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Unique Ligand Binding Sites on CXCR4 Probed by a Chemical Biology Approach: Implications for the Design of Selective Human Immunodeficiency Virus Type 1 Inhibitors

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The chemokine receptor CXCR4 plays an important role as the receptor for the normal physiological function of stromal cell-derived factor 1α (SDF-1α) and the coreceptor for the entry of human immunodeficiency virus type 1 (HIV-1) into the cell. In a recent work (S. Tian et al., J. Virol. 79:12667–12673, 2005), we found that many residues throughout CXCR4 transmembrane (TM) and extracellular loop 2 domains are specifically involved in interaction with HIV-1 gp120, as most of these sites did not play a role in either SDF-1α binding or signaling. These results provided direct experimental evidence for the distinct functional sites on CXCR4 for HIV-1 and the normal ligand SDF-1α. To further understand the CXCR4-ligand interaction and to develop new CXCR4 inhibitors to block HIV-1 entry, we have recently generated a new family of unnatural chemokines, termed synthetically and modularly modified (SMM) chemokines, derived from the native sequence of SDF-1α or viral macrophage inflammatory protein II (vMIP-II). These SMM chemokines contain various de novo-designed sequence replacements and substitutions by D-amino acids and display more enhanced CXCR4 selectivity, binding affinities, and/or anti-HIV activities than natural chemokines. Using these novel CXCR4-targeting SMM chemokines as receptor probes, we conducted ligand binding site mapping experiments on a panel of site-directed mutants of CXCR4. Here, we provide the first experimental evidence demonstrating that SMM chemokines interact with many residues on CXCR4 TM and extracellular domains that are important for HIV-1 entry, but not SDF-1α binding or signaling. The preferential overlapping in the CXCR4 binding residues of SMM chemokines with HIV-1 over SDF-1α illustrates a mechanism for the potent HIV-1 inhibition by these SMM chemokines. The discovery of distinct functional sites or conformational states influenced by these receptor sites mediating different functions of the natural ligand versus the viral or synthetic ligands has important implications for drug discovery, since the sites shared by SMM chemokines and HIV-1 but not by SDF-1α can be targeted for the development of selective HIV-1 inhibitors devoid of interference with normal SDF-1α function.

The direct fusion of viral and target cell membranes required for human immunodeficiency virus type 1 (HIV-1) entry is initiated by the primary receptor, CD4, and a chemokine receptor, usually CXCR4 or CCR5. Chemokine receptors are members of the G-protein-coupled receptor (GPCR) superfamily that possess seven transmembrane (TM) domains. Because of its importance in the development of AIDS, CXCR4 has been explored as a new target for drug discovery to combat the AIDS epidemic (3, 8, 10). As the natural ligands of chemokine receptors, chemokines are small soluble proteins of about 70 amino acid residues that play prominent roles in leukocyte activation and inflammation (5, 11). Most of the known human chemokines are broadly categorized into the CXC and CC chemokines based on the position of two conserved cysteine residues in their amino (N)-terminal domains (3, 11). The natural chemokines of CXCR4 or CCR5 can inhibit HIV-1 infection (4, 13) by blocking HIV-1 gp120 binding sites (2, 14) and/or inducing receptor internalization (1, 9).

Despite their important roles in the pathogenesis of AIDS and other human diseases, the lack of receptor selectivity of natural chemokines has made their direct clinical applications problematic. It is common knowledge that a chemokine receptor can often be recognized by multiple ligands, while a chemokine ligand binds to several different receptors (15), illustrating the apparent redundancy and the lack of selectivity in the chemokine ligand-receptor interaction network. As such, we have been working toward the development of a systematic chemical biology approach based on chemokine protein structures and chemistry to generate synthetically and modularly modified (SMM) chemokines that have higher receptor binding selectivity and improved pharmacological profiles compared with natural chemokines. This SMM chemokine approach was recently applied to generate novel ligands selective for CXCR4 or CCR5 by modifying the N-terminal (1–10) sequence module of viral macrophage inflammatory protein II (vMIP-II) or stromal cell-derived factor 1α (SDF-1α) (unpublished data). Importantly, some of these SMM chemokines,

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such as those containing D-amino acids in particular, can potently inhibit HIV-1 entry via CXCR4 and yet have a less potent effect in causing receptor internalization or preventing SDF-1α signaling, thus suggesting the possibility of using these leads in the development of new selective HIV-1 entry inhibitory drugs.

To develop these SMM chemokine leads into more selective and potent therapeutics, it is critical to elucidate their binding mechanisms with CXCR4. Because mice lacking either SDF-1α (12) or CXCR4 (17, 20) die during embryogenesis, which suggests that SDF-1α and CXCR4 have important physiological functions, understanding the binding sites of SMM chemokines is a crucial first step to optimize their interactions with CXCR4 at sites important for HIV-1 entry and at the same time minimize the contact with CXCR4 sites important for the normal SDF-1α signaling. In addition, these SMM chemokines containing variations in their sequences and structures can serve as probes to help us understand the mechanisms of CXCR4-ligand interactions. Therefore, binding site mapping experiments were conducted to elucidate the binding sites of SMM chemokines on CXCR4 using a panel of site-directed CXCR4 mutants, most of which contain single amino acid substitutions at the TM and extracellular domains. Through these experiments, we sought to address the following questions. (i) Where are the binding sites on CXCR4 TM and extracellular domains for SMM chemokines, particularly D-amino-acid-containing SMM chemokines that are highly potent anti-HIV inhibitors? (ii) Do the binding sites of these unnatural, synthetic D-ligands differ from those of the normal, natural L-ligands, particularly SDF-1α? (iii) Do these D-ligands recognize major HIV-1 binding sites on CXCR4, which can explain their potency in blocking HIV-1 entry? By addressing these issues, we hope to identify potentially different determinants for CXCR4 interactions with natural ligands, SMM chemokines, and HIV-1 gp120 and eventually use such information to design novel inhibitory molecules specifically targeting only CXCR4–HIV-1 gp120 interaction without compromising normal SDF-1α function.

MATERIALS AND METHODS

Total chemical synthesis of SMM chemokines. The automated stepwise incorporation of protected amino acids was performed using an Applied Biosystems 433A peptide synthesizer (Foster City, CA) with a CLEAR amide resin (Peptides International, Louisville, KY) as the solid support. 9-Fluorenylmethoxy carbonyl chemistry was employed for the synthesis. 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydroxybenzotriazole (HOBt) were used as coupling reagents in the presence of diisopropylethylamine. In certain coupling steps with potentially slow reaction rates, double coupling followed by capping of the unreacted amino functional groups was performed. After incorporation of the 50th residue, 2% (vol/vol) dimethyl sulfoxide was introduced to the solution to enhance the coupling reaction. After removing N-terminal 9-fluorenylmethoxy carbonyl protection, the protein was cleaved from the resin support by adding a cleavage cocktail comprised of phenol (4% [wt/vol]), thioanisole (5% [vol/vol]), water (5% [vol/vol]), ethanedithiol (2.5% [vol/vol]), triisopropylsilane (1.5% [vol/vol]), and trifluoroacetic acid (TFA; 82% [vol/vol]). The protein was precipitated by adding ice-cold *tert*-butyl methyl ether and washed repeatedly in cold ether. The crude protein was dissolved in 25% CH_3CN in water containing 0.1% TFA before being lyophilized, and it was dissolved in water and purified by semipreparative reverse-phase high-performance liquid chromatography. Folding of the purified protein was performed in 1 M guanidinium hydrochloride and 0.1 M Trisma base at pH 8.5 (1 mg protein/ml folding buffer), and was monitored by analytical reverse-phase high-performance liquid chromatography using a Vydac C_{18} column (0.46 by 15 cm, 5 μm) with a flow rate of 1 ml/min with solvent A (water with 0.1% TFA), solvent B (20% water in CH_3CN with 0.1% TFA), and a linear gradient of 30 to

TABLE 1. List of SMM chemokines and their sequences and modifications

Analog designation	Modification diagram[b]			Amino acid sequence[a]
SDF-1α	1–8	9–68		KPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLKWIQEYLEKALNK
	NT	SDF-1α core		
vMIP-II	1–10	11–71		LGASWHRPDKCCLGYQKRPLPQVLLSSWYPTSQLCSKPGVIFLTKRGRQVCADKSKDWVKKLMQQLPVTAR
	NT	vMIP-II core		
D(1–8)-SDF-1α	1–8	9–68		*KPVSLSYR*CPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLKWIQEYLEKALNK
	NT	SDF-1α core		
	D-Amino acids			
D(1–10)-vMIP-II	1–10	11–71		*LGASWHRPDK*CCLGYQKRPLPQVLLSSWYPTSQLCSKPGVIFLTKRGRQVCADKSKDWVKKLMQQLPVTAR
	NT	vMIP-II core		
	D-Amino acids			
(1–10)-vMIP-II–(9–68)-SDF-1α	1–10	9–68		**LGASWHRPDK**CPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLKWIQEYLEKALNK
	NT	SDF-1α core		
	vMIP-II			
D(1–10)-vMIP-II–(9–68)-SDF-1α	1–10	9–68		***LGASWHRPDK***CPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLKWIQEYLEKALNK
	NT	SDF-1α core		
	D-vMIP-II			

[a] D-Amino acids are shown in italic, while substituted residues (from either SDF-1α or vMIP-II) are shown in bold.
[b] NT, N terminus.

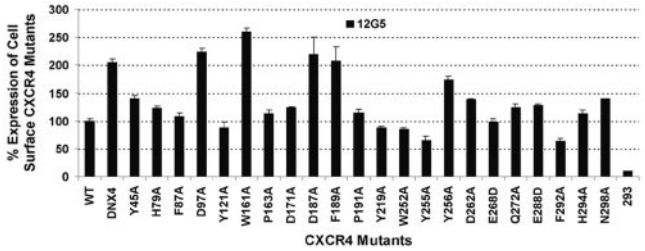

FIG. 1. Cell surface expression of wild-type and mutant CXCR4. Stably transfected 293 cells with wild-type or mutant CXCR4 were analyzed for cell surface expression of CXCR4 by flow cytometry using anti-CXCR4 MAb 12G5. Bars represent the mean fluorescence intensity for cells expressing mutant CXCR4 relative to cells expressing wild-type CXCR4. All data are shown as the mean ± standard deviation from at least three independent experiments.

70% solvent B over 30 min. Protein desaltation and purification were then performed. The purified protein was characterized by matrix-assisted laser desorption ionization–time of flight mass spectrometry.

Biological materials. An HEK 293 cell line, pcDNA3-CXCR4, and an anti-CXCR4 monoclonal antibody (MAb), 12G5, were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, National Institutes of Health, Bethesda, MD). Cell culture media and G418 were purchased from CAMBREX (Walkersville, MD). A mixture of Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 5% penicillin–streptomycin was used to maintain 293 cells.

Site-directed mutagenesis. Wild-type CXCR4 was inserted into the pcDNA3 vector as previously described (18, 19). All of the CXCR4 mutants were prepared with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Most of them were individually substituted for by alanine. The mutations were confirmed by sequencing.

Transfection of adherent 293 cells. Wild-type or mutant CXCR4 was transfected into 293 cells using Tfx-50 reagents (Promega, Madison, WI) according to the manufacturer's instructions. The selective medium containing G418 (800 μg/ml) was used to isolate stably transfected cells. Each stably transfected cell was cloned from a single colony to isolate a colony that displays a comparable expression level with wild-type CXCR4.

Flow cytometry. Transfected 293 cells (5×10^5 cells/well) were washed with fluorescence-activated cell sorting (FACS) buffer (0.5% bovine serum albumin, 0.05% sodium azide in phosphate-buffered saline) twice and incubated with anti-CXCR4 MAb 12G5 (10 μg/ml) for 30 min at 4°C. After being washed with FACS buffer twice, cells were incubated with 10 μg of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Sigma, St. Louis, MO) for 30 min at 4°C. After being washed twice with FACS buffer, cells were fixed in the fixing buffer (2% paraformaldehyde in phosphate-buffered saline) for 30 min at 4°C before being analyzed on a FACScan flow cytometer (19). At least three independent experiments were performed.

Binding site mapping experiments. The experiments were performed using a single concentration (5 μg/ml) of 12G5 in a final volume of 100 μl FACS buffer containing 5×10^5 cells in 96-well plates in the presence of various concentra-

TABLE 2. Residues involved in the chemokine binding and HIV-1 coreceptor activities of CXCR4

CXCR4 domain	CXCR4 mutants	Activity of[a]:						
		SDF-1α	(1–10)-vMIP-II–(9–68)-SDF-1α	vMIP-II	D(1–8)-SDF-1α	D(1–10)-vMIP-II–(9–68)-SDF-1α	D(1–10)-vMIP-II	HIV-1 coreceptor
Wild type		+++	+++	+++	+++	+++	+++	+++
N terminus	DNX4	+	+	+	++	−	+	−
ECL2	D187A	+++	+++	+++	++	+	+++	−
	F189A	+++	+++	+++	+++	+++	+++	−
	P191A	+++	+++	+++	+++	+++	+++	+
ECL3	E268A	+++	+++	+++	+++	+++	+++	+
	Q272A	+++	+++	+++	+++	+++	+++	+
TM1	Y45A	+++	+++	+++	++	+	++	−
TM2	H79A	+++	+++	+++	+++	+++	+++	+
	F87A	++	+++	+	++	−	−	++
	D97A	+++	+++	++	+++	+	++	−
TM3	Y121A	+++	+++	++	+++	+	++	+++
TM4	W161A	+++	+++	+++	+++	+++	+++	++
	P163A	+++	+++	+++	+++	+++	+++	+
	D171A	++	+++	+++	+	++	+	−
TM5	Y219A	+++	+++	+++	+++	++	+++	++
TM6	W252A	+++	+++	+++	+++	++	++	+
	Y255A	+++	+++	+++	+++	++	++	−
	Y256A	+++	+++	+++	+++	+++	+++	+++
	D262A	+++	+++	+++	+++	++	+++	++
TM7	E288D	+++	+++	+++	++	+	+	+
	F292A	+	+++	++	+++	+	++	++
	H294A	+++	+++	+++	+++	+++	+++	+
	N298A	+++	+++	+++	+++	+++	+++	+

[a] The activities that are similar to wild-type activities are denoted by +++, whereas ++, +, or − indicates the degree of reduction in the activity: ++, 10 to 30% reduction; +, 30 to 60% reduction; and −, 60 to 100% reduction. The fusion data are from our previous CXCR4 mutational study (18).

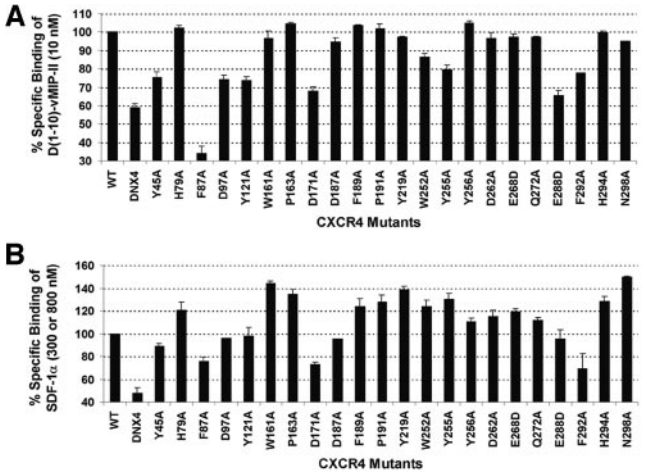

FIG. 2. Specific binding of D(1–10)-vMIP-II (A) and SDF-1α (B) to wild-type CXCR4 and mutants. (A) The point mutation of Tyr^{45}, Phe^{87}, Asp^{97}, Tyr^{121}, Asp^{171}, Trp^{252}, Tyr^{255}, Glu^{288}, or Phe^{292} reduced D(1–10)-vMIP-II binding. The binding activity of D(1–10)-vMIP-II was also impaired by DNX4. (B) F87A, D171A, F292A, and DNX4 decreased the percent specific binding of SDF-1α. The data represent the mean values of three independent assays, with error bars indicating the standard deviations.

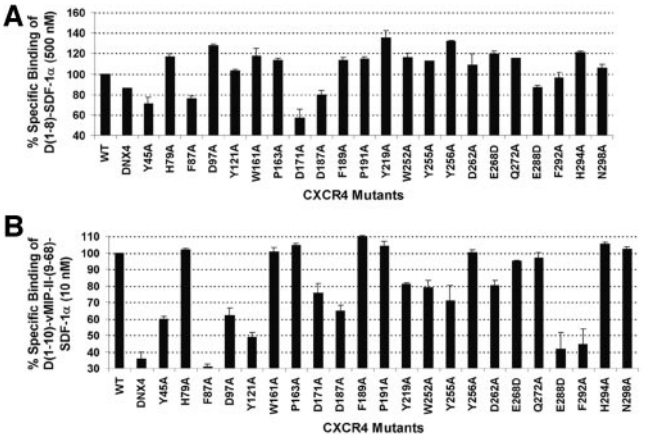

FIG. 3. Binding activities of D(1–8)-SDF-1α (A) and D(1–10)-vMIP-II–(9–68)-SDF-1α (B) to wild-type CXCR4 and mutants. (A) The binding activity of D(1–8)-SDF-1α was reduced by Y45A, F87A, D171A, D187A, E288D, and DNX4. (B) The point mutation of Tyr^{45}, Phe^{87}, Asp^{97}, Tyr^{121}, Asp^{171}, Asp^{187}, Tyr^{219}, Trp^{252}, Tyr^{255}, Asp^{262}, Glu^{288}, or Phe^{292} as well as the deletion of the N terminus impaired D(1–10)-vMIP-II–(9–68)-SDF-1α binding. All data are shown as the mean ± standard deviation from at least three independent experiments.

tions of natural or synthetic ligands. The cells were incubated on ice for 40 min. The cells were washed twice with FACS buffer and stained with 10 μg fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G for 30 min at 4°C. As a negative control, the cells were stained only with the secondary antibody. The cells were washed twice with FACS buffer and resuspended in 100 μl FACS buffer before being analyzed on the Wallac Victor^2 1420 multilabel counter (Turku, Finland). The total ligand binding was calculated by determining the fluorescence counts of 5 μg/ml 12G5 in the absence of competing unlabeled ligands, whereas the nonspecific ligand binding was equal to the fluorescence counts of 10 μg secondary antibody only. The difference between total and nonspecific binding is the specific ligand binding. Only when the point mutation of a CXCR4 residue reduced the percent specific binding activity of a ligand compared with that of the wild-type receptor by more than 10% was the residue considered important for ligand binding. The testing concentration was reduced to as low as 5 nM for high-affinity ligands, i.e., D(1–10)-vMIP-II–(9–68)-SDF-1α and D(1–10)-vMIP-II, whereas only the high concentrations, such as 300 nM and 500 nM, were used to test the binding activity of a low-affinity ligand such as D(1–8)-SDF-1α. At least three independent experiments were performed. Although only the binding data at one testing concentration are shown for simplicity, the data represent the mean values of three independent assays with the error bars indicating the standard deviations.

RESULTS AND DISCUSSION

Selection of SMM chemokines and CXCR4 mutants for binding site mapping experiments. To identify novel ligand functional sites on CXCR4 with our chemical biology approach, the following SMM chemokines were chosen for the present study: (1–10)-vMIP-II–(9–68)-SDF-1α, D(1–8)-SDF-1α, D(1–10)-vMIP-II–(9–68)-SDF-1α, and D(1–10)-vMIP-II, all of which contain amino acid substitutions and/or D-amino-acid replacement at the N terminus. Natural chemokines, SDF-1α and vMIP-II, were used as positive controls and for comparisons. Their sequences and modifications are provided in Table 1. There are two different types of D-amino-acid-containing SMM chemokines investigated in this study, which were based on vMIP-II and SDF-1α, respectively. D(1–10)-vMIP-II was derived from vMIP-II by replacing the N-terminal (1–10) sequence module of vMIP-II with D-amino acids, whereas D(1–8)-SDF-1α and D(1–10)-vMIP-II–(9–68)-SDF-1α were based on SDF-1α and contain the replacement of the N-terminal (1–8) residues of SDF-1α by D-amino acids or all D forms of the N-terminal (1–10) residues of vMIP-II, respectively. Such D-amino-acid-containing SMM chemokines were chosen as probes to study their binding sites because of their improved CXCR4 selectivity, binding affinities, and/or anti-HIV activities (unpublished data).

As for the CXCR4 mutants, with the exception of DNX4, which is a CXCR4 mutant with the entire N-terminus (codons 2 to 25) deleted, all the TM and extracellular loop (ECL) mutants contain a single amino acid substitution, mostly of alanine, in their respective sites. The panel of mutations at residues near or within the TM helices were chosen based on the following considerations: (i) charged residues such as D97, D171, and E288 may interact with the oppositely charged residues of natural ligands or SMM chemokines; (ii) highly conserved residues among chemokine receptors or analogous to corresponding sites in other GPCRs, such as H79, Y121, W161, Y219, and N298, are known to be functionally important for other GPCRs (16); and (iii) residues such as P163 may affect the helical conformations of CXCR4. In addition to these TM mutants, several mutants of the ECL residues were used to investigate the role of the ECL in ligand binding. All of the mutants used for the current binding site mapping experiments were previously tested by our laboratory for their activities in SDF-1α binding and signaling and HIV-1 coreceptor activity (18). Except for the N terminus of CXCR4, which is well documented in the literature for its roles in receptor physiology and pathology (5–7, 19), other regions of CXCR4 such as most of the mutated regions focused on the present study are still poorly understood.

Cell surface expression of CXCR4. We first investigated the question whether a difference in the binding activity of a ligand toward a wild-type versus a mutant receptor could be due to a

TABLE 3. Comparison of CXCR4 binding sites of D-amino-acid-containing SMM chemokines (D-ligands) versus SDF-1α and other L-amino-acid-containing chemokines (L-ligands)

CXCR4 domains	Binding site of:					
	L-Ligands			D-Ligands[a]		
	SDF-1α	(1–10)-vMIP-II–(9–68)-SDF-1α	vMIP-II	D(1–8)-SDF-1α	D(1–10)-vMIP-II-(9–68)-SDF-1α	D(1–10)-vMIP-II
N-terminus	DNX4	DNX4	DNX4	DNX4	DNX4	DNX4
ECL2				**Asp^{187}**	**Asp^{187}**	
ECL3						
TM1				**Tyr^{45}**	**Tyr^{45}**	**Tyr^{45}**
TM2	Phe^{87}		Phe^{87}, Asp^{97}	Phe^{87}	Phe^{87}, Asp^{97}	Phe^{87}, Asp^{97}
TM3			Tyr^{121}		Tyr^{121}	Tyr^{121}
TM4	Asp^{171}			Asp^{171}	Asp^{171}	Asp^{171}
TM5					**Tyr^{219}**	
TM6					**Trp^{252}, Tyr^{255}, Asp^{262}**	**Trp^{252}, Tyr^{255}**
TM7	Phe^{292}		Phe^{292}	**Glu^{288}**	**Glu^{288}**, Phe^{292}	**Glu^{288}**, Phe^{292}

[a] The residues in bold are uniquely involved in the binding activities of D-ligands but not L-ligands.

change in the level of cell surface expression of the receptor. A particular mutant exhibiting a marked reduction in the binding activity could be caused by its poor expression compared with that of wild-type CXCR4. Flow cytometry experiments were performed on all the stably transfected CXCR4 mutants. We found that all the mutants displayed stable expression levels comparable to or higher than those of wild-type CXCR4 (Fig. 1).

D-Amino acid-containing SMM chemokines differ from SDF-1α on CXCR4 binding sites. According to the results for the vMIP-II-based D-amino-acid-containing SMM chemokine, the binding activity of D(1–10)-vMIP-II with its N-terminal (1–10) residues replaced with D-amino acids was reduced by the point mutation of Tyr^{45}, Phe^{87}, Asp^{97}, Tyr^{121}, Asp^{171}, Trp^{252}, Tyr^{255}, Glu^{288}, or Phe^{292} as well as by the deletion of the N terminus (Table 2). While D171A, E288D, and DNX4 decreased the percent specific binding of D(1–10)-vMIP-II by 32 to 41%, F87A reduced D(1–10)-vMIP-II binding by more than 60% (Fig. 2A). Y45A, D97A, Y121A, W252A, Y255A, and F292A all reduced the binding activity of D(1–10)-vMIP-II by 13 to 26%. In contrast, SDF-1α did not require Tyr^{45}, Asp^{97}, Tyr^{121}, Trp^{252}, Tyr^{255}, and Glu^{288} for its interaction with CXCR4, as their mutations had little effect on SDF-1α binding. However, F87A, D171A, and F292A impaired the binding affinity of SDF-1α by 24 to 31%, whereas DNX4 decreased SDF-1α binding by 53% (Fig. 2B). Note that the current results on the binding activity of SDF-1α obtained using the anti-CXCR4 MAb 12G5 are identical to those of ^{125}I-labeled SDF-1α binding experiments (18), demonstrating that the inhibition of 12G5 can substitute for the inhibition of SDF-1α at least for the CXCR4 mutants examined in this study. In fact, according to ^{125}I–SDF-1α competition binding assays (18), all of the CXCR4 mutants had no effect on SDF-1α binding, as they showed comparable 50% inhibitory concentrations (~3 nM) to that of wild type. Only three TM mutants, F87A, D171A, and F292A, drastically reduced the binding activity of SDF-1α, as their binding curves did not reach a plateau of nonspecific binding even at 300 nM of unlabeled SDF-1α. Furthermore, the finding of 50% inhibitory concentrations of each CXCR4 mutant comparable to that of the wild type undermines any notion that some mutations, such as Trp^{161} and Pro^{163}, may be able to increase the binding activity of SDF-1α.

Similar to D(1–10)-vMIP-II, the introduction of D-amino acids in SDF-1α caused the new analogs to interact with a different set of residues on CXCR4 (Table 2). For instance, compared with SDF-1α, D(1–8)-SDF-1α required three new residues, namely Tyr^{45}, Asp^{187}, and Glu^{288}, as their mutations reduced D(1–8)-SDF-1α binding activity by 13 to 29% (Fig. 3A). Also the fact that Phe^{292}, an important residue in SDF-1α binding, was no longer required for D(1–8)-SDF-1α binding further illustrates the differences in the CXCR4 binding sites of SDF-1α versus D(1–8)-SDF-1α. Furthermore, D171A and DNX4 showed a significant difference in their effects on the binding affinities of SDF-1α and D(1–8)-SDF-1α. Whereas D171A reduced SDF-1α binding by 53%, it decreased the percent specific binding of D(1–8)-SDF-1α by a smaller margin, 27%. In contrast, although DNX4 impaired SDF-1α binding by 27%, it caused a greater reduction, 43%, in D(1–8)-SDF-1α binding.

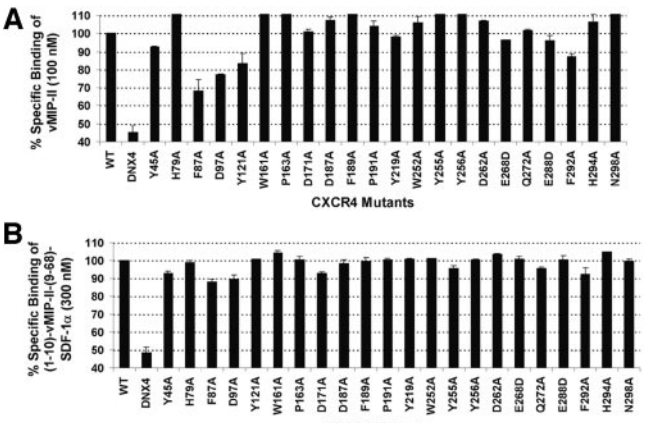

FIG. 4. Inhibition of anti-CXCR4 12G5 binding by vMIP-II (A) and (1–10)-vMIP-II–(9–68)-SDF-1α (B) to wild-type CXCR4 and mutants. The data represent the mean values of three independent assays with the error bars indicating the standard deviations. (A) The binding affinity of vMIP-II was decreased by F87A, D97A, Y121A, F292A, and DNX4. (B) Only DNX4 significantly attenuated the binding activity of (1–10)-vMIP-II–(9–68)-SDF-1α. The other mutants had little effect on (1–10)-vMIP-II–(9–68)-SDF-1α binding.

TABLE 4. Comparison of CXCR4 binding sites of SMM chemokines including D-ligands versus HIV-1 gp120 and SDF-1α

CXCR4 domain	Binding site of[a]:		
	SDF-1α	SMM chemokines	HIV-1 gp120
N terminus	DNX4	DNX4	DNX4
ECL2		**Asp^{187}**	**Asp^{187}**
ECL3			
TM1		**Tyr^{45}**	**Tyr^{45}**
TM2	Phe^{87}	Phe^{87}, **Asp^{97}**	**Asp^{97}**
TM3		Tyr^{121}	
TM4	Asp^{171}	Asp^{171}	Asp^{171}
TM5		Tyr^{219}	
TM6		**Trp^{252}**, **Tyr^{255}**, Asp^{262}	**Trp^{252}**, **Tyr^{255}**
TM7	Phe^{292}	**Glu^{288}**, Phe^{292}	**Glu^{288}**

[a] The residues in bold are those CXCR4 binding sites shared by SMM chemokines and HIV-1 gp120 but not by SDF-1α.

In addition, by replacing the N-terminal (1–8) sequence module of SDF-1α with all D forms of (1–10) residues of vMIP-II, the binding site of the new analog could be deviated further away from that of SDF-1α. Besides the overlapping residues also required for SDF-1α binding, D(1–10)-vMIP-II–(9–68)-SDF-1α needed several additional residues, including Tyr^{45}, Asp^{97}, Tyr^{121}, Asp^{187}, Tyr^{219}, Trp^{252}, Tyr^{255}, Asp^{262}, and Glu^{288} (Table 2). In fact, D(1–10)-vMIP-II–(9–68)-SDF-1α was by far the most selective inhibitor, which seems to be consistent with the largest number of distinct residues observed in our binding site mapping experiments. For example, Y45A, F87A, Y121A, E288D, F292A, and DNX4 impaired D(1–10)-vMIP-II–(9–68)-SDF-1α binding by 40 to 70% (Fig. 3B). The other mutants, including D97A, D171A, D187A, Y219A, W252A, Y255A, and D262A, reduced the binding activity of D(1–10)-vMIP-II–(9–68)-SDF-1α by 19 to 38%. In particular, F87A, F292A, and DNX4, which were also implicated in SDF-1α binding, had a greater impact on the binding activity of D(1–10)-vMIP-II–(9–68)-SDF-1α, as they decreased the percent specific binding of D(1–10)-vMIP-II–(9–68)-SDF-1α by 70, 56, and 64%, respectively. The same set of mutants impaired SDF-1α binding by smaller margins of 24, 31, and 53%, respectively.

D-Amino acid-containing SMM chemokines differ from their L counterparts on CXCR4 binding sites. D(1–10)-vMIP-II and D(1–10)-vMIP-II–(9–68)-SDF-1α also showed major differences in their binding sites from their L counterparts, namely vMIP-II and (1–10)-vMIP-II–(9–68)-SDF-1α. The main sites involved in the binding activities of vMIP-II and (1–10)-vMIP-II–(9–68)-SDF-1α consist of Phe^{87}, Asp^{97}, Tyr^{121}, Phe^{292}, and the N terminus (Table 3). F87A, D97A, Y121A, and F292A reduced vMIP-II binding by 13 to 32%, whereas DNX4 decreased the percent specific binding of vMIP-II by more than 50% (Fig. 4A). In the case of (1–10)-vMIP-II–(9–68)-SDF-1α, all of the mutants had very little effect on its binding, with the exception of DNX4, which decreased (1–10)-vMIP-II–(9–68)-SDF-1α binding by 52% (Fig. 4B). In contrast, there were several distinct TM residues necessary for the binding activities of D(1–10)-vMIP-II and D(1–10)-vMIP-II–(9–68)-SDF-1α only (Table 3). For instance, compared with its L counterpart, D(1–10)-vMIP-II-(9–68)-SDF-1α binding required a large number of distinct residues such as Tyr^{45}, Phe^{87}, Asp^{97}, Tyr^{121}, Asp^{171}, Asp^{187}, Tyr^{219}, Trp^{252}, Tyr^{255}, Asp^{262}, Glu^{288}, and Phe^{292}. Likewise, five additional TM residues, including Tyr^{45}, Asp^{171}, Trp^{252}, Tyr^{255}, and Glu^{288}, were involved in D(1–10)-vMIP-II binding, unlike in vMIP-II binding. Note that D(1–10)-vMIP-II–(9–68)-SDF-1α and D(1–10)-vMIP-II share a great overlap in their binding sites likely due to a common major binding determinant, the D-amino acid sequence derived from the same N terminus of vMIP-II in these two molecules.

D-Amino-acid-containing SMM chemokines significantly overlap with HIV-1 gp120 on CXCR4 binding sites. Based on our previous CXCR4 mutational study (18), Tyr^{45}, Asp^{97}, Asp^{171}, Asp^{187}, Trp^{252}, Tyr^{255}, Glu^{288}, and the N terminus are all known to play key roles in the HIV-1 coreceptor activity of CXCR4 (Table 4). We notice that Asp^{187} is the only ECL residue involved not only in D-ligand binding but also in HIV-1

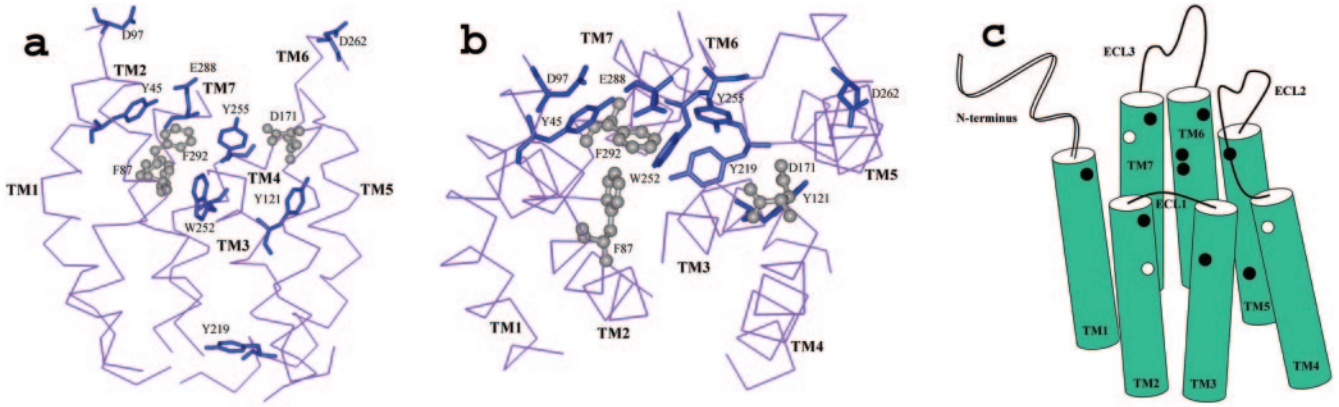

FIG. 5. Distinct functional sites for SDF-1α and SMM chemokines highlighted on a hypothetical structural model of CXCR4. As detailed in Table 4, the residues involved in both SDF-1α and SMM chemokine binding are highlighted in the lighter color and represented in the ball-and-stick format, whereas those selectively involved in SMM chemokine binding are highlighted in the darker color. Only the TM domains, with side (a) and top (b) views, are shown for simplicity. The model was built based on the previously published structural model of CXCR4 by our laboratory (19). (c) Schematic illustration of the locations of residues important for ligand binding on CXCR4 TM and extracellular domains. The residues involved in the binding activities of both SDF-1α and SMM chemokines are highlighted with white spots, whereas those selectively involved in SMM chemokine binding (most of which overlap with HIV-1 binding) are highlighted with black spots. Such overlapping sites between HIV-1 and SMM chemokines may serve as a potential target recognized by new selective anti-HIV inhibitors.

coreceptor activity, suggesting that it will be important to examine further whether the ECL2, a major loop involved in HIV-1 entry but not in SDF-1α binding (19), is an important binding region for D-ligands. We also note that Glu^{288} is required for the binding activities of all of the D-ligands, which makes sense considering that Glu^{288} is located close to the surface of the TM "barrel" (Fig. 5). In addition, the other distinct sites required for both HIV-1 gp120 and D-ligands, such as Tyr^{45}, Asp^{97}, Trp^{252}, and Tyr^{255}, are located on the upper part of the TM barrel close to the extracellular side or to the ECL2. Their role is likely to be involved with direct interactions with different ligands. Based on the present findings, one can hypothesize that certain flexible determinants of HIV-1 gp120, which can reach into CXCR4 TM domains, may be blocked by D-ligands that directly interact with these TM residues. Alternatively, it is possible that the TM mutations may cause changes in the conformations of CXCR4 core domains and thus indirectly affect CXCR4 interactions on its surface with HIV-1 gp120 or D-amino-acid-containing SMM chemokines. In such a case, the potential conformational changes caused by the mutations seem to be selective in hindering CXCR4 interactions with HIV-1 gp120 or D-ligand interaction, since SDF-1α binding to the mutant receptors was not affected. If this notion of conformational changes were true, this would strongly suggest that different conformations of CXCR4 are functionally important for D-amino-acid-containing SMM chemokine and SDF-1α. Despite this preferential overlapping in the CXCR4 binding residues of SMM chemokines with HIV-1 over SDF-1α, we note that several mutants of CXCR4, including H79A, P163A, F189A, P191A, E268A, Q272A, H294A, and N298A, significantly reduced the coreceptor activity of CXCR4 (18) without reducing the binding activities of any ligands (including D-amino-acid-containing SMM chemokines and SDF-1α), indicating that the interaction of CXCR4 with HIV-1 gp120 involves an extensive set of residues, many of which are not required for the interaction with SMM chemokines or SDF-1α. Nevertheless, the findings from the present study provide a basis for the development of new inhibitory agents, as the CXCR4 binding sites shared by both HIV-1 gp120 and D-amino-acid-containing ligands may serve as a major target for the development of new HIV-1 inhibitory agents that can reduce or avoid the side effects in binding to the CXCR4 sites important for its normal ligand, SDF-1α.

Implications for the design of new selective HIV-1 inhibitors. We reported here that SMM chemokines (particularly those unnatural D-amino-acid-containing analogs) share many CXCR4 binding sites with HIV-1 gp120 and yet differ from SDF-1α. These results suggest that these chemically engineered molecules have interesting and unique receptor binding mechanisms distinct from those of the natural chemokines and may be used to selectively disrupt the coreceptor activity of CXCR4. This notion is supported by the finding that some of these D-amino-acid-containing SMM chemokines show greater efficacy than SDF-1α in inhibiting HIV-1 entry via CXCR4 (unpublished data). The distinct residues required for the binding activities of D-amino-acid-containing SMM chemokines include Tyr^{45}, Asp^{97}, Tyr^{121}, Asp^{187}, Tyr^{219}, Trp^{252}, Tyr^{255}, Asp^{262}, and Glu^{288}, many of which play important roles in HIV-1 coreceptor activity. These overlapping functional sites for HIV-1 gp120 and D-amino-acid-containing SMM chemokines, located on CXCR4 TM and extracellular domains, may be used to guide the effort to design selective HIV-1 inhibitors that do not interfere with the normal SDF-1α function.

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REFERENCES

1. **Amara, A., S. L. Gall, O. Schwartz, J. Salamero, M. Montes, P. Loetscher, M. Baggiolini, J.-L. Virelizier, and F. Arenzana-Seisdedos.** 1997. HIV coreceptor downregulation as antiviral principle: SDF-1α-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. J. Exp. Med. **186:**139–146.
2. **Arenzana-Seisdedos, F., J. L. Virelizier, D. Rousset, I. Clark-Lewis, P. Loetscher, B. Moser, and M. Baggiolini.** 1996. HIV blocked by chemokine antagonist. Nature **383:**400.
3. **Berger, E. A., P. M. Murphy, and J. M. Farber.** 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu. Rev. Immunol. **17:**657–700.
4. **Bleul, C. C., M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, and J. Sodroski.** 1996. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature **382:**829–833.
5. **Brelot, A., N. Heveker, O. Pleskoff, N. Sol, and M. Alizon.** 1997. Role of the first and third extracellular domains of CXCR-4 in human immunodeficiency virus coreceptor activity. J. Virol. **71:**4744–4751.
6. **Chabot, D. J., P.-F. Zhang, G. V. Quinnan, and C. C. Broder.** 1999. Mutagenesis of CXCR4 identifies important domains for human immunodeficiency virus type 1 X4 isolate envelope-mediated membrane fusion and virus entry and reveals cryptic coreceptor activity for R5 isolates. J. Virol. **73:**6598–6609.
7. **Doranz, B., M. Orsini, J. Turner, T. Hoffman, J. Berson, J. Hoxie, S. Peiper, L. Brass, and R. Doms.** 1999. Identification of CXCR4 domains that support coreceptor and chemokine receptor functions. J. Virol. **73:**2752–2761.
8. **Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science **272:**872–877.
9. **Förster, R., E. Kremmer, A. Schubel, D. Breitfeld, A. Kleinschmidt, C. Nerl, G. Bernhardt, and M. Lipp.** 1998. Intracellular and surface expression of the HIV-1 coreceptor CXCR4/fusin on various leukocyte subsets: rapid internalization and recycling upon activation. J. Immunol. **160:**1522–1531.
10. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333–348.
11. **Murphy, P. M.** 1994. The molecular biology of leukocyte chemoattractant receptors. Annu. Rev. Immunol. **12:**593–633.
12. **Nagasawa, T., S. Hirota, K. Tachibana, N. Takakura, S. Nishikawa, Y. Kitamura, N. Yoshida, H. Kikutani, and T. Kishimoto.** 1996. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature **382:**635–638.
13. **Oberlin, E., A. Amara, F. Bachelerie, C. Bessia, J. L. Virelizier, F. Arenzana-Seisdedos, O. Schwartz, J. M. Heard, I. Clark-Lewis, D. F. Legler, M. Loetscher, M. Baggiolini, and B. Moser.** 1996. The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. Nature **382:**833–835.
14. **Oravecz, T., M. Pall, and M. A. Norcross.** 1996. Beta-chemokine inhibition of monocytotropic HIV-1 infection. Interference with a postbinding fusion step. J. Immunol. **157:**1329–1332.
15. **Proudfoot, A.** 2002. Chemokine receptors: multifaceted therapeutic targets. Nat. Rev. Immunol. **2:**106–115.
16. **Strader, C. D., T. M. Fong, M. R. Tota, and D. Underwood.** 1994. Structure and function of G protein-coupled receptors. Annu. Rev. Biochem. **63:**101–132.
17. **Tachibana, K., S. Hirota, H. Iizasa, H. Yoshida, K. Kawabata, Y. Kataoka, Y. Kitamura, K. Matsushima, N. Yoshida, S. Nishikawa, T. Kishimoto, and T. Nagasawa.** 1998. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature **393:**591–594.
18. **Tian, S., W. T. Choi, D. Liu, J. Pesavento, Y. Wang, J. An, J. G. Sodroski, and Z. Huang.** 2005. Distinct functional sites for human immunodeficiency virus type 1 and stromal cell-derived factor 1α on CXCR4 transmembrane helical domains. J. Virol. **79:**12667–12673.
19. **Zhou, N., Z. Luo, J. Luo, D. Liu, J. W. Hall, R. J. Pomerantz, and Z. Huang.** 2001. Structural and functional characterization of human CXCR4 as a chemokine receptor and HIV-1 co-receptor by mutagenesis and molecular modeling studies. J. Biol. Chem. **276:**42826–42833.
20. **Zou, Y., A. Kottmann, M. Kuroda, I. Taniuchi, and D. Littman.** 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature **393:**595–599.