Stromal Cell-derived Factor-1α Associates with Heparan Sulfates through the First β-Strand of the Chemokine*

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Biological properties of chemokines are believed to be influenced by their association with glycosaminoglycans. Surface plasmon resonance kinetic analysis shows that the CXC chemokine stromal cell-derived factor-1α (SDF-1α), which binds the CXCR4 receptor, associates with heparin with an affinity constant of 38.4 nM (k_on = 2.16 × 10^6 M^-1 s^-1 and k_off = 0.083 × s^-1). A modified SDF-1α (SDF-1 3/6) was generated by combined substitution of the basic cluster of residues Lys24, His25, and Lys27 by Ser. SDF-1 3/6 conserves the global native structure and functional properties of SDF-1α, but it is unable to interact with sensor chip-immobilized heparin.

The biological relevance of these in vitro findings was investigated. SDF-1α was unable to bind to a CXCR4-independent manner on epithelial cells that were treated with heparan sulfate (HS)-degrading enzymes or constitutively lack HS expression. The inability of SDF-1α 3/6 to bind to cells underlines the importance of the identified basic cluster for the physiological interactions of SDF-1α with HS. Importantly, the amino-terminal domain of SDF-1α which is required for binding to, and activation of, CXCR4 remains exposed after binding to HS and is recognized by a neutralizing monoclonal antibody directed against the first residues of the chemokine. Overall, these findings indicate that the Lys24, His25, and Lys27 cluster of residues forms, or is an essential part of, the HS-binding site which is distinct from that required for binding to, and signaling through, CXCR4.

Based on the relative position of the two first cysteine residues, chemokines are classified in two main subfamilies, CC and CXC chemokines (1). Stromal cell-derived factor-1α (SDF-1) also called pre-B cell-stimulating factor (2) is a CXC chemokine originally purified from bone marrow cell supernatants (3). Two forms, α and β (68 and 72 amino acids, respectively), generated by alternative splicing from a unique sdf1 gene, have been identified (4). The α form is the most abundant (4). Human and murine SDF-1α proteins differ by a single residue at position 18 (valine to isoleucine in the murine protein) (4).

CXCR4 is the only identified receptor for SDF-1α. Furthermore, the interaction between SDF-1α and CXCR4 appears to be unique and non-promiscuous (5–7). SDF-1α stimulates intracellular calcium flux and chemotaxis in monocytes, T lymphocytes, and neutrophils, a characteristic shared with other CXC chemokines (8, 9). However, SDF-1α exhibits structural and molecular characteristics that make it a unique chemokine among members of CC and CXC families. SDF-1α possesses the peculiar capacity of attracting and promoting bone marrow engraftment of CD34+-CXCR4 hematopoietic stem cells (10). In contrast to most chemokines, which are induced by cytokines or mitogenic stimuli, SDF-1α is constitutively expressed in a large number of tissues (4). Importantly, Sdf1 gene knock-outs induce anomalies in hematopoiesis and the development of cardiovascular system provocating pre- or perinatal death of the embryos (5). Apart from these physiological functions, SDF-1α has the selective capacity to inhibit cell entry of CXCR4-dependent human immunodeficiency viruses by occupying and internalizing CXCR4 in T lymphocytes (8, 9, 11, 12). Overall, these findings indicate that SDF-1α and its receptor, both of which are expressed widely outside the lympho-hematopoietic system, accomplish important additional functions that are not typical for chemokines.

The biological activities of chemokines are thought to be influenced by their association with cellular or extracellular matrix glycosaminoglycans (GAG). Usually attached to a core protein to form proteoglycans (13), GAGs are highly sulfated oligosaccharides characterized by a high degree of structural heterogeneity. The common GAG are heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate, and hyaluronic acid (14, 15). The interaction of cytokines with proteoglycans in the extracellular matrix or cell surface have important functional consequences in many biological systems (16, 17). Although the in vivo biological roles of chemokine-GAG complexes are not clear, an increasing body of evidence suggests that GAG immobilize and enhance local concentrations of the chemokines, promoting their oligomerization and facilitating their presentation to the receptors (18, 19). Thus, it has been

spectroscopy; TOCSY, total correlation; Fmoc, fluoremethyloxycarbonyl; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; RANTES, regulated on activation normal T cell expressed and secreted; FACS, fluorescence-activated cell sorter.

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proposed that chemokines like macrophage inflammatory protein (MIP)-1β or interleukin-8 (IL-8) would be tethered to circulating leukocytes complexed to membrane-bound proteoglycans in endothelial cells (20, 21).

Although retention of SDF-1α in heparin affinity columns indicates that the chemokine has the capacity to complex with GAG (22), it is not known whether SDF-1α is capable to interact with GAG under physiological conditions. Moreover, the nature of GAG is not known, and the structural determinants of the protein that eventually would account for such interactions remain unidentified.

The aim of this work was to investigate the capacity of SDF-1α to form complexes with isolated or cell-bound GAG and to characterize the GAG family accounting for these interactions. On the other hand, we wanted to identify structural determinants of SDF-1α involved in the physical contact with GAG. Our findings demonstrate that SDF-1α interacts selectively and with relatively high affinity with HS in vitro. HS is also responsible for the binding of SDF-1α to CXCR4-negative epithelial or endothelial cells. Finally, we identified a cluster of basic residues in the first β-strand of the β-sheet of SDF-1α which is necessary for interaction with HS both in vitro and in intact cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—CHO-K1, CHO-pgsB 618, CHO-pgsD 677, Jurkat, CEM, CEMx174, ECV-304, and HeLa were obtained from the American Type Cell Collection. HS, dermatan sulfate, chondroitin sulfate A, and chondroitin sulfate B were obtained from Sigma (catalogue numbers H5393, C3788, C9819, and C0320, respectively). Heparinase (EC 4.2.2.7), heparitinase I (EC 4.2.2.8), and chondroitinase ABC (EC 4.2.2.4) were purchased from Seikagaku Corp. Heparin was from Sanofi Recherche. An upgraded BiAcore system, F1 sensorchips, amine coupling kit, and biotin hydrazide were purchased from Seikagaku Corp. Heparin was from Sanofi Recherche. An upgraded BiAcore system, F1 sensorchips, amine coupling kit, and biotin hydrazide were purchased from Seikagaku Corp. Heparin was from Sanofi Recherche.

**Flow Cytometric Analysis of SDF-1α Binding to Cells**—Adherent cells were plated 2 days before binding experiments. Cells were detached with 2 mM EDTA in PBS and washed twice with ice-cold binding buffer (RPMI 1640, 20 mM Hepes, 1% bovine serum albumin). 4 × 10^6 cells were resuspended in the presence of the indicated concentration of chemokines in a total volume of 200 μl and incubated for 90 min at 4 °C under agitation. Unbound chemokine was removed by washing with binding buffer, and cell-bound SDF-1α was detected by incubation with the anti-SDF-1α mAb K15C (15 μg/ml, diluted in PBS, 1% bovine serum albumin). After staining with phycoerythrin-conjugated anti-mouse immunoglobulins (Southern Biotechnology), cells were fixed in 1% formaldehyde buffer and analyzed in a FACSscan (Becton Dickinson, CA). To remove cell-surface GAG, CHO-K1 and HeLa cells (10^6 cells) were washed twice and incubated for 90 min at 37 °C with 1 million/ml GAG-degrading enzymes. For trypan treatment, cells were washed once with PBS containing 2 mM EDTA and incubated for 4 min at 37 °C with 0.25% trypan in PBS. After enzyme treatment, cells were washed four times with 5 ml of binding buffer, detached with PBS/EDTA, and then assayed for SDF-1α binding as described above.

**Biotinylation Procedure and Heparin Immobilization**—Fractionated heparin (9 kDa) resuspended in PBS at 1 mg/ml was reacted for 24 h at room temperature with 10 ml biotin-LC-hydrazine. The mixture was then extensively dialyzed against water to remove unreacted biotin and freeze-dried. Two flow cells of an F1 sensorchip were acti-...
binding was determined in the presence of 1 μM unlabeled SDF-1α. Cell pellet-associated radioactivity was counted using a LKB-Wallac microcomputer controlled 1272 CliniGamma counter. The binding data were analyzed using a GraphPad Prism 2.0 software.

**Chemotaxis and Intracellular Ca2+ Mobilization**—CEMx174 cells were resuspended in chemotaxis medium (RPMI 1640 containing 1% human plasma protein (Swiss Red Cross Laboratory) and buffered with 20 mM Hepes, pH 7.3). Cell migration was performed in 48-well chemotaxis chambers (Neuro Probe Inc., Cabin John, MD) as described previously (27). Chemokines diluted in chemotaxis medium were added to the lower and 10^5 CEMx174 cells in the same medium to the upper wells. Polycarbonate membranes with 5-μm pores (PC 5 μm PVPF, Costar, Cambridge, MA) were used to measure cell migration for 2 h at 37 °C. Membranes were removed, and the upper side was washed with PBS, fixed, and stained. Cell migration was assessed at 1000× magnification in five randomly selected fields. For calcium measurements, CEMx174 cells were washed twice with PBS and resuspended in fresh medium (1.25 × 10^6/ml). Calcium mobilization was determined as described previously (8). Single-cell Ca2+ measurements were performed in HeLa cells loaded with Fura-2 (3 μM) as described previously (11).

**Structural Studies**—NMR experiments were acquired at 35 °C on a Varian Unity 500 spectrometer operating at 499.64 MHz for 1H and equipped with a triple resonance 5-mm probe. Data were processed on a Sun workstations using the VNMRS 5.3 program. All two-dimensional proton NMR experiments were acquired in the phase-sensitive mode using the hypercomplex scheme (28). A two-dimensional nuclear Overhauser experiment with pure absorption phase was performed in four quadrants. The SDF-1 3/6 sample was prepared by dissolving 6 mg of freeze-dried powder in 350 μl of 20 mM acetate buffer in 90% H2O/10% D2O, pH 5. The final concentration was 2.1 mM. The
FIG. 2. SDF-1α binds on cell membranes independently of CXCR4. a, CHO-K1 cells were incubated with the indicated concentrations of SDF-1α and, after extensive washing to remove free chemokine, were labeled with K15C mAb and analyzed by flow cytometry. b, flow cytometric analysis of SDF-1α binding on different cell lines. CHO-K1, HeLa (human epithelial cells) ECV-304 (human endothelial cells), and CEM (T cells) were incubated with 300 nM SDF-1α and, after removal of the unbound chemokine, were stained with the K15C mAb. Control (CTRL) corresponds to cells stained with K15C mAb without SDF-1α incubation. CXCR4 expression was assessed using the 6H8 mAb.

RESULTS

CXCR4-independent Binding of SDF-1α to Cells—The capacity of SDF-1α to bind cell membrane molecules other than CXCR4 was investigated using a novel mAb. The K15C mAb was obtained by immunizing mice with a linear peptide derived from the amino terminus of SDF-1α. In enzyme-linked immunosorbent assay experiments, the antibody showed specific reactivity with immobilized SDF-1α (Fig. 1a) as competition occurred when the antibodies were preincubated with free SDF-1α but not with the non-related chemokines RANTES, MIP-1α, or MIP-1β. Apart from SDF-1α or SDF-1β (Fig. 1b) which differ exclusively by four amino acids at the carboxyl terminus domain, the antibody failed to recognize any other known CC or CXC chemokine (data not shown). To characterize further the specificity of epitope recognition by K15C mAb, we performed Western blot experiments using SDF-1α derivatives with progressive deletion of amino acids at the amino terminus of SDF-1α. SDF-1α derivatives lacking the two first residues (Lys1 and Pro2) (29) were not recognized by the antibody. This proves that Lys1 and Pro2 are essential residues in the epitope recognized by K15C mAb (Fig. 1b). Importantly, incubation of K15C mAb fully prevented the biological activity of SDF-1α as assessed by its capacity to block SDF-1α-mediated CXCR4 endocytosis in Jurkat cells (Fig. 1c) or chemotaxis of human T lymphocytes (data not shown).

CXCR4-negative CHO-K1 cells (Fig. 2b) were incubated with 10–1000 nM SDF-1α and labeled with the K15C mAb. The cytofluorographic analysis showed that SDF-1α bound to the cell surface in a concentration-dependent manner (Fig. 2a). The recognition of the SDF-1α-K15C complex by the secondary antibody was specific since no fluorescence was observed when SDF-1α was omitted or replaced by MIP-1β (1000 nM) (Fig. 2a).

The capacity of SDF-1α to bind to cells in a CXCR4-independent manner is not restricted to a particular cell type. Indeed, SDF-1α also bound to CXCR4-negative, endothelial cells (ECV304 cell line, Fig. 2b) with a comparable efficiency as to CHO-K1. Similarly, cell membrane-bound SDF-1α was found when the chemokine was incubated with HeLa cells that constitutively express CXCR4 (Fig. 2b). SDF-1α binding detected by FACS analysis on HeLa cells was not due to interaction of the chemokine with CXCR4 because the K15C mAb exclusively recognizes the critical amino-terminal residues that are engaged in binding to CXCR4 and are required for SDF-1α-induced signal transduction (29). Accordingly, we failed to detect SDF-1α bound to CXCR4-positive, CEM T lymphoblastoid cells (Fig. 2b). CEM cells express high levels of functional CXCR4 receptors, and saturable binding of SDF-1α in these cells has been shown (29). This suggests that in CEM cells, CXCR4 accounts for most of the cellular binding of SDF-1α. Collectively, our results indicate that other structures apart from CXCR4 have the capacity to attach SDF-1α to the cell surface. These interactions apparently do not involve the amino terminus of SDF-1α that is recognized by the K15C mAb and is masked after interaction with CXCR4.

SDF-1α Binds to Cell Surface GAG—To ascertain whether proteoglycans can account for the CXCR4-independent binding of SDF-1α on CHO-K1 or HeLa cells (Fig. 3a), cells were treated for 90 min with enzymes that selective degrade either chondroitin sulfate or HS. The cells were then incubated with 300 nM SDF-1α at 4°C for 90 min. Treatment of both cell types with heparinase or heparitinase, which specifically remove HS,
dramatically reduced the binding of SDF-1α detected by the K15C mAb (Fig. 3a). In contrast, exposure of CHO-K1 or HeLa cells to chondroitinase ABC failed to modify the binding of SDF-1α. Treatment with trypsin, which cleaves the protein core of proteoglycans, also prevented detection of CHO-K1- or HeLa-bound SDF-1α, and probed with the K15C mAb. b, flow cytometric analysis of SDF-1α binding to wild type CHO-K1 cells and GAG-deficient CHO cell mutants. Bound SDF-1α was detected using the K15C mAb. Data are representative of four different experiments.

The capacity of SDF-1α to interact with proteoglycans was further investigated using CHO-derived pgsB618 and pgsD677 cells which lack galactosyltransferase I (pgsB618) or N-acetylglucosaminyltransferase and glucuronyltransferase (pgsD677) activities and consequently are deficient for the synthesis of any GAG or HS, respectively (Fig. 3b). Preincubation of SDF-1α with soluble HS markedly competed binding of the chemokine to CXCR4-negative parental CHO-K1 cells (data not shown).

In conclusion, CXCR4-independent binding of SDF-1α to cell surfaces can be mediated by proteoglycan molecules containing HS as polysaccharide components.

Identification of SDF-1α Residues Allowing Interaction with GAG—CXCR chemokines have been shown to interact with GAGs namely through positively charged sites located in the α-helix at the carboxyl terminus (30–33). We searched for a cluster of residues in SDF-1α with a net positive charge that fulfills criteria to form a GAG-binding site. A putative site is located in the first β-strand of the β-sheet and is exposed on the surface of the molecule (29, 35). To investigate the capacity of this site to interact with GAG, we synthesized a SDF-1α derivative in which the basic residues Lys24, His25, and Lys27 were substituted by Ser (SDF-1α). When applied onto a heparin-Sepharose column, SDF-1α was eluted at low ionic strength of a linear NaCl gradient (Fig. 4a). In contrast, SDF-1α, like RANTES which also associates with GAGs (19), requires considerably higher NaCl concentrations to disrupt the interaction with the heparin-Sepharose. To determine whether the reduced affinity of SDF-1α for heparin correlates with its lower capacity to bind on cells in a CXCR4-independent manner, we incubated CHO-K1 cells with SDF-1α or SDF-1α and determined cell-associated chemokine with the K15C mAb. On Western blots, both SDF-1α and SDF-1α are recognized with similar efficiency by K15C mAb (Fig. 4c). By using increasing concentrations of SDF-1α up to 1000 nM, we could show that the modified chemokine retained less than 10% of the capacity of SDF-1α to bind to cell surface proteoglycans (Fig. 4b). A peptide containing SDF-1α amino acids 1–34 (SDF-1α) had the lowest affinity for heparin, although it included the proposed sequence for interaction with GAG (Fig. 4a). The finding suggests that proper folding of the β-strand containing the BBXB motif is critical for binding to HS. However, at this point, we cannot exclude that an additional domain that is not contained in SDF-1α may contribute to the overall binding of SDF-1α.

By comparing the TOCSY and NOESY spectra of SDF-1α and SDF-1α 3/6 very similar proton chemical shift values are revealed, suggesting that both molecules must fold with a comparable three-dimensional structure. In particular the three-stranded anti-parallel β-sheet, the carboxyl-terminal α-helix, and the rigid turns are similar for both molecules (see Ref. 29, Protein Data Base access code 1sdf). Of particular interest is the structure of the first β-strand of the β-sheet where the modifications were made. Strong His-His interactions are observed between Ala60 and Leu76 and between Gln48 and Arg41 (Fig. 5) as well as weak NH-NH interaction between Ala60 and Val49 indicating that the three-stranded anti-parallel...
β-sheet is preserved in SDF-1 3/6. The strong NH-NH interaction observed between Lys$^{24}$ and His$^{25}$ in SDF-1α is maintained in SDF-1 3/6 between Ser$^{24}$ and Ser$^{25}$ (Fig. 5). In addition, the numerous long range dipolar interactions between Trp$^{57}$ side chain protons and the first strand of the β-sheet are also present (not shown), and all the chemical shift values for this residue are identical for both molecules indicating a similar environment. Although, minor local rearrangements cannot be excluded, it is apparent that the global fold of SDF-1α is essentially maintained in the SDF-1 3/6. The similar affinity of SDF-1α and SDF-1 3/6 for CXCR4 supports this conclusion (Fig. 8a). These findings further strengthen our view that the cluster of basic residues formed by Lys$^{24}$, His$^{25}$, Leu$^{26}$, and Lys$^{27}$ is likely involved in physical contacts with the polyanionic chains of HS and is an essential part of the SDF-1α HS-binding site.
Characterization of GAG/SDF-1α Interactions—The Biacore technology was used to confirm and extend the analysis of the SDF-1α/GAGs interactions in vitro. Surface plasmon resonance (SPR) was used to measure changes in refractive index caused by the binding of SDF-1α (analyte) to the immobilized biotinylated heparin. Injection of SDF-1α (200 nM, 150 μl) over an activated sensor chip containing 120 RU of heparin gave a signal of 350 RU, whereas injection of the chemokine over a control surface (containing streptavidin only) did not lead to any significant signal (Fig. 6a). By using this binding assay, a competition analysis was performed to identify GAG that may mediate chemokin binding to immobilized heparin. SDF-1α was preincubated with different GAG and then injected over the heparin-activated sensorchip. Pretreatment of SDF-1α with heparin or HS significantly attenuated the binding of the chemokine. In keeping with this result, injection of SDF-1α (100 nM) was co-incubated in the absence (open bar) or in the presence of 10 μg/ml (closed bars) of GAG (HP, heparin; HS, heparan sulfate; CSα, chondroitin 4-sulfate; DS, dermatan sulfate; CSc, chondroitin 6-sulfate) and then injected over a heparin-activated sensorchip for 2 min. Response (in RU) at equilibrium was recorded and plotted as the percentage of maximum response (150 RU) (c and d). Overlay of sensorgrams showed binding of chemokines to immobilized heparin. SDF-1α (c) or SDF-1 3/6 (d) was injected over a heparin-activated surface at a flow rate of 50 μl/min for 3 min (from 130 to 310 s), followed by running buffer alone. Each set of sensorgrams was obtained by injecting either SDF-1α or SDF-1 3/6 at (from top to bottom) 208, 139, 92, 62, 41, 27, and 0 nM. The response in RU was recorded as a function of time, and both association and dissociation phases were analyzed with the BiAevaluation 2.1 software.

To investigate further the nature of SDF-1α/HS interactions, we performed a kinetic analysis of SDF-1α binding to heparin. SDF-1α was injected over the Biacore heparin surface in a range of concentrations (usually 0–200 nM) to produce a set of sensorgrams from which association and dissociation phases could be analyzed (Fig. 6, c and d). Biotinylated heparin was immobilized to levels less than 50 pg/mm² (50 RU), and the flow rate was maintained at 50 μl/min so that mass transport problems were minimized (not shown). The sensorgrams could be fitted to an $A + B = AB$ model and analyzed by linear transformation. A plot of $k_1$ versus SDF-1α concentration from the association phase yielded an association rate constant ($k_{on}$) of $2.16 \times 10^5$ M$^{-1}$ s$^{-1}$. A dissociation rate constant ($k_{off}$) of 0.083 s$^{-1}$ was obtained from the direct analysis of the dissociation phase. Thus, binding of SDF-1α to heparin is characterized by an affinity, $K_d (K_d = k_{off}/k_{on})$ of 38.4 nM. By contrast, injection of SDF-1 3/6 in the same concentration range did not produce any binding signal (Fig. 6d). Binding of SDF-1 3/6 was observed at high concentrations (up to 6.5 μM), but the corresponding sensorgrams could not be fitted to any model (data not shown). This finding may be explained by a nonspecific interaction of the modified chemokine with heparin or, alternatively, by the existence of other basic residues apart from the Lys$^{24}$, His$^{25}$, and Lys$^{27}$ cluster that may contribute to the HS-binding site. Altogether, our findings indicate that the Lys$^{24}$, His$^{25}$, and Lys$^{27}$ cluster is critical for physical interaction of SDF-1α with HS.

Since 50 RU of immobilized heparin permitted a maximum binding of 300 RU of SDF-1α, we calculated that each heparin molecule bound to 5 to 6 SDF-1α molecules. Conversely, we estimate that each SDF-1α molecule should occupy an average 6 monosaccharide units along the GAG chain. This finding strongly suggests that the SDF-1α/HS interactions could cause the oligomerization of the chemokine. In keeping with this assumption, incubation of SDF-1α with heparin results in the formation of SDF-1α complexes that can be detected by gel filtration or chemical cross-linking. The calculated apparent molecular weight corresponds to the association of 5–6 molecules of SDF-1α per complex (data not shown).

Functional Properties of SDF-1 3/6, CXCR4 Binding, and Activation—To test if the loss of HS-binding capacity affects the functional properties of SDF-1 3/6, both its ability to bind...
to, and signal through, CXCR4. The affinity of SDF-1 3/6 for CXCR4 was not affected (Kd SDF-1α, 1.67 nM; Kd SDF-1 3/6, 2.12 nM) (Fig. 7a). Moreover, SDF-1 3/6 induced in CEMx174 both chemotaxis (Fig. 7b) and calcium release from intracellular stores (Fig. 7d) as efficiently as SDF-1α (Fig. 7, b and c). Similarly SDF-1α and SDF-1 3/6 did not differ significantly on their capacities to stimulate intracellular calcium mobilization via CXCR4 in HeLa cells (Fig. 8, a and b). In agreement with this observation, enzymatic removal of GAG from the surface of HeLa cells did not affect signal transduction through CXCR4 as assessed by SDF-1α-stimulated calcium mobilization (Fig. 8c). Soluble SDF-1α-HS complexes activate CXCR4 as efficiently as monomeric SDF-1α, confirming that the signaling domain of the protein remains available for interaction with the receptor in the complexes (Fig. 8d). Overall, those findings indicate that SDF-1α-CXCR4- and HS-binding domains are distinct and do not overlap. Moreover, they suggest that the primary consequence of the attachment of SDF-1α to HS on cell surfaces could be the localization and concentration of the chemokine in the surrounding of CXCR4 rather than increasing signaling capacity through the receptor.

**DISCUSSION**

It is conceivable that the interaction with cell surface proteoglycans enhances the physiological activities of SDF-1α. The reported binding of the majority of blood T lymphocytes to the vascular endothelium induced by SDF-1 (36) could be the consequence of haptotactical recruitment and the arrest of circulating cells by the chemokine attached to GAG on the luminal surface of endothelial cells. Like the hematopoietic growth factor GM-CSF (16), SDF-1α could be selectively immobilized by the GAG on the surrounding extracellular matrix or by the membrane of SDF-1α-producing, bone marrow stromal cells. Concentration of SDF-1α in the bone marrow microenvironment could be critical for the pro-hematopoietic activity of the chemokine in embryonic and post-natal life. Similarly, immobilization of SDF-1α by attachment to cell surface or extracellular matrix GAG may be essential for the induction of adhesive cell interactions necessary for tissue modeling (5). The specific interaction of SDF-1α with HS on the plasma membranes of CXCR4-negative epithelial or endothelial cell lines supports the assumption. The inability of SDF-1α to bind CHO mutant cells lacking either any GAGs or, more selectively, HS provides direct evidence that cell membrane-bound HS have the capacity to interact with the chemokine. The remarkable ability of the K15C mAb to recognize the amino-terminal domain of SDF-1α enables us to predict that SDF-1α bound to cell proteoglycans is in a biologically active form. Indeed, the epitope of SDF-1α recognized by K15C mAb is essential for activation of CXCR4 but is not involved in SDF-1α/GAG interaction. SDF-1α variants that lack the two first amino acids (Lys3 and Pro5) fail to induce chemotaxis, intracellular calcium mobilization, or prevent human immunodeficiency virus entry (29). Accordingly, the K15C mAb which recognizes these residues neutralizes the biological activity of SDF-1α. Thus, the specific interaction of the antibody with the HS-complexed chemokine implies that the amino terminus domain of SDF-1α remains exposed and is available for engagement with the interhelical signaling pocket of CXCR4. Moreover, these findings suggest that the SDF-1α domains that interact with CXCR4 or HS are distinct.

The binding of chemokines to GAG is mediated through ionic forces generated by the interactions of negatively charged side chains on GAG with clusters of basic residues in the chemokines (34, 37). Heparin and HS are chemically related molecules, both composed of glucosamines and uronic acids (38). Whereas the majority of glucosamines are N-sulfated and the predominant uronate residues are iduronic acids in heparin, HS contains a variable amount of N-acetylated and N-sulfated glucosamines, and the uronic acids occur as iduranas or glucuronates. Within HS, N-sulfated glucosamines and iduronic acids are usually assembled in short heparin-like sequences separated by extended N-acetylated and glucuronate-rich domains (39). These heparin-like sequences could account for the binding to SDF-1α through direct interaction with clusters of basic, positively charged residues.

The residues responsible for the binding of the CC chemokines (MCP-1 and MIP-1α) to heparin are located in distinct domains outside the carboxyl-terminal α-helix (34, 40). In contrast, mutagenic and biochemical studies of the chemokines IL-8 and PF-4 led to the conclusion that CXC chemokines may bind heparin through a cluster of positively charged residues (mainly Lys) located in the carboxyl-terminal amphipathic α-helix (30–33). Similar conclusions were raised from analysis.
of the carboxyl-terminal α-helix of the chemokines GROα or NAP-2 (41). However, recent findings indicate that the interaction of CXC chemokines with heparin may be mediated by other domains than the carboxyl-terminal α-helix. In PF-4, a loop containing 5 positively charged residues (Arg20, Arg22, His23, Lys27, and Lys49) contributes more than the previously identified carboxyl-terminal end Lys residues to the interaction of the chemokine with heparin (37). This situation is reminiscent of what we find for SDF-1α. Structural differences between SDF-1α and other CXC chemokines were reported showing a distinct packaging of the hydrophobic core, orientation of the α-helix, and distribution and clustering of electric charges (29, 35). Studies of the electrostatic potential map revealed that the carboxyl-terminal α-helix of SDF-1α possesses a predominant negative surface charge (29) that makes its relevance as a potential HS-binding site very unlikely. In contrast, the basic residues Lys34, His35, and Lys27 by Ser confirmed that this cluster of residues represents, or is part of, an exposed region with the required charge complementarity necessary for interaction with heparin and HS. The fact that a truncated, cyclic SDF-1α derivative (amino acids 1–34) encompassing the Lys34-His35-Lys27 sequence failed to associate with heparin strongly indicates that the HS-binding site formed by Lys34-His35-Leu36-Lys27 relies on proper folding of the chemokine. The impaired capacity of SDF-1 3/6 to associate with HS is unlikely to be a consequence of misfolding. Indeed, NMR studies confirm that the global folding of SDF-1 3/6, which occupies and activates CXCR4 as efficiently as SDF-1α, is preserved.

SDF-1α shows an overall positive surface charge (+8) that may contribute to an electrostatic interaction with the highly negatively charged extracellular domains of CXCR4 (net charge −9). On this basis, others (35) have proposed that in addition to the unfolded amino terminus of the chemokine, the cluster formed by residues Lys24, His25, Lys27, and Arg41 could participate in the binding of SDF-1α to CXCR4. The unmodified affinity of SDF-1 3/6 for CXCR4 as compared with SDF-1α does not support this hypothesis and suggests that model should be reconsidered. Moreover, the comparable capacity of SDF-1α and SDF-1 3/6 to activate CXCR4 provided direct evidence that HS- and CXCR4-binding sites of SDF-1α are on opposite faces and do not overlap. According to the three-dimensional structure of SDF-1α, residues Lys24, His25, and Lys27 in the first β-strand fold together in a surface that is distant from the CXCR4 binding and signaling domain. This finding further strengthens our viewpoint that the binding and signaling regions of SDF-1α will remain free for interaction with CXCR4 in SDF-1α-HS complexes.

Although we propose that the cluster formed by Lys24, His25, and Lys27 represents an essential part of the HS-binding site of SDF-1α, we cannot exclude the contribution of other positively charged amino acids. Thus, basic residues (Arg20 and Arg41) located in close proximity in the three-dimensional structure of SDF-1α could, in addition to the putative site Lys24-His25-Leu36-Lys27, form a site with the highest capacity to associate with HS. In this regard, our preliminary evidence indicates that substitution of Arg20 by Ser further decreases the residual low affinity for heparin of SDF-1 3/6. The contribution of Arg41 located in the second β-strand is being investigated.

The relationships between SDF-1α/HS interactions and CXCR4 signaling were addressed. Our results indicate that neither binding nor signaling of CXCR4 are affected by the association SDF-1α with HS. Studies on the consequences of GAG/chemokine interactions for receptor-mediated signal transduction have provided conflicting results. Substitution of basic residues in the carboxyl-terminal α-helix of either PF-4 (31) and MCP-1 (40) prevented binding to GAGs without affecting receptor binding and signal transduction. Similarly, mutation of Lys45 to Ala in MIP-1α which prevents binding to heparin does not affect binding and activation of CCR1 expressed either in parental CHO-K1 or HS-deficient CHO cell mutants (34). In contrast, it was reported that an IL-8 analog with a truncated carboxyl-terminal α-helix does not associate with heparin and lacks receptor binding capacity and signaling activity (30). Furthermore, it was shown that enzymatic removal of cell membrane GAG reduces substantially the capacity of IL-8, RANTES, MCP-1, and MIP-1α to bind CXCR1, CXCR2, or CCR2 expressed on CHO cells (19). A more recent report states that degradation of cell surface-bound GAG affects receptor binding and Ca2+ influx induced by RANTES on peripheral blood leukocytes (42). However, we were unable to confirm these data (data not shown).

The apparent disparity of those findings could be accounted for by the different experimental protocols used. Single amino acid mutations or large deletions of putative GAG-binding domains were made to prevent chemokine/GAG interactions. Enzymatic degradation by glycanases or GAGs-defective cell mu-
plex formation is characterized by a high on-rate constant (k on) which demonstrates that the HS bound to HS. This hypothesis is further supported by the SPR analysis that the chemokine to bind and signal through CXCR4, could be the major biological role of the interaction of SDF-1 with cell-surface HS.

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