-parallel on the outside of this core is an outer layer of three anti-parallel CHR α-helices, each pair of NHR/CHR molecules connected by the immunogenic loop that reverses the polypeptide chain. This trimeric helical hairpin structure is thought to form at a late stage during the membrane fusion process. The gp41 endodomain (see Fig. 1) presents highly-conserved segments with large hydrophobic moments and membrane-interacting capabilities, termed lentivirus lytic peptides (LLP) [9–11], as well as a highly-charged epitope named the Kennedy sequence which has been suggested to be part of an external loop structure [7,12]. The gp41 endodomain has been implicated in membrane association and in the multimerization of gp41 molecules [13].

The mechanism by which proteins facilitate the formation of fusion intermediates is a complex process involving several segments of fusion proteins [14,15]. Although it was initially believed that gp41 interacted with the membrane solely by means of the N-terminal fusion peptide and the transmembrane domain, it has been shown that different regions of the gp41 ecto- and endodomain bind and interact with the surface of model and natural-derived membranes [1,2,6,16–29]. If it were

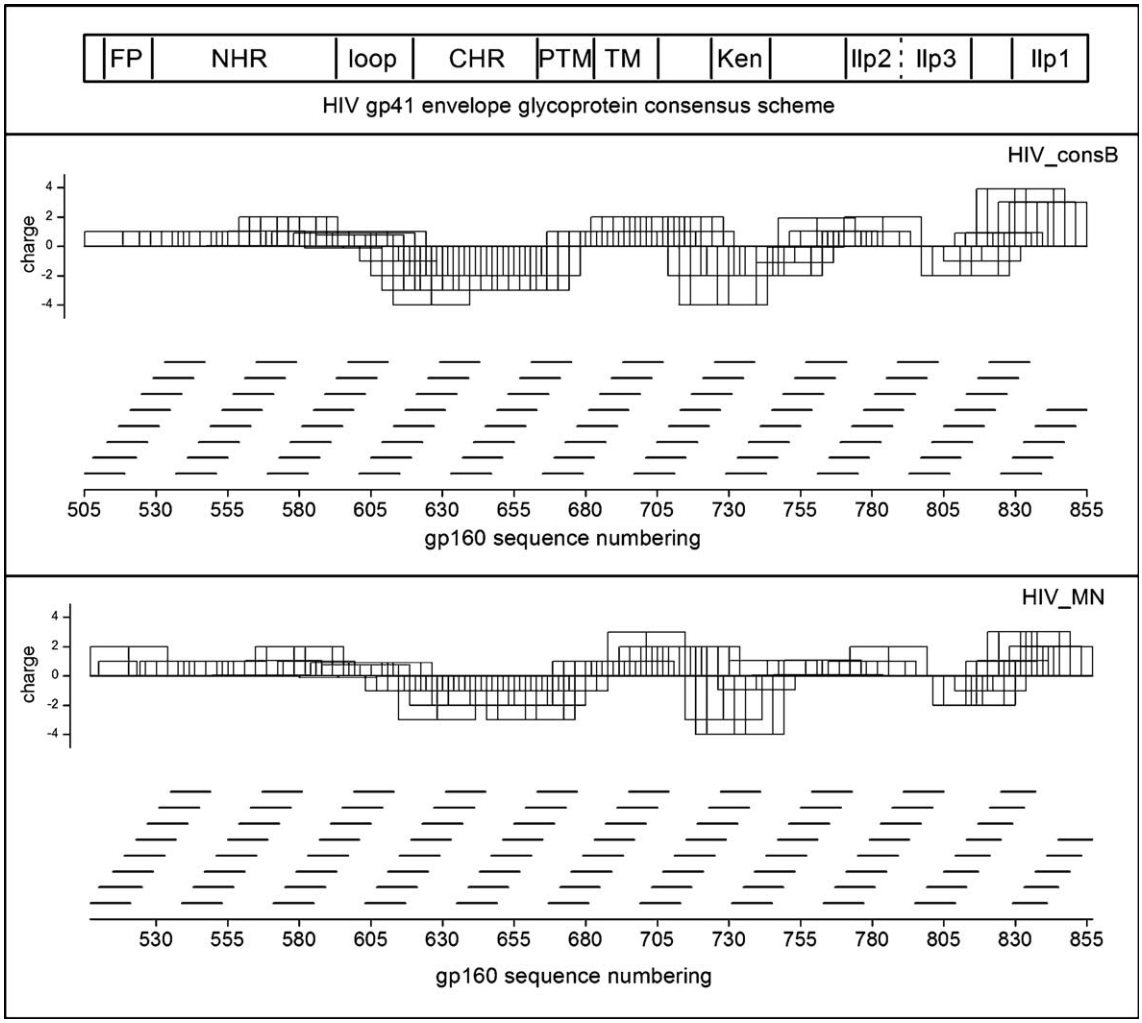


Fig. 1. Scheme of the structure of the gp41 envelope glycoprotein according to literature consensus. The important functional regions are highlighted, i.e., the fusion domain (FP), the N- and C-heptad repeats (NHR, CHR), the immunodominant loop, the pre-transmembrane domain (PTM), the transmembrane region (TM), the Kennedy sequence (Ken) and the lentivirus lytic peptide sequences (LLP). The residue numbers correspond to their positions in gp160 of each respective strain. The sequence and relative location of the [2×85] 15-mer gp41-derived peptide libraries from HIV_MN and HIV_consB used in this study are shown with respect to the sequence of the gp41 (horizontal lines) as well as their charge profile (height of each box) at pH 7.4. Maximum overlap between adjacent peptides is 11 amino acids.

true that some sequences of the gp41 endodomain could be exposed to the outside of the virion (the so-called micro-ectodomain), these segments could interact intra- or intermolecularly with other regions of the gp41 ectodomain (the so-called major-ectodomain, [8]). All these regions of the gp41 glycoprotein have the capabilities of binding and partitioning into the surface of phospholipid model membranes, changing their conformation and inducing the formation of transient non-lamellar structures, indicating that these segments could play an essential role in the viral fusion process. Although the structures of different segments of the HIV gp41 envelope glycoprotein have been known for several years, there are still many questions to be answered regarding its mode of action in membrane fusion. Moreover, viral membrane fusion continues to be an important topic of research since it serves as a model for cellular fusion events and it is an attractive target for therapeutic intervention. In this work, we have made an extensive study of the membrane interacting propensity of the full sequence, i.e., both ecto and endodomains, of HIV gp41 envelope glycoprotein by assaying the membrane rupture, phospholipid-mixing and fusion properties induced by two 15-mer gp41-derived peptide libraries from human immunodeficiency viruses HIV_MN and HIV_consensus_B [30]. The information obtained by performing three different but complementary membrane-dependent assays using peptides from two different HIV clones has led us to identify the different gp41 segments which might be implicated in viral membrane fusion and/or membrane interaction and suggest a general mechanism of viral-provoked membrane merging and fusion typified by Class I membrane fusion proteins.

2. Materials and methods

2.1. 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-phosphadidylinositol (PI), and cholesterol (Chol) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Lissamine™ rhodamine B, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (N-RhB-PE), and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) were obtained from Molecular Probes Inc. (Eugene, OR, USA). 5-Carboxyfluorescein, CF, (>95% by HPLC) was from Sigma-Aldrich (Madrid, ES, EU). Two sets (two batches each) of 85 peptides of 15 amino acids in length (Fig. 1) derived from the gp41 ectodomain of two different env strains, HIV-1MN Env (HIV_MN) and HIV-1 consensus subtype B Env (HIV_consB) [30], having 11-amino acid overlap between sequential peptides, were obtained through the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MD, USA). The purity of the peptides ranged from 75% to 85%. All other reagents used were of analytical grade from Merck (Darmstadt, GER, EU). Water was deionized, twice-distilled and passed through a Milli-Q equipment (Millipore Ibérica, Madrid, ES, EU) to a resistivity better than 18 MΩ cm.

2.2. 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, 1 ml of buffer (10 mM Tris–HCl, 20 mM NaCl, 40 mM CF, 0.1 mM EDTA, pH 7.4) was added to the dry phospholipid mixture and multilamellar vesicles were obtained by vortexing at room temperature. Large unilamellar vesicles (LUV) with a mean diameter of 90 nm were prepared from multilamellar vesicles by the extrusion method [31] using polycarbonate filters with a pore size of 0.1 μm (Nuclepore Corp., Cambridge, CA, USA). Breakdown of the vesicle membrane leads to contents leakage, i.e., CF fluorescence. Non-encapsulated CF was separated from the vesicle suspension through a Sephadex G-75 filtration column (Pharmacia, Uppsala, SW, EU) eluted with buffer containing 10 mM Tris, 100 mM NaCl, 0.1 mM EDTA, pH 7.4. The peptides were dissolved in buffer containing 5% DMSO. For assays of phospholipid-mixing and fusion either (1) 0.6% NBD-PE and RhB-PE or (2) 0.12% NBD-PE and RhB-PE or (3) no phospholipid probes plus 1 ml of buffer 10 mM HEPES, 100 mM NaCl, pH 7.4, buffer were added to the dry phospholipid mixture and multilamellar vesicles were obtained by vortexing at RT (probe percentages correspond to the final total phospholipid contents of the liposomes). Large unilamellar vesicles (LUV) were prepared from multilamellar vesicles by the extrusion method as above, but using polycarbonate filters with a pore size of 0.2 μm (Nuclepore Corp., Cambridge, CA, USA). The use of 0.2 μm pore-size filters gives rise to larger liposomes and henceforth greater fluorescence intensity per surface unit. The phospholipid concentration was measured by methods described previously [32].

2.3. Membrane leakage measurement

Membrane rupture (leakage) of intraliposomal CF was assayed by treating the probe-loaded liposomes (final lipid concentration, 0.125 mM) with the appropriate amounts of peptide on microtiter plates stabilized at 25 °C using a microplate reader (FLUOstar, BMG Labtech, GER, EU), each well containing a final volume of 170 μl. The medium in the microtiter plates was continuously stirred to allow the rapid mixing of peptide and vesicles. Leakage was measured at an approximate peptide-to-lipid molar ratio of 1:15. 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 0.05% (w/w) to the microtiter plates. Fluorescence measurements were made initially with probe-loaded liposomes, afterwards by adding peptide solution and finally adding Triton X-100 to obtain 100% leakage. Leakage was quantified on a percentage basis according to the equation,

$$\%\text{Release} = \frac{F_t - F_0}{F_{100} - F_0} \times 100$$

F_f being the equilibrium value of fluorescence 15 min after peptide addition, F_0 the initial fluorescence of the vesicle suspension and F_{100} the fluorescence value after addition of Triton X-100.

2.4. Phospholipid-mixing measurement

Peptide-induced vesicle phospholipid-mixing was measured by resonance energy transfer [33]. This assay is based on the decrease in resonance energy transfer between two probes (NBD-PE and RhB-PE) when the lipids of the probe-containing vesicles are allowed to mix with lipids from vesicles lacking the probes. The concentration of each of the fluorescent probes within the liposome membrane was 0.6% with respect to the total phospholipid content. 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 spectrofluorimeter using 467 nm and 530 nm for excitation and emission, respectively. Excitation and emission slits were set at 10 nm. One hundred percent 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,

$$\%(\text{Phospholipid mixing}) = \frac{F_t - F_0}{F_{100} - F_0} \times 100$$

F_f being the value of fluorescence obtained at 15 min after peptide addition in the liposomes which contain 0.6% of each probes plus liposomes without probes, F_0 the initial fluorescence of the before vesicles and F_{100} is the fluorescence value of the liposomes which contain 0.12% of each probes.

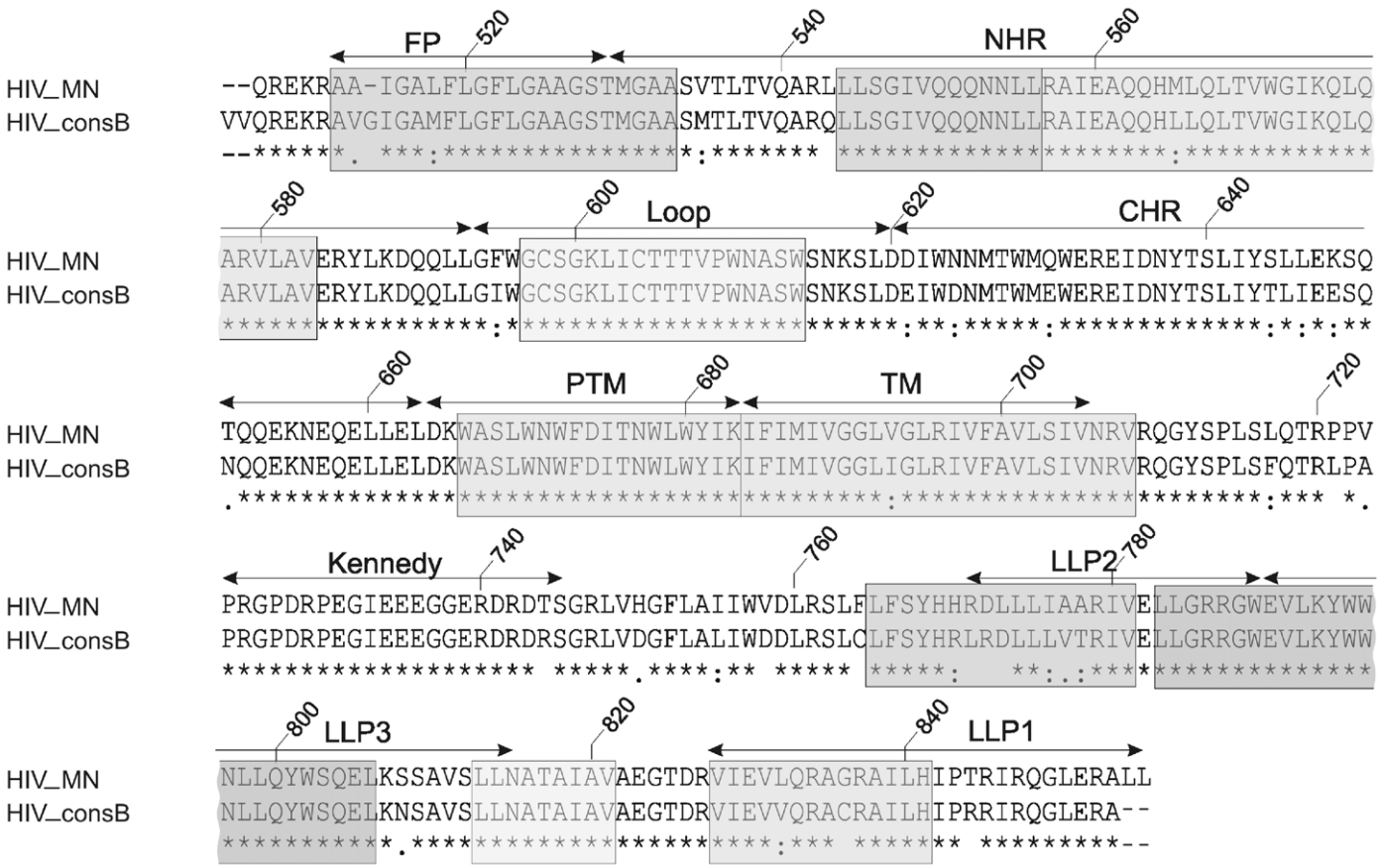
2.5. Inner-monolayer phospholipid-mixing (fusion) measurement

Peptide-induced phospholipid-mixing of the inner monolayer (fusion) was measured similarly to the phospholipid-mixing measurement stated above but using dithionite [34]. Liposomes, prepared as described above, were treated with sodium dithionite to completely reduce NBD-labeled phospholipid located at the outer monolayer of the membrane. Final concentration of sodium dithionite was 100 mM (from a stock solution of 1 M dithionite in 1 M Tris, pH 10.0) and incubated for approximately 1 h on ice in the dark. Sodium dithionite was then removed by size exclusion chromatography through a Sephadex G-75 filtration column (Pharmacia, Uppsala, Sweden) eluted with buffer containing 10 mM Tris, 100 mM NaCl, 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 previously.

3. Results

In order to detect all membranotropic regions of HIV gp41 envelope glycoprotein, we have determined the membrane interacting propensity for the complete sequence of the gp41 glycoprotein including both ecto- and endodomains. We have assayed the membrane rupture, phospholipid-mixing and fusion properties on model membranes induced by two different peptide libraries of 15 amino acids in length with 11-amino acid overlap between sequential peptides derived from the gp41 envelope glycoprotein of human immunodeficiency viruses HIV_MN and HIV_consB. Whereas the use of model membranes with different lipid compositions permit us to distinguish and grade the membranotropic effect elicited by the peptides, the use of two different HIV clones (see Table 1 for the full sequence of the peptides used in this work) permits us to correlate the observed membranotropic effect between two different but related sequences and suggest a general pattern of membranotropic regions for HIV gp41 envelope glycoprotein. The [2×85] peptides we have used in this study, shown in Fig. 1, extend from the N-terminal part of the protein, including the fusion domain (FP), the N-heptad repeat domain (NHR), the immunodominant loop, the C-heptad repeat domain (CHR), the pre-transmembrane domain (PTM), the transmembrane domain (TM), to the C-terminal tail, including the Kennedy and lentivirus peptide sequences (LLP1/3).

Table 1
ClustalW alignment of the gp41 env glycoprotein sequence from HIV-1MN and HIV-1 Cons B, indicating the differences in amino-acid positions, the different domains as well as the most membrane-active regions (the darker the shade of grey, the greater effect)



The sequences represent all the peptides studied in this work. The residue numbers correspond to their positions in gp160 of each respective strain.

3.1. Membrane rupture

When peptides from the HIV_MN gp41-derived peptide library were assayed on liposomes containing PC/Chol at a molar ratio of 5:1, some peptides showed a major leakage effect, but other ones hardly exerted any effect (Fig. 2A). The most notable effect was observed for peptides from the LLP2/3 sequences, which produced leakage values of about 65%, as well as peptides corresponding to the loop region (about 50% leakage). Other peptides induced less but important leakage, such as peptides coming from the FP region (about 30%), from the NHR region (about 20%) and peptides coming from the PTM and TM regions (about 15%) (see Fig. 2A). It is interesting to note the leakage of about 10% observed for peptides corresponding to a region located in between the Kennedy and LLP2 sequences. Similarly to what was found using PC/Chol liposomes, some peptides from the HIV_MN gp41-derived peptide library interacted significantly with liposomes composed of PC/PE/Chol at a molar ratio of 5:3:1 (Fig. 2B). The most important effect was observed for peptides from the LLP2/3 sequences (about 30% leakage), as well as peptides from the loop region (about 40%). Although the general extent of leakage was lower than that found for PC/Chol liposomes, the same trend was observed, since the same peptides that showed a relatively major effect on PC/Chol liposomes were the ones that showed an effect on PC/PE/Chol liposomes. It should be noted that inclusion of phosphatidylethanolamine and/or Cholin membranes reduces peptide-induced membrane leakage as it has been shown previously [21,35,36]. The extent and trend of leakage observed on liposomes composed of PC/PS/Chol at a molar ratio of 5:4:1 (Fig. 2C) was similar to the trend found for both PC/Chol and PC/PE/Chol liposomes. The most important effect was again observed for peptides from the LLP2/3 sequences, which produced leakage values of about 60%; the extent of leakage observed for peptides corresponding to the other aforementioned regions was lower but significant (20–30% leakage). It is worth of noting that an isolated peptide corresponding to the LLP1 region showed about 15% leakage.

Similarly to what has been found above, some peptides from the gp41-derived peptide library from HIV_consB presented a major leakage effect on PC/Chol liposomes, but other ones did not (Fig. 2D). The most notable effect were induced by peptides corresponding to the LLP2/3 and LLP1 sequences (about 60% and 50% leakage, respectively). Other peptides induced smaller but important leakage values, such as peptides coming from the PTM/TM and the FP/NHR regions (Fig. 2D). It is also interesting to note that a peptide located between the Kennedy and LLP2 regions showed leakage values of about 25% (see Fig. 2D). In the case of liposomes composed of PC/PE/Chol at a molar ratio of 5:3:1 (Fig. 2E), the absolute leakage values observed were lower than in the case of the PC/Chol liposomes but they showed the same trend as above. In this case, peptides corresponding to the LLP2/3 and LLP1 sequences presented about 35% and 30% leakage, respectively. Other peptides induced smaller leakage values, such as peptides coming from the PTM/TM and the NHR regions (Fig. 2E). As it was found

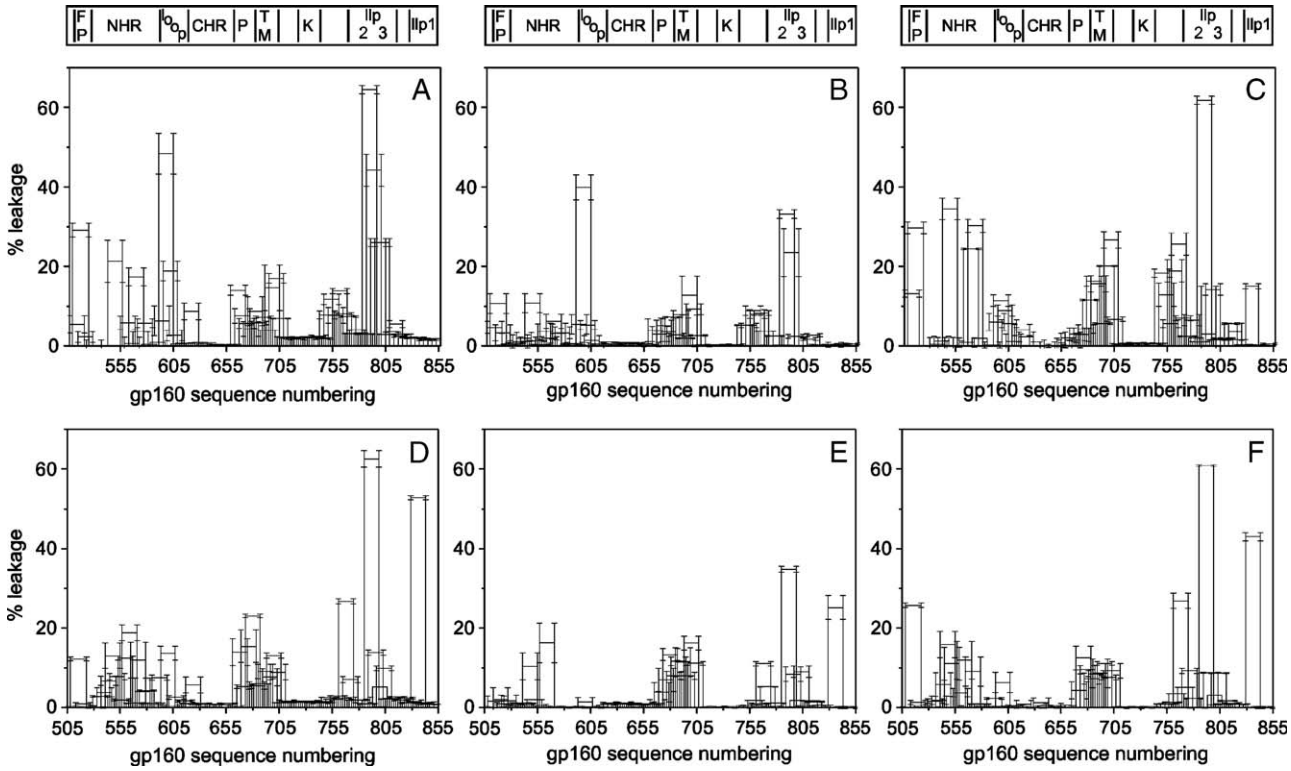


Fig. 2. Effect of the gp41-derived 15-mer peptides from (A–C) HIV_MN and (D–F) HIV_consB on the release of LUV contents for different lipid compositions at a lipid-to-peptide ratio of 15:1. LUVs were composed of (A, D) PC/Chol at a molar ratio of 5:1, (B, E) PC/PE/Chol at a molar ratio of 5:3:1 and (C, F) PC/PS/Chol at a molar ratio of 4:4:1. A scheme of the structure of the gp41 ectodomain according to literature consensus is shown at the top of each figure. Vertical bars indicate standard deviations of the mean of quadruplicate samples.

above, a peptide located in between the Kennedy and LLP2 regions showed a leakage value of about 10%. The extent and trend of leakage observed in liposomes composed of PC/PS/Chol at a molar ratio of 5:4:1 (Fig. 2F) was similar to the leakage values found for both PC/Chol and PC/PE/Chol liposomes. The most notable effect was observed for peptides corresponding to the LLP2/3 and LLP1 sequences (about 60% and 45% leakage, respectively). Other peptides induced smaller but definite leakage values, such as peptides coming from the PTM/TM, NHR and FP regions (Fig. 2F). It is interesting to note that two regions of the gp41 envelope glycoprotein, the CHR and the Kennedy regions, did not present any significant leakage on any of the liposomes tested. It should be recalled that these two regions have the most negative charge of all peptides tested (see Fig. 1).

It is known that the phospholipid profile of the HIV envelope membrane is different to that of the cell plasma membrane of the host cell, the most significant difference being its higher content of Chol and SM [37,38]. It is also important to note that Chol seems to be a specific requirement for HIV infection [39] as well as the PTM region of gp41 has been implicated in the specific interaction of the protein with Chol and SM containing membranes [40]. 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 enveloped viruses [41–44], suggesting that the interaction of gp41 with Chol and SM might play a role in the fusion process. Because of that, we have also studied the interaction of the HIV_MN and HIV_consB 15-mer gp41 envelope glycoprotein peptide libraries with model membranes having a variable Chol and SM composition, namely, PC/SM/Chol at molar ratios of 5:1:1, 26:9:15 and 37:18:45 (Fig. 3). If the ternary phase diagram which has been experimentally obtained for 1-palmitoyl,2-oleoyl-*sn*-glycero-phosphocholine, *N*-palmitoyl-*D*-sphingomyelin and Chol [45] were applicable to PC, SM, and Chol, the lipids used in this study, liposomes containing PC/SM/Chol at molar ratios of 5:1:1, 26:9:15 and 37:18:45 would present a high probability of raft formation.

When the gp41-derived peptide library corresponding to HIV_MN was assayed on liposomes composed of PC/SM/Chol at a molar ratio of 5:1:1, the most notable effect was observed on the loop domain of gp41, since nearly 90% leakage was observed (Fig. 3A). Significant leakage was observed for peptides corresponding to the LLP2/3 sequences (about 60% leakage) as well as peptides corresponding to the NHR/ PTM regions and Kennedy and LLP2 sequences (about 35–45% and 30% leakage, respectively). Significantly, low leakage values of about 10% were observed for the FP domain on these model membranes (Fig. 3A). The most important effect when the gp41-derived peptide library from HIV_consB was assayed on these liposomes was found on

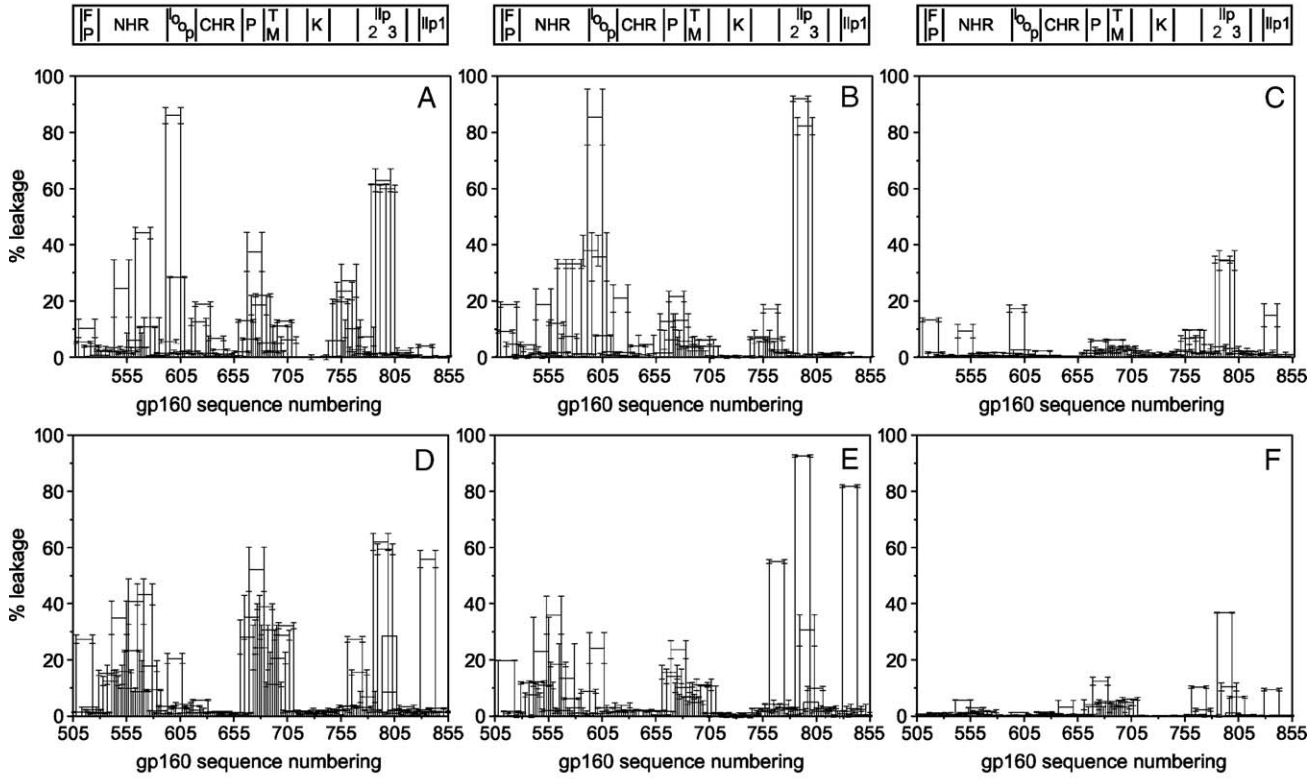


Fig. 3. Effect of the gp41-derived 15-mer peptides from (A–C) HIV_MN and (D–F) HIV_consB on the release of LUV contents for different lipid compositions at a lipid-to-peptide ratio of 15:1. LUVs were composed of (A, D) PC/SM/Chol at a molar ratio of 5:1:1, (B, E) PC/SM/Chol at a molar ratio of 26:9:15 and (C, F) PC/SM/Chol at a molar ratio of 37:18:45. A scheme of the structure of the gp41 ectodomain according to literature consensus is shown at the top of each figure. Vertical bars indicate standard deviations of the mean of quadruplicate samples.

peptides corresponding to the LLP2/3, LLP1 and PTM/TM regions (about 50–60% leakage) (Fig. 3D). Smaller, but significant, values were found for other regions, such as the NHR, FP and loop domains. Some peptides corresponding to a region between the Kennedy and LLP2 sequences presented about 20–30% leakage. A similar pattern was found on liposomes composed of PC/SM/CHOL at a molar ratio of 26:9:15 when the gp41-derived peptide library from HIV_MN and HIV_consB were assayed (Figs. 3B and E, respectively). For the peptide library derived from HIV_MN g41, the greatest leakage values was found for peptides corresponding to the loop region as well as peptides from the LLP2/3 sequences, since important leakage values of about 90% were found. Smaller leakage values were found for the NHR, PTM and FP regions (about 20–35%, 25% and 20%, respectively). When the library from HIV_consB was assayed the greatest effect was found on the LLP2/3 region (about 90%); smaller but important values were obtained for a peptide corresponding to the LLP1 sequence and a peptide located between the Kennedy and LLP2 sequences (about 80% and 50% leakage, respectively). Smaller leakage values were found for the FP, NHR, loop and PTM sequences (Fig. 3E). When liposomes composed of PC/SM/Chol at a molar ratio of 37:18:45 were assayed (Figs. 3C and F), absolute leakage values were much smaller than those observed for the other compositions. The greatest leakage values were obtained for peptides corresponding to the LLP2/3 regions (about 30% and 40% leakage for the HIV_MN and HIV_consB libraries, respectively); peptides corresponding to the FP, NHR, loop and LLP1 regions from HIV_MN library showed leakage values of about 10–15% (Fig. 3C) whereas peptides corresponding to the NHR, PTM, TM and LLP regions from HIV_consB showed smaller leakage values of about 10% (Fig. 3F).

We have also studied liposome leakage produced by the two different peptide libraries on model membranes composed a complex lipid mixtures such as PC:PE:SM:PI:PS:PA:Chol at a molar ratio of 30.5:19.7: 19.25:0.34:7.2:3.06:20 and PC:PE:SM:PI:PS:PA:Chol at a molar ratio of 46.4:21.2:8.8:4.4:9.3:0.81:9.1, respectively (Fig. 4). The greatest effect was found for peptides originated from the LLP2/3 region of the gp41 endodomain of both strains, where major leakage values of about 60–65% were attained. Major values were also obtained for a peptide corresponding to the LLP1 sequence and a peptide located between the Kennedy and LLP2 sequences (about 45–55% leakage) but from HIV_consB and not from HIV_MN (Fig. 4). Smaller leakage values were found for the other regions, since leakage values of about 20–30% were obtained for the FP region and leakage values of about 10–20% were obtained for peptides originated from the PTM and TM regions. It should be noted that these model membranes, apart from containing Chol and SM, included also PE, PS and PA, whose presence reduced leakage to a great extent.

The summary of the normalized membrane rupture data obtained for all the lipid compositions studied here and both

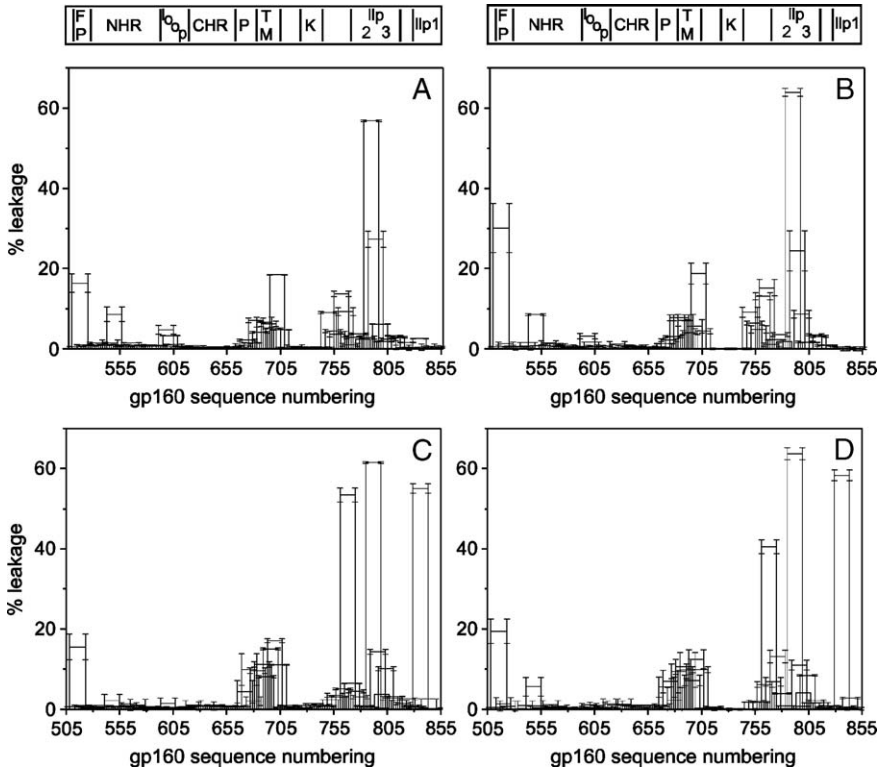


Fig. 4. Effect of the gp41-derived 15-mer peptides from (A, B) HIV_MN and (C, D) HIV_consB on the release of LUV contents for different lipid compositions at a lipid-to-peptide ratio of 15:1. LUVs were composed of simulated viral envelope and plasma cell membranes, namely (A, C) PC:PE:SM:PI:PS:PA:Chol at a molar ratio of 30.5:19.7: 19.25:0.34:7.2:3.06:20 and (B, D) PC:PE:SM:PI:PS:PA:Chol at a molar ratio of 46.4:21.2:8.8:4.4:9.3:0.81:9.1, respectively (see text for details). A scheme of the structure of the gp41 ectodomain according to literature consensus is shown at the top of each figure. Vertical bars indicate standard deviations of the mean of quadruplicate samples.

HIV_MN and HIV_consB libraries is presented in Fig. 7, upper panel. The data has been normalized to the maximum value found for each model membrane studied, so that it is possible to compare all leakage data for all the liposome compositions and the full gp41 sequence, including both the ectodomain and the endodomain. It is possible to detect different segments with large leakage activity, corresponding to the LLP sequences as well as to the PTM and TM domains. Smaller but significant effects are observed at different zones corresponding to the FP, NHR and loop domains. It is also possible to detect slightly differences in leakage for both strains but both of them maintain a similar pattern throughout the full gp41 sequence (Fig. 7).

3.2. Phospholipid-mixing and fusion

Perturbation of membranes is not sufficient to complete the process of viral and cellular membrane fusion, since it also requires the merger of the monolayers, and the stalk formation, according to the stalk model for membrane fusion [46]. However, there is not a clear quantitative criterion to characterize fusion peptides using membrane destabilization [14]. Therefore, we have studied the effect of the peptide libraries derived from the gp41 ectodomain of HIV_MN and HIV_consB on both membrane phospholipid-mixing and fusion using liposomes of different compositions (Figs. 5 and 6).

When the gp41-derived peptide library originated from HIV_MN was assayed on liposomes composed of PC/PS/Chol at a molar ratio of 5:4:1, the most notable effect was observed for two peptides corresponding to the NHR and TM regions, which showed phospholipid-mixing values of about 60% (Fig. 5A). Other peptides which elicited major hemifusion values pertained to the LLP2/3 region and a region adjacent to it (about 30–50% phospholipid-mixing), as well as the FP, the PTM and loop domains (about 30%, 20–25% and 15%, respectively). When liposomes whose composition were PC/SM/Chol at a molar ratio of 5:1:1 were tested, the most important effects were found on peptides corresponding to the PTM/TM and LLP2/3 regions, since phospholipid-mixing values of about 40–50% were observed (Fig. 5B). Other peptides which showed lower values were those corresponding to the FP, NHR, a region adjacent to the LLP2/3 region and a peptide corresponding to the LLP1 region. A relatively similar pattern was obtained when liposomes composed of PC:PE:SM:PI:PS:PA:Chol at a molar ratio of 46.4:21.2:8.8:4.4:9.3:0.81:9.1 were used, since the aforementioned regions where also detected (although showing distinct phospholipid-mixing values, see Fig. 5C). In this case, peptides from the FP and TM regions showed the highest phospholipid-mixing values (about 35%), whereas other peptides showed significant but lower values, such as those corresponding to the NHR, PTM, and LLP2/3 regions.

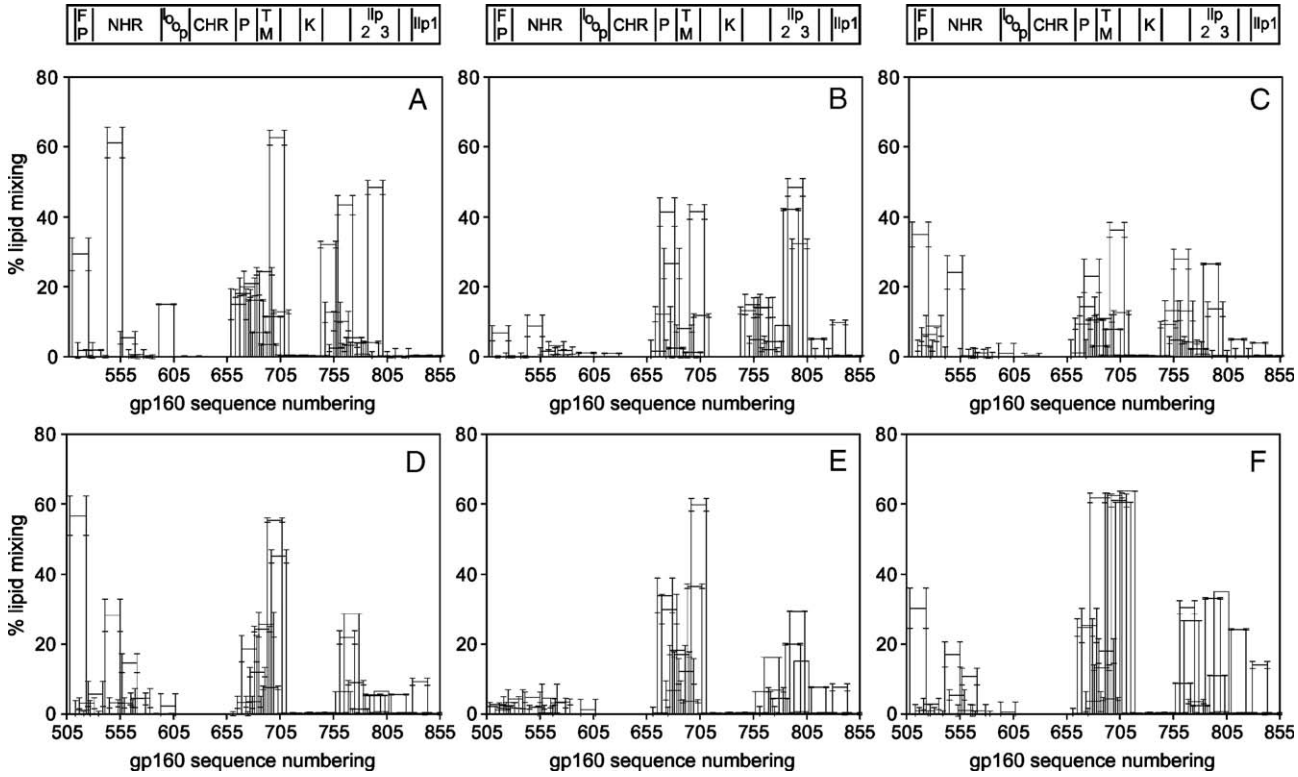


Fig. 5. Effect of the gp41-derived 15-mer peptides from (A–C) HIV_MN and (D–F) HIV_consB on membrane phospholipid-mixing, for different lipid compositions at a lipid-to-peptide ratio of 15:1. Phospholipid-mixing for LUV composed of (A, D) PC/PS/Chol at a molar ratio of 5:4:1, (B, E) PC/SM/Chol at a molar ratio of 5:1:1 and plasma cell membranes composed of (C, F) PC:PE:SM:PI:PS:PA:Chol at a molar ratio of 46.4:21.2:8.8:4.4:9.3:0.81:9.1. A scheme of the structure of the gp41 ectodomain according to literature consensus is shown at the top of each figure. Vertical bars indicate standard deviations of the mean of quadruplicate samples.

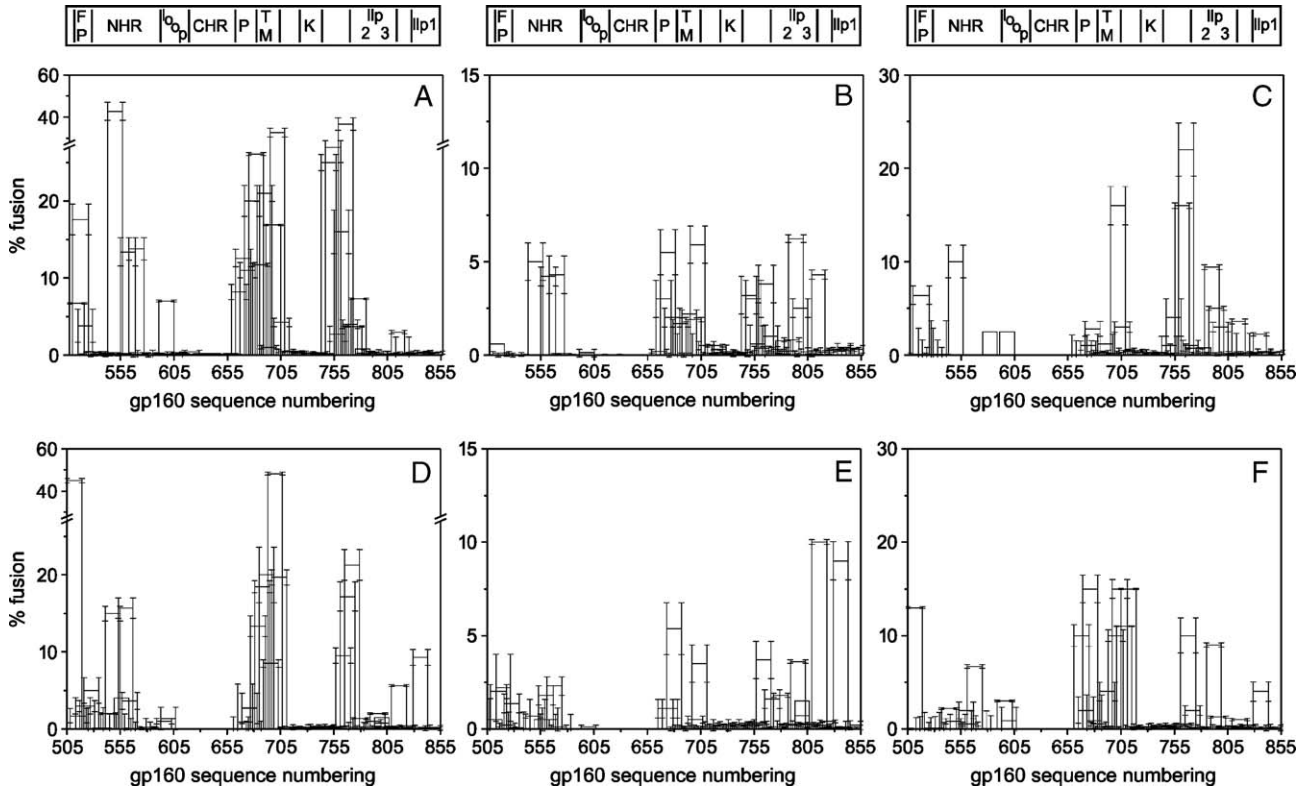


Fig. 6. Effect of the gp41-derived 15-mer peptides from (A–C) HIV_MN and (D–F) HIV_consB on membrane fusion, for different lipid compositions at a lipid-to-peptide ratio of 15:1. Phospholipid-mixing for LUV composed of (A, D) PC/PS/Chol at a molar ratio of 5:4:1, (B, E) PC/SM/Chol at a molar ratio of 5:1:1 and plasma cell membranes composed of (C, F) PC:PE:SM:PI:PS:PA:Chol at a molar ratio of 46.4:21.2:8.8:4.4:9.3:0.81:9.1. A scheme of the structure of the gp41 ectodomain according to literature consensus is shown at the top of each figure. Vertical bars indicate standard deviations of the mean of quadruplicate samples.

When the gp41-derived peptide library originated from HIV_consB was assayed on liposomes composed of PC/PS/Chol at a molar ratio of 5:4:1, a similar pattern was found, since the same regions, although at a different extent, elicited phospholipid-mixing effects (Fig. 5D). The most important effects were observed for peptides corresponding to the FP and TM regions, which showed phospholipid-mixing values of about 50–60%. Other regions which also showed phospholipid-mixing effects were the NHR, PTM and a region adjacent to the LLP2/3 sequences. When liposomes whose composition were PC/SM/Chol at a molar ratio of 5:1:1 were tested, the most important effects were found on peptides corresponding to the TM, since phospholipid-mixing values of about 60% were observed (Fig. 5E). Other regions whose peptides produced significant effect were the PTM and LLP2/3 regions since phospholipid-mixing values of about 30–40% were observed. When liposomes were composed of a complex lipid mixture, i.e., PC:PE:SM:PI:PS:PA:Chol at a molar ratio of 46.4:21.2:8.8:* 4.4:9.3:0.81:9.1, the regions which produced the highest values of phospholipid-mixing were those corresponding to the PTM and TM regions, since phospholipid-mixing values of about 60–65% were found (Fig. 5F). Other regions which showed lower but important phospholipid-mixing values were the FP, the NHR, the LLP2/3, a zone adjacent to LLP2/3 and the LLP1.

Both HIV_MN and HIV_consB gp41 derived peptide libraries induced a similar pattern on membrane fusion to that found for membrane phospholipid-mixing (Fig. 6). When fusion was assayed on liposomes composed of PC/PS/Chol at a molar ratio of 5:4:1 using the gp41 peptide library derived from HIV_MN, there were four regions which displayed large fusion values (Fig. 6A), being the FP (about 18% fusion), the NHR (about 40%), the PTM and TM (about 30–40%) and a region located in between the Kennedy and LLP2/3 sequences (about 30–40%). Small fusion values were obtained for the loop region (about 8%). When the peptide library originated from HIV_consB was assayed on these liposomes, a similar trend was obtained (Fig. 6D). When liposomes composed of PC/PS/Chol at a molar ratio of 5:1:1 were assayed, the fusion elicited by both peptide libraries was significantly reduced (Figs. 6B and E). For the HIV_MN gp41-derived library, the fusion values which were found were about 5% for the NHR, PTM, TM and LLP2/3 regions, whereas for the HIV_consB gp41-derived library, they were about 2–3% for the FP and NHR, about 5% for the PTM/TM and 5–10% for the LLP2/3 regions. As shown in Figs. 6C and F, when liposomes composed of PC:PE:SM:PI:PS:PA:Chol at a molar ratio of 46.4:21.2:8.8:4.4:9.3:0.81:9.1 were tested, the fusion values were significantly reduced compared to those observed for PC/PS/Chol liposomes but slightly higher than those found for PC/SM/Chol liposomes (Fig. 6). Interestingly, the same regions which previously elicited fusion using the other liposome compositions were detected now.

The summary of the normalized phospholipid-mixing and fusion results obtained for both HIV_MN and HIV_consB libraries is presented in Fig. 7, upper panel. These data has been normalized to the maximum value found for each model membrane studied, so that it is possible to compare all phospholipid-mixing and fusion results for all the liposome compositions and the full gp41 sequence. Similarly to what was described above for the leakage data, it is possible to distinguish different segments with large phospholipid-mixing and fusion activity. However, and in contrast to what was found above, the most important effect corresponded to the PTM, TM and FP domains. Smaller but significant effects are observed at specific zones corresponding to the NHR domain and LLP sequences. It is also possible to detect slightly differences in hemifusion and fusion for both strains but, similarly to what was found for leakage, both of them present a nearly identical pattern throughout the full gp41 sequence (Fig. 7).

4. Discussion

We have previously shown the existence of different segments in the HIV gp41 ectodomain with membrane-interacting capabilities [21]. In this work, we have extended our previous work and have made an exhaustive study of the effect on membrane integrity of a peptide scan corresponding to two peptide libraries from the HIV gp41 envelope glycoprotein, i.e., both ecto and endodomains, of two different HIV virus strains, namely HIV_MN and HIV_consB, as well as studied their effect not only on membrane rupture but also on membrane fusion specific approaches such as phospholipid-mixing and fusion. As it is 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, i.e., membrane rupture, phospholipid-mixing 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 gp41 ecto and endodomains according to their effect in an ample representation of membrane systems. Moreover, since two consecutive peptides in the library have an overlap of 11 amino acids, it seems reasonably on thinking on peptide segments rather than on the effect of isolated peptides as we will comment below.

Membrane fusion reactions are involved in many important biological processes, but strong repulsion forces represent

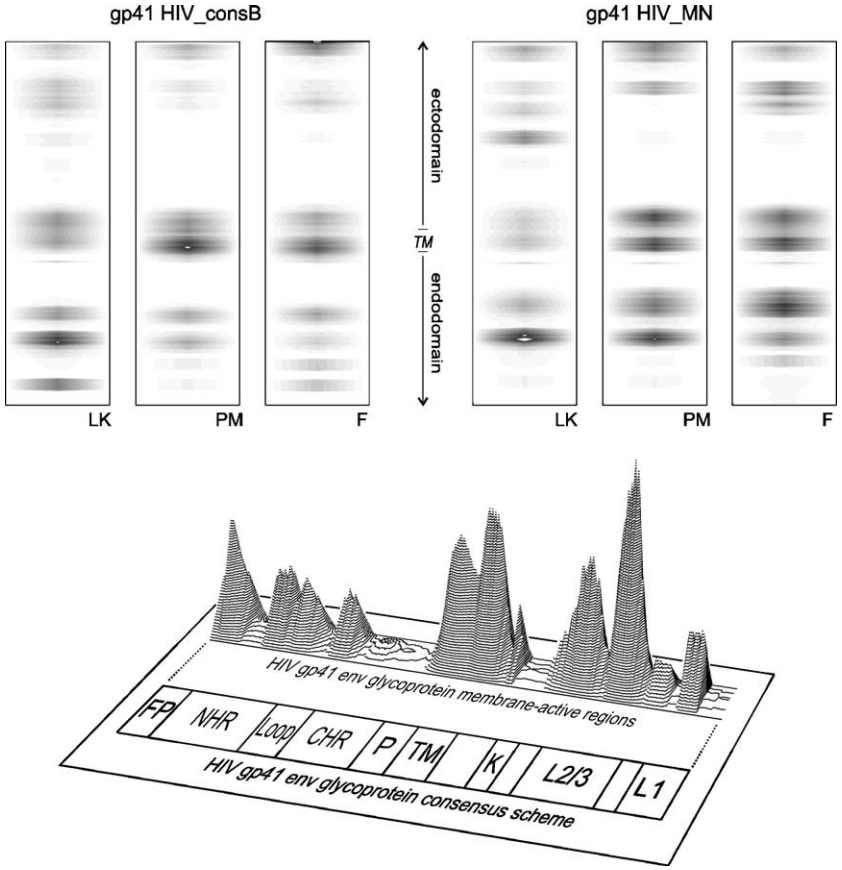


Fig. 7. Summary of all the experimental membrane rupture, phospholipid-mixing and fusion data presented in this work corresponding to the [2 × 85] gp41-derived 15-mer peptides from HIV_consB and HIV_MN (upper panel). The columns show the summation of the normalized (percentage) experimental values corresponding to membrane rupture (leakage, LK), phospholipid-mixing (PM) and fusion (F) of all experimental liposome compositions studied (the darker, the greater membranotropic effect) along the full gp41 sequence. In the lower part of the figure, displayed is the summary of all the normalized experimental data for all experimental results along the consensus scheme of the gp41 glycoprotein highlighting the relationship between the classical and experimentally-identified membrane-active regions.

large energetic barriers [47]. In biological systems, these barriers are overcome by fusion proteins and the simplest membrane fusion reaction is the one produced by the entry of enveloped viruses into the host cells [1,2,48]. Class I fusion proteins, being synthesized as precursors, are cleaved into two subunits which, in the case of HIV are the gp120, the receptor binding subunit, and gp41, the transmembrane fusion subunit. Different structural conformational changes induced by a complex series of protein/protein and protein/phospholipid interactions are present in both proteins. However, little is known about how these conformational changes drive membrane apposition and how they overcome the energy barrier for membrane fusion [49]. Gp41 exists as trimeric spikes, both in its metastable native and stable fusogenic states. In its native state it presumably projects perpendicularly to the viral membrane, whereas in the fusogenic state it runs parallel to both viral and cellular membranes, forming the low-energy six-helix bundle [1,2,48]. The existence of a pre-hairpin intermediate in gp41-induced membrane fusion in which the N-terminal coiled-coil is formed, but the C-terminal helices are not packed, has been also proposed [50]. The transition from the pre-hairpin to the hairpin structure brings the two membranes into close proximity, driving fusion and stabilizing the fusion pore [51,52]. Destabilization of the lipid bilayer and posterior membrane fusion appear to be also the result of the interaction of different segments of gp41 with the membrane [6,16,21,22,27, 28,50,53,54]. These and other observations suggest the hypothesis that, upon interacting with the membrane, HIV gp41 undergoes additional conformational changes in which the trimeric helical hairpin binds to the membrane surface and opens [19]. The possibility that segments of the negatively-charged gp41 endodomain (the Kennedy sequence) could be exposed to the outer surface of the viral membrane would open new implications on the inter-domain interactions related to membrane fusion and viral entry [8]. Understanding the factors that may determine the specificity and stability of the metastable native and the stable fusogenic conformations, being located on the ectodomain or endodomain or both, are required for the understanding of the mechanism of viral membrane fusion and consequent viral entry into cells.

We have obtained information to gather the membrane interacting propensity of all gp41 domains (Fig. 7, lower panel) and therefore have a clue to the possible function of each segment of the protein 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 Class I proteins. The FP and PTM domains, having the highest hydrophobic moment values, showed the highest combined effects, whereas the other domains had a lower extent of effect, but nevertheless a relatively significant one (it should be pointed out that 15mer peptides are too short to cross the membrane as a helix and therefore segments that include part of the TM domain may be more fusogenic in these models than the actual TM segment). Significantly, the gp41 elements which showed no membranotropic effect on any model membrane studied here were the CHR and Kennedy domains. It is interesting to note that peptides corresponding to these domains present a large component of negative charge whereas peptides corresponding to the other gp41 regions present a large positive one. It is known that the CHR and NHR domains (negatively and positively charged, respectively, see Fig. 1) interact with each other in order to form the hairpin structure. Therefore, the aforementioned data would imply that not only hydrophobic interactions are important for the formation of the pre-hairpin/hairpin intermediates, but also charge is another factor which might facilitate the hairpin arrangement and stabilization. If the Kennedy sequence would be exposed to the outer membrane surface of the virion as it has been already suggested [8], it could also participate on the formation of different fusion intermediates.

For membrane fusion to occur, fusion proteins must pull the viral and cellular membranes towards one another, create membrane defects which lower the energy barrier for hemifusion of the outer leaflets and fusion of the membranes and, last but not least, pore formation, stabilization and enlargement [3]. The FP and PTM/TM domains are mainly responsible for the first steps of membrane fusion, but in the last instance other gp41 segments in combination with the previous ones, drive and accomplish membrane fusion. The binding to the surface and the modulation of the phospholipid biophysical properties which take place when the gp41 domains bind to the membrane could be related to the conformational changes which occur upon binding of the envelope glycoprotein to its receptors. It should be also taken in consideration that hairpin formation depends strongly on the interaction between the CHR and NHR domains. 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 [22] and suggests that these changes might be part of the structural transition that transform gp41 from the inactive to the active state, being most probably the dominant form during membrane fusion [55]. It should be noted that the location of the different membrane-interacting segments at the surface of the membrane could effectively reduce the head-group area of the membrane phospholipids and promote the formation of non-bilayer phases [16,48]. The destabilization of the phospholipid bilayer as a result of the interaction of the gp41 segments provides a way to lower the barrier for fusion taking place. All membranotropic segments of gp41 would form a continuous track of membrane-interacting surfaces along the structure of the protein complex providing a low-energy passageway for viral–cellular membrane fusion. Protein-mediated viral fusion is therefore a complex process in which multiple regions from the viral fusion protein interact, destabilize and fuse membranes. At the same time, the comparison of the information obtained from different membrane-fusion proteins support the idea of a common fusion mechanism among enveloped viruses. In addition, the knowledge of all membranotropic regions of HIV gp41 could aid in the search for molecules which could revert or reduce the action of those specific membranotropic peptides, i.e., those molecules which inhibit peptide-induced membrane

leakage or fusion could be potential candidates as inhibitors of membrane-fusion. Work is being carried out in our laboratory to test this hypothesis.

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