Biochemical and Biophysical Research Communications **264,** 42–47 (1999)
Article ID bbrc.1999.1476, available online at http://www.idealibrary.com on IDEAL®

Attachment of C-Terminus of SDF-1 Enhances the Biological Activity of Its N-Terminal Peptide

Jiansong Luo, Zhaowen Luo, Naiming Zhou, James W. Hall, and Ziwei Huang[1]
Kimmel Cancer Institute, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Received September 3, 1999

The N-terminus of stromal cell-derived factor 1 (SDF-1) is known to be a critical site for CXCR4 receptor binding and signaling. However, the functional role of other regions, in particular the C-terminal helix of SDF-1, has yet to be defined. In this study, we designed and synthesized a peptide model of SDF-1 containing its N- and C-terminal regions. The attachment of the C-terminus of SDF-1, which by itself had no activity in receptor binding and signaling, dramatically increased the effect of the N-terminal fragment in inducing chemotaxis and intracellular Ca^{2+} influx in sup T1 cells compared with the peptide containing only the N-terminal sequence. The enhancement in activity was not due to the increase in receptor affinity as the N,C-terminal peptide did not show higher CXCR4 binding than the N-terminal peptide. On the other hand, the intracellular Ca^{2+} influx activated by the N,C-terminal peptide, but not the N-terminal peptide, was completely abolished by the addition of heparin, suggesting that the C-terminal fragment of the peptide binds glycosaminoglycans (GAGs) and exerts an effect to modulate biological activity. These data raise the possibility that the C-terminus in native SDF-1 is one of interaction sites with GAGs and may be associated with biological function of SDF-1. Furthermore, this study demonstrates an approach for the design of novel agonists or antagonists of other chemokine receptors that possess enhanced biological activity.

Stromal cell-derived factor-1 (SDF-1), a protein of 67 amino acids, is a member of the CXC chemokine subfamily of pro-inflammatory mediators and a potent chemoattractant for T cell, monocytes and $CD34^+$ hemopoietic progenitor cells (1). The critical role of SDF-1 in embryonic development is indicated by the observation that mice lacking the SDF-1 gene die *in utero* with severe defects in the ventricular septum of the heart and in the development of pre-B cell and myeloid progenitors (2, 3). As the only physiological ligand identified so far for the chemokine receptor CXCR4, SDF-1 induces chemotaxis and cytoplasmic calcium flux in many cells. Interestingly, defects observed in mice lacking CXCR4 are identical to those lacking SDF-1, suggesting a monogamous relationship between SDF-1 and CXCR4 (2). CXCR4 also serves as one of the principal coreceptors for human immunodeficiency virus type 1 (HIV-1) by mediating the entry of T-cell-tropic virus strains (4). It is known that HIV entry via CXCR4 can be inhibited by SDF-1 (5, 6).

Given the fundamental role of SDF-1 in normal cell physiology and pathology of HIV infection, it has been the subject of intensive structure-function studies. The three-dimensional structure of SDF-1 has been determined by both NMR spectroscopy and X-ray crystallography (7, 8). With an overall folding pattern similar to other chemokines such as MIP-1β, RANTES and IL-8 (9), the structure of SDF-1 consists of three major domains: the N-terminus with mostly extended conformation, a central core region of three antiparallel β-sheet, and the C-terminal amphiphilic α-helix. Studies of synthetic peptides derived from SDF-1 N-terminus and SDF-1 mutants demonstrate that N-terminal residues play a critical role in CXCR4 recognition and signal transduction (7, 10). Analysis of the structure of SDF-1 reveals a cluster of positive charge residues in the central β-sheet region that is hypothesized to be involved in electrostatic interaction with the negative charge residues located at the N-terminus and second extracellular loops of CXCR4 (8). By contrast to the functional importance of the N-terminus and putative role of the central β-sheet region, the C-terminal helix of SDF-1 is still poorly understood in terms of its relevance to SDF-1 biological function. An important question that remains to be addressed is whether the C-terminus contributes to the biological function of SDF-1 or merely serves as a structural template.

[1] To whom correspondence should be addressed at 802 BLSB, 233 South 10th St., Thomas Jefferson University, Philadelphia, PA 19107. Fax: (215) 923-0881. E-mail: zhuang@nana.jci.tju.edu. URL: http://nana.jci.tju.edu.

0006-291X/99 $30.00

In this study, we designed and analyzed a synthetic peptide containing both N- and C-termini of SDF-1 as a minimized model to probe the functional role of SDF-1 C-terminus. The attachment of the C-terminus to the N-terminus generated a new peptide with significantly higher activity in activating chemotaxis and Ca^{2+} mobilization of T cells compared with the peptide containing only the N-terminal region. Studies of the mechanism of the activity-enhancing effect by the SDF-1 C-terminus led to a model for the functional role of this region. These results also suggested a general approach to design novel chemokine analogs with enhanced biological activity.

EXPERIMENTAL PROCEDURES

Peptide synthesis. The peptides were prepared by solid phase synthesis using Fmoc-strategy on a 430A peptide synthesizer (Applied Biosystems, Foster City, CA) and a 9050 Pepsynthesizer Plus (Perseptive Biosystems, Cambridge, MA), as described previously (11, 12). Crude peptides were purified by preparative reverse phase high performance liquid chromatography using a Dynamax-300Å C_{18} 25 cm × 21.4-mm-i.d. column with flow rate of 9 ml/min and two solvent systems of 0.1% TFA/H_2O and 0.1% TFA/acetonitrile. Fractions containing the appropriate peptide were pooled together and lyophilized. The purity of the final products was assessed by analytical reverse-phase high-performance liquid chromatography, capillary electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Chemotaxis. Migration of T cells (sup T1 cells) was assessed in disposable Transwell trays (Costar, Cambridge, MA) with 6.5-mm-diameter chambers and membrane pore size of 3 μM. The peptides were prepared with sterile water, diluted in 0.5% BSA RPMI 1640, and added to the lower well. To the upper wells 100 μl of sup T1 cells at 1×10^7 cells/ml were added. For antibody inhibition, the monoclonal antibody 12G5 (R & D Systems, Minneapolis, MN) was preincubated with the cells at 10 μg/ml for 15 min at 4°C. The antibody was also added to the lower wells at the same concentration. After incubation at 37°C and 5% CO_2 for 4 h, cells that migrated to the lower wells were counted.

Intracellular calcium measurements. Following a modified procedure published by others (10, 13), sup T1 cells were cultured in the RPMI 1640 medium containing 10% FBS. For Ca^{2+}-mobilization studies, 5×10^6 cells/ml were loaded with the fluorescent dye Fura-2 (2 μM, Molecular Probes, Eugene, OR) in Hanks' balanced salt solution (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) at 37°C for 30 min. The same buffer was used to wash the cells three times before the addition of peptides at the indicated concentrations. Cells were resuspended to a concentration of $30-40 \times 10^6$ cells/ml and $1.5-2 \times 10^6$ cells were tested. $[Ca^{2+}]_i$ was measured by using excitation at 340 and 380 nm on a fluorescence spectrometer (Perkin–Elmer LS50). Calibration was performed by using 10% Triton X-100 for total fluorophore release and 0.5 M EGTA to chelate free Ca^{2+}. Intracellular Ca^{2+} concentrations were calculated by using the fluorescence spectrometer measurement program.

RESULTS

Design of peptides containing N- and/or C-termini of SDF-1. To study the functional role of the SDF-1 C-terminus, we synthesized peptides containing this region and analyzed their interaction with the CXCR4 receptor. The structure of SDF-1, like those of other chemokines, displays three distinctive regions, a central core of β-sheet flanked on both sides by an extended N-terminus and a C-terminal helix (Fig. 1a) (7, 8). A minimized peptide model of SDF-1, termed NCT-tide, was designed by deleting the large central β-sheet region and joining the N- (residues 1–14) and C-termini (residues 56–67) with a four-glycine linker (Fig. 1b). The use of this linker is to allow the N- and C-terminal fragments to adopt a spatial orientation similar to the native protein structure. It is hypothesized that this model peptide may mimic two important domains of the SDF-1 protein, the N-terminal region known to be the major site of receptor binding and signaling (7, 10) and the C-terminal helix whose function has yet to be defined. For comparison, two control peptides, termed NT-tide and CT-tide, were also synthesized containing only the N- (residues 1–14) and C-termini (residues 56–67), respectively.

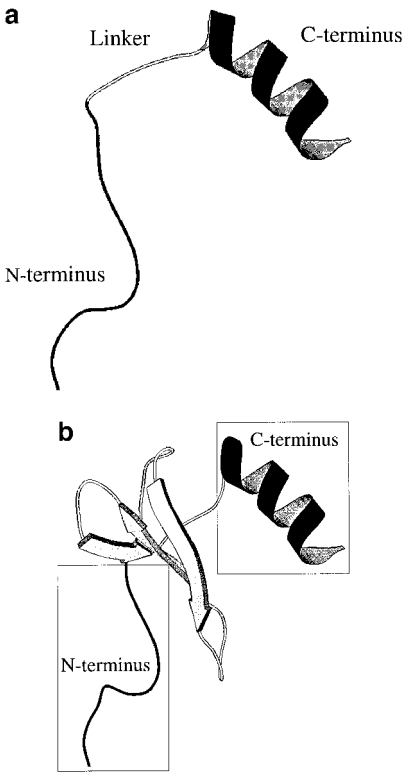

FIG. 1. The N- and C-termini of SDF-1 (a) mimicked by a designed model peptide, NCT-tide (b). NCT-tide consists of N- (residues 5–14) and C-termini (residues 55–67) of SDF-1, which is linked by four glycines. The amino acid sequence of the peptide is as follows: LSYRCPCRFF-GGGG-LKWIQEYLEKALN. Two control peptides NT-tide and CT-tide were synthesized containing the N- (LSYRCPCRFF) and C-termini (LKWIQEYLEKALN) of SDF-1, respectively.

Chemotactic activity of the designed SDF-1 peptide. The synthetic SDF-1 peptides were tested for their ability to induce migration of T lymphocytes. It was found that the N-terminal peptide NT-tide induced

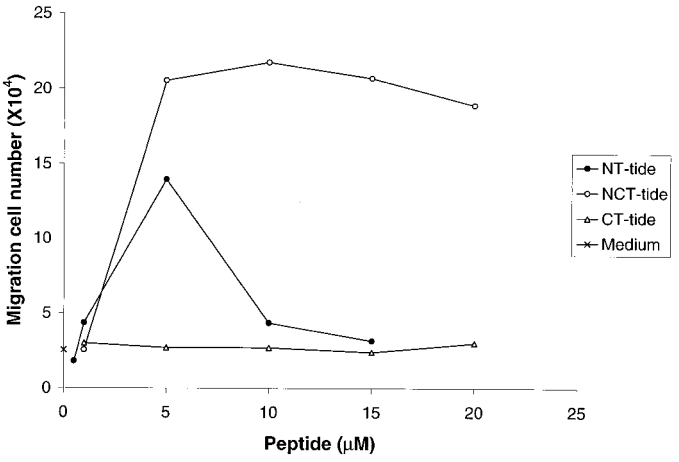

FIG. 2. Migration of sup T1 cells activated by SDF-1 peptides. Peptides were added in the lower well of Transwell tray at the indicated concentration, while 10^6 cells were added in the upper well. After incubation at 37°C for 4 h, cells in the lower well were counted. The data shown here are representative of at least three independent experiments.

dose-dependent chemotaxis of sup T1 cells, reaching the peak of 14×10^4 in 10^6 cells at the concentration of 5 μM (Fig. 2). After that, the chemotaxis of sup T1 cells was sharply decreased with higher concentrations of NT-tide. This activity curve of the peptide was consistent with that of native SDF-1 protein whose activity also showed a similar concentration-dependent pattern in control experiments (data not shown). The C-terminal peptide CT-tide did not show any effect in chemotaxis induction. However, it was interesting to find that NCT-tide, which contains both N- and C-terminal regions of SDF-1, had significantly enhanced chemotaxis of sup T1 cells. It induced dose-dependent migration of cells which reached the maximum of 21×10^4 in 10^6 cells at 10 μM and then slowly declined after that concentration. The potent effect of NCT-tide was in marked contrast to the much weaker activity of NT-tide and complete lack of effect of CT-tide. The chemotactic activity of NCT-tide was reduced upon the addition of an anti-CXCR4 monoclonal antibody 12G5 (14) in a similar manner to that of the SDF-1 protein (Fig. 3). These results suggested that the designed NCT-tide, like native SDF-1, mediates strong chemotaxis via CXCR4.

Signaling activity of the designed SDF-1 peptide. The widely different chemotactic activities of SDF-1 peptides implied their varied abilities to activate receptor-signaling pathways. To confirm this, we measured the effect of the peptides in triggering intracellular Ca^{2+} influx in sup T1 cells (Figs. 4a–4c). As expected from the chemotaxis assay, the SDF-1 C-terminal peptide CT-tide was completely inactive while the N-terminal peptide NT-tide could only weakly induce calcium influx. In contrast, at the same concentration (50 μM) NCT-tide containing both N- and C-termini of SDF-1 induced a significant intracellular calcium influx. These data are consistent with those from chemotaxis experiments described above. Taken together, they suggest that the C-terminal region of SDF-1, which by itself has no effect on CXCR4 receptor activation, can dramatically enhance the efficiency of signal transduction activated by the N-terminus of SDF-1.

Mechanism of activity-enhancing effect of SDF-1 C-terminus. We wanted to understand how the attachment of SDF-1 C-terminus promotes the biological activity of the N-terminal peptide. The first possibility we considered was that the addition of this C-terminus might increase the binding affinity of the peptide toward the receptor. To test this, we studied the competition binding of peptides with mAb 12G5 to CXCR4 receptors on CEM cells. It was found that NCT-tide displayed no higher, but only somewhat comparable CXCR4 binding compared with the N-terminal peptide NT-tide (data not shown). These results clearly argued against the possibility that the C-terminus of SDF-1 could promote the receptor binding of the N-terminus. In view of this, we carried out further experiments to search for other potential mechanisms. In a number of chemokines such as MCP-1 and IL-8, the C-terminal helix is known to mediate glycosaminoglycan binding which modulates the biological activity of these chemokines by recruiting the chemokine and stabilizing its interaction with a receptor (15, 16). SDF-1 can bind heparin with higher affinity than MCP-1 or IL-8 (17). However, the binding sites in SDF-1 for heparin and its biological relevance to SDF-1 function have not yet been established. Here we hypothesized that the

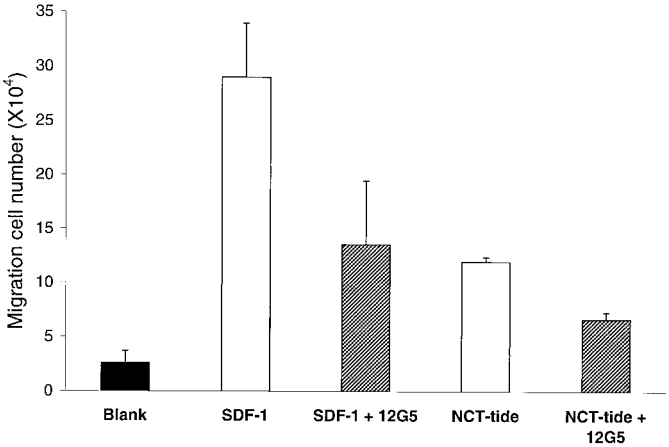

FIG. 3. Selective inhibition of anti-CXCR4 mAb 12G5 to chemotaxis induced by SDF-1 and NCT-tide. The migration of sup T1 cell activated by SDF-1 (50 nM) and NCT-tide (5 μM) was examined in the absence or presence of monoclonal antibody 12G5 (10 μg/ml). The data shown in figure are the mean ± SD of at least three different experiments.

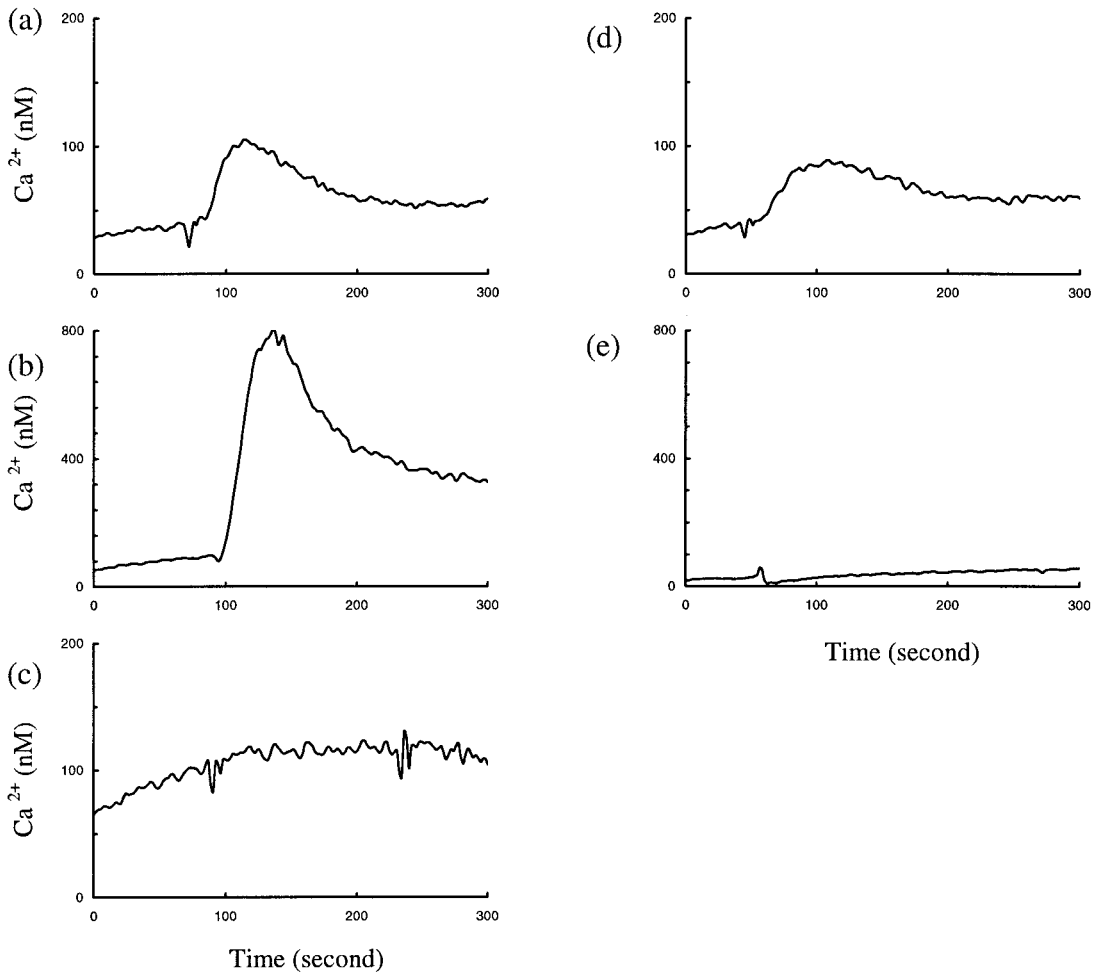

FIG. 4. Intracellular Ca^{2+} influx in CXCR4 expressing cells activated by SDF-1 derived peptides. Sup T1 cells were loaded with Fura-2 (2 μM) and treated with 50 μM of NT-tide (a), NCT-tide (b) or CT-tide (c). For heparin inhibition, sup T1 cells were stimulated with the above concentration of NT-tide (d) and NCT-tide (e) mixed with heparin at the final concentration of 1 U/ml. Data shown here are representative of similar results from three experiments.

C-terminal fragment of NCT-tide might bind heparin and influence the activity of the peptide. This was tested in experiments in which soluble heparin was used to inhibit the signal induced by the peptides. Interestingly, the addition of heparin completely abolished the strong intracellular Ca^{2+} influx of sup T1 cells activated by NCT-tide (Fig. 4e). As a control, the Ca^{2+} influx activated by the N-terminal peptide NT-tide was not affected by heparin (Fig. 4d). These data support the notion that the significant enhancement in signaling and biological activity of NCT-tide is due to binding to glycosaminoglycans.

DISCUSSION

SDF-1 is a chemokine molecule that has critical physiological functions. Like other chemokines, SDF-1 mainly consists of three distinct structural regions: N-terminus, central β-sheet core, and C-terminus (7, 8). Previous studies have established the essential role of the SDF-1 N-terminus as the major site for direct interaction with the receptor and signal transduction (7, 10). By contrast, the functional role of the β-sheet region and C-terminus of SDF-1 is less clear. It has been suggested that a cluster of positive charge residues on the β-sheet is important for SDF-1 binding to the receptor (8). The putative role of these positive charge residues was tested in our recent study using synthetic peptides (manuscript submitted). Here in this study, we investigated the functional role of the SDF-1 C-terminus by designing a minimized SDF-1 peptide model mimicking the native N- and C-termini. We found that this peptide containing both N- and C-termini possessed significantly higher biological activity in inducing calcium influx and chemotaxis in sup T1 cells compared with the control peptide containing only the N-terminus of SDF-1. This suggested that the C-terminal fragment, which by itself has no activity,

served to augment the activity of N-terminal fragment in the designed peptide. The activity-enhancing effect of the C-terminus in the peptide was not due to the increase in receptor binding, but most likely its binding to heparin. These results from the peptide model, in conjunction with the information that native SDF-1 protein has high affinity for heparin (17), tend to suggest a possible role of SDF-1 C-terminus. It is conceivable that the N- and C-termini of SDF-1 engage in separate interactions with the CXCR4 receptor and glycosaminoglycans, respectively. Whereas SDF-1 C-terminus is not directly involved in CXCR4 recognition, its binding to heparin or other glycosaminoglycans could help present the ligand on the cell surface and stabilize its association with the receptor. It is noted that, concomitant with the completion of this study, others have recently reported the binding of the first β-strand of SDF-1 to heparan sulfates (18). Therefore, it may be likely that the association with glycosaminoglycans is mediated by multiple sites on SDF-1.

Chemokines are commonly known to bind GAGs including heparin. The C-terminal helices have been shown to be the GAG binding site in both CXC chemokines such as IL-8 (15) and PF-4 (19) and CC chemokines such as MCP-1 (16) although MIP-1a has been suggested to use a different site for GAG association (20). The involvement of the C-terminus of SDF-1 in GAG interaction as suggested by this study is consistent with the observation of other chemokines described above. However, this study could not rule out the possibility that other regions, particularly the central β-sheet that contains a number of positive charge residues may also contribute to GAG binding. Further experiments will be necessary to establish the role of the C-terminus and/or other sites in GAG binding. The relationship between binding to GAGs and biological activity *in vitro* and *in vivo* varies among different chemokines. In some chemokines such as MIP-1α and MCP-1 (16, 20), GAG binding is not required for signaling and chemotactic activity *in vitro.* However, in other chemokines, the C-termini are critical for maximal biologic potency, which at least in part is due to interactions between the α-helices and GAGs. For example, it has been shown that binding to heparan sulfate or heparin enhances neutrophil responses to IL-8 (15). A more recent study has further demonstrated that the intact C-terminus of IL-8 is required for the endothelial cells binding, transcytosis, and induction of neutrophil emigration (21). A similar observation has been made with another CXC chemokine PF-4 where the heparin-binding C-terminus of PF-4 influences its neutrophil activity (22) and angiostatic activity (19). As to SDF-1, a recent study has indicated the lack of effect of SDF-1 binding to heparan sulfates on CXCR4 signaling (18). Further experiments are needed to delineate the role of heparin binding for *in vitro* and *in vivo* biological function of SDF-1.

The most interesting observation from this study was that the covalent linkage of the heparin-binding C-terminus of SDF-1 dramatically increased the biological effect of its N-terminus derived peptide. This novel finding may have general implication for the design of highly potent peptide analogs of chemokines. Since the N-termini of chemokines are believed to be the main receptor binding and signaling domains, they have been the focus for studies of peptide and other small molecule analogs. However, the activities of such analogs mimicking only the N-terminal fragment are often less than optimal as in the case of the N-terminal peptide in this study and can be enhanced by attachment of other functional parts of the protein such as the C-terminus. As the interaction between cytokines and GAGs has been suggested to retain cytokines at the target sites and protect them from enzymatic cleavage (23, 24), it is conceivable that the addition of C-terminus or other heparin-binding motifs to a chemokine-mimicking analog may increase not only its potency *in vitro* but also *in vivo* efficacy. While in this study a chemokine analog with enhanced agonistic activity was shown, a similar approach should be applicable to the design of chemokine antagonists. Such antagonistic analogs could potentially be used to inhibit chemokine receptors such as CCR5 and CXCR4 that are involved in HIV entry. RANTES is a natural chemokine ligand of CCR5 and has been shown to potently block CCR5-mediated HIV infection (25). It is interesting to note that the anti-HIV activity of RANTES is regulated by the heparan sulfate proteoglycans on the cell surface (26). Thus, it can be envisioned that the attachment of a GAG-binding motif to antagonists of CCR5 or CXCR4 may present a novel approach to the design of anti-HIV inhibitors with higher biological potency.

ACKNOWLEDGMENTS

We thank E. Lolis for providing synthetic SDF-1 protein, R. W. Doms and J. Rucker for providing cell lines, and B. Perussia and L. Azzoni for helping with the Ca^{2+} mobilization assay. This work was supported in part by grants from the National Institutes of Health and the Sidney Kimmel Foundation for Cancer Research.

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