Structure−Function Study and Anti-HIV Activity of Synthetic Peptide Analogues Derived from Viral Chemokine vMIP-II†

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ABSTRACT: The viral macrophage inflammatory protein II (vMIP-II) shows a broad spectrum interaction with both CC and CXC chemokine receptors including CCR5 and CXCR4, two principal coreceptors for the cellular entry of human immunodeficiency virus type 1 (HIV-1). Recently, we have shown that a synthetic peptide derived from the N-terminus of vMIP-II, designated as V1, is a potent antagonist of CXCR4 but not CCR5 [Zhou, N., et al. (2000) *Biochemistry 39*, 3782−3787]. In this study, we synthesized a series of new peptides derived from other regions of vMIP-II and characterized their binding activities with both CXCR4 and CCR5. The results provided further support for the notion that the N-terminus of vMIP-II is the major determinant for CXCR4 recognition and that vMIP-II probably interacts with other chemokine receptors such as CCR5 with different sequence and conformational determinants. To understand the structure−function relationship of V1 peptide, its solution conformation was studied using circular dichroism spectroscopy, which showed a random conformation similar to that of the corresponding N-terminus in native vMIP-II. In addition, we synthesized a series of mutant analogues of V1 containing alanine, glycine, or phenylalanine substitution at various positions. Residues Val-1, Arg-7, and Lys-9 of V1 peptide were found to be critical for receptor interaction, because single alanine replacement at these positions dramatically decreased peptide binding to CXCR4. In contrast, alanine or phenylalanine substitution at Cys-11 led to significant enhancement in peptide affinity for CXCR4. Finally, we showed that V1 peptide inhibits HIV-1 replication in $CXCR4^+$ T-cell lines. These studies provide new insights into the structure−function relationship of V1 peptide and demonstrate that this peptide may be a lead for the development of therapeutic agents.

Chemokines are a family of 8−10 kDa small proteins that act as chemoattractants by signaling through their receptors and activating the target cells. There are two major subfamilies of chemokines: CC and CXC chemokines (*1*). Chemokine receptors are important for both normal physiological function and pathogenesis of HIV infection. CCR5 and CXCR4 are two major coreceptors for the entry of HIV-1[1] into the target cell (*2*). Natural chemokine ligands of CCR5, such as RANTES and MIP-1β (*3*), and CXCR4, such as SDF-1α (*4*, *5*), inhibit HIV-1 entry via their respective receptors. In general, a chemokine can only bind one or more receptors within the same subfamily. However, vMIP-II, a viral chemokine encoded by human herpesvirus 8 (*6*), displays diverse interactions with both CC and CXC chemokine receptors and inhibits HIV-1 entry mediated through CCR3, CCR5, and CXCR4 (*7*, *8*). The broad spectrum receptor binding property of vMIP-II is unique among all known chemokines and thus provides an interesting template to study chemokine ligand−receptor interactions and design novel small molecule anti-HIV agents.

In a recent study, we found that a synthetic peptide designated as V1 derived from the N-terminus (residues 1−21) of vMIP-II is a potent antagonist of CXCR4, but not CCR5, thus indicating the important role of the N-terminus of vMIP-II in its function via CXCR4 (*9*). In the present study, we have extended the synthetic peptide mapping approach to examining the role of other regions of vMIP-II. A series of linear and cyclic peptide analogues were synthesized covering the entire amino acid sequence of vMIP-II except the N-terminus (residues 13−71). The results show that, except for the peptide sequence near the N-terminus (residues 13−34) retaining some CXCR4 binding activity, all peptide analogues derived from other sites of vMIP-II displayed no binding affinity for CXCR4 or CCR5. These data support the notion that the N-terminus of vMIP-II is the major, if not the only, determinant for CXCR4 recognition. In view of the functional importance of the vMIP-II N-terminus, we used V1 peptide described above as a model system to analyze the structure−function relationship of the N-terminus of vMIP-II. A panel of mutant

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[1] Abbreviations: vMIP-II, viral macrophage inflammatory protein II; HIV-1, human immunodeficiency virus type 1; MIP-1α, macrophage inflammatory protein 1α; FACS, fluorescence activated cell sorter; SDF-1, stromal cell derived factor 1; RANTES, regulated upon activation, normal T cell expressed and secreted; CD, circular dichroism; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl.

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analogues of V1 peptide containing alanine and other amino acid substitutions were synthesized and tested for CXCR4 binding. In addition, the solution conformation of V1 peptide was investigated by circular dichroism (CD). The results from these studies provide valuable insight into the structure−function relationship of V1 peptide. Finally, we tested the anti-HIV effect of V1 peptide and found that it could inhibit T-tropic HIV-1 replication. This suggests that V1 peptide is a potential lead for the development of more potent and stable analogues of therapeutic values.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human chemokines SDF-1, MIP-1β, and vMIP-II (R & D systems, Minneapolis, MN) were lyophilized and dissolved as 1 or 2.5 μg/μL stock solutions in sterile phosphate-buffered saline (PBS) and stored at −20 °C in aliquots. The radioiodinated MIP-1β was purchased from DuPont NEN. The specific activity of ^{125}I-MIP-1β was 2200 Ci/mmol. Cell culture media and G418 were purchased from Life Technologies, Inc. The anti-CXCR4 monoclonal antibody (mAb) 12G5 (*10*) was purchased from PharMingen (San Diego, CA). 293 cells (gift from Dr. R. Doms of the University of Pennsylvania) and Sup-T1 cells (provided by the NIH AIDS reagent program) were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. The cell lines used for the HIV-1 replication assay were kindly provided by other laboratories: MT-4 cells by Dr. N. Yamamoto (Tokyo Medical and Dental University, Tokyo, Japan), chronically HIV-1 infected H9 cells (H9/III_B) by the NIH AIDS Research and Reference Reagent Program, and Sup-T1 cells by Dr. J. Hoxie (University of Pennsylvania). These cells were grown in 25 mM HEPES-buffered RPMI 1640 supplemented with 10% fetal bovine serum, 50 units/mL penicillin, 50 μg/mL streptomycin, and 0.125 μg/mL amphotericin B. HIV-1 was prepared from a culture supernatant of H9/III_B cells.

Peptide Synthesis. The peptides were prepared by solid-phase synthesis using Fmoc strategy on a 430A peptide synthesizer (Applied Biosystems, Foster City, CA) and a 9050 Pepsynthesizer Plus (Perseptive Biosystems, Cambridge, MA), as described previously (*11*, *12*). The side chain protecting groups of N^{α}-Fmoc [*N*-(9-fluorenyl)methoxycarbonyl] amino acids were as follows: Arg, Pmc; Asp, OtBu; Cys, Trt; Gln, Trt; His, Trt; Lys, Boc; Ser, tBu, Tyr, tBu; and Trp, Boc (Pmc = 2,2,5,7,8-pentamethylchroman-6-sulfonyl, OtBu = *tert*-butyl ester, Trt = trityl, Boc = *tert*-butyloxycarbonyl, and tBu = *tert*-butyl ester). In every coupling reaction step, a 4-fold excess of N^{α}-Fmoc amino acid, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, and 1-hydroxybenzotriazole and 10-fold excess of diisopropylethylamine were used. The cleavage of peptides from the resin was carried out with the cleavage reagent (trifluoroacetic acid/thioanisole/phenol/water/ethanedithiol/triisopropylsilane, 81.5:5:5:5:2.5:1) for 2 h at room temperature with gentle stirring. Crude peptides were precipitated in ice-cold methyl *tert*-butyl ether, centrifuged, and lyophilized. The crude peptides were then purified by preparative HPLC using a Dynamax 300 Å C_{18} 25 cm × 21.4 mm i.d. column with two solvent systems of 0.1% TFA/H_2O and 0.1% TFA/acetonitrile. Fractions containing the appropriate peptide were pooled together and lyophilized. The purity of the final product was characterized by analytical reverse-phase high-performance liquid chromatography, capillary electrophoresis, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. All peptides were at least 95% pure.

CD Spectroscopy. The experiments were performed following our previously published procedure (*13*, *14*). Fifty micromolar solutions of peptides were prepared in 0.01 M sodium phosphate buffer. The spectra were recorded on an AVIV 62A DS spectrometer (Aviv Instruments Inc., Lakewood, NJ) with a 0.01 cm path-length quartz cuvette and scanned every 2 nm at room temperature.

Flow Cytometry. Following the procedure described in our recent publication (*9*), Sup-T1 cells (2×10^5) were incubated with an anti-CXCR4 monoclonal antibody (mAb) 12G5 (10 μg/mL) and various concentrations of peptides for 40 min at 4 °C and then with FITC-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) for 40 min at 4 °C. Finally, the cells were fixed in 2% paraformaldehyde in PBS and analyzed on a FACScan flow cytometer (Coulter EPICS Elite, Beckman Coulter, Hialeah, FL).

^{125}I-MIP-1β Competitive Binding to CCR5. As outlined in our recent study (*9*), competition binding experiments were performed using a single concentration (0.2 nM) of ^{125}I-MIP-1β in the presence of increasing concentrations of unlabeled ligands in a final volume of 100 μL of binding buffer (50 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1% bovine serum albumin) containing 2×10^5 CCR5 transfected 293 cells. Nonspecific binding was determined by the addition of 100 nM unlabeled MIP-1β. Samples were incubated for 60 min at room temperature. The incubation was terminated by separating the cells from the binding buffer by centrifugation and washing once with 500 μL of cold binding buffer. Bound ligands were quantitated by counting γ emissions.

HIV-1-Induced Cytopathic Effect and Viral p24 Antigen Production. As described previously (*15*), MT-4 cells were seeded at 6×10^4 cells/150 μL per well in a 96-well microplate. HIV-1 was added at 50 $TCID_{50}$ per well in the absence or presence of peptide inhibitors. After 4 days of incubation at 37 °C in a CO_2 incubator, the supernatant was examined for the level of viral p24 antigen using the enzyme immunoassay kit (Beckman Coulter, Hialeah, FL). The cell viability was also determined by the 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The cytotoxic effect of peptides on MT-4 cells was assessed by incubating uninfected cells with peptides without the addition of HIV-1 under the same conditions as above.

HIV-1-Induced Giant Cell Formation. The anti-HIV-1 activity of V1 peptide in Sup-T1 cells was determined by counting the total number of multinucleated giant cells in each well on the fourth day after viral infection. Sup-T1 cells were seeded at 6×10^4 cells per well in a 96-well microplate. HIV-1 was added at 500 $TCID_{50}$ per well in the absence or presence of peptide inhibitors. The cytotoxic effect of peptides on Sup-T1 cells was assessed by incubating uninfected cells with peptides for 5−7 days.

RESULTS

Only vMIP-II N-Terminal Peptides Show CXCR4 Binding Activity. In a previous study, we found that V1 peptide

Table 1: Synthetic Peptides Derived from Various Regions of vMIP-II

peptides	sequences	CXCR4 binding as characterized by 12G5 mAb (IC_{50}, nM)	CCR5 binding as characterized by ^{125}I-MIP-1β (IC_{50}, nM)
V1 (vMIP-II, 1−21)[a]	LGASWHRPDKCCLGYQKRPLP	640	>100 000[b]
V4 (vMIP-II, 13−34)	LGYQKRPLPQVLLSSWYPTSQL	5 200	>100 000
V5 (vMIP-II, 22−44)	QVLLSSWYPTSQLCSKPGVIFLT	>100 000[b]	>100 000
V6 (vMIP-II, 36−57)	SKPGVIFLTKRGRQVCADKSKD	>100 000	>100 000
V7 (vMIP-II, 51−71)	ADKSKDWVKKLMQQLPVTAR	>100 000	>100 000
V8 (vMIP-II, 30−40, cyclic)	CTSQLASKPGC	>100 000	>100 000
V9 (vMIP-II, 41−51, cyclic)	CFLTKRGRQVC	>100 000	>100 000

[a] Reference *9*. [b] At 100 μM, these peptides showed no or very weak competitive binding with 12G5 or ^{125}I-MIP-1β.

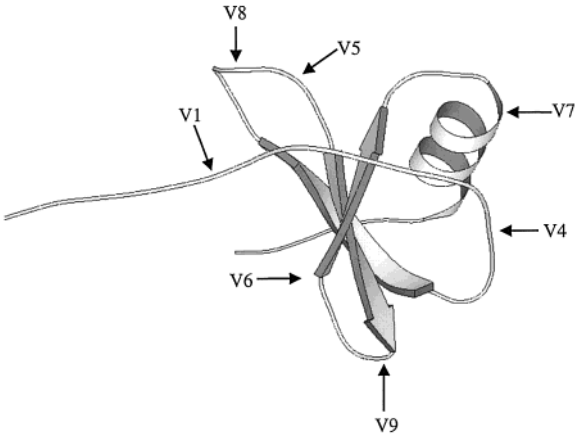

FIGURE 1: Illustration of various regions of vMIP-II studied by synthetic peptides. The coordinates of the crystal structure of vMIP-II were kindly provided by Dr. E. Lolis of Yale University (personal communication). The positions on vMIP-II as pointed to by arrows correspond to the approximately central locations of specific peptide sequences (see Table 1 for peptide sequence).

derived from residues 1−21 of the N-terminus of vMIP-II displayed significant binding affinity for CXCR4 (*9*). Here, four new peptides (V4−V7) were synthesized which, together with V1 peptide, cover the entire sequence of vMIP-II (Table 1 and Figure 1). These peptides correspond to various regions of 21−23 amino acids in length shifted by about 10−15 residues along the sequence of vMIP-II. In addition, two cyclic peptides (V8 and V9) were designed to mimic two loops in vMIP-II, respectively. All peptides were examined for binding to CXCR4 and CCR5. Only V4 peptide, which shares the second half (residues 13−21) of the highly potent V1 peptide, retained binding affinity for CXCR4 (IC_{50} = 5200 nM in competing with CXCR4 binding of mAb 12G5 as compared to that of 640 nM of V1 peptide in the same assay). In contrast, all other peptides (V5−V9) were inactive in CXCR4 binding. These results are consistent with our earlier findings for V1 peptide (*9*) and further support the notion that the N-terminus of vMIP-II is the major, if not exclusive, site responsible for CXCR4 recognition. On the other hand, all peptides including V1 and V4 peptides that bind CXCR4 did not show any binding affinity for CCR5.

Alanine Substitutions of V1 Peptide. To determine specific residues within the V1 peptide sequence important for CXCR4 binding, eight mutant analogues of V1 peptide (V1-L1A to V1-C11AC12A) containing alanine substitutions at various positions were synthesized (Table 2). These V1 mutants were tested for their abilities to compete with the binding of mAb 12G5 to CXCR4 (Table 2 and Figure 2a). As demonstrated by analogues V1-L1A, V1-W5A, V1-R7A, and V1-K9A, mutations around the N-terminal half (residues 1−10) of V1 peptide dramatically decreased the binding of peptide to CXCR4, whereas mutations around the C-terminal half (residues 11−21) of V1 peptide had much less effect on CXCR4 binding as shown by analogues V1-C11A, V1-Q16A, V1-R18A, and V1-C11AC12A. It was noted that a single alanine substitution at the Cys-11 position (analogue V1-C11A) resulted in about an 8-fold decrease in IC_{50} value in inhibiting 12G5 binding to CXCR4, thus indicating a significant increase in CXCR4 binding affinity. Analogue V1-C11AC12A containing alanine replacements of both cysteine residues still retains much CXCR4 binding affinity. These results argue against the possible role of peptide dimerization due to disulfide formation in receptor binding observed in other SDF-1 peptide analogues (*16*). In our previous study (*9*), we found no evidence for V1 dimerization by mass spectrometry and confirmed this here under in vitro cell assay conditions (data not shown).

Other Amino Acid Substitutions of V1 Peptide. We carried out further investigation of the functional role of the two cysteine residues in V1 peptide and the basis for the enhanced CXCR4 binding of analogue V1-C11A. Four additional peptides were synthesized including V1-C11G, V1-C11F, V1-C11AC12G, and V1-C11AC12F. The replacement by either glycine or phenylalanine changes the side chain of positions 11 and 12, thus allowing us to determine the functional requirement of steric size and hydrophobicity at these sites. In addition, glycine substitution introduces more flexibility into the peptide backbone and thus gives information about the conformational effect on receptor binding. The affinity of these analogues for CXCR4 was characterized by their competition with 12G5 mAb in CXCR4 binding (Table 2 and Figure 2b). Both glycine and phenylalanine substitutions at Cys-11 resulted in enhanced CXCR4 binding. It was important to note that analogue V1-C11F containing a more bulky and hydrophobic side chain as compared with that of alanine or cysteine displayed a significant 14-fold increase in potency as compared to the parent V1 peptide. This is the highest affinity for CXCR4 observed among all of the peptides studied here. On the other hand, a decrease in CXCR4 binding potency was observed in analogues V1-C11AC12G and V1-C11AC12F containing glycine and phenylalanine substitutions at Cys-12, respectively.

Solution Conformation of V1 Peptide. To understand the structural basis for the observed biological activity of V1 peptide and its mutant analogues, circular dichroism (CD)

Table 2: Mutant Analogues of V1 Peptide Containing Alanine and Other Amino Acid Substitutions

peptides	sequences[a]	CXCR4 binding as characterized by 12G5 mAb (IC_{50},[b] nM)	*x*-fold increase[c]
V1 (parent)[d]	LGASWHRPDKCCLGYQKRPLP	640	N/A[e]
V1-L1A	**A**GASWHRPDKCCLGYQKRPLP	18 500	~29
V1-W5A	LGAS**A**HRPDKCCLGYQKRPLP	2 300	~4
V1-R7A	LGASWH**A**PDKCCLGYQKRPLP	41 800	~65
V1-K9A	LGASWHRPD**A**CCLGYQKRPLP	>100 000	>156
V1-C11A	LGASWHRPDK**A**CLGYQKRPLP	84	~8 (dec)[e]
V1-Q16A	LGASWHRPDKCCLGY**A**KRPLP	480	<1 (dec)
V1-R18A	LGASWHRPDKCCLGYQK**A**PLP	905	~1.5
V1-C11AC12A	LGASWHRPDK**AA**LGYQKRPLP	2 100	~3
V1-C11G	LGASWHRPDK**G**CLGYQKRPLP	140	~5 (dec)
V1-C11F	LGASWHRPDK**F**CLGYQKRPLP	46	~14 (dec)
V1-C11AC12G	LGASWHRPDK**AG**LGYQKRPLP	10 700	~17
V1-C11AC12F	LGASWHRPDK**AF**LGYQKRPLP	3 700	~6

[a] The numbering of residues in peptides corresponds to that in native vMIP-II. The positions for alanine or other amino acid substitutions are underlined and shown in bold. [b] IC_{50} values were calculated from the anti-CXCR4 mAb 12G5 competitive binding curve derived from at least three independent assays (Figure 2). The data were processed by Prism 2.01 (Graghpad Software, Inc., San Diego, CA). [c] The *x*-fold increase was calculated on the basis of the IC_{50} value of V1 peptide. [d] Reference *9*. [e] N/A = not applicable; dec = decreased.

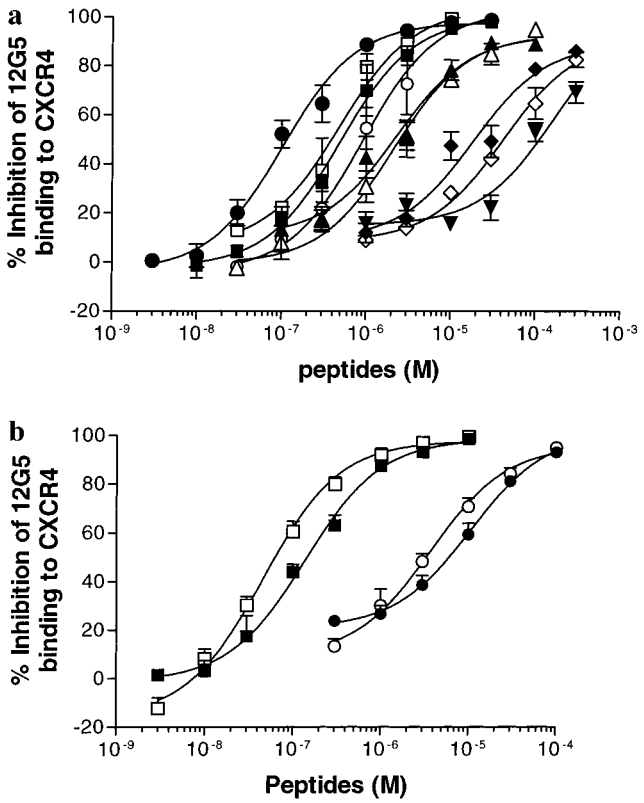

FIGURE 2: (a) CXCR4 binding of peptides containing alanine substitutions: V1 (■), V1-L1A (◆), V1-W5A (▲), V1-R7A (◇), V1-K9A (▼), V1-C11A (●), V1-Q15A (□), V1-R18A (○), and V1-C11AC12A (△). (b) CXCR4 binding of peptides containing other amino acid substitutions at two cysteines: V1-C11G (■), V1-C11F (□), V1-C11AC12G (●), and V1-C11AC12F (○). The binding was characterized using an anti-CXCR4 mAb 12G5 competitive binding assay. The results shown here are the mean values of at least three independent assays.

was used to study the solution conformation of V1 peptide and a representative mutant analogue V1-C11AC12A. As observed by CD (Figure 3), both V1 and V1-C11AC12A peptides have an intense negative band near 200 nm, which is indicative of a random structure for both peptides in aqueous solution (*17*). In addition, the spectra of V1 and V1-C11AC12A are very similar, suggesting that the mutation

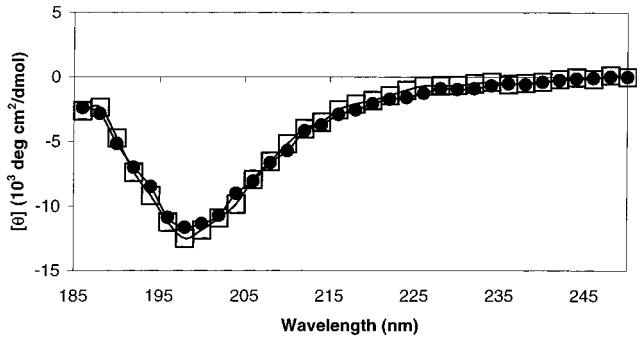

FIGURE 3: CD spectra of V1 (●) and V1-C11AC12A peptides (□). The intense negative band near 200 nm indicated the random conformations of both peptides.

does not affect the overall conformation of the peptide. The solution conformation of these peptides as determined by CD is consistent with the crystal structure (Dr. E. Lolis of Yale University, personal communication) and NMR solution structure (*18*, *19*) of vMIP-II, where the corresponding N-terminus also displays a random structure. Therefore, V1 peptide seems to structurally mimic the N-terminus of the native protein.

Anti-HIV Activity of V1 Peptide. As reported in our previous study (*9*), V1 peptide showed strong inhibition of T- and dual-tropic HIV-1 entry as measured in a cell−cell fusion assay. To further characterize its anti-HIV effect, V1 peptide of various concentrations was added in the 96-well plate which contained MT-4 cells and HIV-1 isolated from the supernatant of $H9/III_B$ cells. After 4 days, V1 showed inhibition of p24 production in a dose-dependent manner. At 75 μM, V1 almost completely abolished the p24 production (Figure 4a). Consistent with this observation, the cell viability assay also showed that V1 protected MT-4 cells from HIV-1 infection (Figure 4b). In addition to the p24 production and cytopathicity assays in MT-4 cells, V1 peptide was tested for its effect on the giant cell formation of Sup-T1 cells infected with HIV-1. After V1 peptide at different concentrations was mixed with HIV-1 for 4 days, the number of giant cells produced decreased substantially with increasing peptide concentration (Figure 4c).

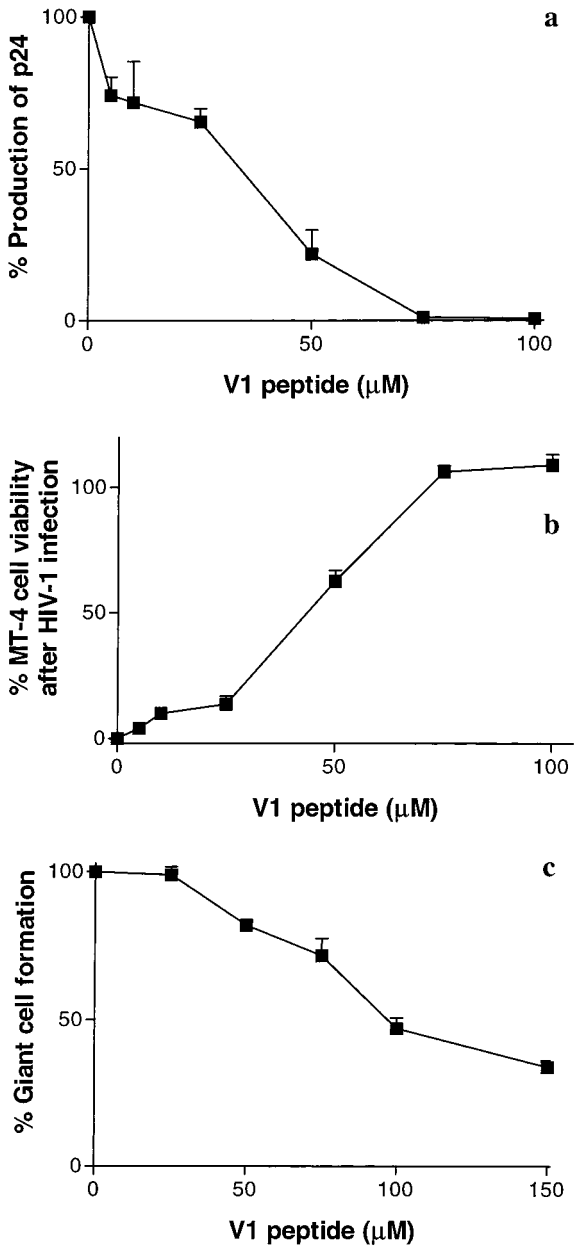

FIGURE 4: Inhibition of HIV-1 replication by V1 peptide. (a) Inhibition of p24 production. (b) Cell viability determined by the MTT method. (c) Inhibition of giant cell formation. The results shown here are the average of at least three independent experiments.

DISCUSSION

The viral chemokine vMIP-II is unique among all known chemokines because it can recognize a wide range of both CC and CXC chemokine receptors including CCR5 and CXCR4, two major coreceptors for HIV-1 entry (*8*). In our previous study (*9*), V1 peptide derived from the N-terminus of vMIP-II was found to be an antagonist for CXCR4 but not CCR5. This finding led us to hypothesize that vMIP-II uses distinctive sites for selective interactions with different receptors, with the N-terminus important for CXCR4 binding and other sites yet to be determined for CCR5 recognition. In this study we further explored the validity of this concept by studying a series of synthetic peptide analogues derived from various regions of vMIP-II. Peptides derived from the N-terminus of vMIP-II displayed CXCR4 binding whereas peptides derived from all other regions were inactive. These results are consistent with our previous study (*9*) and strongly suggest that the N-terminus of vMIP-II is the major determinant for its biological function via CXCR4.

It was found that all synthetic vMIP-II peptides including those derived from the N-terminus did not show any binding activity to CCR5. This is again consistent with a mechanism proposed for the distinctive interactions between vMIP-II and different chemokine receptors (*9*). As such, whereas the N-terminus of vMIP-II has been demonstrated to mediate CXCR4 binding, the sites in vMIP-II involved in CCR5 recognition are still not clear and presumably reside at regions other than the N-terminus of vMIP-II. One may speculate that CCR5 binding requires multiple sites distal in the sequence and dependent on the overall conformation of the whole protein, which may be difficult to be mimicked by small synthetic peptides. If this is the case, there are different requirements in both specific sequence regions and structures for vMIP-II binding to CXCR4 and CCR5. As shown by CD, the solution conformation of V1 peptide appears to mimic the random structure of the N-terminus of native vMIP-II. This may provide a structural basis for the activity of V1 peptide in CXCR4 binding. On the other hand, since the structures of peptides derived from other regions of vMIP-II have not been determined, it is not clear whether these peptides mimic the structures of corresponding sites on vMIP-II. Further studies will be necessary to clarify these issues and define the precise locations of CCR5 binding sites in vMIP-II.

One major goal of this study was to understand the structure−function relationship of V1 peptide. As shown by the CXCR4 binding activities of various alanine-substituted mutants of V1 peptide (Table 2 and Figure 2a), residues around the N-terminal half of V1 peptide play a more important role in CXCR4 binding than those around the C-terminal half. Most notable are residues Val-1, Arg-7, and Lys-9 for which alanine replacements resulted in dramatic decreases in CXCR4 binding (by 29-, 65-, and over 156-fold, respectively). In contrast, alanine mutations at residues Gln-15 and Arg-17 had little effect on receptor binding. These results are consistent with the previous finding that a truncated analogue of V1 peptide containing only residues 1−10 retained much of the CXCR4 binding activity of V1 peptide (*9*).

Alanine substitution at Cys-11 resulted in an 8-fold increase in CXCR4 affinity. To further explore the role of this position and adjacent Cys-12 in receptor binding and search for more potent analogues, we studied additional analogues containing other amino acid substitutions at these positions. For Cys-11, replacements by Ala, Gly, and Phe all resulted in the increase in receptor binding. Most interestingly, V1-C11F displays the highest affinity for CXCR4 among all mutant peptides studied here (IC_{50} = 46 nM in competing with CXCR4 binding of mAb 12G5). This indicates that replacement of Cys-11 by certain residues can enhance peptide binding to CXCR4. It is possible that bulky and hydrophobic side chains at this site form favorable interactions with the receptor. As to Cys-12, replacements by Ala, Gly, and Phe all led to the decrease in CXCR4 affinity. Particularly, V1-C11AC12G containing a conformationally flexible glycine residue at this position had the

most profound effect, thus suggesting that this site may play a structural role in CXCR4 recognition.

The V1 peptide−CXCR4 interaction is likely to be different from those between the receptor and other positively charged CXCR4 binding peptides and organic compounds, such as ALX-40 (*20*), T-22 (*21*), and AMD3100 (*22*). With a common characteristic of having a high positive charge (+8 or +9), all of these CXCR4 ligands presumably bind to the negatively charged surface (−9) of CXCR4 most likely through electrostatic interactions (*23*). A similar argument may also be made for the CXCR4 natural ligand, SDF-1, which has an overall positive charge of +8. Our recent study of synthetic peptides to mimic SDF-1 also supports the important role of positively charged residues of SDF-1 in CXCR4 binding (*24*). In contrast, V1 peptide has an overall charge of only +3.5, much lower than SDF-1 and other CXCR4 antagonists described above. In addition, analogue V1-R18A containing an alanine substitution at Arg-18 has an even lower charge of +2.5 and yet retains significant CXCR4 binding activity. It is noted that a CXCR4 binding peptide derived from the N-terminus of SDF-1 (*25*) also has an overall charge of +2.5. These observations seem to suggest that specific interactions other than the charge interaction are important for CXCR4 binding of these peptides with low positive charges. As such, V1 peptide may provide an interesting template, distinct from other highly charged CXCR4 ligands, to further explore the mechanism of ligand−receptor interaction. In this regard, we recently synthesized analogues of V1 composed of all D-amino acids as chemical probes and obtained novel insights into the stereospecificity of the CXCR4 ligand binding surface (unpublished experiments).

V1 peptide represents a lead for the development of potent and specific CXCR4 antagonists of therapeutic potential. In particular, because of the important role of CXCR4 in HIV infection, such antagonists would be useful for clinical application as anti-HIV agents. Using a gene reporter fusion assay, we have shown that V1 peptide can selectively inhibit T- and dual-tropic HIV-1 gp120-mediated cell−cell fusion via CXCR4 (*9*). Here we have extended this observation to show that V1 peptide can inhibit the replication of HIV-1 in $CXCR4^+$ T cell lines as measured by several different assays including p24 antigen production, cytopathicity, and giant cell formation. These data consistently demonstrate the clinical potential of V1 peptide. With characteristics distinct from other positively charged CXCR4 ligands, modified analogues based on V1 peptide that have higher potency and stability may eventually lead to a new class of compounds for the treatment of HIV infection and other immune diseases.

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