Inhibition of HIV-1 Envelope Glycoprotein-mediated Cell Fusion by a DL-Amino Acid-containing Fusion Peptide

POSSIBLE RECOGNITION OF THE FUSION COMPLEX*

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The N-terminal fusion peptide (FP) of human immuno
deficiency virus-1 (HIV-1) is a potent inhibitor of cell-
cell fusion, possibly because of its ability to recognize
the corresponding segments inside the fusion complex
within the membrane. Here we show that a fusion pep-
dide in which the highly conserved Ile⁴, Phe⁸, Phe¹¹, and
Ala¹⁴ were replaced by their d-enantiomers (IFFA) is a
potent inhibitor of cell-cell fusion. Fourier transform
infrared spectroscopy confirmed that despite these
drastic modifications, the peptide preserved most of
its structure within the membrane. Fluorescence energy
transfer studies demonstrated that the diastereomeric
peptide interacted with the wild type FP, suggesting
this segment as the target site for inhibition of mem-
brane fusion. This is further supported by the similar
localization of the wild type and IFFA FPs to microdo-
mains in T cells and the preferred partitioning into or-
dered regions within sphingomyelin/phosphatidylcho-
line/cholesterol giant vesicles. These studies provide
insight into the mechanism of molecular recognition
within the membrane milieu and may serve in designing
novel HIV entry inhibitors.

Specific fusion proteins, located on the surface of viral mem-
branes, mediate membrane fusion (1). The envelope glycopro-
tein gp160 from HIV,1 containing two non-covalently associ-
ated subunits, gp120 and gp41 (2), mediates the membrane
fusion activity of the virus. The binding of the gp120 subunit
to target cell receptors (3–7) induces a conformational change
in the glycoprotein, which results in the exposure of a previously
hidden hydrophobic N-terminal stretch of gp41, designated the
“fusion peptide” (8, 9). Evidence supporting the role of the FP
domain in mediating membrane fusion came from studies with
intact envelope proteins (10–12), as well as synthetic peptides
used in model and biological systems (13–25). However, the
molecular mechanism of membrane fusion is still poorly under-
stood despite extensive studies done in both biological and
model systems. Nevertheless, the results of many studies sug-
gest common motifs for the diverse biological and model fusion
reactions (13, 26).

Virus-induced membrane fusion is highly sensitive to single
amino acid mutations in the FP domain (27, 28). This can lead
to the conclusion that the structure of FPs is a crucial param-
eter in the fusion process. Indeed, several studies suggest that
the structure of FPs plays a major role in their activities (12,
29). To distinguish between the effects of structure and hydro-
phobicity, we compared an all-L-amino acid FP with its enan-
tiomer composed entirely of d-amino acids (25). Both had the
same activity in liposome fusion assays, although having mirror
image structures (25). Furthermore, the FP of HIV-1 was shown
to inhibit cell-cell fusion (30–32). Cumulative evidence
suggests that the mechanism of inhibition is through interac-
tion with the corresponding region in the intact gp41 (23, 25).
This inhibitory activity of the peptide is chirality-independent,
eliminating the possibility of interaction at the receptor level
since chirality is crucial for recognition of soluble proteins (25).
The chirality independence of membrane-inserted peptide-pep-
tide interaction in vivo has been recently verified by using a
glycophorin A transmembrane domain (33).

The non-chiral nature of the FP-mediated inhibition of HIV-1
cell fusion reinforces the need to understand the role of the
precise structure of the FP in the fusion process. To alter the
structure of the FP while preserving the hydrophobicity (i.e.
same amino acid composition), we introduced d-amino acids into
the FP of HIV-1. For this purpose, we synthesized two 33-residue
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**EXPERIMENTAL PROCEDURES**

**Materials**—BOC-amino acid phenylacetamidomethyl-resin was purchased from Applied Biosystems (Foster City, CA), and BOC-amino acids were obtained from Peninsula Laboratories (Belmont, CA). NBD-fluoride and other reagents for peptide synthesis were obtained from Sigma. Egg phosphatidylcholine (PC) and phosphatidylserine from bovine brain, cholesterol (NBD-PE) were purchased from Molecular Probes (Junction City, OR), and N-(7-nitrobenz-2-oxa-1,3-diazole-4-yl)-dioleoylphosphatidylethanolamine (Rho-PE) and N-(Lissamine-rhodamine B-sulfonyl)-dioleoylphosphatidylethanolamine (Lissamine-rhodamine B-sulfonyl)-dioleoylphosphatidylethanolamine (Rho-PE) were purchased from Molecular Probes (Junction City, OR). TF228 cells, which constitutively express HIV-1uographical, pseudovirions were prepared by transfecting 293T cells (5) with DNA containing the nef, vif, vpr, vpu, vpx, vif, vpr, vpu, vpx, vif, vpr, vpu, vpx, vif, vpr, vpu, vpx, and SupT1 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health from Dr. James Hoxie. Both cell lines were grown in RPMI 1640 medium (with 25% FHEPS, 10% fetal calf serum, 2 mM t-glutamine, penicillin G at 100 units/ml, and streptomycin sulfate at 100 mcg/ml). The vaccinia recombinants vCB4R and vTF7–3 were generous gifts from Dr. Ed Berger (NIAID, National Institutes of Health). All other reagents were of analytical grade.

**Peptide Synthesis and Fluorescent Labeling**—The peptides were synthesized by a solid phase method on phenylacetamidomethyl-amino acid resin (0.15 moles/gram), as described previously (23, 30). Labeling of the N terminus of the peptides was achieved as described previously (35, 36). The synthetic peptides were purified (>95% homogeneity) by reverse-phase high pressure liquid chromatography on a C18 column using a linear gradient of 25–80% acetonitrile in 0.05% trifluoroacetic acid for 40 min. The peptides were subjected to amino acid analysis and mass spectrometry to confirm their composition. Unless stated otherwise, all solutions of peptides were prepared in MeOH to avoid aggregation of the peptides prior to their use. The final concentration of MeOH in each experiment had no effect on the system under investigation.

**HIV-mediated Cell-Cell Fusion Assay**—A β-galactosidase assay (37) was used to measure the inhibitory effect of the peptides. TF228 and SupT1 cells were infected overnight at 10 multiplicity of infection with different recombinant vaccinia viruses. TF228 cells were transfected with pCB4R containing the T7 promoter and lacZ gene, which expresses β-galactosidase enzyme from Escherichia coli. SupT1 cells were transfected with pTF7–3, having T7 RNA polymerase under the control of the natural T7.5 early-late vaccinia virus promoter. Fusion was analyzed by detecting β-galactosidase activity. Since the cytoplasm of SupT1 cells contains T7 RNA polymerase and the cytoplasm of TF228 cells contains the β-galactosidase enzyme under the control of the T7 promoter, the enzyme was produced only in cytoplasmically fused cells. Cells were mixed and incubated for 3 h at 37°C in the presence of peptides. Then cells were lysed with detergent, and β-galactosidase activity was detected by measuring the substrate chlorophenol red-β-naphthylpyranoside. The rate of hydrolysis was measured by reading absorbance at 595 nm. The assay was repeated in the presence of different concentrations of the FP, its IFFA diastereomer, and several other control peptides. Inhibition of the fusion process resulted in a reduction of β-galactosidase activity. There was about 1% fusion (β-galactosidase expression) in the controls: target cells without HIV receptors or cells expressing uncleaved gp160 (37).

**Influenza HA-mediated Cell-Cell Fusion Assay**—Dye transfer between HA-expressing fibroblasts (PF4R) and R18-labeled erythrocytes was monitored spectrofluorometrically using the R18 dequenching method as described previously (38).

**Fusion Peptide Localization on T Cells**—Activated T cells (39) (10⁴) were incubated for 30 min at room temperature with NBD-labeled WT or IFFA (0.5 μM final concentration). The cells were washed twice with phosphate-buffered saline (100 μl) to remove excess unbound peptide. The cells were then observed under a fluorescence confocal microscope. NBD excitation was set at 488 nm with the laser set at 2% power to prevent bleaching of the fluorophore. Fluorescence data were collected from 525 nm and higher.

**Vesicle Preparation**—Giant unilamellar vesicles (GUV) were prepared as previously by Moschol et al. (40). Briefly, a dried film of lipids containing a total of 2 mg of PC:SM:CH:Rho-PE (1:1:0.001) was dissolved in 30 μl of chloroform. The lipids were added to a round flask containing 970 μl of chloroform and 150 μl of methanol. Next, we carefully added 7 ml of double-distilled H₂O along the flask walls. The mixture was evaporated under nitrogen flow (2 bar) while rotating the flask at 30–50 rpm for 5–10 min. Small unilamellar vesicles (SUV) were prepared by sonication from PC:CH (10:1 w/w). The cholesterol was included to reduce the curvature of the small unilamellar vesicles (23).

**Fusion Peptide Localization in Model Membranes**—GUV labeled with Rho-PE were observed under a confocal microscope with excitation set at 488 and 543 nm; emission was collected from 505 to 525 nm and from 610 nm and higher. Unordered regions were observed with excitation set at 543 nm but not with excitation at 488 nm at 610 nm and higher. No signal was detected at 505–525 nm. The NBD-labeled FP and IFFA were added to the GUV, carefully, at a final concentration of 0.5 μM. Binding of the peptides to the GUV was followed until maximal binding was observed after ~10–15 min for PC:SM:CH and 1 h for PC:CH. To eliminate the possibility of quenching the NBD fluorophore due to FRET with the rhodamine, we bleached the rhodamine using the 543-nm laser at 100% intensity for 30 s. No change in NBD emission at 505–525 nm was observed, whereas the rhodamine emission at 610 nm was abolished.

**Peptide Binding to SUV**—The degree of peptide association with lipid vesicles was measured by adding lipid vesicles to 0.1 μM NBD-labeled peptides at 28°C, as has been described previously (41). The fluorescence intensity was measured as a function of the lipid:peptide molar ratio, with excitation set at 467 nm (10-nm slit) and emission set at 530 nm (5-nm slit).

**Resonance Energy Transfer Measurements**—Fluorescence resonance energy transfer was measured using NBD-labeled peptides serving as donors and with Rho-labeled peptides serving as energy acceptors (42, 43). Fluorescence spectra were obtained at room temperature with excitation set at 467 nm using a 10-nm slit width. In a typical experiment, donor peptide (final concentration 0.04 μM) was added to a dispersion of PC:CH (10:1 w/v) SUV (400 μM) in phosphate-buffered saline followed by the addition of acceptor peptide in several sequential doses. Fluorescence spectra were obtained before and after the addition of the acceptor. The efficiency of energy transfer (E) was determined as described previously by Kliger et al. (23).

**Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR)**—Experiments were performed as described previously (44). Briefly, 1 mg of lipids mixed with chloroform, WT FP, or IFFA (1:50) was spread on the prism and dried under vacuum for 30 min. The sample was also tested under hydrated conditions by deuteration.

**RESULTS**

A peptide, representing the N-terminal 33-amino-acid segment of gp41 of HIV-1 (LAV1a strain), WT, and its diastereomer analogue IFFA, were synthesized and labeled at their N-terminal amino acid with either NBD or rhodamine fluorescent probes. The sequences of the peptides and their designations are shown in Table I. The secondary structure of the FP at the site of the substitutions is a β-sheet (44). The rationale behind the diastereomer is that the D-amino acids will destabilize the structure of the FP (the side chains will switch sides) while maintaining the original sequence. We investigated the peptides for their ability to inhibit HIV-1-mediated cell-cell fusion. The results were correlated with structural information.

**Inhibition of Cell-Cell Fusion Induced by WT and IFFA**—The peptides were tested for their inhibitory effect on HIV-1-mediated cell fusion as described under “Experimental Procedures.” The WT peptide and its diastereomer exhibited marked inhibitory effects at concentrations <10 ng/ml. The data also suggest that IFFA is slightly more active than the WT (Fig. 1A). We used a 13-mer peptide corresponding to the N-terminal region of the FP, as well as the FP of the Sendai virus, as negative controls. We further investigated the inhibitory effect of the peptide when added to the ENV-expressing cells prior to adding target cells. An inhibitory effect was observed, similar to that seen in Fig. 1A, when the peptide was added to the mixture of cells. Thus, we assume that the peptide may associate with the surface of the cells and remain there until the fusion process is initiated. At
that point, it inhibits the fusion process. Alternatively, the FP may bind directly to the ENV complex. However, this is less likely since the wild type FP is presumably hidden within the gp120/gp41 complex until receptor binding.

The inhibition of HIV ENV-mediated cell fusion by FP was demonstrated to be HIV-specific. We studied the virus specificity of the IFFA diastereomer in inhibiting viral fusion. Although the IFFA is a potent inhibitor of HIV-1, it could not inhibit influenza HA-mediated cell fusion at concentrations up to 3 μM (Fig. 1B). This result confirms that the inhibitory effect of IFFA is specific to HIV, similar to the WT FP. Furthermore, neither the WT FP nor the IFFA could reach the membrane fusion stage. The inhibition increases fast at the lower nM range, but even up to 1 μM, 30% fusion occurred after 300 s.

Binding of IFFA to Unordered Regions on GUVs—We prepared sphingomyelin:phosphatidylcholine:cholesterol:phosophatidyl-ethanolamine-rhodamine (SM:PC:CH:Rho-PE) (1:1:1:0.001) giant unilamellar vesicles (GUVs) labeled with rhodamine, as described under “Experimental Procedures.” The peptide partitioned into unordered regions. Indeed, the fluorescence of the NBD-labeled peptide remained constant, even when the rhodamine signal was completely abolished. Similar results were obtained with the WT FP (data not shown). Thus, we can conclude that the IFFA diastereomer partitions preferentially into ordered regions, similarly to the WT FP. A similar experiment with PC:CH:Rho-PE (10:1:0.001) GUVs, with no microdomains present, demonstrated a uniform binding pattern for the IFFA (Fig. 4C). The absence of ordered regions is demonstrated by the uniform distribution of the Rho-PE over the surface of the GUVs. However, the time to reach maximal intensity was about four times slower than that observed for SM:PC:CH GUVs.

Co-assembly of WT Fusion Peptide and Its Diastereomer in the Membrane-bound State—The self-association of the peptides in their membrane-bound state was monitored by measuring resonance energy transfer. A dose-dependent quenching of the emission of the NBD-WT donor, consistent with energy transfer, was observed when Rho-WT or Rho-IFFA acceptor (final concentration of 0.025–0.35 μM) was added to a mixture of NBD-WT (0.05 μM) and SUV (400 μM). The energy transfer was calculated and plotted versus the acceptor:lipid molar ratio (Fig. 5). The acceptor peptide was added only after the donor
peptide was already bound to the membrane, thus decreasing association in solution. The free energy of peptide association with SUV was measured as described under “Experimental Procedures.” It was found to be $9.3 \text{ kcal/mole}$ for the WT FP and $8.3 \text{ kcal/mole}$ for the IFFA. Accordingly, the lipid:peptide molar ratio in these experiments was kept high enough to ensure low surface density of donors and acceptors while reducing the energy transfer between unassociated peptide monomers to a minimum. To confirm that the observed energy transfer is due to peptide oligomerization, the transfer efficiencies observed in the experiments were compared with the energy transfer expected for randomly distributed membrane-bound donors and acceptors (Fig. 5, dashed line). The random distribution was calculated as described earlier (42), using 51 Å as the $R_0$ value for the NBD/Rho donor/acceptor pair (43). The levels of energy transfer between the different pairs are significantly higher than those expected for a random distribution of donors and acceptors.

**Secondary Structure of the Peptides as Determined by ATR-FTIR Spectroscopy**—We studied the FTIR spectra of the amide I region of the WT and IFFA peptides in the presence of PC:CH
multibilayers. The samples were exposed to D2O vapors followed by hydration until equilibrium was achieved. The amide I frequencies are known to decrease by up to 10 cm⁻¹ upon H/D exchange. The peaks corresponding to random coil and α-helix partly overlap, but random coils undergo H/D exchange at higher rates than α-helices. Thus, hydration with D2O vapors can enable better distinction between the two components. The contributions of the various secondary structure elements to the amide I peak were obtained by using PEAKFIT (Jandel Scientific, San Rafael, CA) and comparison with accepted values from the literature (Fig. 6) (46, 47). The WT and IFFA both have mainly a β-sheet secondary structure, represented by peaks within the 1620–1640 cm⁻¹ range (Table II). Deconvolution of the IR spectra resulted with 78 and 70% β-sheet content for WT and IFFA, respectively. A second peak, located at ~1648 cm⁻¹, most likely represents a random coil segment (47). A peak at around 1670 cm⁻¹ may represent either a high frequency helix or a β-turn. The area of that peak is 12 and 11% for WT and IFFA, respectively. These results fit well with recent FTIR (44), as well as NMR studies of the FP (48). The studies demonstrated that the FP can associate strongly within a membrane environment and adopt both parallel or antiparallel β-sheet structures. This is in contrast to the FTIR by Gordon et al. (49), which demonstrated an α-helical structure for the FP at low concentrations and a β-sheet structure only at high loading. However, they used a shorter 23-residue peptide.

The amide II band is even more susceptible to D2O hydration. Upon H/D exchange, its frequency shifts by about 100 cm⁻¹. After exposure of the samples to D2O vapors, a strong effect was detected for the diastereomer peptide, whereas a smaller shift was observed for the WT (data not shown). These results indicate that D-amino acid substitutions destabilize the secondary structure of the peptide in the membrane-bound state to a certain extent. Although the overall structure of IFFA is maintained, there is higher flexibility, and thus, lower aggregation and an increase in the exposure to D2O. It is interesting to note that the peaks corresponding to the β-sheet components of the diastereomer are wider than those of the WT. This phenomenon was observed previously for diastereomers with primarily α-helix structures (50).

**Fig. 6. ATR-FTIR spectra of the peptides in PC:CH multibilayers.** The peptide/lipid molar ratio was 1:50. Panel A represents the FTIR spectra of WT FP, and panel B represents the spectra of IFFA FP. The spectra were analyzed using the curve fitting of the amide I band area assuming Voight line shapes for the IR peaks. The resulting deconvolution peaks are displayed under the fitting.

**Table II**

| Structure                | Peak location (cm⁻¹) | Area (%) |
|--------------------------|----------------------|----------|
| Aggregated β-sheet       | 1622                 | 55       |
| β-Sheet                  | 1636                 | 23       |
| 3_10 helix/β-turn        | 1670                 | 12       |
| Random coil              | 1648                 | 10       |

Recently, a peptide named T20/Fuzeon/enfuvirtide corresponding to the C-terminal helical region of gp41 was approved by the FDA for use as a fusion inhibitor (51). This peptide highlighted a new family of inhibitors that target the viral entry into the host. The FP of HIV gp41 has similar properties to the Fuzeon in that it targets a transient triggered state of gp41. It was previously shown to inhibit HIV infection in vitro with EC₅₀ concentrations in the nM range (23). This inhibition proved to be chirality-independent when an all-D-analogue of the FP demonstrated similar antiviral activity (25). Nevertheless, the FP inhibitory activity is sequence-specific; the HIV-1 FP cannot inhibit the activity of Sendai virus (data not shown), and the FP of Sendai virus could not inhibit HIV-1 ENV-mediated cell fusion (23). Furthermore, no cross-reactivity was observed between HIV-1 FP and HIV-2 ENV-mediated cell fusion (25).

We synthesized a diastereomer analogue of the WT FP with 4 residues in a highly conserved region replaced by D-amino acids. The motivation behind the IFFA diastereomer synthesis was to verify the importance of the precise structure of the FP when compared with its inhibitory potential. The major secondary structure of the WT FP is a β-sheet at the sites of the substitutions. Introduction of D-amino acids will result in a stretch of amino acids with their side chains facing the same direction instead of alternating. This is bound to cause some steric hindrance and thus affect the structure. Nevertheless, the IFFA diastereomer was able to maintain a high inhibitory activity against HIV ENV-mediated cell-cell fusion. This inhibition is specific to HIV since the IFPA could not inhibit influenza HA-mediated fusion (Fig. 1B).

Interestingly, we found that the overall ATR-FTIR spectra of both the WT and the IFFA FPs were similar. The main secondary
The need for widening the antiviral, especially anti-HIV, arsenal is great. Fuzexon addresses just such a need. It inhibits a new step in the virus life cycle, and thus, it has a synergistic effect with the customary anti-HIV cocktails. However, aggregation and susceptibility to proteolytic cleavage are the main drawbacks of peptide inhibitors derived from membrane proteins. The short biological half-life of the Fuzeon peptide demonstrates this problem (several shots of large doses are needed per day). We demonstrated that the solubility of the FP can be increased by incorporating \( \mathbf{D} \)-amino acids while retaining full inhibitory activity within the membrane. Furthermore, altering the chirality of membrane-inserted peptides is not as important as in aqueous solution, merely resulting in minor modifications to the secondary structure. This makes the diastereomer peptide inhibitors attractive alternatives to the regular \( \mathbf{L} \)-amino acids in strategic positions can increase proteolytic resistance. This, in turn, might improve the pharmacological properties of the peptides.

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