

Antiviral Activity and Conformational Features of an Octapeptide Derived from the Membrane-Proximal Ectodomain of the Feline Immunodeficiency Virus Transmembrane Glycoprotein

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Feline immunodeficiency virus (FIV) provides a valuable animal model by which criteria for lentivirus control strategies can be tested. Previous studies have shown that a 20-mer synthetic peptide of the membrane-proximal ectodomain of FIV transmembrane glycoprotein, designated peptide 59, potently inhibited the growth of tissue culture-adapted FIV in feline fibroblastoid CrFK cells. In the present report we describe the potential of this peptide to inhibit the replication of primary FIV isolates in lymphoid cells. Because antiviral activity of peptide 59 was found to map to a short segment containing three conserved Trp residues, further analyses focused on a derivative of eight amino acids (⁷⁷⁰W-I⁷⁷⁷), designated C8. Peptide C8 activity was found to be dependent on conservation of the Trp motif, to be removed from solution by FIV absorbed onto substrate cells, and to be blocked by a peptide derived from the N-terminal portion of FIV transmembrane glycoprotein. Structural studies showed that peptide C8 possesses a conformational propensity highly uncommon for peptides of its size, which may account for its considerable antiviral potency in spite of small size.

Initiation of cell infection by lentiviruses is a complex process involving multiple interactions between the glycoproteins (gp) of the viral envelope (Env) and cell surface molecules, with the transmembrane (TM) gp mediating the final event of membrane fusion and entry (6, 46, 68). In recent years, improved understanding of the structural features and functions of human immunodeficiency virus type 1 (HIV-1) TM gp41 (8, 15, 36, 38, 42, 44, 45, 59) has permitted the development of antiviral peptides derived from amino-terminal (N peptides) and carboxy-terminal (C peptides) ectodomains of this molecule, which effectively inhibit both HIV-1 entry into cells and HIV-1-induced cell fusion in vitro (17, 28, 29, 31, 32, 57, 65; A. R. Neurath, K. Lin, N. Strick, and S. Jiang, *Letter, AIDS Res. Hum. Retrovir.* **11**:189-190, 1995). Some such peptides are currently under clinical evaluation for their therapeutic and prophylactic potentials (35). For example, in recent studies T-20, a 36-amino-acid C peptide also known as DP-178, has been found to be safe and powerfully suppress viral loads in HIV-1-infected individuals (33). It may also have applications for gene therapy (26). T-20 is believed to owe its antiviral activity primarily to hindrance of the receptor-triggered conformation rearrangements of TM gp41 that mediate virion and cell membrane fusion (9, 30, 34, 36, 56, 64). Although T-20 exerts its antiviral activity specifically against HIV-1 (65), similar results have been reported with simian immunodeficiency virus-derived peptides, supporting the concept that the viral entry functions associated with the TM gp ectodomain are essentially conserved among lentiviruses (39, 59).

Feline immunodeficiency virus (FIV) is a naturally occurring pathogen that causes an AIDS-like syndrome in domestic cats (50), and it is a valuable model system by which criteria for antilenticular vaccine and drug development can be investigated (reviewed in references 5, 20, 21, and 66). The surface and TM gp of FIV and HIV-1 exhibit a common structural framework and appear to play similar roles in cell entry (6, 11, 19, 23, 46–48, 54, 60, 67, 68). We have previously screened 20- to 23-mer peptides covering the entire Env of FIV and found especially potent in vitro antiviral activity associated with peptides derived from two regions, one located in the N-terminal domain of the surface gp and the other located membrane proximally in the ectodomain of the TM gp (37). In subsequent studies (40), we focused on peptides of the surface gp, because the most efficacious of the TM peptides, referred to as peptide 59 (Fig. 1), had not appeared promising as a therapeutic strategy for naturally occurring infections since it had potently inhibited tissue culture-adapted (TCA) FIV in fibroblastoid cells (CrFK) but not a primary isolate in feline lymphoid cells. Prompted by the HIV-1 findings discussed above, we have now reexamined the antiviral properties of peptide 59 under modified experimental conditions. Removal of unadsorbed input virus from the test cultures revealed that peptide 59 and smaller derivatives potently inhibited the replication of both TCA and fresh isolates of FIV in feline lymphoid cells. Because the activity of peptide 59 mapped to a short stretch encompassing three conserved Trp residues, further analyses were carried out with a peptide of eight amino acids containing such a Trp motif (⁷⁷⁰W-I⁷⁷⁷), designated C8. Peptide C8 exerted a powerful antiviral effect on all the FIV isolates tested, and this activity was dependent on an intact Trp motif, was removed by FIV that had interacted with substrate cells, and

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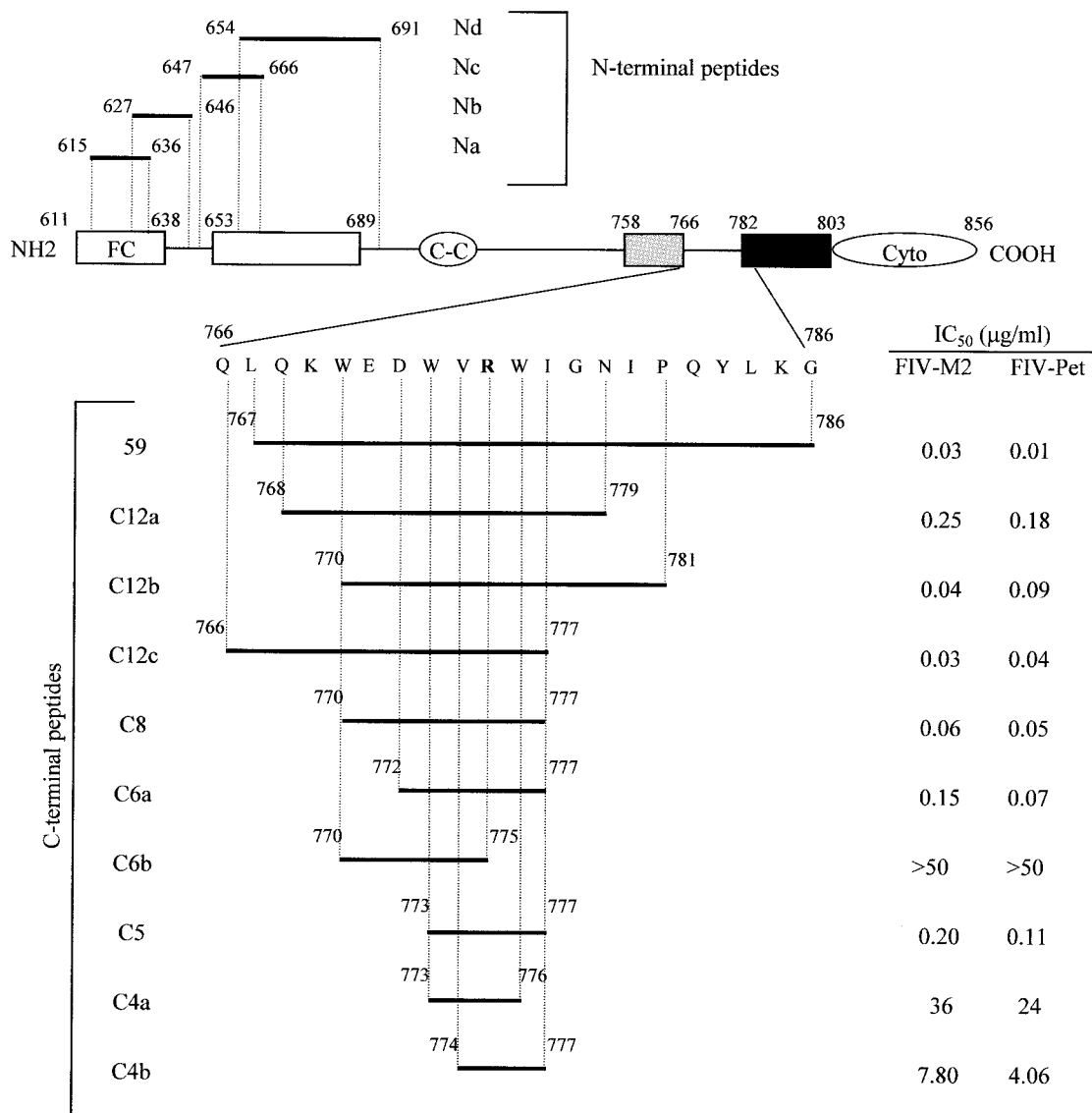


FIG. 1. Schematic representation of the TM gp of FIV and derived synthetic peptides used in this study. Superscript numbers indicate amino acid positions starting with the first methionine of Env, according to the sequence of prototype FIV-Pet, clone 34TF10 (62). Domains are indicated as previously described (53). C peptides include peptide 59, described in a previous report (37), and nine shorter peptides. At position 775, the latter had a Gly instead of the Arg present in peptide 59, to better conform to the great majority of FIV published sequences (55). The IC₅₀ for FIV-M2 and FIV-Pet of the C peptides found in one representative experiment out of three performed are also shown. N peptides were used only to investigate the possible site of action of peptide C8. Abbreviations and symbols: FC, fusion peptide; □, leucine zipper region; C-C, cysteine loop; ▨, membrane-proximal α-helix; ■, membrane-spanning segment; Cyto, cytoplasmic region.

was blocked by a peptide derived from the N-terminal segment of the FIV TM gp. Additional investigations have shown that peptide C8 possesses a conformational propensity highly uncommon for peptides of this size, which might explain why it is potently inhibitory in spite of its reduced size.

MATERIALS AND METHODS

Cells and viruses. MBM cells are a line of CD3⁺, CD4⁻, and CD8⁻ T lymphocytes originally established from the peripheral blood mononuclear cells (PBMC) of an FIV-negative and feline leukemia virus-negative cat (10). They are routinely grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 5 μg of concanavalin A, and 20 U of recombinant human interleukin-2 per ml. The viruses used included 11 primary isolates of subtype B (FIV-M2, -M3, -M19, -M22, -M45, -M73, -M82, -M88, -M91, -M92, and -M97) described in

a previous report (10), one primary isolate of subtype A (FIV-GL8; kind gift of Os Jarrett, Glasgow, United Kingdom), and one TCA strain of subtype A (FIV-Pet; kindly provided by J. K. Yamamoto, Gainesville, Fla.). The viral stocks consisted of cell-free supernatant of chronically infected FL4 cells (FIV-Pet [70]) or of acutely infected MBM cells (all other viruses). Titration of the viral stocks was carried out in MBM cells and, depending on experiment design, included or did not include washout of unadsorbed virus after 5 h at 37°C to reproduce the conditions used in the FIV inhibition assay (see below). Internal inactivation of the viruses used in peptide binding experiments was carried out with 2,2'-dithiodipyridine (also known as aldrithiol-2 [AT2]) as previously described (24). In brief, viral stocks were incubated with 300 μM AT2 at 4°C for 2 h, pelleted in the cold at 20,000 × g for 90 min, and resuspended at 1 mg/ml in phosphate-buffered saline.

Peptide synthesis and purification. Synthetic peptides (Fig. 1) were prepared manually using a conventional solid-phase strategy using N-α-fluorenylmethoxy-

carbonyl-protected amino acids and a *p*-alkoxybenzyl alcohol resin as solid support, as previously described (37). All were N-terminally acetylated and C-terminally amidated in order to improve enzymatic stability. Crude peptides were purified to homogeneity by semipreparative reverse-phase high-pressure liquid chromatography, with purity greater than 95%, and lyophilized. Final products were characterized by analytical high-pressure liquid chromatography and electrospray mass spectrometry.

Assays for FIV inhibition. Cell-free virus inhibition assays were performed in 96-well flat-bottom microplates against 10 50% tissue culture infectious doses (TCID₅₀) of FIV, using MBM cells as substrate and supernatant p25 antigen quantification as an end point. In some experiments, larger doses of virus were also used. Virus was appropriately diluted and mixed with an equal volume of the test peptides diluted from 0.0005 to 50 µg/ml (final concentrations) or with diluent alone. The mixtures were then immediately inoculated (100 µl/well) into two to four wells containing 10⁵ MBM cells in 100 µl of culture medium. After 5 h at 37°C, the inocula were rinsed out and replaced with fresh medium, while after 4 additional days 100 µl of supernatant was removed from each well and replaced with fresh medium. Deviations from this standard protocol are described below. In any case, the cultures were stopped on day 8, when p25 production by control wells inoculated with virus alone was well evident. The enzyme-linked immunosorbent assay for measuring p25 has been described previously (37). Peptide inhibition of virus growth was calculated using the formula (mean p25 concentration in wells inoculated with FIV + peptides/mean p25 concentration in wells inoculated with FIV alone) × 100. Fifty percent inhibitory concentrations (IC₅₀) were calculated using the predicted exponential growth function in Microsoft Excel, which uses existing *x-y* data to estimate the corresponding peptide concentration (*x*) from a known value (*y*), which in this case was 50% infectivity. Mean IC₅₀ ± standard deviations (SD) were calculated using all replicates. All experiments were repeated at least twice.

The assay for cell-associated FIV inhibition was performed as described above, except that the viral inoculum was 10⁵ fresh Ficoll-separated PBMC pooled from three specific-pathogen-free cats infected with FIV-M2 6 months earlier, the wells were not rinsed after contact, and the medium added on day 4 contained appropriate concentrations of test peptides. Parallel wells showed that such inoculum titrated approximately 10² infectious units in substrate MBM cells but produced no detectable p25 antigen if incubated alone.

CD and NMR. Circular dichroism (CD) spectra were recorded using a Jasco J-710 spectropolarimeter, with a cell of 1 mm in path length. The CD measures were performed in the near-UV CD range with 1-nm bandwidth, four accumulations, and scanning speed of 20 nm/min, at room temperature. The pH of the aqueous sample was adjusted to 6.6 by adding small amounts of phosphate buffer solution. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-600 spectrometer. Samples were prepared by dissolving the appropriate amount of the test peptides in 0.5 ml of H₂O-phosphate buffer (pH 6.6) to obtain a 1 mM solution. Lyophilized peptides were dissolved in 8 mM sodium phosphate buffer containing 80% dimethyl sulfoxide (DMSO). One-dimensional NMR spectra were recorded in the Fourier mode with quadrature detection, and the water signal was suppressed by a low-power selective irradiation in the homogated mode. Double-quantum-filtered correlated spectroscopy, total correlated spectroscopy (TOCSY), and nuclear Overhauser enhancement spectroscopy (NOESY) experiments (4, 27, 51) were run in the phase-sensitive mode using quadrature detection in 1 by time-proportional phase increase of initial pulse. Data block sizes were 2,048 addresses in t₂ and 512 equidistant t₁ values. Before Fourier transformation, the time domain data matrices were multiplied by shifted sin² functions in both dimensions. NOESY experiments were run at 270 K in DMSO-water. A mixing time of 70 ms was used for the TOCSY experiments, while mixing times in the range of 150 to 300 ms were used for NOESY experiments. The qualitative and quantitative analyses of double-quantum-filtered correlated spectroscopy, TOCSY, and NOESY spectra were obtained using the interactive program package SPARKY. The nuclear Overhauser effect (NOE)-based distance restraints were obtained from NOESY spectra collected with a mixing time of 250 ms.

Structure calculation. The NOE cross peaks were integrated with the SPARKY program and converted into upper distance bounds using the CALIBA module of DYANA software (25). Cross peaks which overlapped by more than 50% were treated as weak restraints in the DYANA calculation. An ensemble of 50 structures was generated with distance geometry-simulated annealing by program DYANA using 75 NOE-based distance constraints. No dihedral angle restraints nor hydrogen bond restraints were used. The necessary pseudoatom corrections were applied for nonstereospecifically assigned protons at prochiral centers and for the methyl group. After discarding redundant and duplicated constraints, the final list included 51 intraresidue and 113 interresidue constraints, which were used to generate an ensemble of 50 structures by the

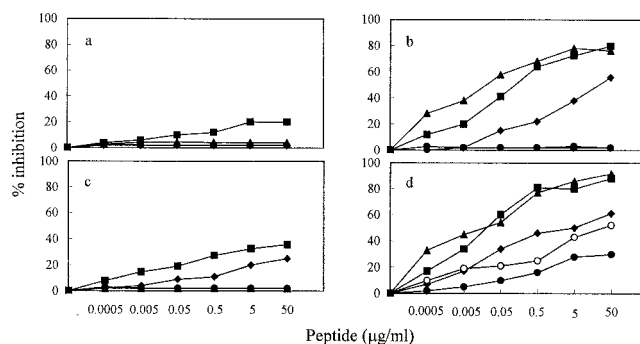


FIG. 2. Effect of varying the experimental conditions on FIV inhibition by peptide 59. Test viruses were the primary isolate FIV-M2 (a and b) and the TCA strain FIV-Pet (c and d). The peptide was mixed with virus 1 h before infection (■) or added to cells 1 h before (◆), simultaneously with (▲), or 1 h after the virus. In the latter case, virus-cell contact was carried out at 4°C (○) or 37°C (●). Inoculum virus-peptide mixtures were washed out after 5 h of contact (b and d) or left in the cultures throughout the incubation period (a and c). A 20-mer control peptide derived from a different region of the FIV TM was devoid of activity (not shown).

standard protocol of simulated annealing in torsion angle space implemented in DYANA (using 6,000 steps) The best 20 structures with low values of target functions (0.83 to 1.19) and small residual violations (maximum violation = 0.38 Å) were refined by in vacuo minimization in the AMBER 1991 force field, using the program SANDER of the AMBER suite (version 5.0; University of California, San Francisco [63]). To mimic the effect of solvent screening, all net charges were reduced to 20% of their real value, and moreover a distance-dependent dielectric constant ($\epsilon = r$) was used. The cutoff for nonbonded interactions was 12 Å. The NMR-derived upper bounds were imposed as semiparabolic penalty functions, with force constants of 16 Kcal/mol Å²; the function was shifted to linear when the violation exceeded 0.5 Å. The best 10 structures after minimization had AMBER energies ranging from -441.4 to -391.1 kcal/mol. The final structures were analyzed using the Insight 98.0 program. Computations were performed on SGI Indigo II computers.

RESULTS

Inhibition of FIV replication in feline lymphoid cells by peptide 59. To establish the optimal conditions for the antiviral action of peptide 59 in vitro, we examined the effects of varying the conditions of virus-peptide-cell contact. As shown by Fig. 2, removing the inoculum virus-peptide mixtures after virus-cell adsorption greatly affected outcome. When the inocula were left in the MBM cell cultures throughout the incubation period, TCA FIV-Pet inhibition was less evident (Fig. 2c) than that previously observed with CrFK cells (37), and FIV-M2 inhibition was virtually absent (Fig. 2a). In contrast, removing the inocula after 5 h of contact markedly augmented FIV-Pet inhibition (Fig. 2d) and rendered FIV-M2 inhibition also very clear (Fig. 2b). Time of peptide addition proved also critical. Pretreating cells and, in particular, adding the peptide 1 h after the virus markedly reduced the effect. In contrast, mixing virus and peptide immediately before inoculation, or 1 h earlier, yielded equally optimal inhibition. In subsequent experiments virus and peptides were therefore mixed just before addition to cells and washed off 5 h later.

FIV inhibition by peptides of reduced size. Peptide 59 contains a motif of three Trp residues separated, from each other, by 2 amino acids (Fig. 1). The fact that several Trp residues are highly conserved in the membrane-proximal ectodomain of the

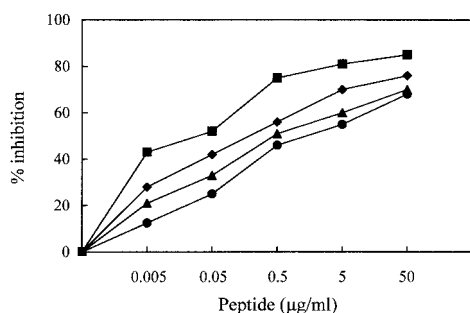


FIG. 3. Inhibition of graded doses of cell-free FIV-M2 by peptide C8. Peptide diluted from 0.005 to 50 $\mu\text{g/ml}$ (final concentration) was mixed with 10 (■), 32 (◆), 100 (▲), and 320 (●) TCID_{50} of virus. Except for virus dose, the procedure was as described in Materials and Methods. The experiment was repeated three times with comparable results.

TM gp of lentiviruses suggests that these residues exert an essential role for viral infectivity (59). Moreover, an inhibitory C peptide of HIV-1 corresponding in location to peptide 59 was shown to lose much activity following deletion of the Trp-rich domain (64, 65). For these reasons, the derivatives of peptide 59 that were assayed for anti-FIV activity (Fig. 1) had all, or most, of the Trp residues conserved. As summarized by Fig. 1, all peptides comprising eight (peptide C8) or more amino acids inhibited both FIV-M2 and FIV-Pet, with IC_{50} ranging between 0.03 and 0.25 $\mu\text{g/ml}$. On the other hand, of the two hexamer peptides examined, the one with Trp-770 truncated (C6a) showed reduced activity, while the one with Trp-776 truncated (C6b) was totally ineffective. Finally, the 5-mer peptide having both Trp-773 and Trp-776 included in it still had some activity, and the two 4-mer peptides were essentially ineffective. Thus, peptide size could be reduced considerably with little or no loss of antiviral activity. Subsequent studies focused on peptide C8, i.e., on the shortest among the above peptides that was fully active and had all three Trp conserved. This peptide was tested for its ability to inhibit graded doses of FIV. The results of a representative experiment are shown in Fig. 3. Overall, in three experiments the mean $\text{IC}_{50} \pm \text{SD}$ were 0.07 ± 0.01 , 0.31 ± 0.07 , 0.92 ± 0.05 , 2.02 ± 0.1 $\mu\text{g/ml}$ against 10, 32, 100, and 320 TCID_{50} , respectively. Similar to peptide 59, peptide C8 had no detectable effects on MBM cell viability and proliferation (data not shown).

Importance of the Trp motif for antiviral activity of peptide C8. To assess the importance of the Trp residues for antiviral activity of peptide C8, we performed an Ala scan study, in which modified peptides were compared for anti-FIV activity with the wild-type peptide and a scrambled form of it (C8_{scr}), thus obtaining information about each side chain. Test viruses were the ones used above plus FIV-GL8, a primary isolate of subtype A. As shown in Table 1, Ala substitution for Trp-770 markedly reduced effectiveness, while Ala substitution for Trp-773 or Trp-776 completely eliminated it. All other Ala substitutions had little or no effect, whereas C8_{scr} was completely inactive. Thus, these findings suggest that an intact Trp motif is crucial for a robust antiviral activity.

Peptide C8 inhibition of different primary FIV isolates. As described above, primary isolate FIV-GL8 had appeared

TABLE 1. Effect of alanine scan on FIV-inhibitory activity of peptide C8

| Peptide | Mean $\text{IC}_{50} \pm \text{SD}$ ($\mu\text{g/ml}$) ^a | | |
|------------------------------------------------|-----------------------------------------------------------------------|-----------------|-----------------|
| | FIV-M2 | FIV-GL8 | FIV-Pet |
| C8 | 0.07 ± 0.02 | 0.46 ± 0.20 | 0.05 ± 0.01 |
| C8_{scr} | >50 | >50 | >50 |
| $\text{C8}_{\text{W} \rightarrow \text{A770}}$ | 4.30 ± 0.70 | >50 | 2.10 ± 0.10 |
| $\text{C8}_{\text{E} \rightarrow \text{A771}}$ | 0.16 ± 0.03 | 0.22 ± 0.05 | 0.12 ± 0.01 |
| $\text{C8}_{\text{D} \rightarrow \text{A772}}$ | 0.10 ± 0.02 | 0.40 ± 0.22 | 0.03 ± 0.06 |
| $\text{C8}_{\text{W} \rightarrow \text{A773}}$ | >50 | >50 | >50 |
| $\text{C8}_{\text{V} \rightarrow \text{A774}}$ | 0.07 ± 0.03 | 0.54 ± 0.02 | 0.15 ± 0.12 |
| $\text{C8}_{\text{G} \rightarrow \text{A775}}$ | 0.09 ± 0.03 | 0.38 ± 0.04 | 0.25 ± 0.06 |
| $\text{C8}_{\text{W} \rightarrow \text{A776}}$ | >50 | >50 | >50 |
| $\text{C8}_{\text{I} \rightarrow \text{A777}}$ | 0.15 ± 0.01 | 0.64 ± 0.08 | 0.13 ± 0.04 |

^a Values obtained in two to three independent assays.

somewhat more resistant to C8 inhibition than primary isolate FIV-M2 (Table 1). It appeared therefore of interest to extend the study to a larger number of primary isolates. Ten additional local isolates selected at random from our collection were infectivity titrated in MBM cells and then assayed for susceptibility to C8. All were effectively inhibited, with IC_{50} ranging between 0.03 and 0.63 $\mu\text{g/ml}$ (data not shown), thus revealing a considerable breadth of activity although with some variation depending on the FIV isolate tested.

C8 inhibition of cell-associated FIV. Since lentiviruses can spread directly via cell-to-cell contact (5), it was of interest to verify whether peptide C8 might block this form of infection. PBMC of FIV-M2 infected cats were cocultivated with MBM cells in the presence or absence of C8. Figure 4 shows that C8, but not C8_{scr} , inhibited cell-associated infection with an IC_{50} of 0.015 $\mu\text{g/ml}$. This observation agrees with HIV-1 studies showing that inhibitory peptides block cell-associated infectivity at lower concentrations than that required for cell-free virus (65).

Peptide C8 binds to cell-associated FIV. In the experiments in Fig. 2, the antiviral effects of peptide 59 were reduced when the peptide was preincubated with substrate cells prior to their infection, suggesting that it may bind to, or be taken up by, the cells, as reported for certain anti-HIV-1 peptides (61). To investigate this possibility, peptide C8 was incubated with either MBM cells alone, FIV particles alone, or both, and the

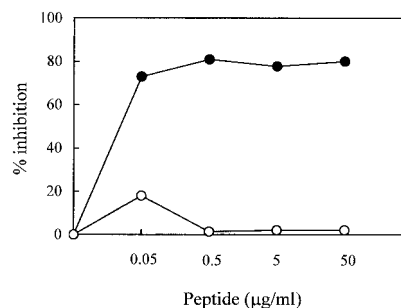


FIG. 4. Inhibition of cell-associated FIV infection by peptide C8. PBMC, freshly harvested from long-term FIV-M2-infected cats and containing approximately 10^2 infectious units as determined in substrate MBM cells, were mixed with C8 (●) or C8_{scr} (○). Test cultures were then processed as described for inhibition of cell-free virus except that the wells were not rinsed after contact and the medium added on day 4 contained appropriate concentrations of test peptides.

TABLE 2. Effect of preincubation with FIV, substrate cells, or virus-cell mixtures on FIV-inhibitory activity of peptide C8

| Expt no. | Virus used | Mean IC ₅₀ ± SD (μg/ml) of C8 preincubated with ^a : | | | | |
|----------|------------|---------------------------------------------------------------------------|----------------------------|--------------------------|----------------------------------------------------|-------------------------------------------------|
| | | Nothing | Virions ^b alone | Cells ^c alone | Virions + cells ^d (no entry allowed) | Virions + cells ^e (entry allowed) |
| 1 | FIV-M2 | 0.04 ± 0.02 | 0.03 ± 0.01 | 0.08 ± 0.03 | >1 | 0.04 ± 0.03 |
| 2 | FIV-GL8 | 0.46 ± 0.10 | 0.77 ± 0.20 | 0.38 ± 0.03 | >1 | 0.46 ± 0.13 |
| 3 | FIV-Pet | 0.03 ± 0.02 | >1 | 0.06 ± 0.04 | >1 | 0.03 ± 0.01 |

^a Peptide C8 (0.8 ml of a 5-μg/ml solution) was preincubated with the indicated material at 4°C for 1 h, centrifuged at 600 × g for 15 min (to remove cells) followed by 20,000 × g for 90 min (to remove virions), diluted from 0.001 to 1 μg/ml, and then assayed for inhibition of the same FIV strain used in C8 preincubation, by the standard procedure. Values obtained in two to three independent assays.

^b FIV inactivated with AT2 (5 μg protein).

^c Viable MBM cells (5 × 10⁵).

^d FIV inactivated with AT2 (5 μg of protein) and incubated with MBM cells (5 × 10⁵) at 4°C for 1 h before C8 addition, to permit virus adsorption but not entry (24).

^e FIV inactivated with AT2 (5 μg of protein) and incubated with MBM cells (5 × 10⁵) at 37°C for 3 h before C8 addition, to permit both virus adsorption and entry (24).

residual inhibitory activity in the culture supernatant was determined after 1 h at 4°C. The FIV particles used in this study were rendered noninfectious by the internal inactivating agent AT2 (24). This agent has previously been shown to inactivate HIV and SIV without affecting Env structure and functions, including cell entry (2, 58). We also investigated the effect of conformational changes in the viral Env on the capacity to bind peptide C8. This was done by comparing the adsorption capacity of virus-cell mixtures preincubated at either 4°C for 1 h or 37°C for 3 h. The results are shown in Table 2. Preincubation with MBM cells alone had a negligible effect on the antiviral properties of peptide C8. Likewise, two primary isolates of FIV, FIV-M2 and FIV-GL8, also appeared unable to bind the peptide. However, the conformational changes which occurred in Env following binding of these primary FIV isolates to cells markedly enhanced the capacity to remove peptide C8 activity, as shown by the fact that virus-MBM cell mixtures preincubated under conditions which permitted virus adsorption to cells but not under conditions which also permitted cell entry effectively removed C8 activity from solution. On the other hand, TCA FIV-Pet removed much peptide C8 activity, even without having previously interacted with cells. Thus, these findings showed that FIV has the capacity to bind peptide C8 but, in the case of primary isolates, this occurs only after virus has adsorbed onto cells.

In a second approach, we examined whether C8 activity was affected by mixing with peptides derived from the N-terminal portion of FIV TM gp (N peptides), as suggested by previous studies on HIV-1 which indicated that inhibitory C peptides act by binding to specific N-terminal segments of the TM gp41 (9, 30, 34, 36, 56, 64). The synthetic N peptides used comprised amino acid positions 615 to 636 (Na), 627 to 646 (Nb), 647 to 666 (Nc), and 654 to 691 (Nd) (Fig. 1). Since these N peptides were also weakly inhibitory for FIV replication (reference 37 and data not shown), the mixtures contained a fixed volume of the N peptides diluted to the respective IC₅₀. As shown in Table 3, peptide Nb produced a 25-fold reduction of the expected IC₅₀ value of C8, whereas the other N peptides left C8 activity essentially unchanged. The antiviral effect in control cultures in which C8 was substituted with either C8_{W→A776} or C8_{scr} was as with the N peptides alone. Thus, the presence of a specific N-terminal segment of the TM gp of FIV eliminated C8-mediated inhibition of FIV replication.

Conformational analysis of peptide C8. The above findings were conducive to the idea that peptide C8 had a stable conformation in spite of its small size. This possibility was addressed by comparing the CD and NMR properties of C8 and the inactive Ala analogue C8_{W→A776}. A preliminary screening of the conformational preferences of the two peptides as a function of the environment was performed by CD spectroscopy. CD spectra were run in water and DMSO-water (80:20, vol/vol). The spectra in water showed that both peptides, like most similarly short linear peptides, were present in extended, random-coil conformations. On the contrary CD spectra recorded in the near-UV CD range showed well-defined bands at the longer wavelength adsorption maxima of tryptophan, anticipating the NMR finding that the backbone and consequently the indole side chains are in a defined structural orientation (see below).

Previous findings have shown that the DMSO-water solution, due to its high viscosity, affects the equilibrium among isoenergetic conformers, thus selecting the more-ordered ones (1), and produces physicochemical conditions compatible with those of biological fluids (16). Based on these findings and the above CD data, analysis of the conformational preferences of C8 and C8_{W→A776} by NMR spectroscopy was performed in DMSO-water. Figure 5 shows amide and fingerprint regions of the NOESY spectra (270 K; 600 MHz; mixing time, 300 ms). The presence of a high number of well-resolved cross peaks, unusual for peptides as short as the ones under scrutiny, is

TABLE 3. Effect of mixing with N peptides on FIV-inhibitory activity of peptide C8

| Test peptide | Mean IC ₅₀ ± SD (μg/ml) of mixture of test peptide and N peptide ^a | | | | |
|----------------------|------------------------------------------------------------------------------------------|------------------------|------------|-------------|-------------|
| | None | Na (1.20) ^b | Nb (1.20) | Nc (1.20) | Nd (0.05) |
| C8 | 0.06 ± 0.01 | 0.06 ± 0.01 | 1.5 ± 0.80 | 0.07 ± 0.03 | 0.01 ± 0.01 |
| C8 _{W→A776} | >50 | 1.50 ± 0.10 | 1 ± 0.20 | 1 ± 0.24 | 0.06 ± 0.02 |
| C8 _{scr} | >50 | 1.50 ± 0.15 | 1 ± 0.23 | 1 ± 0.20 | 0.06 ± 0.02 |
| None | >50 | 1.20 | 1.20 | 1.20 | 0.05 |

^a Test peptides diluted to contain final concentrations ranging from 0.0005 to 50 μg/ml were mixed with fixed IC₅₀ of the indicated N peptides, incubated at 4°C for 30 min, and then assayed for cell-free FIV inhibition by the standard procedure. Values obtained in two to three independent assays.

^b The IC₅₀ of the indicated N peptide is given in parentheses in micrograms per milliliter.

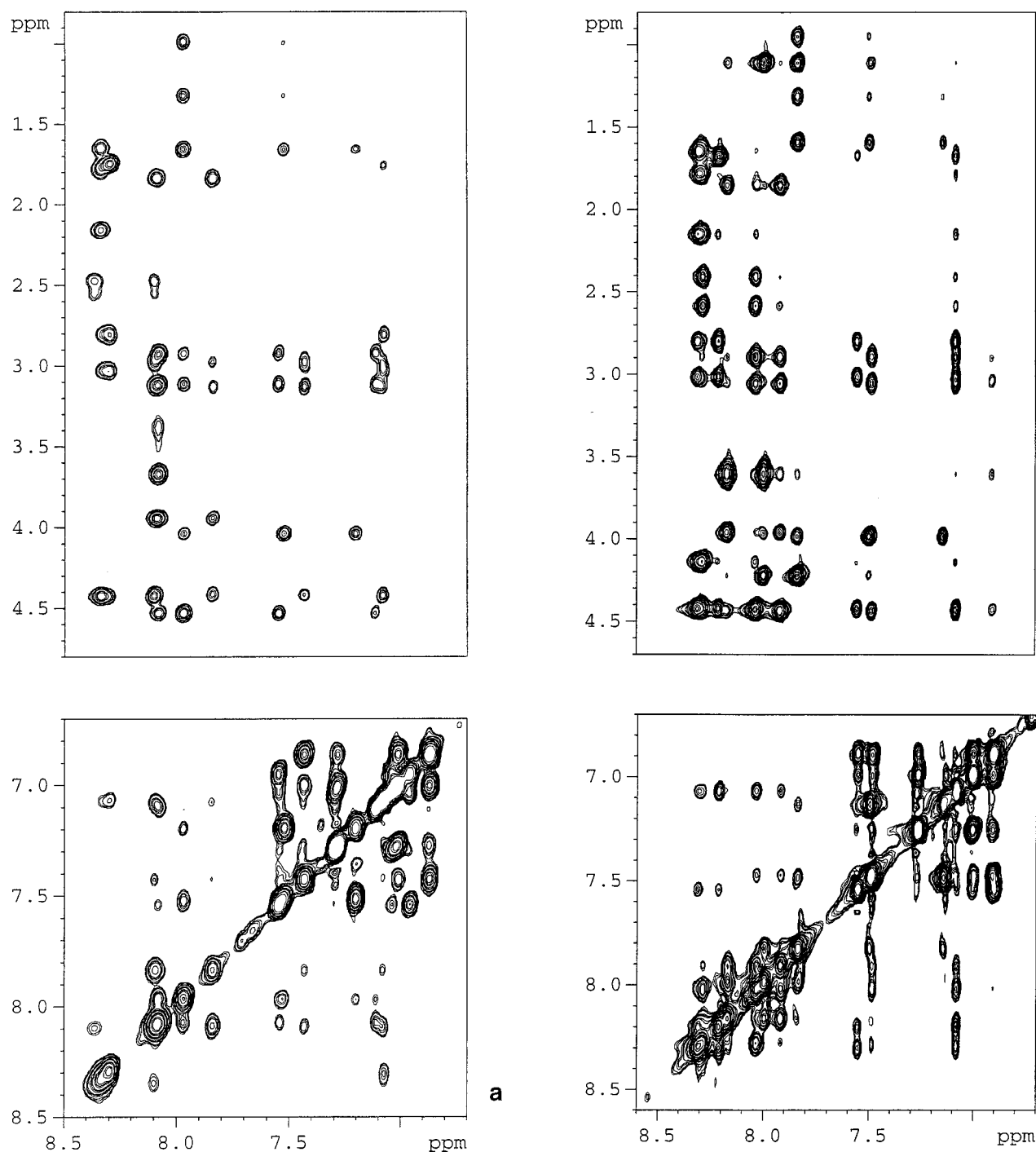


FIG. 5. NMR spectra of peptide C8 and its inactive Ala analogue $C8_{W \rightarrow A776}$. Shown are the amide (top) and the fingerprint (bottom) regions of the NOESY spectra of C8 (a) and $C8_{W \rightarrow A776}$ (b) collected in DMSO-water at 270 K (600 MHz; mixing time, 300 ms).

evident: particularly notable are a series of sequential (NH_i, NH_{i+1}) NOEs and of diagnostically critical ($i, i+3$) effects, which are strongly indicative of the predominance of ordered conformers.

Three-dimensional structures were calculated from NMR data by simulated annealing in torsion angle space using the DYANA software package and refined by a minimization pro-

cedure using the SANDER module of AMBER software (version 5.0). Figure 6 shows the overlap of the best 20 structures of peptides C8 and $C8_{W \rightarrow A776}$. The fit was calculated on the backbone heavy atoms of all residues 770 to 777. Peptide C8 structure is characterized by a well-defined α -turn structure in the 773 to 776 segment, stabilized by the presence of H bonds between the C=O of Asp-772 and the NH of Gly-775, as well

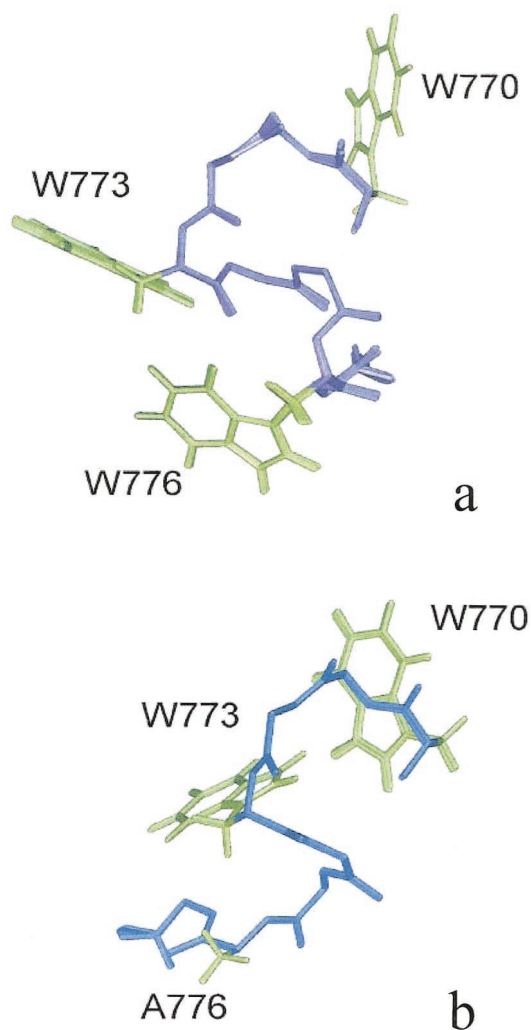


FIG. 6. Three-dimensional structure of peptide C8 and its inactive Ala analogue $C8_{W \rightarrow A776}$. Shown are the superpositions of the best 20 structures of peptides C8 (a) and $C8_{W \rightarrow A776}$ (b) obtained from DYANA structure calculations and following minimization using the SANDER module of AMBER software (version 5.0). The backbones are displayed in violet for C8 and in blue for $C8_{W \rightarrow A776}$. The side chains of Trp residues are shown (green) as well as the side chains of the Ala-776 of $C8_{W \rightarrow A776}$.

as between the C=O of Trp-773 and the NH of Trp-776 (Fig. 6a). Also, peptide C8 conformation is characterized by a well-defined orientation of the side chains, including the Trp rings. Peptide $C8_{W \rightarrow A776}$ is characterized by a folded, ordered conformation in the four N-terminal residues, whereas it shows a prevailing extended-coil structure in the remaining C-terminal residues (Fig. 6b). Consistently, overlapping of the NMR structures of C8 and $C8_{W \rightarrow A776}$ leads to a good backbone atoms fitting in the four N-terminal residues and shows divergent conformations in the C-terminal portion. Inspection of the side chain arrangement of peptide C8 shows that the aromatic rings of Trp-773 and Trp-776 are located on the same side of the structure, whereas in the inactive analogue $C8_{W \rightarrow A776}$ the region corresponding to Trp-776 in C8 is occupied by the extended N-terminal backbone.

DISCUSSION

Peptide 59, a 20-mer C peptide of FIV that had previously proven inhibitory for TCA FIV *in vitro* (37), was found to effectively inhibit a primary FIV isolate as well, provided that test conditions were appropriately set. Prerequisite for inhibition—absolute for the fresh isolate but well evident also for the TCA virus—was that the inoculum virus-peptide mixtures were removed from the test cultures after adsorption, which suggests that peptide activity is short-lived, possibly due to rapid uptake and/or degradation by cells. Analysis of smaller derivatives of this peptide mapped the inhibitory activity to a small segment containing three Trp residues, separated from one another by two amino acids. Peptide C8, *i.e.*, the shortest derivative of peptide 59 that had this Trp motif wholly conserved and was still fully active, was further investigated for inhibitory potency and breadth, critical residues, and site of action.

Peptide C8 inhibited all the primary isolates tested as cell-free virus across two FIV clades with moderate variations in IC_{50} and was also active against cell-associated virus. In particular, cell-free FIV-M2 was inhibited also when used at concentrations as high as 320 $TCID_{50}$ per well. Furthermore, its activity proved markedly dependent on an intact Trp motif, as revealed by testing a series of analogues in which each individual amino acid Ala was substituted. In addition, peptide C8 activity was removed by preincubation with two primary FIV isolates adsorbed onto substrate cells but not by preincubation with the same viruses alone, cells alone, or cells which had permitted virus entry, demonstrating that its target is viral in nature but becomes significantly accessible or functional only after FIV has undergone postadsorption changes. That TCA FIV-Pet bound substantial C8 peptide even without prior interaction with substrate cells might be a consequence of the differences evolved by TCA relative to freshly isolated lentiviruses, including a looser Env organization (3, 43), and possibly explains the great sensitivity of this virus to peptide inhibition. Finally, peptide C8 was found to be blocked in its antiviral action by a peptide corresponding to a conserved segment at the N terminus of FIV TM gp, near the fusion domain, suggesting that this might be the site of its action. Proving unequivocally the latter aspect will, however, require a finer dissection of the phenomenon.

Collectively, the above findings are markedly reminiscent of observations with HIV-1-inhibitory C peptides (28, 29, 31, 32, 65; Neurath *et al.*, letter). In particular, HIV-1-inhibitory C peptides act on gp41 that has undergone receptor-activated conformational changes and not on native, resting gp41 (6, 8, 15, 35, 42, 65). Also, the already-mentioned T-20, as well as C34, T649, and other peptides, seems to act by binding the N-terminal domain of gp41, as shown by peptide mixing experiments similar to the ones reported here and other evidence (9, 30, 34, 36, 56, 64). In turn, such binding is believed to inhibit the otherwise-ensuing association between structures at the N terminus of gp41, including a hydrophobic cavity and the C terminus of the same molecule, thus ultimately preventing fusion and entry. Note, however, that recent findings that T-20 activity can be modulated by HIV-1 coreceptor specificity, which is known to be defined by V3 sequences in the surface

gp, suggest the existence of additional elements regulating T-20 target exposure (12, 13).

A peculiar aspect of the present results is the small size of some FIV-inhibitory C peptides studied here. As a rule, oligopeptides of such small size are devoid of consistent activities due to absent or poor conformational stability. Indeed, all consistently effective HIV fusion-inhibitory C peptides described to date are composed of 30 or more residues (31, 64). Our structural studies of peptide C8, however, suggest that at least this peptide might be unusually conformationally stable. In fact, NMR analysis of peptide C8 dissolved in DMSO-water to recreate physicochemical conditions compatible with biological environments (1, 16) revealed a remarkable degree of conformational propensity, highly uncommon in similarly short peptides. Moreover, the presence in C8 of three equally distanced Trp residues was found to determine a folded compact structure which appeared to correlate with antiviral activity, since substitution of an Ala for Trp-776 greatly affected conformational features and eliminated antiviral activity as well. The orientation of the aromatic side chains of Trp-773 and Trp-776 on the same side of the C8 structure, which was observed in the main conformer, is suggestive of a possible direct involvement of these residues in hydrophobic interaction with the C8 target site. As determined by mutagenesis and crystal structure analyses, the target cavity of HIV-1 gp41 where C peptide inhibitors are believed to exert their action is susceptible to filling by small-molecule compounds. Nevertheless, small compounds capable of filling such a cavity—identified by combinatorial chemistry and other techniques—have exhibited no anti-HIV-1 activity, and this has been attributed to conformational instability (7, 9, 18, 22). Attempts to increase the conformational stability of small peptides have included chemical cross-linking, introduction of helix-capping motifs, and substitutions with amino acids of high helical propensity with variable results (31, 32). In any case, a stable helical structure appears to be important for both binding affinity to the gp41 target and antiviral activity (22).

In HIV-1, the C-terminal membrane-proximal ectodomain of gp41 not only has served for designing antiviral peptides but also is viewed as an extremely interesting target for broad range antibody-mediated neutralization due to its critical role in viral entry and wide conservation (49, 69, 71). Unfortunately, antibodies reactive with this region are very infrequent in HIV-1 patients and have never been elicited with candidate vaccines, so far (69). In our hands, the corresponding domain of FIV is also poorly immunogenic, as determined in infected cats and in mice repeatedly injected with derived peptides (41). However, groups using other strains of FIV have shown that the same domain is widely recognized by infected cat sera and also immunogenic when presented to cats as a fusion protein (14, 52). In addition, cat immunization with a peptide based on such a domain was seen to delay infection following FIV challenge (53). Thus, the C-terminal membrane-proximal ectodomain of the TM gp should be further evaluated also for the design of FIV immunoprophylactic agents. The small size and structural features of peptide C8 make it an ideal lead compound for the development of novel peptide-mimetic anti-FIV drugs and vaccines.

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