C-Terminal Cyclization of an SDF-1 Small Peptide Analogue Dramatically Increases Receptor Affinity and Activation of the CXCR4 Receptor

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In an effort to improve the activities and bioavailabilities of stromal cell-derived factor-1 (SDF-1, CXCL12) sdf-(1−67)-OH (**1**), we have prepared a linear peptide analogue [sdf-(1−31)-NH_2 (**2**)] and two lactam analogues [*cyclo*(Lys^{20}-Glu^{24})-sdf-(1−31)-NH_2 (**3**) and *cyclo*(Glu^{24}-Lys^{28})-sdf-(1−31)-NH_2 (**4**)], consisting of the N-terminal region (amino acids 1−14) joined by a four-glycine linker to the C-terminal region (amino acids 56−67) of **1**. Analogues **2** and **4** had eight residues of α-helix, as estimated from its circular dichroism (CD) spectra, in contrast to 10 residues in analogue **3**. Cyclization of analogue **2** at residues 20 and 24 to give analogue **3** resulted in only a slight change to the $\theta_{222}/\theta_{209}$ ratio (0.81 to 0.86, where 1.09 is considered a perfect α-helix), although an increase in the α-helix length of analogue **3** was observed. In contrast, cyclization between residues 24 and 28 by lactamization to give analogue **4** only slightly affected the helical content but clearly resulted in a more classical α-helical structure ($\theta_{222}/\theta_{209} = 0.98$). Cyclization of the linear analogue **2** enhanced the SDF-1 receptor CXCR4 binding approximately 114-fold, where the IC_{50} values derived from ^{125}I-SDF-1 competitive binding assays with CEM cells were found to be 39.5 ± 5.9 nM, 28.9 ± 6.3 μM, 225.8 ± 11.8 nM, and 254.1 ± 5.4 nM for analogues **1**−**4**, respectively. Intracellular calcium mobilization ($[Ca^{2+}]_i$) induced after interaction with CXCR4, as measured by EC_{50}, was significantly reduced in analogue **4** compared to **3**, and approached the EC_{50} of native SDF-1, indicating a correlation between the degree of α-helix and biological activity. Therefore, the biological activity of small peptide SDF-1 analogues is highly dependent on the conformation of its C-terminal region.

Introduction

Chemokines are an important class of polypeptides in the hematopoietic and immunological systems that act to recruit leukocytes to sites of inflammation and infection by interacting with specific receptors on the cell surface of their target cell.[1−3] Stromal cell-derived factor one (SDF-1, CXCL12) is a member of the CXC family of chemokines that has been found to be constitutively secreted from the bone marrow stromal cells.[4] The biological effects of SDF-1 are mediated by the chemokine receptor CXCR4 (also known as fusin or LESTR), which is expressed on mononuclear leukocytes including hematopoietic stem cells.[5] To date, SDF-1 is the only natural ligand to be identified for CXCR4.[6] Genetic elimination of SDF-1 is associated with perinatal lethality, including abnormalities in cardiac development, B-cell lymphopoiesis, and bone marrow myelopoiesis.[7] Although many chemokines have proinflammatory roles, SDF-1 appears to have a fundamental role in the trafficking, export, and homing of bone marrow cells.[7−10] It is produced constitutively, and particularly high levels are found in bone marrow stromal cells.[6,8−12] A basic physiological role is implied by the high level of conservation of the SDF-1 sequence between species.[11] In vitro SDF-1 stimulates chemotaxis of a wide range of cells including monocytes and bone marrow-derived progenitor cells.[7−10,12]

SDF-1 is also structurally distinct from the other chemokines in that it has only about 22% amino acid sequence identity with other CXC chemokines.[12] A basic physiological role for SDF-1 is implied by the high level of conservation of the SDF-1 sequence between species. It has recently been demonstrated that SDF-1 addition to long-term cultures (LTCs) of normal human marrow can selectively and reversibly block the cycling of primitive erythroid and granulopoietic colony forming cells (i.e., precursors of erythrocytes and neutrophils, respectively) in the adherent layer.[13] To date, SDF-1 is the only chemokine identified to regulate the cycling of both LTC-IC (long-term culture initiating cells) and high proliferative potential colony forming cells (HPP-CFC).[13]

A variety of diseases require treatment with agents that are preferentially cytotoxic to dividing cells. Cancer cells, for example, may be targeted with cytotoxic doses of radiation or chemotherapeutic agents. A significant side effect is the impact of such treatments on normal tissue cells in active mitosis. The indiscriminate destruction of hematopoietic stem and progenitor cells can lead to a reduction in normal mature blood cell counts,

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such as leukocytes, lymphocytes, and red blood cells. A decrease in leukocyte count, signifying a loss of immune function, increases a patient's risk of opportunistic infection (bacterial and fungal). Neutropenia (low blood neutrophil counts) is particularly important because of the ability of neutrophils to clear fungal infection. While new drugs improved the treatment option, high mortality and morbidity are still associated with severe fungal infections.[14] Thrombocytopenia (low blood platelet counts) is associated with bleeding risks, is compromised of mucosal barriers, and further increases the potential of infection. Platelet transfusions are expensive and can lead to antibody development making further transfusion ineffective.[15,16] Interruption of therapy is often indicated because of pancytopenia that can in turn lead to the development of drug resistant malignant cells and ultimately in relapse. There is accordingly a need for therapeutic agents and treatments that facilitate the preservation of normal hematopoietic stem and progenitor cells in patients during treatment with cytotoxic agents. SDF-1, with its effect on the cycling of hematopoietic cells, may be used for this purpose. Bone marrow transplantation has been used in the treatment of a variety of hematological, autoimmune, and malignant diseases. In conjunction with bone marrow transplantation, ex vivo hematopoietic (bone marrow) cell culture may be used to expand the population of hematopoietic progenitor or stem cells. It may be desirable to purge an ex vivo hematopoietic cell culture of cancer cells with cytotoxic treatments, while preserving the viability of the hematopoietic progenitor or stem cells. There is accordingly a need for agents and methods that facilitate the preservation of hematopoietic progenitor or stem cells in ex vivo cell cultures exposed to cytotoxic agents.

Given the fundamental role of SDF-1 as a potent chemoattractant for T cells, monocytes, and $CD34^+$ hematopoietic progenitor cells[10,17] and in the normal cell physiology and pathology of HIV infection, the structure–function analyses of SDF-1 have been the subject of intense study. The three-dimensional structure of SDF-1 has been determined by both NMR spectroscopy and X-ray crystallography.[18–20] The structure of SDF-1 consists of three major domains: the N-terminus with a mostly extended conformation, a central core region consisting of three antiparallel β-sheets, and a C-terminal amphililic α-helix. The N-terminal residues of SDF-1 are known to be a critical site for CXCR4 receptor binding and activation.[18,21] Specifically, an SDF-1 peptide analogue corresponding to the N-terminal 1–17 residues has been reported to have SDF-1 activity.[22] Structural analysis has revealed a cluster of positively charged residues in the central β-sheet region that is hypothesized to be involved in electrostatic interaction with the negatively charged residues located at the N-terminus and second extracellular loops of CXCR4.[20] By contrast to biological function of the N-terminus and central charged β-sheet region, the C-terminal α-helix region is still poorly understood. Luo et al.[23] described an analogue in which a portion of the N-terminal region (residues 5–14) of native SDF-1 is linked by four glycines to the C-terminal residues 55–67. The four-glycine linker approximates the distance between these C-terminal and N-terminal regions in the native folded SDF-1. This peptide was shown to enhance the CXCR4-dependent biological activity compared to the N-terminal peptide. This model showed clearly that linkage of C-terminal to its N-terminal region enhances the CXCR4 induction properties of the N-terminal region. Lactam formation on the polar side face has been shown to increase activity and bioavailability in hormones such as PTH,[24] particularly if these lactams are between natural sequence ion pairs of the *i,i+4* type. We describe here novel truncated analogues of native SDF-1 where the N-terminal (1–14) sequence is linked by four glycines to the C-terminus residues 55–67 (sdf-(1–31), **2**). These novel 31-residue analogues are cyclized by lactamization between residues K20-E24 or residues E24-K28. We demonstrate that these cyclized analogues have enhanced in vitro biological activity and that the level of biological activity correlates with the degree of α-helix formed by lactamization.

(1) KPVSLSYRCPCRFFESHVARANVKHLKILNTPN-
CALQIVARLKNNNRQVCIDPKLKWIQEYLEKALN-OH

1 5 10 15 20 25 30

(2) KPVSLSYRCPCRFFGGGGLKWIQEYLEKALN-NH_2

(3) KPVSLSYRCPCRFFGGGGLKWIQEYLEKALN-NH_2

(4) KPVSLSYRCPCRFFGGGGLKWIQEYLEKALN-NH_2

Figure 1. Sequences of SDF-1 and SDF-1 analogues: **1**, sdf-(1–67)-OH; **2**, sdf-(1–31)-NH_2; **3**, *cyclo*(Lys^{20}-Glu^{24})-sdf-(1–31)-NH_2; **4**, *cyclo*(Glu^{24}-Lys^{28})-sdf-(1–31)-NH_2.

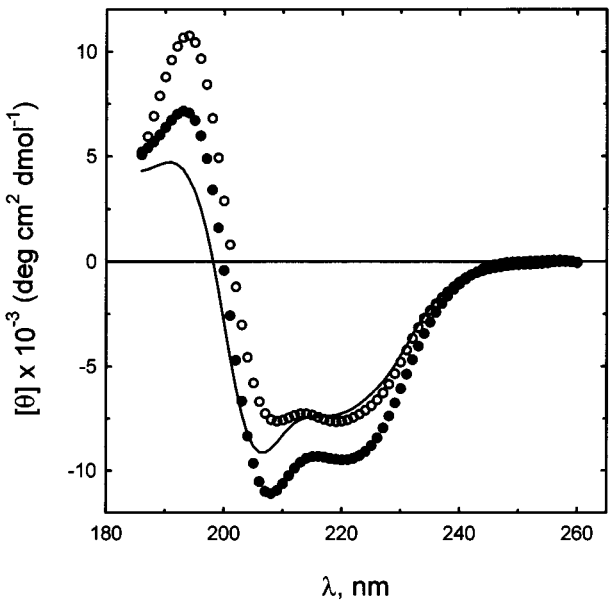

Figure 2. CD spectra of SDF analogues. Each analogue, at a concentration of 0.14 mM, was dissolved in 25 mM sodium phosphate, pH 6.0. sdf-(1–31)-NH_2 (**2**) (—), *cyclo*(Lys^{20}-Glu^{24})-sdf-(1–31)-NH_2 (**3**) (●), *cyclo*(Glu^{24}-Lys^{28}-)-sdf-(1–31)-NH_2 (**4**) (○).

Results

Circular Dichroism Spectroscopy. Lactams used in this study are derivatives of linear analogues sdf-(1–31)-NH_2 (**2**) (Figure 1). The circular dicroism (CD) spectra of *cyclo*[Lys^{20}-Glu^{24})-sdf-NH_2 (**3**) and *cyclo*[Glu^{24}-Lys^{28})-sdf-NH_2 (**4**) are shown in Figure 2, together with the spectrum of the parent linear analogue (**2**). Since

Table 1. CD Parameters for SDF-1 Analogues at pH = 6.0

SDF-1 analogue	θ_{209} ($\times 10^{-3}$)	$-\theta_{222}$ ($\times 10^{-3}$)	$\theta_{222}/\theta_{209}$	helical residues
2	8.6	7.0	0.81	8
3	10.9	9.5	0.87	10
4	7.6	7.4	0.97	8

estimating the secondary structure of short peptides from their CD spectra with algorithms that use basis sets derived from protein structures is not considered valid, we have used here the ellipticity at 222 nm, $[\theta]_{222}$, to measure the α-helix content as previously described.[24] The two lactams (peptides **3** and **4**) with *i*, *i+4* spacings are expected to be helix stabilizing.[25] To compare these spectra with that of a commonly used α-helix spectrum,[26] we have used the ratio of the absolute value θ_{222} to θ_{209}. The θ_{222} to θ_{209} ratio has a value of approximately 1.09 in a perfect α-helix model. The CD spectrum of both the lactams in Figure 2 are substantially different than that of their linear analogue. The CD data illustrated that analogue **3**, cyclized between K20 and E24, had two to three extra residues in α-helix, compared to the linear form, as shown by the θ_{222} values (Table 1). The $\theta_{222}/\theta_{209}$ value changes only slightly in analogue **3** as compared to **2**, even though there is an increase in the length of the α-helix. In contrast, cyclization between residues 24 and 28 (analogue **4**) affects only slightly the helical content of the analogue but results in a clearly more classical α-helical structure.

SDF-1 Receptor Affinity Determinations. The affinity of the SDF-1 peptide analogues was demonstrated with a SDF-1 receptor (CXCR4) binding assay. A competitive dose response for binding to CXCR4 by native SDF-1 and the peptide analogues against ^{125}I-SDF-1 is shown in Figure 3. CEM cells were assessed for ^{125}I-SDF-1 binding in the presence or absence of SDF-1 or peptide analogues following 2 h of incubation. The inhibition of ^{125}I-SDF-1 binding in the presence of SDF-1 and the peptide analogues is indicative of CXCR4 binding.[22,27] The ligand affinity is expressed as the concentration necessary to inhibit 50% binding (IC_{50}) of the radio-labeled ligand (^{125}I-SDF-1). The IC_{50} for native SDF-1 was determined to be 39.6 ± 5.9 nM. Lactam formation between either Lys^{20} and Glu^{24} (**3**, IC_{50} = 226 (±11) nM) or Glu^{24} and Lys^{28} (**4**, IC_{50} = 254 (±5) nM) resulted in CXCR4 affinity approaching that of native SDF-1. The nonlactamized linear analogue **2** demonstrated a substantially reduced affinity for the SDF-1 receptor, where the IC_{50} was determined to be 29 (±6) μM, illustrating that lactam formation is necessary for receptor association of the SDF-1 peptide analogues.

The Association between α-Helix Stabilization through Lactamization and Intracellular Calcium Mobilization. Induction of chemokine seven-transmembrane receptors, including CXCR4, is often associated with the mobilization of calcium within the cytosol, depending on the cell type.[21−23,28,29] Intracellular calcium mobilization has been shown to be associated with G-protein-dependent regulation of phospholipase C (PLC), which, in turn, mediates the hydrolysis of phosphatidylinositol diphosphate (PIP_2) to inositol triphosphate (IP_3) that mediates the release of calcium ions into the cytosol through calcium channels in the endoplasmic reticulum.[30] This receptor-dependent increase in intracellular calcium concentration has previously been used to quantitatively determine the biological activity of SDF-1 and SDF-1 polypeptide analogues.[22,27] The data in Figure 4A show that native SDF-1 and the lactam peptide analogues induced a rise in cytoplasmic calcium concentration. The magnitude of calcium concentration increase was both rapid and transient, peaking within 10 s of stimulation (data not shown) as indicated elsewhere.[22] The response to SDF-1 and the lactam analogues was concentration-dependent, with stimulation being observed in the nanomolar range, and the maximum calcium release peaking between 800 nM and 1 μM for analogues **3** and **4**. In contrast to analogues **3** and **4,** the linear analogue **2** showed minimal stimulation of intracellular calcium mobilization (data not shown). Figure 4B demonstrates the minimal receptor stimulatory effect of linear peptide sdf-(1−31)-NH_2 (**2**), indicating that formation of an α-helix, here through lactamization, is necessary for biological activity, similar to the observations reported for CXCR4 binding.

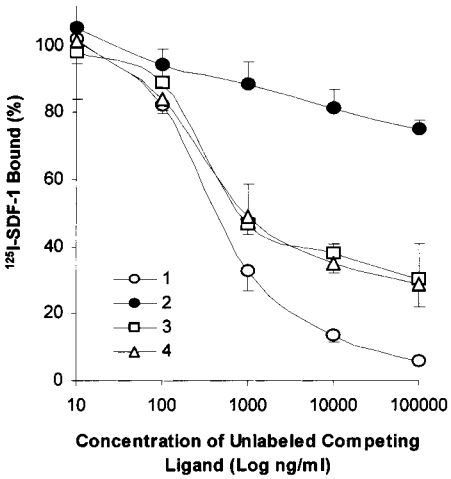

Figure 3. SDF-1 receptor competition binding analysis. SDF-1 (**1**) and the indicated peptide analogues (competing ligands) were added at the concentrations illustrated in the presence of 4 nM ^{125}I-SDF-1 and CEM cells (1×10^6/mL). Cells were assessed for ^{125}I-SDF-1 binding following 2 h of incubation. The results are expressed as the percentage of maximal specific binding that was determined without competing ligand and are the mean ± 1 SD of $n = 3$ experiments.

Discussion

The CXCR4 chemokine differs from all known human chemokines in that SDF-1 is the only chemokine that functions as the endogenous ligand for this receptor, and it has exceptionally strong sequence conservation between species.[9,11,12] Tissue distribution of SDF-1 suggests that it may have a role in immune surveillance rather than inflammation as it has been demonstrated to principally function in trafficking, export, and homing of bone marrow cells.[12,22] CXCR4 is also a major coreceptor of HIV-1.[31]

The SDF-1 N-terminus was identified as being critical for CXCR4 binding and activation. The ^{1}H NMR structure of SDF-1 has a flexible N-terminal region that is entirely solvent-accessible with no detectible secondary structure.[18,20] This terminal region binds sufficiently that receptor activation is possible. This was demonstrated by Loetscher et al. who showed that SDF-1

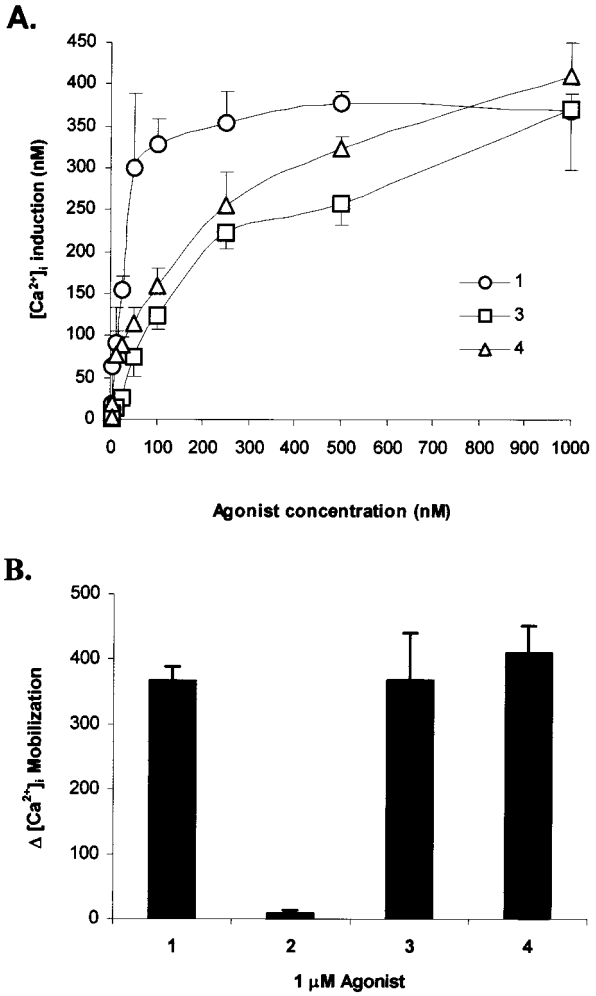

Figure 4. Induction of intracellular calcium mobilization ($[Ca^{2+}]_i$) by SDF-1 and peptide analogues. (A) Fura-2,AM loaded THP-1 cells (1×10^6/mL) were stimulated with SDF-1 or the lactamized peptide analogues (compounds **3** or **4**) at the concentrations indicated. (B) Fura-2,AM loaded THP-1 cells (1×10^6/mL) were stimulated with SDF-1 or the peptide analogues at the maximal concentration where both compounds **3** and **4**, as well as SDF-1, induced the maximum $[Ca^{2+}]_i$ (1 μM). The values represent the mean ± 1 SD of n = 3 experiments.

peptides corresponding to 1−8, 1−9, 1−9 dimer, and 1−17 bind and activate CXCR4, although with low potency compared with native SDF-1.[22] SDF-1 is the only chemokine to date where an N-terminus peptide analogue was shown to be sufficient for receptor binding.[22] Other chemokines require additional sequences that interact with their receptors to provide sufficient binding energy. These characteristics of SDF-1 present a novel avenue to investigate the structural basis for SDF-1/CXCR4 interaction and activation of CXCR-dependent biological responses. Some bioactive peptides have α-helical structures, often amphiphilic, that are necessary for binding to their receptors.[24,32] These sequences may have ionic pairs, spaced four residues apart, which help to stabilize the helical structure. Cyclization by lactam formation between these residues on the polar face can lead to dramatic increases in activity, as observed with these SDF-1 analogues.

There are several striking conclusions from the data presented here. First, stabilization of the C-terminal α-helix in an analogue lacking the entire central β-sheet region of SDF-1 is sufficient to confer almost full receptor activation on the SDF-1. This implies that the function of the central region as far as receptor activation is concerned is mainly to stabilize the C-terminal α-helix. This result is not necessarily in contradiction with data showing the critical nature of specific residues in the central region. It simply means that a particular structure of the central region is required in the context of SDF-1. Second, these data show that the ability of the C-terminal region to bind negatively charged polysaccharides, such as heparin, is not required for receptor binding and activation. Such binding has been proposed to be important for binding of SDF-1 to its target cells.[33,34] This results from the observation that activity is gained even though lactam formation removes one of the C-terminal Lys positive charges. It has been previously demonstrated that some chemokines, for instance, RANTES, MIP-1α and MIP-1β, bind to glycosaminoglycans (GAG) via the C -terminal region.[35,36] The residues identified as responsible for GAG binding are in the region of SDF-1 deleted and replaced by GGGG in the analogues presented here. GAG's are also found on leukocytes and therefore may play a role in presenting the chemokines to the G-protein-coupled chemokine receptors on the same cell. Notably, loss of cell surface glycosaminoglycan reduces the affinity of these cells for many chemokines.[37] SDF-1 protein has high affinity for heparin.[12] The importance of GAG's in SDF-1 attachment to monocytic and lymphoid cells was demonstrated in a recent study.[34] On the other hand, SDF-1 C-terminus has been shown to significantly improve the CXCR4 associated biological activity,[23] and this activity-enhancing effect of the C-terminus was not due to the increase in receptor binding but most likely its binding to heparin. Either the observed binding of this chemokine to such polysaccharides as heparin or GAG is unimportant biologically or it has some function unrelated to receptor binding function. The latter could include the mechanism for establishing chemokine gradients in vivo.[38] We are currently investigating the role of heparin and cell surface GAG's on the binding and CXCR4 associated biological activity of the SDF-1 analogues in our laboratory, and this will be the subject of a future discussion.

There is an interesting comparison of these analogues topographically to hormones in another 7-transmembrane G-protein receptor family. The other family includes hormones that activate receptors that include secretin, glucagon, parathyroid hormone, vasoactive intestinal peptide, and growth hormone-releasing peptide.[39] They typically have minimal active sequences with about 30 residues and a C-terminal amphiphilic helix with about 10 residues. The N-terminal region commonly has an α-helix, with the two helices joined by a flexible sequence. The cyclization of $i,i+4$ sequences has been reported to result in not only increased receptor activation[24,32,40−43] but an improved in vivo activity.[44] It appears that the way in which SDF-1 binds to and activates its receptor may be similar to these hormones, despite the much larger size.

It has previously been shown that only the N-terminal peptide, extending as short as eight amino acid residues, is necessary for SDF-1 peptide-CXCR4 binding,[22] and that the addition of the C-terminal domain, extending

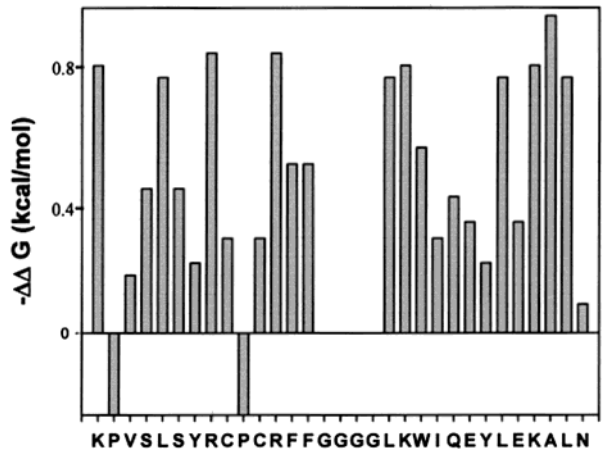

Figure 5. Helix potentials of SDF-1, plotted using values of O'Neil and DeGrado.[45]

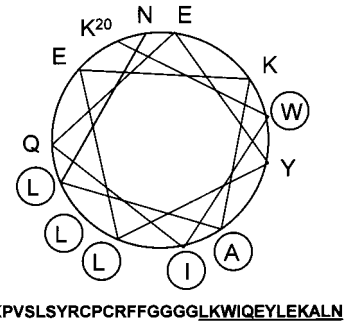

Figure 6. Helical wheel for C-terminal amphipathic α-helix of SDF-1. The sequence shown in the wheel is underlined in the SDF-1 sequence shown at bottom. Hydrophobic residues are circled.

from amino acids 55−67 to a N-terminal peptide composed of residues 5−14, augments the CXCR4-dependent biological activity, although to a lesser degree than the native protein.[23] We similarly report that the SDF-1 analogue **2** containing both the N-terminus and C-terminus binds to the SDF-1 receptor, but with substantially less affinity (724-fold less) than SDF-1. Our observation that lactamization of the C-terminus of the SDF-1 analogues (**3** and **4**) results in greater binding (more than 100-fold compared to **2**) indicates that although the N-terminus is required for binding, the affinity for CXCR4 interaction is augmented through the C-terminus, which requires an α-helical conformation.

Within experimental error, the data suggests that *cyclo*(Glu^{24}-Lys^{28})-sdf-(1−31)-NH_2 (**4**) has a slightly higher activity than *cyclo*(Lys^{20}-Glu^{24})-sdf-(1−31)-NH_2 (**3**). The CD data indicates that these two analogues do not have the same structure, and the small difference in their activities may be directly related to the different stabilized structures, assuming that the residues involved in the lactams do not directly interact with the receptor. A helix potential plot for **2**, using the values of O'Neill and Degrado,[45] shows that most of the helix is in the C-terminus of the molecule (Figure 5). In addition, most of the CD signal comes from this region because the possible helix near the N-terminus has only about seven residues. Helices with less than 7−11 residues have little or no expected CD because of the known dependence of CD per residue on the length of the helix.[46] The increase in α-helix for the 20−24 cyclization (**3**) as opposed to the 24−28 one (**4**) is anticipated in view of the helix potentials and the contributions of the charged residues to the helix macrodipole. Given that the number of residues estimated from the CD is not exact, we suggest that a plausible interpretation of the spectrum of analogue **4** is that the cyclization stabilizes a more perfect helix about residue 23 and 30. This seems to be the most favorable structure for receptor binding. The 20−24 cyclization of analogue **3** results in a net increase of α-helix because the lactam is between residues that are less favorable for helix in the linear form (see helix potential, Figure 6) or in analogue **4**. A more likely reason is that the cyclization of analogue **3** is more helical is that it removes a strong Lys → Glu contribution to the helix macrodipole in comparison to the more C-terminal lactam. The latter not only leaves the unfavorable one intact but also removes a favorable Glu → Lys contribution. Although stabilization of the conformation of the C-terminal helix is suggested to be the most important reason for the higher activities of the two cyclized analogues, contributions, positive or negative, resulting from changes in the side-chain conformations of the residues involved in the lactams cannot be excluded.

An NMR comparison between a 31-residue parathyroid hormone analogue and a cyclic lactam analogue between residues 22 and 26 has been reported.[47] This study showed that the effect of the cyclization is on a small region near the sequence contained within the lactam ring. Small linear peptides such as the one studied here exist in a large number of conformations that are poorly defined by the NMR data and only have an average CD spectrum. The cyclic analogue is partially constrained and therefore has a lower entropy. If this constraint results in an average conformation more similar to that of the receptor-bound form, then it is expected to bind more tightly to the receptor. The observation in several biologically important peptides that an appropriate cyclization can dramatically improve receptor-activating activity[24,32] is consistent with this explanation.

Lactam formation in **4** resulted in a higher rate of calcium mobilization compared to the **3** analogue, as illustrated in Figure 4. The concentration of SDF-1 and peptide analogues necessary to effectively induce 50% maximal calcium ion mobilization (EC_{50}) was determined for each of the compounds, except **2**, which could not be determined. The EC_{50} for native SDF-1 (**1**) and compounds **3** and **4** were 26.7 (±10.6) nM, 147.9 (±13.5) nM, and 106.3 (±14.1) nM, respectively. Hence, the data would suggest that the observed difference in the conformation stabilized by the lactams of peptides **3** and **4**, as indicated by their respective $\theta_{222}/\theta_{209}$ values, and their relative biological activities likely relates to their position in the α-helix. This correlation may be carried further to in vivo results obtained in collaboration with our laboratory. It was demonstrated that SDF-1 represses the cycling of hematopoietic progenitor cells, including BFU-E (erythroid precursors) and CFU-GM (granulacyte-monocytes precursors).[13] We have observed that analogue **3** is equally effective as SDF-1, and was 10-fold more efficacious at repressing BFU-E and CFU-GM cell cycling than the linear peptide analogue **2**. The

properties of SDF-1 analogue(s) in the inhibition of cell cycle activities have important clinical/medical applications. It can be used to protect the hematopoietic compartment during chemotherapy. It is also possible to apply this property in the area of gene therapy. Hematopoietic stem cells, because of their self-renewal capability, are ideal for gene therapy. The cells can be purified in clinically significant quantities.[48−50] Successful transduction and expression of the target sequence in a small number of pluripotent stem cell is sufficient to propagate the gene indefinitely. The best system for gene transduction is the use of retroviral vectors, where transduction efficiencies are high[51] and large quantity of clinical grade material can be produced with sufficient titer.[52] However, gene therapy using hematopoietic stem cells and retroviral vectors has been hampered by a lack of stimulating factors that stimulate stem cell mitosis without commitment to differentiation.[50,53] Recent data have shown that self-renewal potential of mouse stem cells can be maintained in the first divisions but were lost during subsequent mitosis.[50,53,54] Conceivably, SDF-1 agonist(s) can be used to stop stem cell cycling after a brief period of growth factor stimulation and transduction. In this way, the high efficiency transduction of retroviral vector and the self-renewal capacity of stem cells are both utilized. The hypothesis is currently tested in laboratory studies.

Conclusions

This study illustrates the effects of the formation of a lactam between residues 20 and 24, or 24 and 28, on the hydrophilic face of the 31-mer analogue of SDF-1, developed by replacing the β-sheet amino acid sequence (residues 15−54 of the native protein) and terminating the C-terminus as an amide. The results demonstrate that the large intervening sequence of SDF-1 is not essential for CXCR4 receptor binding or CXCR4-mediated biological activity. Further, the greater the stability of the α-helix stabilizing lactam, as determined by circular dicroism, the greater the biological activity of the analogue.

The rationale for developing small peptide analogues of SDF-1 from the clinical perspective include increasing bioavailability, such as by reducing metabolism resulting from enzymatic cleavage through minimization of the protein size and converting the C-terminus acid to an amide; reducing immunogenicity that is currently associated with full length recombinant therapies; and the complications and costs associated with synthesizing larger polypeptides. The results presented in this work show that, by reducing the size of native SDF-1 by replacing the 40 amino acids associated with the β-sheet with a four-glycine linker and adding a lactam within the C-terminus, particularly between residues 24 and 28, to stabilize the resulting α-helix, activation of the CXCR4 receptor is effectively mediated. These observations may be particularly important to potential therapeutic uses of these peptides in the treatment associated with myelosuppressive therapies (for example, chemotherapy and radiation therapy) in order to protect hematopoietic stem cells and during peripheral blood progenitor cell collection and bone marrow harvesting.

Experimental Section

Chemicals. *N*-α-Fmoc-L-amino acids and *N*-α-Boc-L-dipeptides: Boc-Lys(Boc)-Pro-OH and Boc-Lys(Boc)-Gly-OH were obtained from Chem-Impex Inc. (Wood Dale, IL). The following protection groups were used: Lys(Boc), Arg(Pbf), Glu(OtBu), Ser(tBu), Asn(Trt), Gln(Trt), Cys(trt), Tyr(tBu), and Trp(Boc). *N*-α-Fmoc-Glu(All) and *N*-α-Fmoc-Lys(Alloc) were obtained from Applied Biosystems (Framingham, MA). TentaGel-R RAM (0.21 mmol/g) was purchased from Peptides International (Louisville, Kentucky). NovaSyn-N resin (0.20 mmol/g) was purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). Tetrakis(triphenylphosphine)palladium(0) and sodium diethyldithiocarbamate were from Aldrich Chemical Co. (Milwaukee, WI). Guanidine HCl and Tris were from Fluka (Oakville, ON). Syntheses was performed on a continuos-flow peptide synthesizer (Pioneer Peptide Synthesis System or Perseptive Biosystems Model 9050 Plus). Mass spectra were obtained either by MALDI (voyager STR) or electrospray MS (Micromass Quattro I).

sdf-(1−67)-OH (1). This peptide was synthesized by Fmoc protocol using 0.5 g of Fmoc-Asn preloaded resin (NovaSyn-N: 0.2 mmol/g). The remaining synthesis was performed with TBTU/HOBt/2,4,6-Collidine activation using a 4-fold excess of activated amino acids and double-coupling each residue. The last two residues (Lys^1-Pro^2) were added manually by coupling Boc-Lys(Boc)-Pro-OH for 4 h with a 4-fold excess of amino acid. The peptide was cleaved from the resin by shaking with 7.5 mL of reagent K (6.19 mL of TFA, 0.38 mL each of water, 90% phenol/water, thioanisole, and 0.19 mL of 1,2-ethanedithiol) for 4 h at 20 °C. The cleaved peptide mixture was removed by filtration and precipitated by addition to cold ether. The crude product was purified on a Vydac C_4 column (10 μm, 1 × 25 cm), using 0.75%/min gradient (20−50%) of acetonitrile in 0.1% TFA/water. The purity of the product was estimated by analytical HPLC on a Vydac C_4 column (10 μm, 0.4 × 25 cm), using a 0.5%/min gradient of acetonitrile in 0.1% TFA/water. The estimated purity was >90%. The molecular weight was 7836.34 (±0.23) (calculated, 7835.26). After lyophilization, the full-length peptide was air oxidized at 0.92 mg/mL in 2 M guanidine HCl, 0.1M Tris, pH 8.6, at room temperature.[55] Folding was complete after stirring overnight and was monitored by HPLC and mass spectrometry. The solution was dispensed into a Spectrum Spectra/Por*7 dialysis membrane (Houston, TX) (M_r cutoff, 3500), and the bag was placed in 2 L of distilled water. Over a period of 2 days, 6 L of water was pumped into the vessel containing the dialysis bag. After lyophilization, the refolded SDF-1 (**1**) was purified on a Vydac C_4 column (10 μm, 1 × 25 cm), using 0.75%/min gradient (20−50%) of acetonitrile in 0.1% TFA/water and 1.8 mg of pure material was obtained. The purity of the product was estimated by analytical HPLC on a Vydac C_4 column (10 μm, 0.4 × 25 cm), using a 0.5%/min gradient of acetonitrile in 0.1% TFA/water. The estimated purity was >95%. The molecular weight was 7831.73 (±0.62) (calculated, 7831.26).

sdf-(1−31)-NH_2 (2). Starting with TentaGel R RAM resin (0.5 g, 021 mmol/g), the synthesis was performed with TBTU/HOBt/2,4,6-collidine activation at room temperature with extended protocol mode (5 min Fmoc-deprotection and 60 min coupling). All residues were single coupled, and a 4-fold excess of activated amino acids was used. The last two residues (Lys^1-Pro^2) were added manually as Boc-Lys(Boc)-Pro-OH for 4 h and using a 4-fold excess of amino acid. The peptide resin was washed with DCM (13 mL) and then cleaved from the resin by shaking with 7.5 mL of reagent K (6.19 mL of TFA, 0.38 mL each of water, 90% phenol/water, thioanisole, and 0.19 mL of 1,2-ethanedithiol) for 4 h at 20 °C. The cleaved peptide mixture was removed by filtration and precipitated by addition to cold ether.

The crude product was dissolved in 15 mL of 20% acetonitrile/water and 0.1% TFA and chromatographed on a Vydac C_{18} column (10 μm,1 × 25 cm), using a 0.5%/min gradient (30−50%) of acetonitrile in 0.1% TFA/water. The purity of the final product was estimated by analytical HPLC on a Vydac C_{18} column (10 μm, 0.4 × 25 cm), using a 0.5%/min gradient of

acetonitrile in 0.1% TFA/water. The purity was >99%. The molecular weight by MALDI mass spectrometry was 3560.38. (±0.19) (calculated for M + 1, 3562.23).

***cyclo*(Lys^{20}-Glu^{24})-sdf-(1−31)-NH$_2$ (3).** This peptide was synthesized as described for peptide **2** with Lys(Alloc)-OH and Glu(OAll)-OH substituted at positions 20 and 24, respectively. After completion of the addition of the residue 19 Fmoc-Leu, the peptide-resin was suspended in 1.7 mL of a solution of tetrakis(triphenylphosphine)palladium(0) (0.32 mmol), 5% acetic acid, and 2.5% NMM in chloroform under argon and then shaken at 20 °C for 6 h to remove the allyl and alloc protecting groups.[56] The peptide resin was then washed with 0.5% sodium diethyldithiocarbamate trihydrate (DEDT) in DMF (50 mL), followed by DMF (50 mL) and DCM (50 mL). The peptide (0.06 mmol) was cyclized by shaking with 0.18 mmol of 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP)/0.18 mmol NMM in 2 mL of DMF for 14 h at 20 °C. The peptide-resin was filtered, washed with DMF, and repacked into the column. The peptide was cleaved from the resin with reagent K. The purity was >98%, and the product had a mass of 3542.49 (calculated, M + 1 = 3542.23).

***cyclo*(Glu^{24}-Lys^{28})-sdf-(1−31)-NH$_2$ (4).** This peptide was synthesized as for peptide **3**, with Glu(OAll)-OH and Lys-(Alloc)-OH substituted at positions 24 and 28, respectively. The product had an estimated purity of >98% and a mass of 3542.47 (calculated, M + 1 = 3542.23).

Circular Dichroism Spectroscopy. Spectra were obtained on a JASCO J-600 spectropolarimeter at 20 °C. At least four spectra were averaged, and the data smoothed by the JASCO software. The instrument was calibrated with ammonium(+)-10 camphorsulfonate. Peptide concentrations were determined from the absorption at 280 nm, using a calculated extinction coefficient of 8100 M^{-1} from the single Trp and two Tyr. Data are expressed per peptide bond.

Receptor Binding Assay. A competition binding assay using I^{125} labeled SDF-1 as a competitive ligand was used to determine the affinity of a compound for the SDF-1 receptor (CXCR4). The competition binding was carried out by incubating the reaction mixture (final volume 150 μL) consisting of CEM cells (1×10^6/mL), ^{125}I-SDF-1 radioligand, and the binding buffer (RPMI with 23.8 mM $NaHCO_3$, 10 μg/nL bovine serum albumin, 0.1% (15.38 mM) sodium azide, and 25 mM HEPES) with different concentrations of peptide (0−100 nM) at room temperature for approximately 2 h. Following incubation, cells bound with radioligand were washed with phosphate-buffered saline containing 10% sucrose, followed by measurement of bound ^{125}I-SDF in a γ counter (LKB-Wallac Clini-Gamma 1272).

Calcium Mobilization Assay. Chemokine receptors are G-protein-coupled, seven-transmembrane receptors. They signal by interaction with specific G-proteins. Receptor-mediated responses to chemokines are associated with rapid and transient elevations of intracellular Ca^{2+} mobilization. All determinations of $[Ca^{2+}]_i$ were made at 37 °C. Fluorescence emission from 1.5 mL of Fura,2-AM loaded THP-1 cells (1×10^6/mL) in the presence or absence of ligand was recorded continuously by excitation at 340 nm and emission at 500 nm. Fluorescence intensities (F) were corrected by subtraction of the scatter intensity (F_{scat}). Intracellular calcium concentrations $[Ca^{2+}]_i$ were determined using the following equation: $[Ca^{2+}]_i = K_D \cdot [F - F_{min}]/[F_{max} - F]$, where F_{max} and F_{min} are the fluorescence intensities at maximal (0.02% Triton X-100) and minimal (50 mM EGTA) calcium concentrations, and K_D is the dissociation constant of Fura,2-AM and is equal to 224 nM.

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