Liposome destabilization induced by the HIV-1 fusion peptide Effect of a single amino acid substitution

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Abstract The 23-residue synthetic peptide representing the N-terminus of HIV-1 gp41 is known to induce either leakage or fusion of lipid vesicles depending on the experimental conditions. In this paper we report that a polar amino acid substitution V→E at position 2, known to block gp41 activity in vivo, makes the peptide unable to destabilize and/or fuse membranes. Moreover this variant, unlike the parent peptide, is never found in the membrane-associated β conformation.

Key words: Membrane fusion; Fusion peptide; Peptide conformation; HIV-1

1. Introduction

It has been proposed that a pivotal event during the HIV-1 fusion mediated by the envelope protein gp120/41 would be the exposure of a putative fusogenic sequence, a conserved hydrophobic segment of about 25 amino acids located at the N-terminus of gp41 [1]. The interaction of this hydrophobic stretch or 'fusion peptide' [2] with the target membrane could induce some kind of molecular destabilization essential for the completion of the process. The involvement of the N-terminus of gp41 in HIV fusion in vivo has been demonstrated by mutational analysis. Syncytium formation in transfected cell systems is enhanced by mutations within this sequence that increase its hydrophobicity [3], while substitutions that increase the charge can block the process [4]. More recently, Freed et al. [5] have reported that a single polar substitution V→E at position 2, known to block syncytium formation [4], leads to a mutant envelope glycoprotein (named 41.2 after the mutant) that completely interferes with syncytium formation and infection mediated by the wild-type fusion protein. The interfering effect is detected even in the presence of excess wild-type glycoprotein, suggesting that a higher-order protein complex must be involved in membrane fusion.

With the aim of testing the membrane destabilizing effects of the HIV-1 fusion peptide, several studies have been conducted in model membranes using synthetic peptides representing this sequence. Rafalski et al. [6] showed destabilization of POPG liposomes and membrane mixing of small sonicated vesicles. Slepushkin et al. [7] showed the ability of a family of gp41 peptides to cause increased conductance of planar lipid bilayers, liposome lysis and lipid mixing. The HIV-1 fusion peptide has been shown to induce lysis of human erythrocytes and $CD4^+$ lymphocytes [8]. A 16-amino acid peptide has been reported to induce lipid mixing and leakage of contents in PC:PE containing vesicles [9].

In an attempt to establish a relationship between peptide membrane activity and structure, we have recently carried out a study in a liposomal system using a 23-residue synthetic peptide (HIVarg) derived from the LAV_{1a} strain of HIV-1 [10]. The peptide alone could not aggregate negatively charged large unilamellar POPG vesicles. When Ca^{2+} or Mg^{2+} were first added to the POPG LUV, inducing aggregation but not fusion of the vesicles, the subsequent addition of the peptide initiated vesicle fusion. HIVarg also induced leakage of small molecules from the vesicles by forming membrane pores but only in the absence of cations. We observed that, for HIVarg and POPG liposomes, pore formation and membrane fusion required different peptide conformations represented by an α-helix and a β-structure, respectively [10].

Here, using a similar approach, we have evaluated the effect of the V→E substitution at position 2 on both membrane activity and conformation of a related HIV-1 fusion peptide. The former substitution was selected in view of the already mentioned dramatic effects produced on gp41 activity in vivo [4,5].

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoylphosphatidylglycerol (POPG) and the fluorescent probes, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). 8-Aminonaphtalene-1,3,6-trisulfonic acid sodium salt (ANTS) and *p*-xylene-bis(pyridinium)bromide (DPX) were from Molecular Probes (Junction City, OR, USA). Triton X-100 was obtained from Sigma (St. Louis, MO, USA). Fluoraldehyde reagent was from Pierce (Rockford, IL, USA). All other reagents were of analytical grade.

2.2. Peptide synthesis

The sequences, AVGIGALFLGFLGAAGSTMGAAS (HIV_{wt}) and AEGIGALFLGFLGAAGSTMGAAS (HIV_{E2}) representing the N-terminus of the HIV gp41 (HXB-2 viral clone) and its 41.2 mutant [4,5], respectively, were synthesized as their C-terminal carboxamides by Dr. T. Saermark (EC Concerted Action Programme) as previously described [11]. Purification was carried out by reverse-phase HPLC (estimated homogeneity >90%). Final amino acid analysis confirmed the correct composition. Peptide stock solutions were prepared in DMSO (spectroscopic grade).

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Abbreviations: ANTS, 8-aminonaphtalene-1,3,6-trisulfonic acid; DMSO, dimethylsulfoxide; DPX, *p*-xylenebis(pyridinium)bromide; FTIR, Fourier transform infrared spectroscopy; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfo nic acid; HIV, human immunodeficiency virus; HIV_{wt}, synthetic N-terminal sequence (23 aa) of HIV-1 gp41; HIV_{E2} synthetic N-terminal sequence (23 aa) of HIV-1 gp41 bearing the V→E substitution at position 2; LUV, large unilamellar vesicles; *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine; PE, phophatidylethanolamine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine.

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2.3. Vesicle preparation

Large unilamellar vesicles (LUV) of POPG were prepared according to the extrusion method of Hope et al. [12] as previously described [10].

2.4. Fluorimetric assays for vesicle fusion and leakage

All fluorescence measurements were conducted in thermostatically controlled cuvettes using a Perkin-Elmer LS50-B spectrofluorimeter. The medium in the cuvettes was continuously stirred to allow the rapid mixing of peptides and vesicles.

Membrane fusion was demonstrated as the mixing of the lipidic components of the vesicles. Lipid mixing was monitored using the resonance energy transfer assay, described by Struck et al. [13]. Vesicles containing 0.6 mol% of *N*-NBD-PE and *N*-Rh-PE were mixed with unlabeled vesicles in a 1:4 ratio. The NBD emission was monitored at 530 nm with the excitation wavelength set at 465 nm. A cutoff filter at 515 nm was used between the sample and the emission monochromator to avoid scattering interferences. The fluorescence scale was calibrated such that the zero level corresponded to the initial residual fluorescence of the labeled vesicles, and the 100% value to the complete mixing of all the lipids in the system. The latter value was set by the fluorescence intensity of vesicles, labeled with 0.15 mol% each of the fluorophores, at the same total lipid concentration as in the fusion assay.

Release of vesicular contents to the medium was monitored as in [10], by the ANTS/DPX assay [14]. LUV containing 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl and 5 mM HEPES, were obtained by separating the unencapsulated material by gel-filtration in a Sephadex G-75 column eluted with 5 mM HEPES, 100 mM NaCl (pH 7.4). Osmolarities were adjusted to 200 mosm. Fluorescence measurements were performed by setting the ANTS emission at 520 nm and the excitation at 355 nm. A cutoff filter (470 nm) was placed between the sample and the emission monochromator. The 0% leakage corresponded to the fluorescence of the vesicles at time zero; 100% leakage was the fluorescence value obtained after addition of Triton X-100 (0.5% v/v).

2.5. Peptide binding to vesicles

Peptide binding to vesicles was estimated by flotation analysis of the peptide–liposome complexes in D_2O buffer (5 mM HEPES, 100 mM NaCl, pH 7.4). Peptide dissolved in DMSO was added to 1 ml POPG LUV (1 mM) prepared in D_2O buffer (peptide-to-lipid ratio, 1:100). After 30 min incubation at room temperature, centrifugation of the peptide–lipid complexes in a Beckman Optima TLX ultracentrifuge in a TL100 rotor (627,000×*g*, 120 min) gave rise to a homogeneous band of vesicles floating on top of the D_2O buffer. The tubes were fractionated from the top and peptide distribution was subsequently quantitated by the fluoraldehyde assay following the instructions of the manufacturer. Vesicle distribution was determined after phosphate analysis [15] of the same fractions.

2.6. Infrared spectroscopy

Measurements were essentially conducted as in [10] on peptide–lipid complexes obtained in D_2O buffer following the above binding protocol. Infrared spectra were recorded in a Nicolet 520 spectrometer equipped with a DTGS detector. Samples, containing ≈4 mg peptide/ml, were placed between two CaF_2 windows separated by 50 μm spacers. 200 sample scans and 200 reference scans were taken for each spectrum, using a shuttle device. Spectra data were transferred to a personal computer where solvent subtraction and band-position determinations were performed as previously reported [16].

3. Results

In Fig. 1, the effects of HIV_{wt} and HIV_{E2} on POPG LUV stability are comparatively shown. The peptides in this example were added at a relatively high dose (peptide-to-lipid, 1:15) in order to rule out the existence of different threshold peptide-to-lipid ratios for the induction of destabilization. Higher doses were likely to induce destabilization via unspecific mechanisms. As shown in panel A, HIV_{wt} induced extensive leakage of ANTS from the vesicles (75% after 90 s) whereas the addition of HIV_{E2} resulted in a much smaller release (15% after 90 s). Thus the substitution severely affected the ability of the peptide

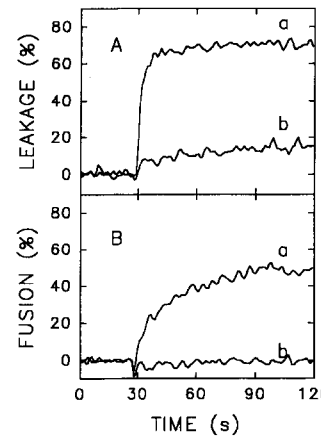

Fig. 1. Effect of V→E substitution on HIV-1 fusion peptide-induced POPG LUV destabilization. HIV_{wt} (curves a) and HIV_{E2} (curves b) were added to vesicle suspensions and their effects on vesicle stability compared. Vesicle concentration was 50 μM. Peptides were added at time = 30 s (peptide-to-lipid ratio of 1:15). Fluorescence was recorded continuously. (A) Kinetics of leakage of ANTS/DPX. (B) Kinetics of membrane lipid mixing (resonance energy transfer assay). The medium contained 5 mM Ca^{2+} in this case.

to disrupt the permeability barrier of the POPG vesicles. The results in panel B demonstrate that HIV_{E2} was unable to induce mixing of the membrane lipids in the presence of 5 mM Ca^{2+} as compared to HIV_{wt}. 90 s after addition of HIV_{E2}, virtually no membrane mixing was detected by the resonance energy transfer assay whereas HIV_{wt} induced the mixing of 50% of the vesicular lipids.

We also checked in the POPG LUV system the ability of HIV_{E2} to interfere with HIV_{wt}-induced leakage and fusion (data not shown). Several HIV_{wt}-to-HIV_{E2} ratios ranging from 1:1 to 1:10 and different protocols were tested. Addition of premixed HIV_{wt} and HIV_{E2} displayed the same effects as HIV_{wt} alone, and pre-addition of HIV_{E2} to the vesicles did not affect either leakage or membrane mixing induced by HIV_{wt}. HIV_{E2} addition at any time after the onset of HIV_{wt}-induced destabilization also did not affect the course of the kinetics in either case. We thus conclude that HIV_{E2} was unable to interfere with the activity of HIV_{wt}.

The binding data in Fig. 2 demonstrate that the differences detected in the previous experiments were not due to differences in the vesicle-binding ability of the peptides. Both in the absence (panels A and C) and in the presence (panels B and D) of 5 mM Ca^{2+}, the distribution patterns of HIV_{wt} and HIV_{E2} were very similar after flotation. Therefore we can assume the existence of a comparable amount of both variants associated to vesicles. The data also suggest a higher amount of peptide associated to the top fraction of vesicles in the presence of the cation. Under the latter conditions, HIV_{wt} induced vesicle fusion.

Infrared analysis of the peptide–lipid complexes floating in D_2O buffer corroborated the earlier binding data. The spectra in Fig. 3 displayed an intense band centered at 1734/1735 cm^{-1}, corresponding to the COO^- stretching vibration of the phospholipid ester bonds, together with bands in the conformation-sensitive amide I region corresponding to the peptides. The relative intensities of the peptide and lipid bands indicated a comparable degree of binding for both peptides. Conversely, these bands suggested the existence of a distinct conformational

behaviour for each peptide. In the absence of Ca^{2+} (panel A) both HIV_{wt} (curve a) and HIV_{E2} (curve b) spectra displayed a main band centered at 1657 cm^{-1}, indicating a preferential α-helical conformation adopted by both peptides in the membrane. However, in the presence of Ca^{2+} (panel B), spectrum a included a conspicuous band, centered at 1626 cm^{-1}, indicative of the presence of a population of peptide backbones in β-conformation. This band was absent in spectrum b. We concluded that HIV_{wt}, but not HIV_{E2}, could adopt a β-conformation in the membrane under conditions allowing fusion.

4. Discussion

Taken together, the results described above demonstrate that a single substitution in HIV_{wt} that did not affect its binding capacity, brought about dramatic effects as to its ability to destabilize membranes and inhibited its adoption of a β-conformation in the membrane under conditions allowing fusion. First we found that, in contrast to HIV_{wt}, HIV_{E2} was unable to promote extensive leakage of vesicle contents (Fig. 1). Such inability can not be easily interpreted in terms of structure since both HIV_{wt} and HIV_{E2} showed similar α-helix contents when associated to vesicles (Fig. 3). Clearly additional requirements, e.g. the formation of peptide aggregates or pores [10], must be met in order to effectively disrupt the permeability barrier of the vesicles. Nevertheless, the absence of HIV_{E2}-induced vesicle fusion can be correlated with its inability to adopt in the membrane the β structure adopted by the wild-type peptide under conditions allowing this process (i.e. in the presence of 5 mM Ca^{2+}). The lack of liposome fusion induced by another HIV-derived peptide carrying the same mutation has been recently observed as well (J. Wilschut, L. Yang, S.Q. Nie and K.C. Lin, submitted for publication). Given the short length of the peptide and the position of the substitution, it appears again that peptide–peptide interactions to form higher order complexes, in which the glutamate residue could cause extensive conformational disruption, might be involved in membrane destabilization. Whereas on isolated vesicles the peptides in those complexes are probably assembled forming pores, they must form some different kind of structures when cations pre-aggregate the membranes [10].

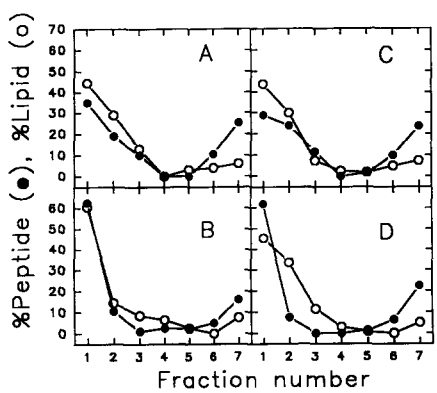

Fig. 2. Binding of HIV_{wt} (A,B) and HIV_{E2} (C,D) to POPG LUV in absence (A,C) and in presence (B,D) of 5 mM Ca^{2+}. Attachment of the peptides to the liposomes was assessed by co-flotation analysis in D_2O buffer (5mM HEPES, 100 mM NaCl, pH 7.4). Fraction 1 corresponds to the top of the tube. Filled circles correspond to percentage of the total peptide subjected to flotation, and open circles to the total lipid. More than 90% of the total peptide was collected in the bottom fraction in the absence of vesicles (data not shown).

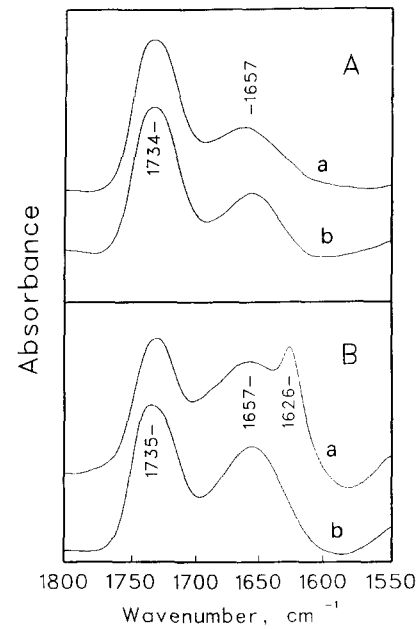

Fig. 3. Infrared spectra of HIV_{wt} (curves a) and HIV_{E2} (curves b) bound to POPG vesicles. The peptides (peptide-to-lipid ratio 1:100) were added to LUV (1 mM) under the experimental conditions used in the fusion and leakage assays (see section 2). (A) In the absence of 5 mM Ca^{2+}. (B) In the presence of 5 mM Ca^{2+}.

The situation in vivo is qualitatively different. It has been suggested that the gp41 external domain is responsible for surface oligomerization of the HIV-1 envelope protein [17]. Thus, the interfering effect on infectivity and fusion exerted by the 41.2 mutant could be explained in terms of pre-existing protein complexes that contain mutant monomers [5]. Our results suggest that those mutant monomers would bear a defective fusion peptide which lacks affinity towards its companions and are unable to induce membrane destabilization. We speculate here that the presence of those defective fusion peptides could be enough to distort the correct architecture of the fusion sites built by the otherwise functional protein complexes. This could also explain the dramatic effect displayed by this single substitution on gp41 activity in vivo.

One interesting finding in this study is that the active peptide, in contrast to the inactive one, was able to adopt a β structure in the membrane. Initial studies on fusion peptides have been conducted under the prevailing assumption of the α-helix representing the characteristic fusogenic conformation of these peptides [6,18–20]. However, this notion has been questioned as a general rule by Gallaher et al. [21] and recent reports favor β structures as the putative fusogenic conformations of certain fusion peptides in vesicular systems [10,22,23]. It appears that in those systems, factors like the use of appropiate vesicles as LUV to measure peptide-induced membrane destabilization (for a discussion on this issue see [24]) and the determination of peptide conformation under conditions comparable to those used to measure membrane fusion and destabilization, can become extremely important in order to establish the correct function–structure relationship [10,23]. The fact that a single substitution abolishing fusion leads to the absence of the β

structure while keeping intact the ability of the peptide to adopt the α-helix conformation, argues in favor of the former structure as representing a crucial element for the activation of fusion in our system. To our knowledge, this is the first time that a substitution in the HIV-1 fusion peptide, known to block gp41 fusion activity, is related to the loss of a functional conformation. Moreover, our findings highlight the relevance that the adoption by the fusion peptide of such a structure could have in vivo for HIV-1 fusion.

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