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Critical Role in CXCR4 Signaling and Internalization of the Polypeptide Main Chain in the Amino Terminus of SDF-1α Probed by Novel N-Methylated Synthetically and Modularly Modified Chemokine Analogues

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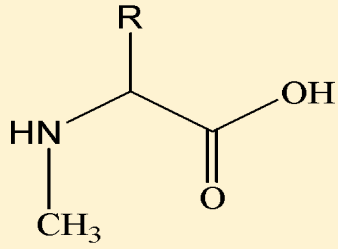
ABSTRACT: The replication of human immunodeficiency virus type 1 (HIV-1) can be profoundly inhibited by the natural ligands of two major HIV-1 coreceptors, CXCR4 and CCR5. Stromal cell-derived factor-1α (SDF-1α) is a natural ligand of CXCR4. We have recently developed a synthetic biology approach of using synthetically and modularly modified (SMM)-chemokines to dissect various aspects of the structure−function relationship of chemokines and their receptors. Here, we used this approach to design novel SMM-SDF-1α analogues containing unnatural N-methylated residues in the amino terminus to investigate whether the polypeptide main chain amide bonds in the N-terminus of SDF-1α play a role in SDF-1α signaling via CXCR4 and/or receptor internalization. The results show that SDF-1α analogues with a modified N-methylated main chain at position 2, 3, or 5 retain significant CXCR4 binding and yet completely lose signaling activities. Furthermore, a representative N-methylated analogue has been shown to be incapable of causing CXCR4 internalization. These results suggest that the ability of SDF-1α to activate CXCR4 signaling and internalization is dependent upon the main chain amide bonds in the N-terminus of SDF-1α. This study demonstrates the feasibility and value of applying a synthetic biology approach to chemically engineer natural proteins and peptide ligands as probes of important biological functions that are not addressed by other biological techniques.



As the natural ligands of chemokine receptors, chemokines activate and recruit a large variety of cell types in inflammation. On the basis of the positions of two conserved cysteine residues in their N-termini, chemokines can be classified into two main subfamilies. They are the CXC and CC chemokines. Because of their active roles in the immune system, chemokines and their receptors are implicated in a wide range of human diseases, most notably in acquired immune deficiency syndrome (AIDS).[1−3] For the development of AIDS, HIV-1 has to enter the target cells via the direct fusion of viral and target cell membranes initiated by CD4, the primary receptor on the target cell, and a coreceptor, usually CXCR4 or CCR5.[4−7] Shortly after infection and during the asymptomatic stage of the disease, M-tropic strains of HIV-1 primarily use CCR5 as an entry coreceptor.[8−10] However, over the course of infection, T-tropic strains that predominantly use CXCR4 eventually replace M-tropic strains.[11,12] Natural chemokines of CXCR4 or CCR5 can inhibit HIV-1 infection[13,14] by blocking HIV-1 envelope glycoprotein gp120 binding sites and/or by inducing receptor internalization.[15,16]

Because of the importance of chemokines and their receptors in numerous physiological and pathological processes, most notably in AIDS, we have been working toward the development of a systematic strategy of synthetic biology based on full-length chemokine structures to synthesize a new family of unnatural chemokines called SMM-chemokines.[17,18] Using synthetic chemistry, unnatural amino acids or novel chemical modifications are introduced into the important functional sequence modules of native chemokines to yield new mechanistic probes of receptor functions and inhibitors of pathological processes. Previously, this SMM-chemokine approach has been applied to convert the nonselective viral macrophage protein-II (vMIP-II)[19] into highly selective ligands for CXCR4 or CCR5 in terms of their binding, signaling, and antiviral activities.[17] Also using a similar approach, we have demonstrated distinct signaling pathways involved in neuronal apoptosis associated with HIV-associated dementia (HAD) activated by different chemokine receptor ligands that are either agonists or antagonists.[20] More recently, to investigate whether receptor internalization caused by SDF-1α, a natural ligand of

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CXCR4, plays a role in its anti-HIV activity, we again applied the SMM-chemokine concept to generate a functional probe of SDF-1α that retains significant CXCR4 binding yet does not induce CXCR4 internalization.[21] The antiviral study of this functional probe analogue versus wild type SDF-1α showed that, despite the significant CXCR4 binding activity, this probe analogue displayed a complete loss of effect in causing CXCR4 internalization and greatly diminished antiviral activity, thus suggesting that receptor internalization plays an important role in the anti-HIV activity of SDF-1α and possibly other natural chemokines.

Prior to the recent publication of high-resolution crystal structures of CXCR4 by Wu et al.,[22] several groups have endeavored to characterize interactions of CXCR4 with HIV-1, natural ligands, and de novo designed inhibitors using molecular modeling, chimeras, and site-specific mutagenesis. These studies demonstrated that the N-terminus and the second (ECL2) and third (ECL3) extracellular loops of CXCR4 are required for HIV-1 coreceptor activity.[23−33] They also indicated the important roles of multiple extracellular and transmembrane (TM) domains of CXCR4 for ligand interactions and receptor signaling.[24,25,29,33−38] In addition, a separation of binding and signaling functions was revealed by these chimeric and mutational studies, and it has been exploited in validating the accuracy of a two-site model that was initially developed for the C5a chemoattractant and its receptor. This model has the chemokine core domain being the "site 1" docking domain and the chemokine N-terminus being the "site 2" signaling trigger.[39] According to this model, the motif composed of amino acids 12−17 of SDF-1α, RFFESH loop, first docks onto the N-terminal domain of CXCR4, and this contact allows the subsequent interaction of the flexible N-terminus of SDF-1α with the receptor groove formed by TM domains and/or extracellular loops, thereby triggering the receptor function.[39−41] The N-terminus of SDF-1α, being relatively flexible and unstructured in solution, has been confirmed as being essential for CXCR4 recognition and signal transduction.[40,42,43] We incorporated this concept and further hypothesized a two-pocket model of CXCR4.[18,33,44] According to our model, the natural CXCR4 chemokine antagonist vMIP-II and its synthetic modified analogues DV1 and RCP168 are thought to recognize a flexible binding pocket in CXCR4, whereas the natural CXCR4 chemokine agonist SDF-1α reaches more deeply into another different and stricter signaling pocket.

EXPERIMENTAL PROCEDURES

Materials. 4-(Hydroxymethyl)phenoxymethylpolystyrene (HMP) resin, Fmoc-Lys(Boc)-NovaSyn TGA resin, *N*-(9-fluorenylmethoxycarbonyl) (Fmoc)-L-amino acids, other reagents, and solvents required for solid phase peptide synthesis (SPPS) were purchased from Novabiochem (San Diego, CA) and Applied Biosystems (Foster City, CA). Other chemicals were obtained from Acros-Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (Milwaukee, WI). The radioiodinated SDF-1α was purchased from PerkinElmer Life Sciences (Boston, MA). Plasmid pcDNA-CXCR4, antibody 12G5, and human kidney cell line 293 were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The Sup T1 cell line was obtained through the ECACC (European Collection of Cell Cultures). Cell culture media and G418 were purchased from CAMBREX (Walkersville, MD). While Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 5% penicillin-streptomycin (P/S) was used to maintain 293 cells, RPMI 1640 with 10% FBS and 5% P/S was used to culture Sup T1 cells.

Total Chemical Synthesis of SDF-1α Analogues. The automated stepwise incorporation of protected amino acids was performed using an Applied Biosystems 433A peptide synthesizer with a CLEAR amide resin (Peptides International, Louisville, KY) as the solid support. Fmoc chemistry was employed for the synthesis. 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydroxybenzotriazole (HOBt) were used as coupling reagents in the presence of diisopropylethylamine (DIEA). 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% (v/v) DMSO was introduced into the solution to enhance the coupling reaction. After N-terminal Fmoc protection had been removed, the protein was cleaved from the resin support by adding a cleavage cocktail comprised of phenol [4% (w/v)], thioanisole [5% (v/v)], water [5% (v/v)], ethanedithiol [2.5% (v/v)], triisopropylsilane [1.5% (v/v)], and trifluoroacetic acid [TFA, 82% (v/v)]. 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 using semipreparative reverse phase high-performance liquid chromatography (RP-HPLC). Folding of the purified protein was performed in 1 M guanidinium hydrochloride and 0.1 M trisma base (pH 8.5, 1 mg of protein/mL of folding buffer) and was monitored by analytical RP-HPLC using a Vydac C-18 column (0.46 cm × 15 cm, 5 μm) with a flow rate of 1 mL/min (solvent A being water with 0.1% TFA and solvent B being 20% water in CH_3CN with 0.1% TFA) and a linear gradient from 30 to 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.

Transfection of Adherent 293 Cells. Wild-type CXCR4 was transfected into 293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The selective medium containing G418 (800 μg/mL) was used to isolate stably transfected cells that were subsequently singly cloned.

Flow Cytometry. Transfected 293 cells (5×10^5 cells/well) were washed with FACS buffer (0.5% bovine serum albumin and 0.05% sodium azide in PBS) and incubated with monoclonal antibody (mAb) 12G5 (10 μg/mL) for 30 min at 4 °C. After being washed with FACS buffer, the cells were incubated with 10 μg of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) for 30 min at 4 °C. The cells were washed with FACS buffer and fixed in the fixing buffer (2% paraformaldehyde in PBS) for 30 min at 4 °C before being analyzed on a FACScan flow cytometer.

Competition Receptor Binding Assays Using Labeled Chemokines. Ligand binding experiments were performed using a single concentration (0.2 nM) of [^{125}I]SDF-1α in a final volume of 100 μL of binding buffer [50 mM Hepes (pH 7.4), 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.1% bovine serum albumin] containing 5×10^5 cells in 96-well plates in the presence of various concentrations of unlabeled chemokines. Nonspecific

binding was assessed by adding 150 nM unlabeled SDF-1α. Samples were incubated for 60 min at room temperature. The cells were washed with 200 μL of binding buffer. Bound ligands were determined by counting gamma emissions. The binding data were analyzed using Prism (GraphPad Inc., San Diego, CA). At least three independent experiments were performed.

Intracellular Calcium Measurement. Sup T1 cells (10^7 cells/mL) were loaded with 2 μM fura-2/AM (Molecular Probes, Eugene, OR) and 0.01% Pluronic F-127 (Sigma) in Hank's balanced salt saline [140 mM NaCl, 5 mM KCl, 10 mM Hepes (pH 7.4), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/mL glucose, and 0.025% BSA] for 20 min at room temperature. The cells were washed and resuspended in the same buffer to a density of 10^6 cells/mL. Fura-2 fluorescence was measured on the fluorescence spectrophotometer (ISA SPEX FluoroMax-2) using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. At least three independent experiments were performed.

Internalization Assays. Transfected 293 cells (3×10^5 cells/well) were plated onto 24-well tissue culture plates pretreated with 0.01% poly-L-lysine (Sigma). Nontransfected 293 cells were used as the background. Fixing the cells for 5 min with 4% paraformaldehyde and PBS stopped the reactions. After the nonspecific binding had been blocked with 1% BSA and PBS and the cells incubated for 45 min, mAb HA.11 (Covance Inc., Princeton, NJ) was added. The cells were washed with PBS and reblocked with 1% BSA and PBS for 15 min. The cells were incubated with goat anti-mouse conjugated alkaline phosphate (Bio-Rad, Richmond, CA) for 1 h. The cells were washed with PBS before colorimetric alkaline phosphate substrate BCIP-NBT (Bio-Rad) was added. The plate was continuously shaken until an adequate color change occurred (~1 h). The absorbance readings were taken using the Wallac Victor^2 1420 Multilabel counter.

Molecular Dynamics (MD) Simulation. The crystal structure of CXCR4 in complex with the cyclic peptide antagonist CVX15 [Protein Data Bank (PDB) entry 3OE0] and the solution NMR structure of SDF-1α (PDB entry 2SDF) were utilized to construct the models of SDF-1α analogues via Sybyl x1.3 (Tripos, Inc.), which were further refined before MD simulations. In the CXCR4 crystal structure, both bound ligand and lipid molecules were deleted. In the SDF-1α NMR structure, only residues 1−16 were kept, whereas the other residues were deleted. MD simulations were first performed using Sybyl x1.3 and the Tripos force field for 2 ns after SDF-1α or its analogues had been manually docked into CXCR4. The MD simulations were gradually increased to 300 K over 50 ps. The system was then equilibrated at 300 K for an additional 50 ps. Finally, the MD simulations were performed while the temperature was kept at 300 K. During the MD simulations, only the residues in the extracellular loops of CXCR4 and all the residues of ligands were allowed to move, whereas the remaining residues were frozen at their respective positions in their crystal structures.

RESULTS AND DISCUSSION

The inclusion of unnatural amino acids with well-defined conformational preferences into the peptide backbone is an active area of research for understanding the peptide-based molecular architecture and the structure−activity relationship.[45−48] These changes can have significant impacts on many biological and chemical properties, including receptor binding, signaling, and internalization. In this study, we sought

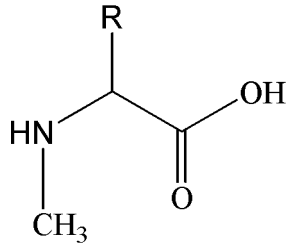

Figure 1. Chemical structure of an N-methylated amino acid.

to investigate whether the polypeptide main chain amide bonds in the N-terminus of SDF-1α and the hydrogen bonds that they may form with CXCR4 play a role in the ligand binding and signaling activities, and receptor internalization. Using the SMM-chemokine approach, we designed specifically modified analogues of SDF-1α with a single modification in a particular amide bond by introducing an unnatural N-methylated amino acid at position 2, 3, or 5 in the N-terminus of SDF-1α (Figure 1). Three N-terminal residues of SDF-1α were replaced with N-methylated amino acids, resulting in three unnatural N-methylated SMM-SDF-1α analogues: Pro-2 with *N*-methylalanine to give the analogue SDF-1α-NMeA, Val-3 with *N*-methylvaline to give SDF-1α-NMeV, and Leu-5 with *N*-methylleucine to give SDF-1α-5NMeL. Molecular modeling and dynamic simulation studies of SDF-1α with CXCR4 suggested that Val-3 and Leu-5 are both involved in the H-bond interactions with the receptor (Figure 5A). Therefore, N-methylation of the amide bond at either of these two residues should abolish such hypothesized H-bond interactions and allow us to examine the validity of the prediction by molecular modeling studies and the role of these main chain amide bonds in ligand functions. With respect to the modification at position 2, even though Pro-2 cannot participate in the H-bond interactions with the receptor, SDF-1α-NMeA, which replaces Pro-2, was synthesized to investigate whether a more flexible and less bulky structure at this position may have an impact on receptor binding and signaling, as SDF-1α-NMeA opens the prolyl ring of Pro-2 and is more flexible locally. The methyl group is also less bulky and hydrophobic than the prolyl ring.

As discussed above, the incorporation of N-methylated amino acids at the N-terminus of SDF-1α specifically and locally disrupts the H-bond interactions of the modified main chain amide bonds without affecting the other H-bonds of the ligand with the receptor. This should influence the binding and/or signaling activities of the modified analogues toward CXCR4 if the modified amide bond group plays a role in the ligand functions. Interestingly, according to the binding data (Table 1 and Figure 2), SDF-1α-NMeA (IC_{50} = 30 nM) and SDF-1α-NMeV (IC_{50} = 42 nM) retained significant CXCR4 binding, whereas SDF-1α-NMeL retained some CXCR4 binding (IC_{50} = 84 nM). These results suggested that the CXCR4 binding pocket can accommodate a one-carbon unit (methyl group). On the other hand, the amide bond group at position 5 may be important for the interaction of SDF-1α with CXCR4, as the introduction of a methylated group at this position decreases the binding affinity of SDF-1α-NMeL by the greatest degree among the three N-methylated analogues.

To test our hypothesis that the incorporation of N-methylated amino acids at the N-terminus of SDF-1α may influence the signaling activities of the modified SDF-1α analogues, intracellular Ca^{2+} mobilization assays were performed. In contrast to SDF-1α that can activate the intracellular

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Table 1. List of N-Methylated SDF-1α Analogues, Sequences, and IC_{50} Values

SDF-1α analogue	sequence	IC_{50} (nM)
SDF-1	KPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLKWIQEYLEKALNK	4
SDF-1α-NMeA	K-NMe-A-VSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLKWIQEYLEKALNK	30
SDF-1α-NMeV	KP-NMe-V-SLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLKWIQEYLEKALNK	42
SDF-1α-NMeL	KPVS-NMe-L-SYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLKWIQEYLEKALNK	84

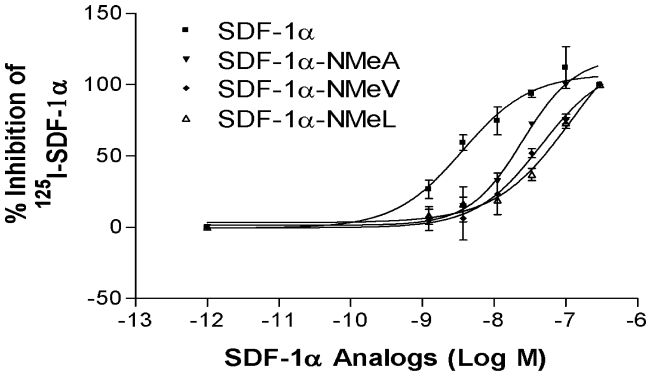

Figure 2. CXCR4 binding activities of SDF-1α (■), SDF-1α-NMeA (▼), SDF-1α-NMeV (◆), and SDF-1α-NMeL (△) as characterized by [^{125}I]SDF-1α competition binding assays. The binding data were analyzed using Prism (GraphPad Inc.). All data are shown as means ± the standard deviation from at least three independent experiments.

Ca^{2+} influx in Sup T1 cells expressing CXCR4, none of the N-methylated SDF-1α analogues at 50 or 500 nM was able to induce any mobilization of Ca^{2+} in Sup T1 cells (Figure 3). Whether modified analogues may interfere with the normal Ca^{2+} signaling activated by SDF-1α was also examined by adding 50 nM SDF-1α after the 5 min treatment with the SDF-1α analogues. Consistent with the binding data, SDF-1α-NMeA inhibited the Ca^{2+} mobilization induced by SDF-1α most effectively. Taken together, the results suggested that SDF-1α signaling is sensitive to and restrictive of its main chain amide bonds in the N-terminus of SDF-1α, as changes in these amide bonds may directly affect the H-bond interactions with CXCR4 that are important for signaling.

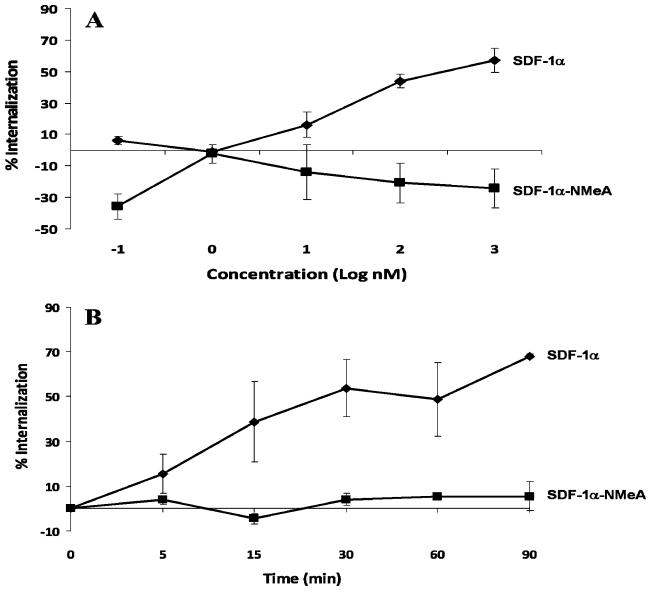

Figure 4. Receptor internalization activities of SDF-1α and SDF-1α-NMeA. (A) An increase in the concentration of SDF-1α-NMeA from 0.1 nM to 1 μM failed to cause CXCR4 downregulation, while 1 μM SDF-1α induced 50% receptor loss. (B) The longer incubation of cells with SDF-1α-NMeA did not elicit any significant internalization of CXCR4. All data are shown as means ± the standard deviation from at least three independent experiments.

Another important biological activity of SDF-1α is its ability to cause CXCR4 internalization. Unlike SDF-1α, which induced 50% receptor loss when its concentration was increased up to 1 μM, SDF-1α-NMeA, chosen as the representative molecule for this study, with concentrations

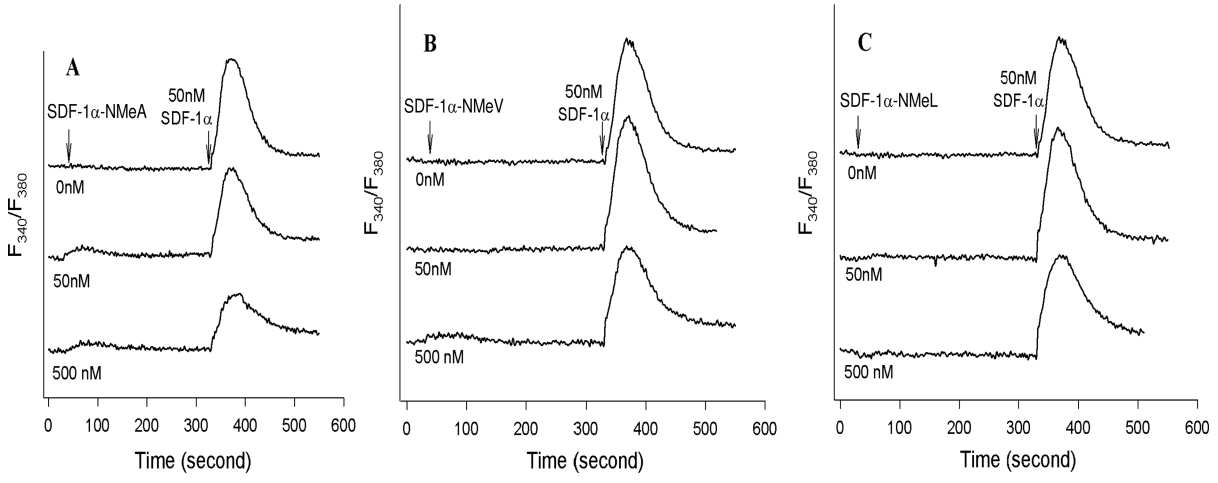

Figure 3. Signaling activities of SDF-1α-NMeA, SDF-1α-NMeV, and SDF-1α-NMeL. The intracellular Ca^{2+} influx in Sup T1 cells was measured in response to 50 or 500 nM SDF-1α-NMeA (A), SDF-1α-NMeV (B), or SDF-1α-NMeL (C). For inhibition assays, Sup T1 cells were preincubated with 50 or 500 nM SDF-1α-NMeA, SDF-1α-NMeV, or SDF-1α-NMeL for 5 min before being stimulated with 50 nM SDF-1α. At least three independent experiments were performed.

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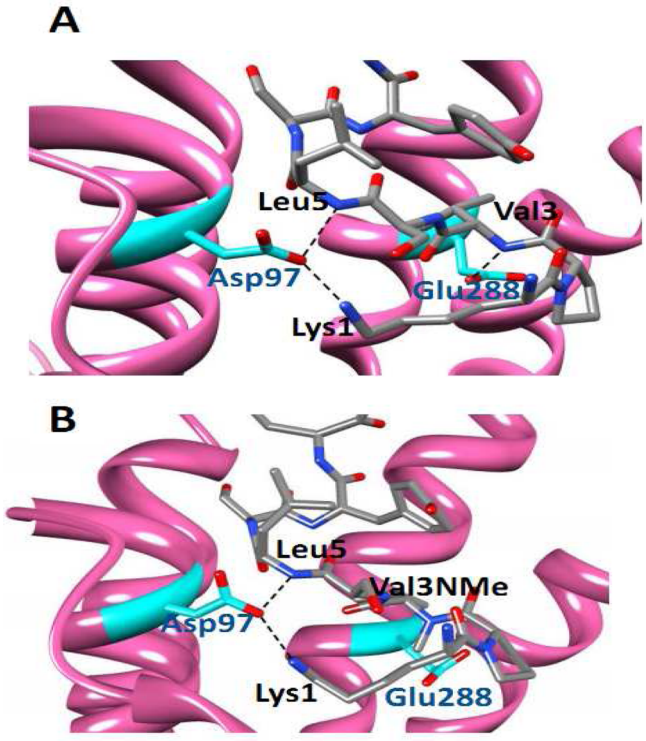

Figure 5. Binding conformations and interactions of SDF-1α (A) and SDF-1α-NMeV (B) with CXCR4 predicted by molecular dynamics simulations. These structural models were generated from the molecular dynamics calculations as described in Experimental Procedures. For the natural chemokine SDF-1α (A), the amide groups of Val-3 and Leu-5 showed H-bond interactions with Glu-288 and Asp-97 of CXCR4, respectively, which are known to be important residues for CXCR4 signaling function.[22] The methylation of SDF-1α backbone amide group of Val-3 can disrupt the H-bond interaction with Glu-288 of CXCR4 (B). Similarly, the methylation of the SDF-1α backbone amide group of Leu-5 can disrupt the H-bond interaction with Asp-97 of CXCR4 (structure not shown). CXCR4 is represented as a pink ribbon. The two critical residues, Asp-97 and Glu-288, that are involved in H-bonds with the ligand as described above are colored cyan. Both SDF-1α and SDF-1α-NMeV are represented as gray sticks. H-Bonds are represented as black dashed lines.

ranging from 0.1 nM to 1 μM failed to cause CXCR4 downregulation (Figure 4A). The impact of time was also examined. As shown in Figure 4B, SDF-1α-NMeA did not elicit any significant internalization of CXCR4 even after incubation for 90 min. In contrast, SDF-1α induced rapid receptor downregulation, as 70% of the receptors were internalized after incubation for 30 min. This finding may be of interest for the future design of therapeutics based on SDF-1α. For example, changes in the N-terminal main chain of SDF-1α may compromise CXCR4 signaling and internalization, which may lead to decreased anti-HIV activity, because CXCR4 internalization caused by a potential therapeutic is thought to be involved in its anti-HIV activity.

Molecular modeling was performed to unravel the binding conformations and interactions between CXCR4 and SDF-1α analogues (Figure 5). Only the first 16 amino acids in the N-terminus of SDF-1α were used for MD simulations, as these residues are critical for CXCR4 binding and signaling.[40] For the natural chemokine SDF-1α, the amide group of Val-3 showed a H-bond interaction with Glu-288 of CXCR4, which is known to be an important residue for CXCR4 signaling function.[22] However, after the methylation of the backbone amino group of Val-3, this H-bond interaction with CXCR4 was abolished, which may explain the loss of CXCR4 signaling and activation. On the other hand, the CXCR4 binding pocket is deep and wide enough to accommodate a methyl group on the SDF-1α backbone, which is consistent with the significant binding affinity retained. SDF-1α-NMeA and SDF-1α-NMeL similarly disrupted H-bond interactions with CXCR4, which seems to be the cause for the loss of CXCR4 activation (data not shown). In summary, using the SMM-chemokine approach, we generated several SDF-1α analogues with a modified main chain amide bond, yet maintained the significant binding activity, to demonstrate the importance of the polypeptide main chain amide bonds in the signaling activity of SDF-1α via CXCR4 and receptor internalization. These results support a two-pocket model of CXCR4–ligand interactions recently proposed by us, in which antagonists recognize a flexible binding pocket in CXCR4 while agonists reach more deeply into a different and more restrictive signaling pocket.[40] Furthermore, this study demonstrates a synthetic biology approach to chemically engineering natural proteins and peptide ligands as probes of important biological functions that cannot be studied by conventional biological techniques.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

SMM, synthetically and modularly modified; AIDS, acquired immune deficiency syndrome; HIV, human immunodeficiency virus; SDF-1α, stromal cell-derived factor-1α.

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