Structural and Functional Roles of HIV-1 gp41 Pretransmembrane Sequence Segmentation

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INTRODUCTION

HIV-1 relies on the fusogenic activity of the gp120/41 envelope protein to introduce its genetic material into target cells, thereby initiating the infectious cycle (reviewed in Doms and Moore, 2000; Eckert and Kim, 2001). In the currently accepted model, HIV-1 fusion cascade begins with the binding of surface subunit gp120 to target cell receptors. Activation causes the gp41 ectodomain to undergo structural rearrangements toward a final structure that has been crystallographically solved (Chan et al., 1997; Weissenhorn et al., 1997). These include the formation and/or reorientation of a triple-stranded α-helical coiled coil by the amino-terminal heptad repeat domain (HR1). The resulting intermediate filamentous structure is postulated to be simultaneously anchored to viral and target cell membranes, through the transmembrane domain (TMD) and the fusion peptide (FP), respectively. This structure collapses into a folded-in-half six-helix bundle. In this trimeric hairpinlike structure the carboxy-terminal helices (HR2) pack in the reverse direction against hydrophobic grooves outside the coiled coil, so that the amino- and carboxy-termini are placed at the same end of the molecule. It is thus inferred that the production of the trimeric hairpin might bring membranes into close apposition.

It has been argued that the free energy released by the formation of the helical bundle (estimated to be in the order of 40–60 kT; Melikyan et al., 2000) would suffice to induce membrane fusion (in the order of several tens of kT for the activation energy of hemifusion intermediate formation; see an overview in Lentz et al., 2002). However, it is not straightforward how conformational rearrangements in gp41 would couple mechanistically with the actual membrane merger. In particular, bundle structures determined by x-ray crystallography do not include the pretransmembrane (preTM) sequences linking the HR2 to membrane-embedded anchors (Chan et al., 1997; Weissenhorn et al., 1997). The gp41 preTM linker comprises ~20 amino acids (residues 664–683 of the HIV-1 gp160 precursor). Based on the protease sensitivity of the preTM segment, it has been proposed that this region is a flexible, structurally irregular linker (Weissenhorn et al., 1997). However, if fully extended, preTM sequence might keep the six-helix bundle 70 Å apart from the viral membrane (Fig. 1), i.e., distant from the close contacting dehydrated areas required for membrane fusion initiation.

The gp41 membrane-proximal region is accessible to anti-gp41 broadly neutralizing mAbs such as 2F5, 4E10, and Z13 (Zwick et al., 2001). The antigenic nature of the region is most likely conformationally constrained and varies along the HIV-1 fusion pathway (Sattentau et al., 1995; Gorny and...
that a preTM amphipathic helical segment could segregate hydrophobic-at-interface moment. This parameter indicates problem by performing the computation of the gp41 preTM under-stand as yet. We have employed a new approach to the functional and immunogenic properties of preTM is not understood. Peaks above the 0 level (horizontal line) correspond to positive bilayer-to-water free energy values.

**MATERIALS AND METHODS**

**Materials**

1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) was purchased from Avanti Polar Lipids (Birmingham, AL). Calcein AM and CMAC (CellTracker Blue) were from Molecular Probes (Junction City, OR). Bisulfosuccinimidylsulphate (BS^2) was from Pierce (Rockford, IL). D_2O, octaethyleneglycol monododecyl ether (C_{12}EO_{10}), N-acetyl-l-tryptophanamide (NATA), and Triton X-100 were obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade. The sequences, DIKASLWNWFNIY VLYK (HIVc) representing the preTM stretch of HIV-1 (BH10 isolate) gp41, its fluorescent derivative (Rho-HIVc) labeled with rhodamine at its N-terminus, and DKAASLANAFNITNWLKWYIK (HIVc(1-3)A) sequence representing a defective gp41 phenotype (Salzwedel et al., 1999), were synthesized as their C-terminal carboxamides and purified (estimated homogeneity >90%) at the Synthesis Facility of the University Pompeu-Fabra (Barcelona, Spain). Peptide stock solutions were prepared in dimethylsulfoxide (DMSO) (spectroscopy grade).

**Hydrophobic moment calculations**

To compute the hydrophobic-at-interface moment, we considered the membrane interface-to-water (ΔG_{iwu}) transfer free energies for each amino acid (Wimley and White, 1996) as the moduli of the vectors that project from the main axis of the secondary structure element following the direction of the amino acid side chains (Saéz-Cirión et al., 2003). The hydrophobic moment (μ_H) of a sequence of N residues was then calculated as the addition of the N hydrophobicity vectors corresponding to the constituent amino acids, following the equation (Eisenberg et al., 1982, 1984):

\[
\mu_H = \left( \sum_{n=1}^{N} H_n \sin(\delta n) \right)^2 + \left( \sum_{n=1}^{N} H_n \cos(\delta n) \right)^2 \right)^{1/2},
\]

where \(H_n\) is the hydrophobicity of the n residue, and δ is the angle formed between side chains of consecutive residues, i.e., 100° for helical conformations.

**Preparation of vesicles**

Large unilamellar vesicles (LUV) were prepared following the extrusion method of Hope et al. (1985) in 5 mM Hepes, 100 mM NaCl (pH 7.4) buffer. Lipid concentrations of liposome suspensions were determined by phosphate analysis (Böttcher et al., 1961). Mean diameter of vesicles was estimated to be in the range of 100–110 nm by quasielastic light scattering using a Malvern Zeta-Sizer instrument.

**Secondary structure determination**

Infrared spectroscopy (IR) measurements were conducted essentially as described in Pereira et al. (1997). Samples consisted of floated peptide-lipid complexes obtained in D_2O buffer after ultracentrifugation. Solvent samples...
were also obtained from the fraction not containing lipid or peptide and subsequently used as background controls. Infrared spectra were recorded in a Nicolet 520 spectrometer equipped with an MCT detector. Samples were placed between two CaF₂ windows separated by 50-μm spacers. A total of 1000 scans (sample) and 1000 scans (reference) were taken for each spectrum, using a shuttle device. Spectra were transferred to a computer where solvent subtraction and band-position determinations were performed as previously reported (Arrondo et al., 1993).

**Monolayer penetration**

Surface pressure was determined in a fixed-area circular trough (μTrough S system, Kibron, Helsinki). Measurements were carried out at room temperature and under constant stirring. The aqueous phase consisted of 1 ml 5 mM Hapes, 100 mM NaCl (pH 7.4). POPC, dissolved in chloroform, was spread over the surface and the desired initial surface pressure (π₀) was attained by changing the amount of lipid applied to the air-water interface. Peptide was injected into the subphase with a Hamilton microsyringe. At the pressures used, peptides alone induced negligible increases in surface pressure at the air-water interface.

**Partitioning into membranes**

Peptide partitioning into membranes was evaluated using POPC LUV (Wimley and White, 1996) by monitoring the change in emitted Trp fluorescence. Corrected spectra were recorded in a Perkin Elmer MFP-66 spectrophotometer with excitation set at 280 nm and 5-nm slits. Partitioning curves were subsequently computed from the fractional changes in emitted Trp fluorescence when titrated with increasing lipid concentrations. The signal was further corrected for dilution and inner filter effects as described in White et al. (1998) using the soluble Trp analog, NATA, which does not partition into membranes. The apparent mole fraction partition coefficients, Kapp, were determined fitting the experimental values to an hyperbolic function:

\[
F/F₀ = 1 + \left[\frac{(F_{\text{max}}/F₀) - 1}{K} + [L]\right]/[L],
\]

where [L] is the lipid concentration and K is the lipid concentration at which the bound peptide fraction is 0.5. Therefore, \(K_{\text{app}} = [W]/K\) where [W] is the molar concentration of water.

**Peptide oligomerization**

Clustering of HIV, peptide was monitored by the self-quenching effect produced in aggregates of Rho-labeled peptide as described in Ben-Efraim et al. (1999) and Saéz-Cirión et al. (2002). Changes in fluorescence intensity were measured at 581 nm (5-nm slits) with excitation set at 550 nm (5-nm slits). Maximal quenching (or 0% quenching) was inferred from samples solubilized with Triton X-100 (0.5% v/v). Occasionally, maximal dequenching measurements were carried out in SDS-solubilized samples, always with good correlation. Oligomerization was also evaluated using Tricine-SDS-PAGE. Samples consisted of 45 μg peptide incubated in presence or absence of 20 mM BS⁵ bifunctional cross-linking reagent (Knoller et al., 1991). Cross-linked complexes were subjected to gel electrophoresis. Gels were stained with Gelcode blue stain reagent (Pierce, Rockford, IL).

**Cell-cell fusion assays**

TF228.1.16 cells expressing HIV-1 Env (BH10 strain, a gift from Dr. Z. Jonak, SmithKline Beecham) and HeLaT4⁺ cells expressing CD4 and CXCR4 (obtained from AIDS Research and Reference Reagent Program, NIH) were maintained as described in Melikyan et al. (2000). TF228.1.16 cells (referred to as effector cells) were loaded with fluorescent dye calcein (AM, green emission). The target (HeLaT4⁺) cells were loaded with CMAC (blue emission). Fusion was quantified by fluorescence microscopy by measuring the fraction of cells positive for both dyes out of the total number of effector/target cells in contact, as described in Melikyan et al. (2000). Briefly, effector and target cells were mixed in the presence or in absence of HIVc or HIVw₁₋₃A peptides, allowed to settle on polysylane-coated eight-well chambered slides, and incubated at 37°C for 2 h. Images of calcein and CMAC fluorescence were acquired by intensified charge-coupled device (CCD) camera using appropriate filter sets, digitized, pseudocolored, and overlaid on each other to facilitate detection of cell fusion.

**Results**

**Interfacial hydrophobicity distribution in gp41 preTM sequence**

As shown in Fig. 1A, the gp41 membrane-proximal domain, or pretransmembrane sequence, is located between the carboxy-terminal helix (residues 624–665 of HIV-1 Env precursor) and the transmembrane region (residues 684–706 of HIV-1 Env precursor). In a compelling site-directed mutagenesis analysis of the preTM region, Salzwedel and coworkers (Salzwedel et al., 1999) described the production and functional characterization of 25 gp41 variants. Among these variants, mutants displayed in Fig. 1B were selected based on the following criteria. We analyzed the deletions and substitutions reported by these authors (full deletion and insertions of epitopes were not analyzed) and established the relationship existing between the capacity of a particular preTM sequence to partition into the membrane interface (Wimley and White, 1996) and the fusogenic activity of gp41 (Salzwedel et al., 1999). When we plot the fusion index versus ΔG for transfer from membranes into water (data not shown), it follows that the wild-type (wt) sequence is the most active, in accord with its largest ΔG value, whose magnitude is linearly related to the fusion index for most substitutions. However, for the group of mutants displayed in Fig. 1B, the fusion index is much lower. Thus, these sequences showed comparable capability to partition into membranes to that shown by corresponding functional sequences but displayed defective fusion phenotypes. This suggested that, in addition of the overall value, the actual distribution of hydrophobicity (i.e., a structure-related factor) might affect the function of this sequence.

The interfacial hydropathy plots displayed in Fig. 1B indeed suggest the existence within gp41 preTM of two positive peaks, each comprising ~10 amino acids. Since helical anchors immersed into one monolayer interface might be shorter than TMD anchors (Picot et al., 1994), we calculated average hydrophobicity-at-interface in the plots using small five amino acid windows. Deletions in the preTM region correlated with ablation or merging of the two peaks, and concomitant loss of function. Likewise, the analysis of the triple substitutions and scrambled SC7 mutant suggested that the presence of both peaks might promote fusogenicity. Thus results displayed in Fig. 1B suggest that gp41 preTM region contains two interfacial segments,
a pattern recently described to occur also in 6K, the alpha-virus protein involved in catalyzing membrane fission during viral budding (Sanz et al., 2003). An interfacial sequence containing two hydrophobic-at-interface segments also exists in vesicular stomatitis virus (VSV) glycoprotein membrane-proximal region (data not shown), which is also involved in fusion and budding processes (Robinson and Whitt, 2000; Jeetendra et al., 2002).

The N-terminal gp41 preTM subdomain (peak 1 in Fig. 1 B) would also generate a face with strong affinity for membranes, if folded into an α-helix (Fig. 2). Hydrophobic moments measure the periodicity of residue distribution along the secondary structure elements (Eisenberg et al., 1982, 1984). Preferential orientation of the hydrophobic residues toward one face of the structure element has been proposed to favor hydrophobic interactions between protein structural components and between membranes and protein sequences. Considering that preTM may be part of the soluble gp41 ectodomain (see Discussion), its hydrophobic moment was computed using the membrane interface-to-water transfer free energies (ΔG_{iw}) for each amino acid (Wimley and White, 1996; White and Wimley, 1999) as the moduli of the vectors that project following the direction of the amino acid side chains. It must be noted that, when partition from water into interfaces of whole residues is taken into consideration, the relative amino acid hydrophobicities seem to be different from those derived from bulk-phase partitioning of side chains (see for extensive discussions Deber and Goto, 1996; White and Wimley, 1999; Nieva and Suárez, 2000). For instance, nonpolar Val and Ala residues, commonly present in TMD sequences, would not promote partitioning into membranes, whereas aromatic Trp, Tyr, and Phe residues would be efficient promoters. This difference also affects the hydrophobic moment calculation (Fig. 2). According to plots in Fig. 2 A, a helical sequence that segregates residues to enable maximal partitioning from water into membranes would be barely detected as amphipathic using Eisenberg hydropathy scale.

![Figure 2](image_url)

**FIGURE 2** Hydrophobic moments (as a function of the δ angle) calculated according to different hydropathy scales. (A) Comparison using WW hydrophobicity-at-interface (left panel) or Eisenberg hydrophobicity (right panel). The selected sequences WWEEWEEW (solid lines) and IIRRIRRIRR (dotted lines) contain, alternating with helical periodicity, most hydrophobic and most hydrophilic residues in WW and Eisenberg scales, respectively. (B) Hydrophobic moment of the HIV-1 gp160 664–674 segment (3 helical turns) calculated as a function of the δ angle. Sequences derived from wild-type (solid lines) and W(1–3)A mutant (dotted lines) proteins are compared using three different hydrophobicity scales (WW, Eisenberg, and KD). Dashed line in WW panel corresponds to the 673–683 gp160 stretch.
Plots in Fig. 2B show the calculated WW moments as a function of the angle between residues (δ). The plot for the amino-terminal preTM segment (peak 1 in Fig. 1B) displayed a maximum at ~100°, indicating that the adoption of an α-helical conformation generates a Trp-rich surface, whereas the carboxy end (peak 2 in Fig. 1B) seemed not to be amphipathic. The W(1–3)A triple substitutions within HIV-1 gp41 abrogate its ability to promote cell-cell fusion and virus entry without affecting maturation, transport, or CD4-binding ability of the protein (Salzwedel et al., 1999). These triple Ala substitutions induced the disappearance of the helical interfacial peak of the preTM region. Accordingly, the W(1–3)A mutant also showed a largely reduced moment at δ = 100° (top panel in Fig. 2B). A difference between wt and mutant sequences was not evident when Eisenberg or KD hydropathy scales were applied to calculate the hydrophobic moment (bottom panels in Fig. 2B).

In agreement with the previous observations, the sum of moments with fixed δ = 100° in five-residue windows detected a peak comprising 10 amino acids (residues 663–672 of HIV-1 gp160) at the N-terminus of HIV-1 gp41 preTM (Fig. 3). As shown in Fig. 3, an amphipathic helical motif was present in different HIV-1 isolates and in other lentiviruses. This suggests that the N-terminal segment of gp41 preTM may become an extension of the preceding helical HR2 segment of gp41 ectodomain. This helical HR2 extension shows a tendency to partition into membrane interfaces and is segregated from its C-terminal segment that directly links preTM to the TMD.

IR analysis of gp41 preTM structure in solution and membranes

The structural changes induced by W(1–3)A substitutions were investigated in solution (Fig. 4A) and membranes (Fig. 4B) by looking at the infrared amide I band located between 1700 and 1600 cm⁻¹ (Arrondo and Goñi, 1998, 1999). Deconvolved spectra of HIVc and HIVW(1–3)A peptides are shown. For HIVc (top spectra) the presence of lipids produces shifts of the 1650 cm⁻¹ and the 1629 cm⁻¹ components to 1621 and 1626 cm⁻¹ (bottom spectra), respectively. On the other hand, in HIVW(1–3)A (bottom spectra), the 1626 cm⁻¹ band is shifted to 1621 cm⁻¹ and two other components at 1644 and 1656 cm⁻¹ are present in the presence of lipids. The main component in solution is at 1650 cm⁻¹ for HIVc, whereas it is at 1626 cm⁻¹ for HIVW(1–3)A.

The band at 1650 cm⁻¹ (≈60% of total area of HIVc in solution) can be unambiguously assigned to a canonical α-helix. Assignment of component at 1629 cm⁻¹ (≈30% of total area) is not straightforward. Bands below 1635 cm⁻¹ together with high frequency components are usually assigned to β-sheet in proteins. However, in oligopeptides short α-helices have been found to display a band around 1635 cm⁻¹ (Martinez and Millhauser, 1995); and using ¹³C-labeled peptides it has been shown that α-helices with a solvated backbone also give rise to signals in that range of wave numbers. Besides, helix-helix interactions in coiled coils also exhibit bands in the region usually attributed to β-sheet (Reisdorf and Krimm, 1996), because of the cross-hydrogen bonds that can be formed. These cross-hydrogen bonds have been described in bacteriorhodopsin (Ubarretxe-Belandia and Engelman, 2001) that also exhibits an anomalous infrared spectrum (Rothschild and Clark, 1979). The presence of lipids produces comparable shifts in both components (to 1654 and 1635 cm⁻¹, respectively) but does not significantly alter the overall structure (55 and 36% of total area, respectively). The former effect can be interpreted either as changes in the geometry of the structural element, or a shift from aqueous to a less polar environment with less strong hydrogen bonds.

To explore further the origin of the 1635 cm⁻¹ band, we analyzed IR spectra of HIVc helices in a membrane milieu. Fluorescence data indicated that rhodamine-labeled HIVc in lipid vesicles displays a dose-dependent tendency to self-associate (Sáez-Cirión et al., 2002). This is illustrated by results obtained in POPC LUV containing increasing peptide loads (Fig. 5A). Since detergent-solubilized HIVc is monomeric as judged by SDS-PAGE (see Fig. 7A), Rh-HIVc fluorescence quenching levels reflect different self-
association degrees at the membrane surface. Thus these model membranes provide a tool to assess helical structures as both monomers or multimers. In HIVc, decreasing the peptide/lipid ratio from 1:50 to 1:1000, where the peptide is most likely monomeric (Fig. 5A), results in a more symmetrical band at 1653 cm⁻¹ (Fig. 5B), with no contribution from the component around 1635 cm⁻¹, thus reinforcing the assignment of this band to helix-helix contacts in our system.

At the 1:50 ratio where self-association is prominent (Fig. 5A), the α-helical band splits into two components, absorbing at 1656 and 1649 cm⁻¹, besides the presence of a band at 1639 cm⁻¹, as was postulated previously for coiled coils (Reisdorf and Krimm, 1996). The 1:250 peptide/lipid ratio exhibits an intermediate pattern, but is dominated by the band attributed to canonical α-helix at 1653 cm⁻¹.

The structure of HIV_W(1–3)A in solution differs from that of HIVc (Fig. 4A). It has its most intense band at 1626 cm⁻¹ (≿47% of total area), indicating an extended conformation of the peptide. Moreover, in the case of this mutant, peptide-lipid binding produces the opposite effect. Rather than keeping a structural organization, the shift downwards to 1621 cm⁻¹ of the major component (≿40% of total area), points to a more massive aggregation of the peptide in membranes. The smaller components at 1644 cm⁻¹ (≿35%) and 1656 cm⁻¹ (≿25%) would indicate the presence of unordered structure and β-turns respectively, as has been shown in all-β proteins such as lectins (Chehin et al., 1999).

In conclusion, the wild-type sequence seems to self-associate into helical multimers both in solution and in membranes. In addition, the effect of lipids on both peptides are quite contrasting. Whereas the main conformation of HIVc is not affected upon its insertion in the lipid bilayer maintaining the solution secondary structure, HIV_W(1–3)A stability is disturbed by the presence of lipids, resulting in aggregation-denaturation of the mutant peptide. This con-
firms the predictions in Figs. 2 and 3 and highlights the specific role of Trp residues in stabilizing the sequence folding also in solution.

**Gp41 preTM interaction with membranes**

The ability of HIV$_c$ and HIV$_{W(1–3)A}$ peptides to penetrate membrane bilayers was first studied by the monolayer technique (Fig. 6 A). The penetration capacity depending on the monolayer surface pressure may be inferred from the monolayer exclusion pressure value ($\pi_{ex}$), or initial pressure ($\pi_0$) at which no increment in pressure is observed after injection of the peptide into the subphase ($\Delta \pi = 0$). The $\pi_{ex}$ values obtained from plots in Fig. 6 A were 37.2 mN m$^{-1}$ and 34.2 mN m$^{-1}$ for HIV$_c$ and HIV$_{W(1–3)A}$, respectively. Both values are within the range of lateral pressures postulated to arise from the lipid packing density that exists in natural membranes and unstrained bilayers ($\pi_0 \approx 30$ mN m$^{-1}$; Marsh, 1996).

Consistent with these observations both HIV$_c$ and HIV$_{W(1–3)A}$ were also able to penetrate efficiently lipid bilayers of POPC LUV (Fig. 6, B and C). Results in Fig. 6 B demonstrate that the intrinsic fluorescence of both peptides increased in the presence of these vesicles (dotted versus solid lines). This is indicative of the Trp residues sensing a low-polarity environment of the lipid bilayer. However, due to the higher Trp content, a higher tendency for HIV$_c$ to spontaneously transfer from water into membranes is expected (Wimley and White, 1996). In accordance with the predictions, titration of the peptides in solution with increasing amounts of lipid vesicles (Fig. 6 C) demonstrated a higher apparent partitioning capacity for the wt-representing peptide, ($K_{x(app)} > 10^7$), compared to the mutant, ($K_{x(app)} \approx 10^6$). The comparable saturation levels observed for both peptides also suggest that membrane-associated species are probably sensing a similar membrane, low-polarity environment. Taken together these data demonstrate that the W(1–3)A mutation did not significantly affect the capacity of the preTM to insert into membranes upon binding (Fig. 6, A and B) but reduced its affinity for the interface (Fig. 6 C).

**Gp41 preTM self-association in solution**

Experiments were conducted to explore homo-oligomerization of the HIV$_c$ sequence. As shown in Fig. 7 A, HIV$_c$ showed a single band on SDS-PAGE with a molecular weight corresponding to that of a monomer ($\approx 2600$ Da). This is consistent with the capacity of the detergent to solubilize Rho-HIV$_c$ peptide oligomers. After subjecting the peptide to chemical cross-linking, oligomers became apparent on the gel. The cross-linking reaction developed similarly when the reagent was present at time $t = 0$, or when added...
after 90 min of preincubation of the peptide in solution. These oligomers were not observed in the case of HIV\textsubscript{W(1–3)A} peptide. In summary, the SDS-PAGE analysis suggests the formation of HIV\textsubscript{c} homo-oligomers in solution.

This self-association process of HIV\textsubscript{c} in solution was subsequently studied using the fluorescently labeled Rho-HIV\textsubscript{c} sequence (Fig. 7 B). When fluorophore molecules are in close proximity, rhodamine emission diminishes (Hoekstra et al., 1984; Ben-Efraim et al., 1999). Thus, Rho quenching efficiency correlates with the aggregation state of the peptide (Ben-Efraim et al., 1999; Sáez-Cirión et al., 2002). Experiments in Fig. 7 B show that Rho emission decreased with time of incubation in solution (inset). When a fraction of Rho-HIV\textsubscript{c} labeled peptide was replaced by unlabeled HIV\textsubscript{c} peptide, Rho quenching was hampered (compare bars a and b in Fig. 7 B). This is consistent with the observed fluorescence attenuation due to peptide self-association.

**Synthetic preTM peptide specifically inhibits gp41-induced fusion**

The ability of preTM to self-associate in solution and membranes raised the possibility of preTM homo-oligomer formation at some stage during gp41-induced fusion process. To explore this hypothesis we tested whether the preTM peptides could interfere with cell-cell fusion induced by HIV-1 envelope (Fig. 8). Co-incubation of the effector and target cells at 37°C in the absence of peptides resulted in extensive fusion, as evidenced by cytoplasmic content mixing between cells. When HIV\textsubscript{c} was present in the external solution, it inhibited fusion in a dose-dependent manner showing an apparent IC\textsubscript{50} value of 577 nM (Fig. 8, open circles). In contrast, the mutant HIV\textsubscript{W(1–3)A} peptide did not suppress fusion even at concentrations as high as 10 µM (filled circles). In control experiments, neither of these two peptides inhibited influenza hemagglutinin (HA)-mediated fusion between HA-expressing cells and red blood cells (data not shown). This implies that the inhibitory effect of wt preTM peptide was through its interaction with HIV envelope protein and not through its partitioning into cell membranes and causing nonspecific perturbations. Neither of the two peptides showed a lytic activity on the cells within the concentration range tested here (data not shown).
DISCUSSION

PreTM role in membrane anchoring

Truncation of the gp41 carboxy terminus beyond Lys683 (i.e., last residue of the preTM region) causes loss of membrane anchoring and secretion of the glycoprotein in a soluble form (Dubay et al., 1992; Owens et al., 1994; Salzwedel et al., 1999). This might be interpreted as an indication that the preTM sequence is not sufficient to membrane anchor gp41 with a deleted TMD. However, an inspection of the energetics of helix insertion suggests that a single preTM region might potentially function as a stable interfacial anchor. Estimations of the free energy of insertion of a single preTM region might potentially function as a stable interfacial anchor. Estimations of the free energy of insertion of a single preTM region might potentially function as a stable interfacial anchor. Estimations of the free energy of insertion of a single preTM region might potentially function as a stable interfacial anchor. Estimations of the free energy of insertion of a single preTM region might potentially function as a stable interfacial anchor. Estimations of the free energy of insertion of a single preTM region might potentially function as a stable interfacial anchor. Estimations of the free energy of insertion of a single preTM region might potentially function as a stable interfacial anchor. Estimations of the free energy of insertion of a single preTM region might potentially function as a stable interfacial anchor.

The fact that preTM region has been shown to bind cholesterol in the context of the full-length envelope protein (Vincent et al., 2002) gives support to this hypothesis. Importantly, recent hydrophobic photolabeling studies have revealed fusion-dependent incorporation of a label into the transmembrane domain and membrane-proximal region of HR2 (Raviv et al., 2003), suggesting the existence after fusion of a membrane-embedded preTM structure connecting both gp41 elements.

PreTM role in oligomerization

One possible means to enhance preTM solubility within the prefusogenic gp41 intermediate is to cluster interfacial residues into an occluded surface. This might be achieved through oligomerization of amphipathic helices. Our hydrophobicity-at-interface distribution analysis suggests that the functional preTM sequence is segmented into two subdomains: one amphipathic at the N-terminal side and one interfacial at the C-terminus (Figs. 1–3). This supports a supplementary role for the preTM sequence in sustaining the prefusogenic gp41 oligomeric organization existing in the virion (Center et al., 2002). However, we caution that membrane partitioning does not seem to be significantly affected by the tendency of preTM peptides to self-associate in solution. We have not observed differences in partitioning at peptide concentrations ranging from 0.1 to 1 μM, and the kinetics of incorporation into membranes of Rho-labeled sequences were similar at 0.1 and 4 μM (i.e., 40 times more concentrated; data not shown). Moreover, the C-terminal segment (residues 674–683 of gp160) contributes enough partitioning energy to enable association with and penetration into membranes, even of denatured sequences (Fig. 6).

At any rate, the amphipathic-at-interface N-terminal stretch (residues 664–673) seems to preserve an overall α-helical structure in solution and in membranes (Figs. 4 and 5). IR (Fig. 5) and NMR (Schibli et al., 2001) structural data reveal a monomeric helical structure that would be prevalent in membranes at low doses. This structure self-associates at higher membrane loads or in the presence of raft-type lipids (Sáez-Cirión et al., 2002). Results in this work confirm that preTM may establish helix-helix interactions in solution and in membranes (Figs. 4, 5, and 7).

As depicted in Fig. 9A, the molecular model for the elongation of HR2 including the preTM amino subdomain shows that hydrophobic HR2 residues implied in packing against the trimeric HR1 coiled coil, and aromatic-interfacial residues might occupy opposite faces of an helix. Thus, a putative oligomeric preTM structure might also help preventing HR2 packing against trimeric HR1 coiled coil. In fact, recent studies demonstrate that HR2 regions are exposed and accessible to inhibitors in native and prefusogenic intermediates of gp41 (Root and Hamer, 2003; Koshiba and Chan, 2003). This mechanism would also be in agreement with the disappearance of mAb 2F5 binding site upon HR1/HR2 complex formation (Gorny and Zolla-Pazner, 2000). The epitope recognized by 2F5 mAb comprises residues 656–671 (Parker et al., 2001), i.e., it includes almost completely the amphipathic preTM amino subdomain. This epitope is recognized at the native prefusogenic form of gp41 and is thought to become occluded upon fusion activation when the six-helix bundle forms (Sattentau et al., 1995; Gorny and Zolla-Pazner, 2000; Finnegan et al., 2002).

Thus, interfacial hydrophobicity could play a role by eliciting the preTM-membrane interaction required to release conformational constraints in HR2 and facilitate HR1-HR2 interactions. Of note, a W(1–5)A mutant in which the 5 Trps have been substituted with Alas induces a defective fusion process arrested at the level of pore opening (see below) (Muñoz-Barroso et al., 1999). This mutant, for which we foresee a defect in preTM-membrane association, is actually more susceptible to inhibition by T20 (Muñoz-Barroso et al., 1999), indicating a defect in bundle formation.
The preTM role in fusion activity and inhibition

Different cell-cell fusion assays demonstrate that gp41 lacking preTM sequence is not fusogenic, i.e., it is incapable of mediating both membrane-and-volume mixing and syncytium formation (Muñoz-Barroso et al., 1999). The fusion activity of W(1–5)A mutant, namely, the formation of non-expanding small fusion pores, is similar to that exhibited by GPI-linked hemagglutinin (GPI-HA) of influenza virus (Markosyan et al., 2000). This mutation provokes the elimination of positive hydrophobic-at-interface peaks from preTM region (data not shown). Thus, it appears that in the absence of interfacial hydrophobicity at the preTM region, gp41 displays the activity of ectodomains, which are membrane linked through lipidic tethers. Based on this outcome and our results, we suggest two mutually non-exclusive mechanisms according to which preTM interfacial hydrophobicity could play a role at fusion-pore enlargement (illustrated in Fig. 9, B and C):

Modulation of monolayer net curvature

Long interfacial preTM sequences seem to be functional in fusion (Salzwedel et al., 1999; Muñoz-Barroso et al., 1999; Sua´rez et al., 2000a; Jeetendra et al., 2002) and also in membrane fission processes that take part during viral budding (Robinson and Whitt, 2000; Sanz et al., 2003). A low-energy interfacial topology would confer net positive curvature to the preTM-containing membrane monolayer, a factor that might help budding. Conversely, according to the “stalk-pore” hypothesis of fusion, positive curvature in contacting monolayers (cis-monolayers) would hinder the formation of a “stalk” (an initial lipidic intermediate that locally connects the cis-monolayers of two apposed membranes) (Chernomordik et al., 1995; Siegel and Epand, 1997; Razinkov et al., 1998; Lentz et al., 2000, 2002). Thus, in the framework of this theory, we have to infer that preTM must insert into membranes at later stages of the process. For instance, interfacial preTM structure might promote positive curvature by expanding the cis-monolayer in trans-monolayer contacts (TMCs), a second lipidic intermediate in the fusion pathway (Fig. 9 B). As a result, expansion of fractured single-bilayer diaphragm in the TMC intermediate would be favored (Siegel and Epand, 1997; Lentz et al., 2000). In this way, preTM interfacial hydrophobicity would elicit the eventual opening of fusion pores connecting the initially separated aqueous environments.

Self-assembly of gp41 monomers in membrane surfaces

It has been postulated that an assembly of gp41 oligomers could establish the molecular scaffold that enables membranes to merge. As described by Markosyan et al. (2002), recombinant bundles did not inhibit fusion. Thus, it is unlikely that bundles interact with each other at the fusion site. We propose that the preTM embedded in the viral membrane interface is responsible for self-assembly of gp41
at the fusion site (see also Sáez-Cirión et al., 2002) and, therefore, a defined hydrophobicity distribution along the sequence might condition the rate of formation and/or eventual structure of gp41 complexes. The inhibition data reported in this work (Fig. 8) are consistent with this hypothesis. Our estimates indicate that 99% of the added peptide partitions into membranes under the experimental conditions used in cell-cell fusion experiments. Thus, a likely mechanism of the inhibitory activity of the HIVɛ peptide is through its binding to the gp41 cognate preTM region in its membrane-bound state, thereby interfering with functional oligomerization of HIV-1 Env (Fig. 9 C). This inhibition mechanism would be reminiscent of that previously proposed by Kliger et al. (2001) for the T20 peptide.

CONCLUSIONS

Unraveling gp41 preTM structure-function relationship was instrumental in our design of inhibition of cell-cell fusion and may implement anti-HIV drug and vaccine development (Zwick et al., 2001). The hypothesis that stems from our predictions (Figs. 1–3) is that the interfacial hydrophobicity distribution might condition this element's structure function, particularly its tendency to oligomerize as an α-helix in solution and membranes. Experimental data generated so far, including mutagenesis and biophysical characterizations, are consistent with this hypothesis. However, we caution that prefusogenic and postfusogenic gp41 atomic structures, including the membrane-proximal region, are still unavailable. Moreover, even if in the past biophysical characterization of synthetic peptides representing different regions of the HIV-1 gp41 subunit has led to fundamental conclusions on the structural organization and putative mechanism of action of this protein (Doms and Moore, 2000; Eckert and Kim, 2001), we cannot rule out that the structure of these sequences might turn out to be different within full-length gp41.

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