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Identification of membrane-active regions of the HIV-1 envelope glycoprotein gp41 using a 15-mer gp41-peptide scan

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Abstract

The identification of membrane-active regions of the ectodomain of the HIV-1 envelope glycoprotein gp41 has been made by determining the effect on membrane integrity of a 15-mer gp41-derived peptide library. By monitoring the effect of this peptide library on membrane leakage, we have identified three regions on the gp41 ectodomain with membrane-interacting capabilities: Region 1, which would roughly correspond to the polar sequence which follows the fusion domain and extends to the N-terminal heptad repeat region; Region 2, which would correspond to the immunodominant loop; and Region 3, which would correspond to the pre-transmembrane region of gp41. The identification of these three regions supports their direct role in membrane fusion as well as facilitating the future development of HIV-1 entry inhibitors.

Keywords: gp41; HIV-1; Virus-cell entry; Membrane fusion

1. Introduction

The attachment of enveloped viruses to cells and the consequent fusion of viral and cellular membranes, the critical early events in viral infection are mediated by envelope glycoproteins (gp) located on the outer surface of the viral membrane [1,2]. The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein, gp160, synthesized as a precursor, is proteolytically cleaved into the receptor binding subunit, gp120, and the fusogenic subunit, gp41, which remain non-covalently associated with one another. The native conformation of gp41 is metastable and is stabilized by gp120. Binding of gp120 to the cell-surface receptors and co-receptors, triggers a series of conformational changes in gp41, resulting 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 [1–5]. At the N terminus of gp41, a stretch of about 15 hydrophobic residues (see Fig. 1A), named the fusion peptide [6,7] is believed to insert into and destabilize the membrane, thus facilitating viral and cell membrane fusion [8]. Consecutive to the fusion peptide and preceding the transmembrane domain, two heptad repeat regions (NHR and CHR, respectively) have been identified [9]. 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, protease-sensitive loop that reverses the polypeptide chain [4,5,10]. This trimeric helical hairpin structure is thought to form at a late stage during the membrane fusion process [4,5].

It has been proposed that 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 [11]. At this stage, the C-

Abbreviations: CF, 5-carboxyfluorescein; Chol, cholesterol; CHR, C-terminal heptad repeat region; gp, glycoprotein; HIV-1, human immunodeficiency virus type 1; LUV, large unilamellar vesicles; NHR, N-terminal heptad repeat region; PA, egg L-α-phosphatidic acid; PC, egg L-α-phosphatidylcholine; PE, egg L-α-phosphatidylethanolamine; PI, bovine liver L-α-phosphatidylinositol; POPC, palmitoyloleoylphosphatidylcholine; PS, bovine brain phosphatidylserine; PSM, palmitoylsphingomyelin; SM, egg sphingomyelin; T_m, main transition temperature

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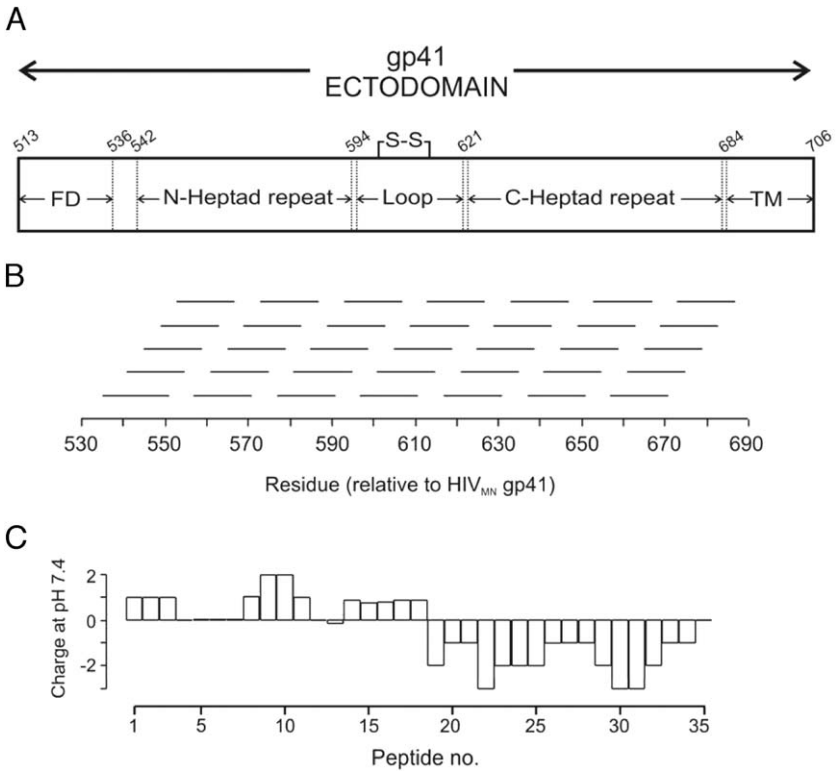

Fig. 1. (A) Scheme of the structure of gp41, the fusogenic glycoprotein subunit of HIV-1, according to literature consensus. The sequence numbering follows isolate HIV-1$_{MN}$ and the important functional regions are highlighted, i.e., the fusion domain, FD, the N- and C-heptad repeats, the immunodominant loop and the transmembrane region, TM. (B) Sequence of the 15-mer peptides used in this study with respect to the sequence of gp41. Maximum overlap between adjacent peptides is 11 amino acids. (C) Charge profile at pH 7.4 of the 15-mer peptides.

peptide inhibitors could bind to the exposed coiled-coil, thus preventing the subsequent refolding and blocking fusion [12]. The pre-hairpin intermediate spans both membranes, with the gp41 transmembrane domain anchored in the viral membrane, and the fusion peptide inserted into the target cell membrane. Inhibition by N- and C-peptides is thought to act at this level, by preventing transition to the hairpin/fusion structure [13,14]. The transition from the pre-hairpin to the hairpin structure brings the two membranes into close proximity, driving fusion. Although much information has been gathered in recent years, we do not yet know the exact mechanism of membrane fusion and the processes which are behind them.

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 gp41 constructs lacking these two segments bind and interact with the surface of phospholipid bilayers as well as other regions of the ectodomain, not only derived from gp41, but also from other viral fusion proteins [15–20]. These and others observations suggested the hypothesis that, upon interacting with the membrane, HIV-1 gp41 undergoes an additional conformational change in which the trimeric helical hairpin binds to the membrane surface and opens [15]. The mechanism by which proteins facilitate the formation of fusion intermediates is a complex process involving several segments of fusion proteins [21,22]. These regions, either directly or indirectly, might interact with biological membranes, contributing to the viral envelope and cell membrane merging. Although the detailed structure of different segments of the gp41 ectodomain of HIV-1 have been known for several years, there are still many questions to be answered regarding its mode of action in accelerating membrane fusion. Moreover, HIV-1 entry is an attractive target for anti-HIV-1 therapy. Therefore, we have initiated the first steps towards the finding of inhibitors of gp41 membrane fusion by identifying the regions of gp41 which might interact with phospholipid membranes. The first one has been the identification of membrane-active regions by determining the effect on membrane integrity of a 15-mer gp41-derived peptide library. By monitoring the effect of this peptide library on membrane integrity, i.e., leakage, we have identified three different regions of gp41 with membrane-interacting capabilities, supporting their direct role in membrane fusion, and therefore might help in the understanding of the molecular mechanism of membrane merging as well as making possible the future development of HIV-1 entry inhibitors which may lead to new vaccine strategies.

2. Materials and methods

2.1. Materials and reagents

Egg L-α-phosphatidylcholine (PC), egg sphingomyelin (SM), cholesterol (Chol), bovine brain phosphatidylserine (PS), egg L-α-phosphatidylethanolamine (PE), egg L-α-phosphatidic acid (PA) and bovine liver L-α-phosphatidylinositol (PI) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). 5-Carboxyfluorescein (CF), >95% by HPLC, was from Sigma-Aldrich (Madrid, Spain). A set of 35 peptides of 15 amino acids in length derived from HIV-1_{MN} gp41, most of which with 11 amino acid overlaps between sequential peptides, were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MD, USA). Peripheral blood lymphocytes were obtained from healthy volunteers using standard procedures (Hospital Universitario de Elche, Alicante, Spain). Lipid extraction from human lymphocytes was performed according to the Bligh and Dyer procedure using a ratio of 1:1:0.9 (v/v/v) between chloroform/methanol and the corresponding aqueous sample [23]. All other reagents used were of analytical grade from Merck (Darmstadt, Germany). Water was deionized, twice-distilled and passed through a Milli-Q equipment (Millipore Ibérica, Madrid, Spain) to a resistivity better than 18 MΩ cm.

2.2. Sample preparation

Aliquots containing the appropriate amount of lipid in chloroform/methanol (1:1, v/v) were placed in a test tube, the solvents 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. After that, 2 ml of buffer containing 20 mM Hepes, 50 mM NaCl, 0.1 mM EDTA, pH 7.4 buffer was added, and multilamellar vesicles were obtained. Large unilamellar vesicles (LUV) with a mean diameter of 90 nm were prepared from multilamellar vesicles by the extrusion method [24] using polycarbonate filters with a pore size of 0.1 μm (Nuclepore Corp., Cambridge, CA, USA) using 10 mM HEPES, 100 mM NaCl, 0.1 mM EDTA, pH 7.4 buffer and CF at a concentration of 40 mM. 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, Sweden) eluted with buffer containing 10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4 [20].

2.3. Leakage measurement

Leakage of intraliposomal CF was assayed by treating the probe-loaded liposomes (final lipid concentration, 0.5 mM) with the appropriate amount 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 ratio of 1:25. Changes in fluorescence intensity were recorded with excitation and emission wavelengths set at 492 and 517 nm, respectively. One hundred percent release was achieved by adding Triton X-100 to a final concentration of 0.5% (w/w) to the microtiter plates. Fluorescence measurements were made initially with probe-loaded liposomes, afterwards by adding peptide solution and finally adding Triton X-100 to obtain 100% leakage. Leakage was quantified on a percentage basis according to the equation,

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

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. The phospholipid concentration was measured by methods described previously [25].

3. Results and discussion

Although it was previously thought that the N-terminal fusion peptide of gp41 was the only responsible for cell membrane interaction leading to membrane fusion, it has been shown that other gp41 regions bind, interact and undergo conformational changes in the presence of membranes [21,22]. To explore the specific interaction of the 15-mer gp41-ectodomain derived peptide library with phospholipid model membranes, we have studied their effect on the release of an encapsulated fluorophore, 5-carboxyfluorescein using the experimental set up described in Materials and methods. The 35 peptides we have used in this study, derived from the gp41 ectodomain, are shown in Fig. 1B with respect to the gp41 sequence, whereas Fig. 1C shows its charge at pH 7.4. As it can be observed in Fig. 1B, the peptide scan extends from the sequence which follows the fusion domain to the sequence which precedes the transmembrane domain, which also includes a small amino acid stretch of both hydrophobic domains. The peptide scan library encompasses also the NHR, CHR and loop regions (Fig. 1A and B).

Fig. 2 shows the effect of the peptide scan on membrane integrity, i.e., leakage, for four different liposome compositions, namely EPC, EPC/Chol at a molar ratio of 5:1, EPC/BPS/Chol at a molar ratio of 4:4:1 and EPC/EPE/Chol at a molar ratio of 5:3:1. For liposomes composed of EPC (Fig. 2A), it is clearly evident that some peptides hardly exerted any effect on EPC liposomes, but other ones showed a significant effect. The most notable effect was observed for peptide no. 15, which produced a leakage of about 90%, i.e., a near complete rupture of the liposomes. Other peptides induced less leakage though a significant one, such as

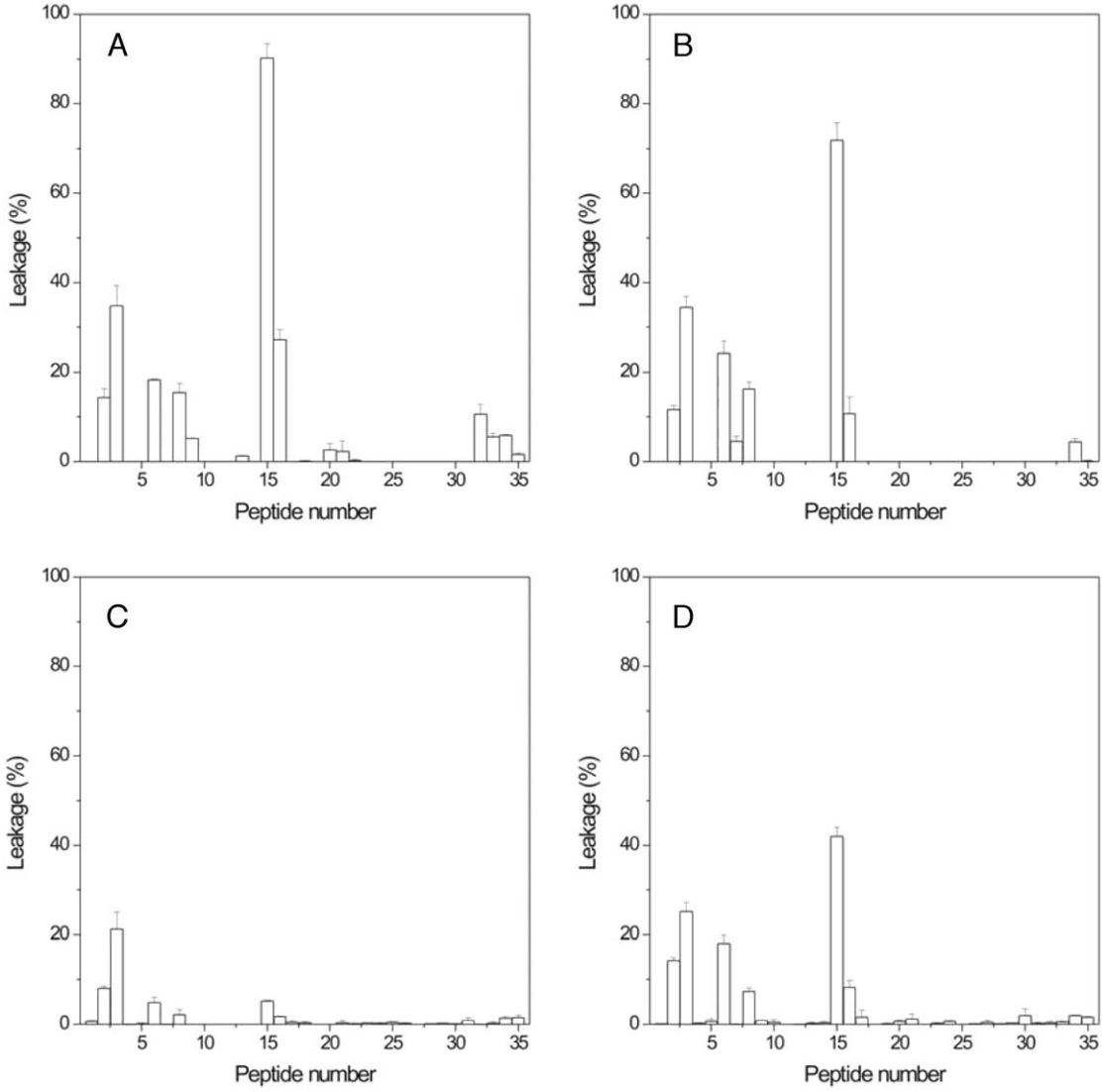

Fig. 2. Effect of the gp41-derived 15-mer peptides on the release of LUV contents for different lipid compositions. Leakage data for LUV composed of (A) EPC, (B) EPC/Chol at a molar ratio of 5:1, (C) EPC/BPS/Chol at a molar ratio of 4:4:1 and (D) EPC/EPE/Chol at a molar ratio of 5:3:1. Vertical bars indicate standard deviations of the mean of triplicate samples.

peptides no. 3 and 16, which produced about 35% and 30% leakage. Other peptides showed leakage values between 10% and 20% (peptides no. 2, 6, 8, and 32). Similarly to what was found using EPC liposomes, some peptides interacted significantly with liposomes composed of EPC/Chol at a molar ratio of 5:1 (see Fig. 2B), although there were several ones that have hardly any effect. As above, the most significant effect was observed for peptide no. 15, which showed leakage values of about 75%. The leakage extent for this type of liposomes was in general slightly lower than that found on EPC liposomes; interestingly, the same peptides that showed a significant effect on EPC liposomes were the ones that showed a significant one for EPC/Chol liposomes. Peptides no. 3 and 6 were the ones which presented a greater extent of leakage but also peptides no. 2 and 8 presented significant leakage values (see Fig. 2B). It should be noted that inclusion of Chol in membranes reduced peptide-induced membrane leakage as it has been shown before [26]; however, the presence of Chol seems to be a specific requirement for HIV-1 infection (Ref. [27]; see below). The extent of leakage observed in liposomes composed of EPC/BPS/Chol at a molar ratio of 4:4:1 (Fig. 2C), was much lower than that found for both EPC and EPC/Chol liposomes. Except for peptide no. 3, which presented leakage values slightly above 20%, the other peptides presented small leakage values or no leakage at all (i.e., peptide no. 15, which in either EPC or EPC/Chol liposomes showed significant leakage values). It is worth to note that the major difference between EPC/BPS/Chol liposomes and EPC or EPC/Chol liposomes is the negative net charge the former has (compare the net charge of the different peptides at pH 7.4 shown in Fig. 1C). Liposomes composed of EPC/EPE/Chol at a molar ratio of 5:3:1 showed slightly lower leakage values than those found for EPC or EPC/Chol

liposomes but greater than those found for EPC/BPS/Chol liposomes (Fig. 2D). For this liposome composition, peptide no. 15 produced leakage values of about 40%, whereas peptides no. 2, 3 and 6 showed leakage values between 10% and 30%. It is worth to note that peptides no. 32–34 showed a limited extent of leakage for EPC liposomes but hardly detected values for the other liposomes (Fig. 2), the major difference being Chol content (see below). By observing the leakage values driven by the 15-mer peptide library on these different liposome compositions, two delimited regions could be discerned, one which would roughly correspond to the polar sequence which follows the fusion domain and extends to the NHR region (Region 1, comprising peptides 2, 3, 6 and 8, Fig. 5) and another one which would correspond to the immunodominant loop (Region 2, comprising peptides 15 and 16, Fig. 5).

It was previously thought that the lipid composition of the HIV-1 envelope was similar to that of cell plasma membranes; however, its phospholipid profile and fluidity is notably different from plasma membranes of the host cells, the most significant difference being is higher content of Chol and SM [28]. Both lipids, which have a propensity to form an ordered domain separate from the more liquid plasma membrane, rich in other lipids, have been related to the occurrence of laterally segregated membrane microdomains or "lipid rafts" [29–31]. Earlier studies have demonstrated an important relationship between membrane fusion and Chol and SM membrane content for several enveloped viruses. For example, Semliki Forest virus requires Chol for membrane fusion [32,33] and the presence of SM enhances the membrane-destabilizing capacity of the pre-transmembrane sequence of Ebola glycoprotein [34]. HIV-1 uses Chol and sphingolipids during budding [35], and several domains within the gp41 C-helix either interact with Chol or with sphingolipids or both [36–38]. Interestingly, CD4 receptors are presumably located in lipid rafts [39] and relocation of CD4/viral proteins outside rafts might be important steps in the fusion process [40]. All these data strongly suggest that the interaction of gp41 with Chol and SM might play a major role in the fusion process [2]. Because of that, we have studied the interaction of the 15-mer gp41-ectodomain peptide library with model membranes having a variable Chol and SM composition, namely, EPC/SM at a molar ratio of 5:1 and EPC/SM/Chol at molar ratios of 17:2:1, 5:1:1, 26:9:15 and 37:18:45 (Fig. 3). Recently, a ternary phase diagram for palmitoylsphingomyelin (PSM), palmitoyloleoylphosphatidylcholine (POPC) and Chol, a typical raft-containing lipid mixture, has been determined [41]. If the ternary phase diagram for POPC/SM/Chol were applicable to EPC, SM, and Chol, the lipids used in this study, liposomes containing EPC/Chol at a molar ratio of 5:1 and liposomes containing EPC/SM/Chol at a molar ratio of 17:2:1 would be predominantly in the liquid-disordered phase, whereas liposomes containing EPC/SM/Chol at molar ratios of 5:1:1, 26:9:15 and 37:18:45 would present a coexistence of liquid-ordered and liquid-disordered phases, i.e., a high probability of raft formation [42,43].

For liposomes composed of EPC/SM at a molar ratio of 5:1 (Fig. 3A), it is clearly evident the significant effect on leakage produced by some peptides, whereas any effect was observed for other ones. Interestingly, peptides no. 15 and 16, which showed a significant effect on EPC-containing LUVs (peptide no. 15 significantly more than peptide no. 16, see Fig. 2) also produced a significant effect on these EPC/SM LUVs, since a complete rupture of the liposomes was observed (ca. 100% leakage for peptide no. 15, about 60% for peptide no. 16). Other peptides, such as no. 3, 8 and 9, produced a significant leakage, i.e., between 20% and 40%, similarly to what was found before (see Fig. 2). Other peptides, which did not produce any significant leakage in the other liposome compositions described above, now produced a significant one, i.e., peptides no. 32 and 33, which showed leakage values ranging from 20% to 40%. It is interesting to note that peptides no. 32 and 33 correspond to the pre-transmembrane sequence of gp41, region which has been implicated in the specific interaction of gp41 with Chol and SM containing membranes [37]. Liposomes composed of EPC/SM/CHOL at a molar ratio of 17:2:1 showed slightly lower leakage values than those found using liposomes containing EPC/SM at a molar ratio of 5:1 but the pattern was essentially the same (Fig. 3B). Liposomes composed of EPC/SM/CHOL at molar ratios of 5:1:1, 26:9:15 and 37:18:45 showed similar results. For example, peptides no. 15 and 16 presented the higher effect on leakage, peptides no. 3, 8 and 9 smaller but significant leakage values, similarly to peptides no. 32 and 33, whose leakage values ranged from about 15% to about 40%. This pattern is essentially the same for all liposomes containing Chol and SM. Therefore, and in contrast to what was shown above, three, instead of two, delimited regions are apparent in the leakage diagrams shown in Fig. 3: two regions identical to the ones which were described above (Regions 1 and 2, Fig. 5) and a new one, which would correspond to the pre-transmembrane region of gp41 (Region 3, comprising peptides no. 32, 33 and 34, Fig. 5).

As we have noted above, lipid composition of plasma cell membranes differ significantly from HIV-1 envelopes [28] and we have found different effects on leakage when including SM in liposome composition. Taking into account this fact, we have also studied the effect of the 15-mer peptide library on liposomes whose composition resemble the lipid composition of un-infected cell plasma membranes [28], i.e., LUVs whose composition is PC/PE/SM/PS/PI/PA/Chol at a molar ratio of 47:23:10:10:5:0.5:3 (Fig. 4A). Leakage values are similar to those found for EPC-containing liposomes but dissimilar to those containing SM (compare with Figs. 2 and 3). The most effective peptide in provoking leakage for these liposomes is again

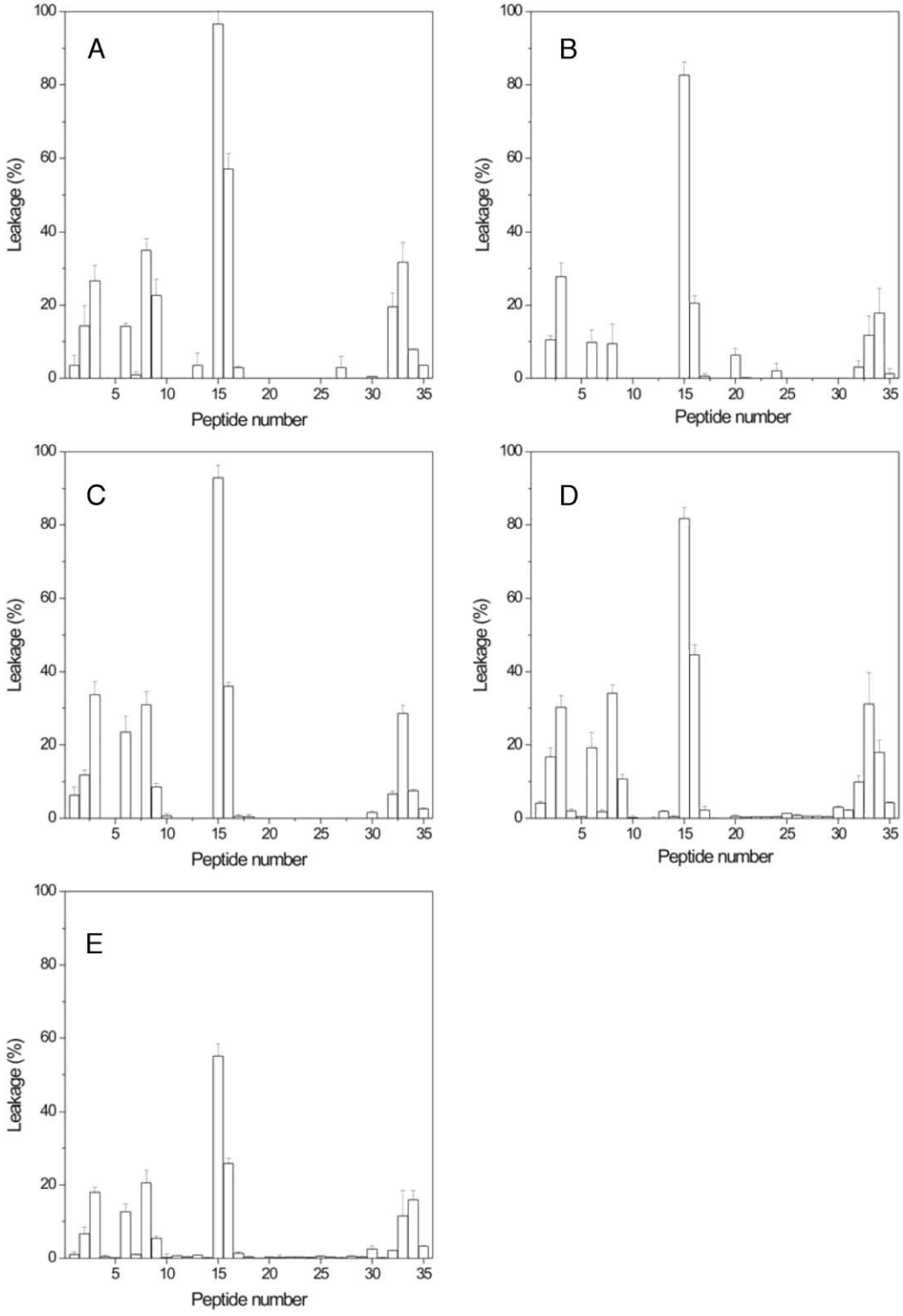

Fig. 3. Effect of the gp41-derived 15-mer peptides on the release of LUV contents for different lipid compositions. Leakage data for LUV composed of (A) EPC/SM at a molar ratio of 5:1, (B) EPC/SM/Chol at a molar ratio of 17:2:1, (C) EPC/SM/Chol at a molar ratio of 5:1:1, (D) EPC/SM/Chol at a molar ratio of 26:9:15 and (E) EPC/SM/Chol at a molar ratio of 37:18:45. Vertical bars indicate standard deviations of the mean of triplicate samples.

peptide no. 15, whose leakage value is above 40%. Other peptides which show a significant effect are peptides no. 3 and 8, whose leakage values lie between 20% and 40% (see Fig. 4). Interestingly, hardly any effect is observed for peptides which correspond to the pre-transmembrane region of gp41. It is interesting to note that these liposomes, apart from containing Chol and SM, also include PS, whose presence reduced leakage to a great extent (see

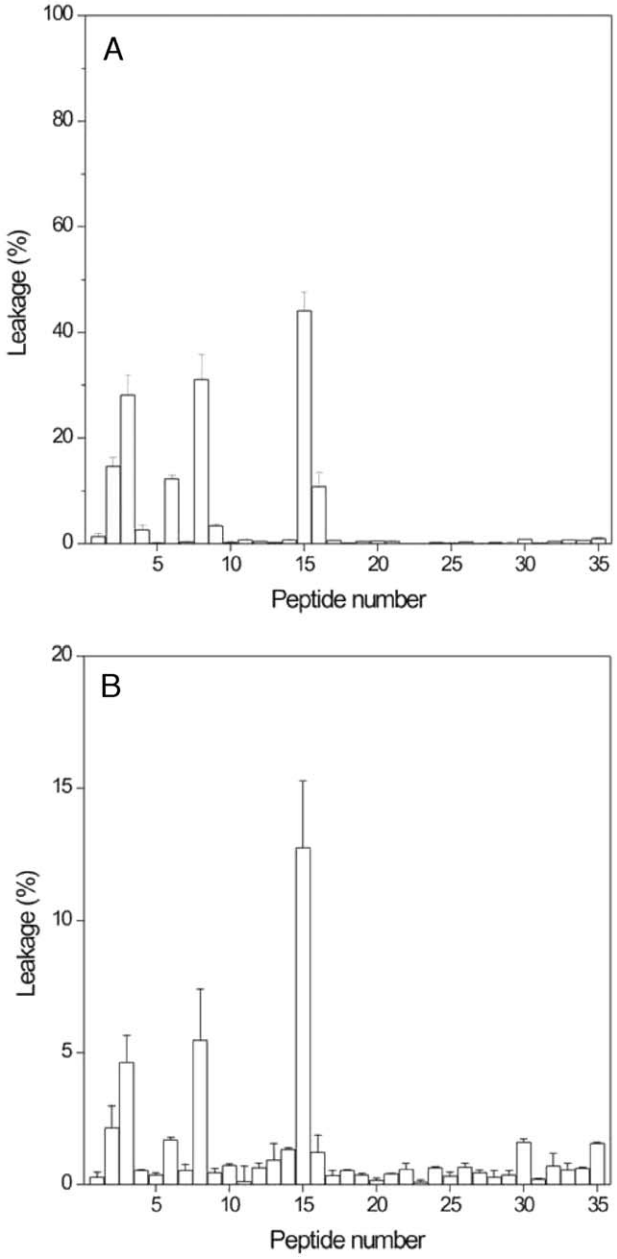

Fig. 4. Effect of the gp41-derived 15-mer peptides on the release of LUV contents for (A) PC/PE/SM/PS/PI/PA/Chol at a molar ratio of 47:23:10:10:5:0.5:3 and (B) lipidic extract of lymphocytes. Experimental conditions are described in the text. Vertical bars indicate standard deviations of the mean of triplicate samples.

Fig. 2C). We have also tested leakage on LUVs whose lipids were obtained from a lipidic extract of lymphocytes (see Fig. 4B; note the increase in leakage scale). It can be observed that the extent of leakage has been very much reduced compared to the other LUV systems studied above. However, similar patterns of leakage to those described above can be discerned, delimiting in this case two regions, the polar sequence which follows the fusion domain and the immunodominant loop, as described above (Regions 1 and 2, Fig. 5).

Several lines of evidence indicate that, in addition to classical fusion peptides, different regions of the gp41 ectodomain are essential for membrane fusion to occur. For example, synthetic peptides that partially overlap the CHR segment and a consecutive Trp-rich region have been shown to be potent inhibitors of gp41-mediated membrane fusion [3,11,44], deletion of short sequences within the CHR region reduce fusogenicity, whereas longer deletions abolish it completely [45,46], the CHR region is highly conserved in the vast majority of otherwise highly variable HIV-1 isolates [46], some peptides coming from both NHR and CHR regions as well as from the immunodominant loop show membrane binding, causing membrane leakage in different experimental conditions [19,20,47] and the inhibitory effect of some peptides appears conditioned by their ability to partition into membranes and aggregate within them [48]. In concordance with these findings, our results demonstrate that peptides originated from three different gp41 regions (Fig. 5), namely Region 1, which corresponds to the 15–20 residues peptide segment which follows the fusion peptide at the N-terminus, Region 2, which corresponds to the immunodominant loop, and Region 3, which corresponds to the 10–15 residues peptide segment which precedes the transmembrane domain at the C-terminus, are capable of modifying the biophysical properties of phospholipid membranes, a property which could provide an additional driving force for the merging of the viral and target cell membranes, supports their direct role in membrane fusion and therefore might be essential for the assistance and enhancement of the viral and cell fusion process. Our results should also facilitate the development of HIV-1 entry inhibitors, which might lead to new vaccine strategies, an important focus for clinical intervention.

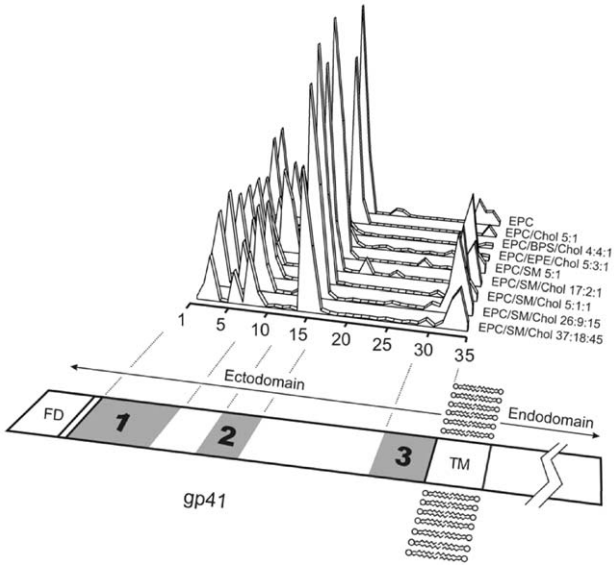

Fig. 5. Scheme of the HIV-1 gp41 structure, along with a summary of the data obtained in this work. Regions 1, 2 and 3, as specified in the text, are shown in dark grey.

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