N-terminal Peptides of Stromal Cell-derived Factor-1 with CXC Chemokine Receptor 4 Agonist and Antagonist Activities*

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Peptides corresponding to the N-terminal 9 residues of stromal cell-derived factor-1 (SDF-1) have SDF-1 activity. SDF-1, 1–8, 1–9, 1–9 dimer, and 1–17 induced intracellular calcium and chemotaxis in T lymphocytes and CEM cells and bound to CXC chemokine receptor 4 (CXCR4). The peptides had similar activities to SDF-1 but were less potent. Whereas native SDF-1 had half-maximal chemotactic activity at 5 nM, the 1–9 dimer required 500 nM and was therefore 100-fold less potent. The 1–17 and a 1–9 monomer analog were 4- and 36-fold, respectively, less potent than the 1–9 dimer. Both the chemotactic and calcium response of the 1–9 dimer was inhibited by an antibody to CXCR4. The basis for the enhanced activity of the dimer form of SDF-1, 1–9 is uncertain, but it could involve an additional fortuitous binding site on the 1–9 peptide in addition to the normal SDF-1, 1–9 site. A 1–9 analog, 1–9[Abu 9], dimer, was found to be a CXCR4 antagonist. Overall this study shows that the N-terminal peptides are CXCR4 agonists or antagonists, and these could be leads for high affinity ligands.

Stromal cell-derived factor-1 (SDF-1) is a member of the chemokine family of structurally related proteins with cell chemotactic activity (1). Although many chemokines have pro-inflammatory roles, SDF-1 appears to have a fundamental role in the trafficking, export and homing of bone marrow cells (2, 3). It is produced constitutively, and particularly high levels are found in bone marrow stromal cells (4, 5). A basic physiological role is implied by the high level of conservation of the SDF-1 sequence between species (4). In vitro SDF-1 stimulates chemotaxis of a wide range of cells including monocytes and bone marrow-derived progenitor cells (2, 5). Particularly notable is its ability to stimulate a high percentage of resting and activated T lymphocytes (5, 6). It is the only known ligand for CXCR4, a 7-transmembrane receptor that has been variously described as LESTR (7), HUMSTR (8), and fusin (9). CXCR4 is widely expressed on cells of hemopoietic origin and is a major coreceptor for HIV-1 (9). Consistent with this dual role of CXCR4, SDF-1 blocks HIV-1 entry into CD4+ cells (10, 11).

The SDF-1 sequence indicates that it belongs to the CXC family of chemokines, but it has only about 22% identity with other chemokines (5). Despite the divergent primary structure, the recently described three-dimensional structure indicates that it has a similar fold to other chemokines (12). Furthermore, structure-activity analysis of SDF-1 (12) indicated the importance of N-terminal residues 1–8 for binding and of residues 1 and 2 for receptor activation. Residues 12–17 located in the loop region also contribute to binding. In the SDF-1 structure, the region N-terminal to the CXC motif is highly disordered, but the loop region immediately following the CXC motif is well defined at least in its backbone atoms. These two regions have been identified as being important in other CC and CXC chemokines (12–15). As with other chemokines, N-terminal modification of SDF-1 led to dissociation of binding and activity (12). Thus despite the difference in primary structure, from both a structural and a functional perspective, the general mechanism of receptor binding is similar for SDF-1 and other chemokines.

The key role of the N-terminal region of the SDF-1 protein in receptor binding and activation suggests that the N-terminal region alone could be sufficient for binding or activity. Here we show that peptides corresponding to the N-terminal region bind CXCR4 and have SDF-1 activity. A dimer of SDF-1, 1–9 was the most potent of the peptides tested.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—The peptides were prepared as described previously (13). The peptides were purified by HPLC and analyzed by mass spectrometry. Tertiary butyloxycarbonyl-α-aminoacyluride acid was used to prepare the analog SDF-1, 1–9(Aba 9), which had CH2-CH3 in place of CH2-SH. The 1–9 and 1–17 peptides were dimerized via a disulfide bridge formed by gentle oxidation of the cysteines using 10% MeSO in water. Following HPLC purification dimer formation was verified by mass spectrometry.

**Cell Preparation and Culture**—Human peripheral blood mononuclear cells were isolated from donor blood buffy coats by centrifugation on Ficoll-Paque. The cells were treated with phytohemagglutinin (1.0 μg/ml) and expanded in the presence of interleukin-2 (100 units/ml) for 7–17 days as described (16). These cells are referred to as “T lymphocytes.” CEM cells, a human lymphoblastoid CD4+ T cell line (ATCC, Manassas, VA), was cultured in RPMI medium containing 15 μg/ml of 8-azaguanine (Aldrich) and 10% fetal calf serum.

**Chemotaxis**—Migration of T lymphocytes was assessed in 48-well chambers (NeuroProbe, Cabin John, MD) using collagen-coated polystyrene-free polycarbonate membranes with 3-μm pores (16). Migrated cells were counted in five randomly selected fields at 1000× magnification after migration of 1 h. Disposable Transwell trays (Costar, Cambridge, MA) with 6.5-mm diameter chambers and membrane pore size of 3 μm were used to assay chemotaxis of CEM cells.

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Chemotactic activity assays were performed in duplicate. Chemotaxis was determined by subtraction of cells migrated in medium alone. All stimulations were performed at 60-s intervals. Where indicated the cells were preincubated with the agonist. Fluorescence changes were recorded from 0–60 s (18). Receptor desensitization was tested by monitoring changes during sequential additions at 60-s intervals. Where indicated the cells were preincubated with the 12G5 antibody prior to chemokine treatment.

RESULTS

SDF-1 N-terminal Peptides Are Chemotactic—The N-terminal region of SDF-1 is directly involved in receptor recognition and activation (12), and therefore we tested peptides corresponding to the N-terminal region for activity. The sequences of the peptides are shown in Fig. 1. Both the 1–8 and 1–9 peptides induced dose-dependent chemotaxis of CEM cells (Fig. 2a). The concentrations required for 50% of the maximal response (EC_{50}) are summarized in Table I. The 1–9 peptide was about 1,000-fold less potent than native SDF-1. However, the 1–9 was 7-fold more potent than the 1–8 peptide. The peptides were also tested on T lymphocytes (Fig. 2b), and the results were similar to those obtained with CEM cells, except that the T lymphocytes were less responsive to SDF-1 or the peptides. The results clearly show that the 1–9 and 1–8 peptides have SDF-1-like activity but relatively low potency. The chemoattractant activity of 1–9 was fully inhibited by the SDF-1 antagonist, SDF-1, 1–67[P2G] (12), but not by an interleukin-8 antagonist that blocks CXCR1 (19) (Fig. 3a). These findings suggest that despite its lower potency the 1–9 peptide is similar in its mechanism of CXCR4 activation to native SDF-1.

To explore the possibility that the low potency of the N-terminal peptide is because of its lack of an independent second binding site, we tested whether activity could be enhanced by co-addition of a folded fragment corresponding to SDF-1, 9–67, which lacks residues 1–8. Thus the entire SDF-1 structure was available to the receptor but as two separate molecules. SDF-1, 9–67 alone did not bind CXCR4 at the concentrations used (12). Chemotaxis, mobilization of cytosolic free calcium, and receptor binding of 1–8 or 1–9 were not affected by the addition of SDF-1, 9–67 (not shown). Thus no synergy could be demonstrated. This does not rule out the possibility that a second site could be involved in full-length SDF-1.

Activity of SDF-1(1–9) Dimer—We tested two possible mechanisms that could account for the difference in activity between the 1–9 and 1–8 peptides. The first was whether 1–9 forms a superactive dimer. This hypothesis was advanced when we detected the 1–9 dimer in solutions of 1–9. The second was whether the 1–9 and 1–8 peptides form a super active dimer. This hypothesis was supported by the observation that dimer detection was observed in solutions of 1–9. The 1–9 dimer was detected at concentrations of 1–9 that were at least 30-fold less potent than native SDF-1.

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The agonist, in Hepes-buffered RPMI 1640 supplemented with 10 mg/ml bovine serum albumin (0.6 ml), was added to the lower well, and 0.1 ml of CEM cells (1 x 10^5/ml) in the same medium without agonist was added to the upper wells. The concentrations required for 50% of the maximal response (EC_{50}) were calculated relative to native SDF-1.

![Fig. 1. Sequences of native SDF-1 and the N-terminal SDF-1 peptides.](image)

![Fig. 2. Chemotactic activity of SDF-1 peptides.](image)

![Table I. Summary of the relative potencies of SDF-1 peptides](table)

| Peptides | Binding K_d (nM) | Fold increase | Chemotactic activity EC_{50} (nM) | Fold increase |
|----------|------------------|---------------|----------------------------------|---------------|
| SDF-1    | 9 ± 3            | 1             | 17,800                           | 1             |
| SDF-1, 1–8 | 9,300 ± 7,500 | 1,500         | 7,500                            | 1,500         |
| SDF-1, 1–9 | 13,900 ± 3,600 | 1,500         | 1,500                            | 1,500         |
| SDF-1[Ab] | 1,500 ± 500     | 1             | 1,500                            | 1             |
| SDF-1, 1–9 dimer | 730 ± 90 | 82          | 1,000                            | 1,000         |
| SDF-1, 1–9[P2G] dimer | 2,580 | 290         | 100                              | 100           |
| SDF-1, 1–17 | 850 ± 26 | 94          | 400                              | 400           |

* K_d values were calculated from CEM cell binding curves derived in two to six experiments with results similar to those in Fig. 4 using Scatchard methods.

* The fold increase in K_d was calculated relative to native SDF-1.

* The chemotaxis EC_{50} was calculated from the CEM cell data in Fig. 2a. Results are presented as the means ± S.D. from two experiments.

* Fold increase in chemotaxis EC_{50} was calculated relative to native SDF-1.

* K_d not determined (see “Results”).

* EC_{50} not detectable.
number of CC chemokine receptors, including receptors for MCP-1 and RANTES. Like native SDF-1, the peptides did not compete for the binding of either MCP-1 or RANTES (not shown).

An SDF-1, 1–9 Analog Is an Antagonist—A low molecular weight antagonist for SDF-1 could provide a lead for therapeutics. We had previously shown that a full-length analog, SDF-1, 1–67[P2G], is a potent receptor antagonist (12). Therefore we tested the corresponding 1–9 analog, SDF-1, 1–9[P2G], and found that its dimer lacked detectable activity (Fig. 2a), but it competed for SDF-1 binding with similar affinity to 1–9 dimer (Fig. 4). The 1–9[P2G] dimer inhibited SDF-1 activity in a dose-dependent manner (Fig. 3b). 50 μM of 1–9[P2G] dimer was required to inhibit the activity of 10 nM of SDF-1 by 50%, a ratio of 5,000. Therefore, as with full-length SDF-1 (12), this modification converted the 1–9 into an antagonist. Thus we have identified a peptide antagonist of CXCR4.

SDF-1 Peptides Are Specific for CXCR4—Native SDF-1 and the N-terminal peptides induced a rapid and transient rise in cytoplasmic calcium concentration, [Ca$^{2+}$]i, in T lymphocytes (Fig. 5a) as well as CEM cells (Fig. 6). The rate and magnitude increased with the concentration. Whereas a response to SDF-1 was observed at 1 × 10–9 M, the peptides induced [Ca$^{2+}$]i changes in the micromolar range. Receptor usage by the SDF-1 peptides was assessed by monitoring [Ca$^{2+}$]i changes after sequential stimulation. As shown in Fig. 5a, treatment of T lymphocytes with SDF-1 completely abolished the responsiveness to the 1–9 peptide, and conversely, the 1–9 peptide also markedly attenuated the response to native SDF-1. The 1–9 dimer (50 μM) completely desensitized the response to subsequent native SDF-1 (not shown). No effect on the response to the 1–9 peptide was observed when T lymphocytes were pre-stimulated with MCP-1, RANTES, macrophage inflammatory protein-1β, γ-interferon inducible protein-10, or monokine induced by interferon-γ (Fig. 5b). The selectivity of these chemokines (1) implies that SDF-1 peptides desensitize CXCR4 but not other chemokine receptors including CXCR3 and CC chemokine receptors 1, 2, and 5. No response to eotaxin, I-309, or thymus- and activation-regulated chemokine (Fig. 5b) was obtained with these cells under the conditions used, and as expected, they did not desensitize 1–9.

Specificity of the peptides was further examined using a CXCR4 blocking monoclonal antibody (17). The chemotaxis and calcium induction by SDF-1 and SDF-1, 1–9 dimer were blocked by the antibody (Fig. 6). The response of a control
chemokine, secondary lymphoid tissue chemokine, which does not bind CXCR4, was not affected by the antibody (Fig. 6b).

Taken together these data show that the 1–9 peptide binds and activates CXCR4 and demonstrate that it is specific for CXCR4.

DISCUSSION

We have shown that N-terminal SDF-1 peptides bind and activate CXCR4. Peptides corresponding to 1–8, 1–9, 1–9 dimer, and 1–17 bind and activate CXCR4 but have low potency compared with SDF-1. Apart from their potency, the agonist peptides were indistinguishable from SDF-1 in all the activities measured. The same substitution, P2G, converted SDF-1 and the 1–9 dimer to a specific SDF-1 antagonist. The binding and activities of the N-terminal peptides were blocked by an antibody directed to CXCR4, confirming that the peptides bind CXCR4.

Although residues in the N-terminal region of chemokines are critical for receptor activation, N-terminal peptides that have been tested do not bind and stimulate chemokine function. This study demonstrates that SDF-1 is an exception. There are several possible reasons for the difference between SDF-1 and other chemokines. A substantial body of work had lead to a model in which there are two chemokine receptor-binding sites, and the initial interaction occurs with a site in the loop region that follows the CXC or CC motif (12–15, 19–21). This is proposed to facilitate the subsequent binding of the N-terminal region to a buried site in the receptor (12, 15). Whereas interaction of a structured loop region with the receptor could be required for the activity of most chemokine N-terminal peptides (15), our data indicate that this is not essential for the SDF-1 peptides. Alternatively, the lack of binding of the N-terminal peptides of other chemokines is because of the failure to adopt a receptor bound conformation. The 1H NMR structure of SDF-1 shows that the N-terminal region is entirely solvent-accessible and has no detectable secondary structure and therefore is expected to be highly flexible. However, it is reasonable to propose that the N-terminal region adopts a well defined conformation when SDF-1 binds to CXCR4. Similarly for the N-terminal peptide, the receptor bound conformation could be represented in solution. Nevertheless a prerequisite for binding of the N-terminal peptide to CXCR4 is that the receptor-binding site must be accessible. For other chemokines there is no evidence that this site is accessible. One possibility is that for binding of the N-terminal domain, other chemokine-receptor interactions are first required. This exposes the receptor site and allows the N-terminal domain to bind. In this model N-terminal peptides would not bind. In CXCR4 the loops of the receptor could be arranged such that this binding site is accessible to the peptide, whereas for most chemokines even if the N-terminal peptide existed in the bound conformation the pathway to the receptor could be sterically blocked without the remainder of the chemokine. Thus the difference between SDF-1 and other chemokines might not be in the peptide ligand but in the receptor.

The affinity and potency of the N-terminal SDF-1 peptides
are lower than those of the native protein. Thus the peptides can bind CXCR4, but not as efficiently as native SDF-1. Similar arguments to those above could account for the difference in potency. The low affinity could be because of the lack of the loop-binding site on the N-terminal peptide or because of multiple conformations of the peptide and/or a requirement for binding cooperativity in SDF-1.

The disulfide-linked dimer of SDF-1, 1–9 peptide was considerably more potent than the 1–9 peptide monomer. Although 1–9 was isolated as the monomer it had higher activity than an analog that could not dimerize, suggesting that the 1–9 spontaneously starts to form dimer in solution. In native SDF-1, Cys-9 normally participates in a disulfide bridge. Thus, the disulfide bridge itself could directly enhance binding to the receptor. A second alternative is that dimerization could change the conformation of the 1–9 resulting in an enhanced binding. The fact that native SDF-1 binds as a monomer and not as a dimer (12) indicates that only one 1–9 can bind to the receptor site. A third possibility is that one half of the dimer binds the activation site, but the other half could bind to other receptor sites, perhaps because of fortuitous complementarity. This last mechanism is consistent with the finding that the 1–17 has similar binding to the 1–9 dimer. The 1–17 contains the RFFESH motif, which is a receptor-binding site on SDF-1. Further experiments will be required to determine the detailed mechanisms involved.

Stable low molecular weight nonpeptide ligands are preferred for therapeutic applications. SDF-1 is the ligand for a co-receptor for HIV and is involved in hematopoietic cell homing. Several nonchemokine molecules have been found to inhibit HIV, and it was shown that they target CXCR4 (22–24). However, none of these have SDF-1 activity. Antagonists of chemokine receptors, perhaps because of fortuitous complementarity. This last mechanism is consistent with the finding that the 1–17 has similar binding to the 1–9 dimer. The 1–17 contains the RFFESH motif, which is a receptor-binding site on SDF-1. Further experiments will be required to determine the detailed mechanisms involved.

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REFERENCES
1. Baggiolini, M., Dewald, B., and Moser, B. (1997) Annu. Rev. Immunol. 15, 675–705.
2. Aiuti, A., Webb, J. J., Bleul, C., Springer, T., and Guierrez-Ramos, J. C. (1996) J. Exp. Med. 185, 111–120.
3. Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S.-I., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996) Nature 382, 635–638.
4. Shirouzu, M., Nakano, T., Inazawa, J., Tashiro, K., Tada, H., Shinozara, T., and Honjo, T. (1996) Genomics 28, 485–500.
5. Bleul, C. C., Fuhlerbrigg, K. C., Casasnovas, J. M., Aiuti, A., and Springer, T. A. (1999) J. Exp. Med. 184, 1101–1109.
6. Campbell, J. J., Hendrick, J., Zlotnik, A., Siani, M. A., Thompson, D. A., and Butcher, E. C. (1998) Science 279, 381–383.
7. Lochtcher, M., Geiser, T., O’Reilly, T., Zwahlen, R., Baggiolini, M., and Moser, B. (1994) J. Biol. Chem. 269, 232–237.
8. Federrappli, B., Duncan, A. M. V., Delaney, A., Schappert, K., Clark-Lewis, I., and Jirik, F. R. (1993) Genomics 16, 707–712.
9. Feng, Y., Broeder, C. C., Kennedy, P. E., and Berger, E. A. (1996) Science 272, 872–877.
10. Oberlin, E., Amara, A., Bacheleir, F., Bennin, C., Virelizier, J.-L., Azzanesi-Seisdedos, F., Schwartz, O., Heard, J.-M., Clark-Lewis, I., Legler, D. F., Loetscher, M., Baggiolini, M., and Moser, B. (1996) Nature 382, 833–835.
11. Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodrkozi, J., and Springer, T. A. (1996) Nature 382, 829–833.
12. Crump, M., Gong J.-H., Loetscher, P., Rajarathnam, K., Amara, A., Azzanesi-Seisdedos, F., Virelizier, J.-L., Baggiolini, M., Sykes, B. D., and Clark-Lewis, I. (1997) EMBO J. 16, 6996–7007.
13. Clark-Lewis, I., Dewald, B., Loetscher, M., Moser, B., and Baggiolini, M. (1994) J. Biol. Chem. 269, 16075–16081.
14. Gong, J.-H., Ugucioni, M., Dewald, B., Baggiolini, M., and Clark-Lewis, I. (1996) J. Biol. Chem. 271, 10521–10527.
15. Clark-Lewis, I., Kim, K.-S., Rajarathnam, K., Gong, J.-H., Dewald, B., Moser, B., Baggiolini, M., and Sykes, B. D. (1995) J. Leukocyte Biol. 57, 703–711.
16. Loetscher, P., Seitz, M., Clark-Lewis, I., Baggiolini, M., and Moser, B. (1994) FASEB J. 8, 1055–1060.
17. Endres, M. J., Calpham, P. R., Marsh, M., Auhua, M., Davis-Turner, J. C., Knight, A., Thomas, J. P., Stoebenau-Haggart, B., Choe, S., Banze, P. H., Wells, T. N. C., Power, C. A., Sutterwala, S. S., Duns, R. W., Landay, N. R., and Hoxie, J. A. (1996) Cell 87, 745–756.
18. von Tscharnier, V., Prodhom, B., Baggiolini, M., and Reuter, H. (1986) Nature 324, 369–372.
19. Jones, S. A., Dewald, B., Clark-Lewis, I., and Baggiolini, M. (1997) J. Biol. Chem. 272, 16166–16169.
20. Monteciaro, F. S., and Charo, I. F. (1996) J. Biol. Chem. 271, 19084–19092.
21. Lowman, H. B., Slagle, P. H., Deforge, L. E., Wirth, C. M., Giller-Castro, B. L., Bourell, J. H., and Fairbrother, W. J. (1996) J. Biol. Chem. 271, 14344–14352.
22. Doranz, B. J., Grevit-Ferbas, K., Sharron, M. P., Mao, S.-H., Goetz, M. B., Daar, E. S., Doms, R. W., and O’Brien, W. A. (1997) J. Exp. Med. 186, 1395–1400.
23. Murakami, T., Nakajima, T., Koyanagi, Y., Tachibana, K., Fujii, N., Yamamura, H., Yoshida, N., Waki, M., Matsuura, A., Yoshie, O., Kishimoto, T., Yamamoto, N., and Nagasawa, T. (1997) J. Exp. Med. 186, 1389–1393.
24. Schols, D., Struyf, S., Van Damme, J., Este, J. A., Henson, G., and Clercq, E. D. (1997) J. Exp. Med. 186, 1383–1388.