Differential Processing of Stromal-derived Factor-1alpha and beta

Explains Functional Diversity

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Abstract

The chemokine SDF-1, which is constitutively expressed in most tissues as SDF-1 α and β resulting from alternative gene splicing, regulates hematopoiesis, lymphocyte homing, B-lineage cell growth and angiogenesis. Since SDF-1α and β are constitutively and ubiquitously expressed, their degradation must serve an important regulatory role. Here we show that SDF-1α and β are secreted as full-length molecules. When exposed to human serum, full-length SDF-1α (1–68) undergoes processing first at the COOH terminus to produce SDF-1α 1–67 and then at the NH₂ terminus to produce SDF-1α 3–67. By contrast, full length SDF-1β (1–72) is processed only at the NH₂ terminus to produce SDF-1β 3–72. Dipeptidyl-peptidase/CD26 is responsible for serum cleavage of SDF-1 α and β at the NH₂ terminus. Serum processing of SDF-1α at the COOH terminus, which has not been previously reported, reduces the ability of the polypeptide to bind to heparin and to cells, and to stimulate B cell proliferation and chemotaxis. The additional processing at the NH₂ terminus renders both forms of SDF-1 unable to bind to heparin and to activate cells. The differential processing of SDF-1 α and β provides biological significance to the existence of two splice forms of the chemokine, and adds a tool to precisely regulate SDF-1’s biological activity by changes in specific activity.

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Introduction

The chemokine stromal-derived factor-1 (SDF-1) is a key regulator of physiological cell motility during embryogenesis and post-natally, and it has been implicated in pathological cell motility associated with tumor metastasis.\(^1\)\(^-\)\(^6\) The biological effects of SDF-1 are mediated by a specific G protein-coupled receptor, CXCR4, which has also been shown to serve as a co-receptor for T-cell tropic HIV-1.\(^7\)\(^-\)\(^13\) SDF-1 is constitutively expressed in stromal cells, endothelial cells, dendritic cells and other cells.\(^1\)\(^,\)\(^4\)\(^,\)\(^14\)\(^-\)\(^16\) On the basis of sequence analysis, SDF-1 is remarkably conserved among species. A single conservative substitution at position 18 (Ile to Val) distinguishes human and murine SDF-1.\(^17\) Two splice forms of SDF-1 have been identified, SDF-1\(\alpha\) and \(\beta\), which have identical amino acid sequence except for the presence of four additional amino acids at the carboxy-terminus of SDF-1\(\beta\).\(^1\)\(^,\)\(^17\) The significance of the existence of two splice forms of SDF-1 has remained unclear.

Structure-function analysis of SDF-1\(\alpha\) (1-67) has identified the NH\(_2\)-terminal amino acids (residues 1 to 8) as critical to CXCR4 binding and activation. Modification of the first two amino acids (K-1 and P-2) alone resulted in loss of receptor activation, and deletion of the first eight amino acids resulted in loss of receptor binding activity.\(^18\) However, the NH\(_2\)-terminus alone, which is a highly mobile region of SDF-1\(\alpha\), was found to be insufficient for receptor binding and activation, and an additional site consisting of a RFFESH motif (residues 12 to 17) was identified as necessary for SDF-1\(\alpha\) docking to CXCR4.\(^18\)\(^,\)\(^19\) Furthermore, the cluster of basic residues K-24, H-25, K-27, and
R-41 was proposed to provide surface charge complementarity for the negatively charged extracellular portion of CXCR4, and to contribute to a heparan sulfate binding site anchoring SDF-1α (1-67) to cell surface proteoglycans.

Recently, proteolytic degradation of endogenous SDF-1 in the bone marrow was identified as playing a critical role in mobilization of hematopoietic progenitor cells to the peripheral circulation. Endogenous SDF-1 provide a retention signal for hematopoietic stem and progenitor cells, which express CXCR4, such that its local degradation would release the cells from this site. In vitro, SDF-1α can be enzymatically cleaved by metalloproteinases, CD26/dipeptidyl-peptidase IV, serine proteases, and leukocyte elastase to generate distinct N-terminally truncated forms of the molecule. However, it is unclear whether these enzymes play a role in the physiologic processing of SDF-1 in vivo.

In vitro, SDF-1α can inhibit T-cell tropic HIV-1 infection of target cells acting as a competitor for CXCR4-mediated viral entry. Recently, we found endogenous SDF-1 to be inactive in normal adult serum, and this could explain why endogenous SDF-1 is generally ineffective as a natural anti-HIV-1 agent. Here, we examined SDF-1 inactivation further. These studies reveal that serum enzymes can selectively cleave SDF-1α both at the carboxy and NH₂ terminus, and SDF-1 β at the NH₂ terminus only, generating molecules with differing specific activity. The different sensitivity of SDF-1 α and β to proteolytic processing provides a mechanism for chemokine functional regulation and reveals a functional difference between the two splice forms of the chemokine.
Materials and Methods

Reagents

Recombinant SDF-1α and SDF-1β were from R&D Systems Inc. (Minneapolis, MN). Synthetic forms of SDFα lacking the C-terminal lysine (amino acids 1-67) or the C-terminal lysine (K) plus the 2 NH2-terminal amino acids lysine (K) and proline (P) (amino acids 3-67) were from Upstate (Lake Placid, NY) or from Dr. Clark-Lewis (University of British Columbia, Vancouver, Canada). SDF-1 protein concentration in PBS was verified by OD measurements at 280 nm and the extinction coefficient of 8200. Antigen-affinity purified goat anti-human SDF-1α and β (BAF-310), antigen-affinity purified goat anti-human SDF-1 β (BAF 311), mouse IgG1 control (hybridoma clone 11711.11), mouse IgG1 monoclonal anti-human/mouse SDF-1 antibody (MAB350), and mouse IgG1 monoclonal anti-human/mouse SDF-1 antibody (MAB 310) were from R&D Systems Inc.; rabbit anti-human SDF-1 antigen-affinity purified antibodies were from PeproTech, Inc. (Rocky Hill, NJ). The CD26/dipeptidyl-peptidase inhibitor AB192 was a gift of Dr. A.M. Lambeir (Univ. of Antwerp, Antwerp, Belgium). Heparin (porcine, intestinal mucosa) was from Sigma (Chemical Co., St. Louis MO).

Cells, flow cytometry, calcium flux, cell proliferation and chemotaxis

Primary human umbilical vein endothelial cells (HUVEC), were prepared from umbilical cord and propagated through passage 5 as previously described. The human Jurkat and BL-41 cell lines were cultured in RPMI1640 (Gibco-Invitrogen Co.) medium supplemented with 10% fetal bovine serum (Biofluids, Rockville, MD). The murine bone marrow stromal cell line MS-5, a gift of Dr. A. Berardi, Ospedale Bambin Gesu, Rome Italy) was cultured in alpha-MEM medium (Gibco-Invitrogen Corporation)
supplemented with 5% fetal bovine serum (Biofluids, Rockville, MD) and 2x10^{-2} M 2-mercaptoethanol. Conditioned medium was prepared in serum-free Opti-MEM (Gibco-Invitrogen Co.) over 24 hr incubation. Flow cytometry was performed as described previously. Briefly, HUVEC (passage 4) were incubated with SDF-1α, SDF-1 β, synthetic SDF-1 (1-67 or 3-67), or recombinant SDF-1 α that had been treated (10 min or 20 h) with 10% human serum. For detection of surface SDF-1, cells were stained with mouse monoclonal anti-SDF-1 antibody (clone MAB350, R&D Systems, 5 µg/ml for 45 minutes at 4°C) followed by a PE-labeled goat anti mouse F(ab’)2 fragment (30 minutes at 4°C; Jackson Immuno Research, West Grove, PA). Calcium flux was detected using a ratio fluorescence spectrometer (Photon Technology International, South Brunswick, NJ). For proliferation assays, the murine DW34 cells, (a gift of Dr. P. Kincade, Oklahoma Medical Research Foundation, Oklahoma City, OK), were cultured in triplicate at 2x10^4 cells/well in RPMI 1640 medium containing 10% fetal calf serum and 50 µM 2-mercaptoethanol in 96-well flat bottom plates for 30 hr at 37°C. Cultures were pulsed with 0.5µCi thymidine (Perkin-Elmer, Boston, MA) for 6 hr, cells harvested, and radioactivity measured. For chemotaxis assays, the human Burkitt lymphoma cell line BL-41 (0.5x10^6 cells/transwell) was incubated (RPMI 1640 medium containing 10 mM HEPES buffer and 0.5% BSA) in the upper chamber of transwells (5 µm pore size, Costar, Cambridge, MA) for 4 hr at 37°C.

Immunoprecipitation, SDS-PAGE and immunoblotting

Cell lysates were prepared in lysis buffer (10mM Tris pH 7.4, 0.5% NP-40, 150 mM NaCl, and protease inhibitor cocktail (Roche Complete, Mannheim, Germany) and dilution (1:2) in tricine sodium dodecyl sulfate (SDS) sample buffer (Novex, San Diego,
CA). Immunoprecipitation, performed as described,\textsuperscript{30} utilized mouse monoclonal anti-
SDF-1 antibody (10 µg/mL, R&D Systems Inc., MAB310), followed by addition of
protein G beads to the antibody/protein mixture. Immunoblotting, performed as
described,\textsuperscript{30} utilized rabbit anti-human SDF-1α antigen-affinity purified polyclonal
antibody (PeproTech, Inc., Rocky Hill, NJ), goat IgG anti-human SDF-1α antigen-
affinity purified antibody (BAF310, R&D Systems), or goat IgG anti-human SDF-
1β antigen-affinity purified (BAF310, R&D Systems).

**SDF-1/heparin binding assays**

Porcine intestinal mucosa heparin (Sigma, Chemical Co., St. Louis, MO) suspended in
0.1M MES (2-N-morpholinoethanesulfonic acid pH5.5) at 1810U/ml was reacted at room
temperature with 1.25mM biotin LC hydrazide (Pierce Biotechnology, Rockford, IL) and
1.25mM EDC (Pierce) for 18hrs. The mixture was extensively dialyzed against PBS to
remove unreacted biotin. Biotinylated heparin (1.8U/ml in PBS) was injected onto the
flow cell of the Sensor Chip SA (Biacore, Uppsala, Sweeden) that is coated with
streptavidin. 375 resonance unites (RU) of biotinylated heparin was immobilized. The
flow cell was then conditioned with several injections of 1.5M NaCl. SDF-1/heparin
binding assays were performed by surface plasmon resonance using a Biacore 3000
system (Biacore). Test samples were diluted in hepes buffer saline containing 3 mM
EDTA and 0.005% Surfactant P20 (HBS-EP, Biacore) maintained at 25 °C and injected
over the heparin-coated or control flow cell surfaces at a flow rate of 50 µl/min.

Association and dissociation phases were evaluated over 2 min. To evaluate the
dissociation phase, the formed complexes were washed with HBS-EP (Biacore) at a flow
rate of 50 µl/min over 2 min. Sensorchips were regenerated with three time pulses of
1.5M NaCl for 30 seconds. Kinetic constants were obtained from the sensorgrams by fitting the data using BIAevaluation software. Dissociation constants (Kd) were calculated from the ratio of dissociation and association rate constants (Kd=koff/kon).

**SDF-1 evaluation by HPLC-ESI-MS**

Intact and truncated forms of SDF-1α and SDF-1 β were analyzed by HPLC-ESI-MS. Capillary HPLC was performed using a MAGIC 2002 binary gradient pump (Michrom BioResources, Inc., Auburn, CA, USA), operating at 40 µL/min. A Michrom capillary precolumn splitter assembly reduced the flow being delivered to a C-18 capillary column (BetaBasic-18, 100x0.32 mm, 3 µm; ThermoHypersil-Keystone Scientific; Bellefonte, PA, USA) to approximately 2 µL/min. Solvent A was HPLC grade water with 0.1% formic acid, and solvent B was HPLC grade acetonitrile with 0.1% formic acid. Five microliter of sample solution was injected into a Michrom Cap-Trap (Michrom BioResources, Inc., Auburn, CA, USA), washed with 20 µL of HPLC grade water, and eluted onto the column by the following gradient: 10% B (0.00min)→10%B (5.00min)→50%B (50min)→100% B (75min)→100% B (100min). The eluent was introduced into a Finnigan LCQ ion trap mass spectrometer (ThermoQuest; San Jose, CA, USA) by a modified nanospray device for mass analysis. No sheath or auxiliary gases were used. The temperature of the heated capillary was set at 200°C. The spray voltage was 2.5 kV. The scan range was set from 150 to 2000 m/z for full scan mode. Centroid data were collected and interpreted using the Xcalibur software package. Masses of the intact and truncated forms of SDF-1α and SDF-1 β were determined using the BIOMASS deconvolution algorithm.
Statistical analysis

Data were compared by Student’s t-test; P values <0.05 were considered significant.

Results

Comparison of SDF-1 produced by MS-5 cells and SDF-1 present in serum

We looked for SDF-1α and SDF-1β in cell lysates and culture supernatants of MS-5 stromal cells. By immunoprecipitation followed by immunoblotting with antibodies that recognize both SDF-1α and SDF-1β or antibodies that preferentially recognize SDF-1β, specific bands corresponding in size to full-length SDF-1α and SDF-1β are identified in MS-5 cell lysates and culture supernatants (Fig. 1A), suggesting that MS-5 stromal cells produce full length SDF-1α and SDF-1β. By the same method, we identified SDF-1-related bands in human sera (Fig.1B). Notably, these bands appeared to be of somewhat lower molecular weight relative to the full-length recombinant SDF-1α and SDF-1β molecules used as controls, suggesting the occurrence of serum cleavage. To test for this possibility, recombinant SDF-1α (2 µg in 40 µl PBS) and SDF-1β (2 µg in 40 µl PBS) were incubated (20 h at room temperature) either alone or with human serum (4 µl in 40 µl PBS containing SDF-1) and subsequently analyzed by mass spectrometry. SDF-1α incubated for 20 h in serum revealed a relative molecular mass of 7606 Da (average, av.), which is 353 lower than that of the control SDF-1α [7959 Da. (av.); residues 1-68, calculated 7959 (average for 4 oxidized cysteines)] and is consistent with the recovery of SDF-1α encompassing residues 3-67, reflecting the loss of two residues (KP, 225 Da) at the NH2 terminus and of one residue (K, 128 Da) at the carboxy terminus (Fig. 2B). In addition, mass spectrometry analysis of SDF-1β incubated for 20 h in serum revealed a
relative molecular mass of 8297 Da (av.), which is lower than that of the control SDF-1β (mass 8522 Da (av.); residues 1-72), and is consistent with the recovery of a truncated SDF-1β species encompassing residues 3-72, reflecting the loss of two NH$_2$ terminal residues (KP) (Fig. 2C).

A kinetic analysis of SDF-1 α conversion from full length (1-68) to the cleaved form 3-67 (lacking KP at the NH$_2$ terminus and K at the carboxy terminus) in the presence of serum is shown in Fig. 3. At the 10 min time point, the mass spectrum of SDF-1 α shows a mass of 7831 Da (av.), corresponding to the loss of a K at either termini. At the 60 min time point, in addition to a main SDF-1 α-related peak (mass 7831 Da (av.); loss of one K), a smaller peak is noted with a relative mass of 7606 Da (av.), corresponding to the SDF-1α 3-67 species, lacking both KP at the NH$_2$ terminus and K at the carboxy terminus. At the 180 min and 20 hr time points, SDF-1 α has mostly converted to the cleaved form 3-67. These results provide evidence for a two-step SDF-1 α processing by serum resulting in the final cleaved product, in which the carboxy-terminal K is rapidly removed followed by the cleavage of he NH$_2$ terminal KP.

**Characterization of SDF-1 cleavage by serum**

CD26/dipeptidyl peptidase IV can cleave dipeptides from the NH$_2$ terminus of polypeptide chains, preferably after a proline or alanine. SDF-1α, which possesses the residues KPV- at the NH$_2$ terminus, can be a substrate for CD26/dipeptidyl peptidase IV. To test whether CD26/dipeptidyl peptidase IV is responsible for serum cleavage of SDF-1α and SDF-1β, we used AB192, a diaryl phosphonate ester, which is a potent and irreversible specific inhibitor of CD26/dipeptidyl peptidase IV. In the presence of 50 µM AB192, most SDF-1α incubated for 20 h with 10% serum had a mass of 7831 Da.
(av.), corresponding to SDF-1α 1-67, whereas in absence of the inhibitor all SDF-1α had a mass of 7606 Da (av.), corresponding to SDF-1 3-67 (Fig. 4A). This protection of SDF-1α cleavage by AB192 provides evidence that CD26/dipeptidyl peptidase IV is responsible for serum cleavage of SDF-1α at the NH₂ terminus, and that the sequence of cleavage events is SDF-1α 1-68 to 1-67 to 3-67.

We tested for the effects of heat on the ability of serum to cleave SDF-1. When SDF-1α was treated for 20 h with human serum that had been heated (56 °C, 30 min), the mass spectra showed the conversion of full length SDF-1α (mass 7959 Da (av.); residues 1-68) to a species with relative mass of 7734 Da. (av.), corresponding to SDF-1α 3-68, consistent with the loss of the NH₂ terminal residues KP (Fig. 4B). Based on these results, we conclude that the serum activity which removes the carboxy-terminal K from SDF-1α is heat sensitive. A comparison of the time course of SDF-1α conversion to the 3-68 amino acids species (in the presence of heat-treated serum) with the time-course of SDF-1α conversion to the 3-67 amino acids species (in the presence of fresh serum) revealed minimal differences with respect to the processing at the NH₂ terminus (not shown), suggesting that cleavage at the carboxy and NH₂ termini are independently regulated.

To test for metal dependency of serum processing of SDF-1 we used EDTA, which was added to fresh serum at 20 mM 15 min prior to testing. Serum containing EDTA was then added to recombinant SDF-1α for 10 min at room temperature. As shown (Fig 4C), most SDF-1α exposed to serum plus EDTA had a mass of 7959 Da (av.), corresponding to the full length molecule (1-68). Instead, 10 min incubation with fresh serum without EDTA reduced SDF-1α to a mass of 7831 Da (av.), corresponding to
a loss of one K. We therefore conclude that serum truncation of SDF-1α at the carboxy terminus is, at least in part, metal dependent.

Several sera were tested at 10% concentration for their ability to cleave SDF-1α over 20 hr incubation at room temperature, including 3 normal human sera, 1 serum from an individual who had received G-CSF for hematopoietic cell mobilization, a bovine serum, a rabbit serum, and a mouse serum. When analyzed by mass spectrometry, all sera were indistinguishable in their ability to process SDF-1α from full-length molecule (1-68) to the cleaved 3-67 species lacking KP at the NH₂ terminus and K at the carboxy terminus (not shown). Thus, these sera contain catalytically active CD26/dipeptidyl peptidase IV, which cleaves SDF-1α and SDF-1β at the NH₂ terminus to generate chemokines lacking the NH₂ terminal peptides KP. Sera also contain an activity that selectively cleaves SDF-1α at the carboxy terminus to generate a chemokine lacking the carboxy terminal K.

**Heparin binding by SDF-1 species and effects of heparin on serum SDF-1 processing**

The binding of full-length SDF-1α and SDF-1β to heparin was evaluated by surface plasmon resonance using the BIAcore system. In preliminary experiments (not shown), injection of SDF-1α (200 nM) over heparin-coated sensor chip containing 375 RU of heparin gave a signal of 2000 RU, whereas similar injection over the control chip caused minimal signal (not shown). Also, pre-incubation of SDF-1α with heparin (200 µg/ml) prior to injection over the heparin-activated sensor chip reduced markedly SDF-1α binding demonstrating the specificity of SDF-1/heparin interaction. Using this system, full-length SDF-1α and β and the SDF-1α 1-67 fragment displayed significant dose-
dependent binding to immobilized heparin, whereas SDF-1α 3-67 displayed little binding (Fig. 5A). Kinetic constants derived from the sensograms revealed that the dissociation equilibrium constants \( (K_d = k_{off}/k_{on}) \) for SDF-1β (1-72) and for SDF-1α (1-68) were similar at 17 and 24 nM, respectively. However, the \( K_d \) for SDF-1α 1-67 was measured at 83 nM, which is more than three-fold higher than that displayed by full length SDF-1α (1-68), suggesting that the carboxy terminal K contributes, in part, to heparin binding. Since the \( K_d \) for SDF-1α 3-67 was measured at 6.4 µM, this form of SDF-1α has lost most of the heparin-binding capacity.

Heparan sulfate proteoglycans, which are structurally related to heparin, are found ubiquitously on cell surfaces. We therefore examined whether the different affinities for heparin exhibited by SDF-1 species might correlate with differences in binding to cell surface proteoglycans. Primary human umbilical vein endothelial cells (HUVEC) naturally express low level SDF-1 on the cell surface detected by specific antibodies (Fig. 5B). Incubation (1 h, 4°C) with full length SDF-1α (1-68) resulted in a dose-dependent increase of surface SDF-1. This binding of SDF-1α to the cell surface is mostly unrelated to receptor engagement as CXCR4 is saturated by SDF-1α at low nanomolar concentrations. Similar results were derived from full length SDF-1 β (not shown).

Synthetic SDF-1α 1-67, or SDF-1α 1-67 generated by serum cleavage (not shown), bound less efficiently than the full-length molecule to HUVEC, such that approximately twice as much protein was required to achieve similar cell surface chemokine levels (Fig. 5B). However, synthetic SDF 1 3-67 (Fig. 5B) or SDF-1 3-67 generated by serum cleavage (not shown) bound poorly to HUVEC even at the highest concentration tested. Thus, the different species of SDF-1 differ in their ability to bind to cell surface.
Since primary endothelial cells naturally express low level surface SDF-1, which appears to be full-length based on its motility on SDS-PAGE and is functional as a regulator of endothelial cell morphogenesis and angiogenesis, we examined whether SDF-1 binding to cell surface proteoglycans might affect SDF-1 cleavage by serum. We took advantage of the observation that the rabbit anti-SDF-1 α and β antibodies (PeproTech) recognize full-length (1-68) SDF-1 α but not the truncated SDF-1α species 1-67, whereas the goat anti-SDF-1 α and β antibodies (R&D Systems) recognize both SDF1 α species equally well. Using these antibodies for detection, we examined serum cleavage of SDF-1α in the presence or absence of heparin. SDF-1α (50 ng) in PBS alone (10 µl) or PBS with heparin (1000 or 50 µg/ml) was exposed for 10 min to human serum (10%). As expected (Fig. 5C), 10 min exposure to fresh serum resulted in cleavage of the carboxy terminal K from SDF-1α as evidenced by low-level recognition by the rabbit antibody and the slightly increased mobility of the specific band visualized on re-probing with the goat anti-SDF-1 antibodies (lane 2). In the presence of heparin at the higher (1000 µg/ml) concentration (lane 6), little SDF-1 cleavage was detected as evidenced by recognition with the rabbit antibodies and relative protein mobility (Fig. 5C, compare lanes 2 and 6). Thus, SDF-1 can bind to heparin and presumably heparan sulfate proteoglycans, which can protect the chemokine from carboxy terminal serum cleavage.

Receptor activation and biological function of SDF-1 species

Binding of chemokines to their receptors leads to a transient rise in Ca²⁺ ions. Recombinant full length SDF-1α and SDF-1β similarly stimulated a dose-dependent rise in Ca²⁺ ions in the T-cell line Jurkat, which expresses CXCR4 (Fig. 6A). The synthetic 1-67 SDF-1α was indistinguishable from the full-length molecule in its ability to cause a
dose-dependent rise in $\text{Ca}^{2+}$ ions in the target Jurkat cells, whereas the synthetic 3-67 SDF-1α failed to induce a rise in $\text{Ca}^{2+}$ ions in the target Jurkat cells even at the highest concentration (250 ng/ml). This confirms the critical importance of the NH$_2$-terminal residues KP for SDF-1α receptor activation, and suggests that the loss of the carboxy terminal K does not impair SDF-1α ability to activate the CXCR4 receptor.

The pre-B cell clone DW34 responds to SDF 1 with increased proliferation. We used DW34 cell proliferation as an in vitro biological assay for SDF-1 (Fig. 6B). Full-length recombinant SDF-1α and SDF-1β similarly induced a dose-dependent rise in DW34 cell proliferation (up to 240 ng/ml, higher concentrations were less stimulatory). The SDF-1α species 1–67 (generated by 10 min exposure to 10% fresh human serum; final human serum concentration 0.1%) also induced a dose-dependent rise in cell proliferation (up to 240 ng/ml, higher concentrations were less stimulatory). However, approximately twice as much SDF-1α 1-67 was required to achieve levels of stimulation comparable to those induced by full length SDF-1α (p<0.01). Control SDF-1α 1–68 incubated for 10 min with 10% heat inactivated serum (which does not cleave the molecule at this time point) was indistinguishable from the full length SDF-1α, providing evidence that decreased proliferation by the serum processed 1-67 SDF-1α was likely attributable to the cleavage. SDF 1α 3–67 (generated by 20 h incubation with fresh human serum) retained minimal ability to stimulated DW34 cell proliferation. The significant loss of SDF-1α biological activity associated with serum processing was confirmed (Fig. 6C) by comparing DW34 cell stimulation by full-length recombinant SDF-1α (1–68) and by the synthetic SDF-1α species 1-67 (p<0.01) and 3-67 (p<0.01).

In additional testing, we compared the chemotactic activity of recombinant SDF-1α 1-68
and synthetic SDF-1α 1-67. The human Burkitt lymphoma cell line BL-41, which expresses CXCR-4 at high levels, 36 displayed a dose-dependent chemotactic response to both SDF-1α species, but the response to SDF-1α 1-67 was significantly reduced (p<0.01) compared to SDF-1α 1-68 at suboptimal concentrations of 5 and 25 ng/ml (Fig. 6D). Thus, two different biological assays detect a loss of activity associated with COOH-terminal processing of SDF-1α.

**Discussion**

We show that SDF-1α and SDF-1β, are expressed and secreted by cells as apparently intact polypeptides but are present in the circulation as processed forms as a result of proteolytic processing. When exposed to serum, rHu SDF-1α (1–68) undergoes processing first at the COOH terminus to produce SDF-1α 1–67 and then at the NH2 terminus to produce SDF-1α 3–67, whereas rHuSDF-1β (1–72) is processed only at the NH2 terminus to produce SDF-1β 3–72. In functional assays, proteolytic removal of the COOH-terminal K from SDF-1α reduces the polypeptide’s ability to bind to heparin and to cells, and to stimulate pre-B cell proliferation and B-cell chemotaxis. The additional processing at the NH2 terminus reduces markedly SDF-1’s ability to bind to heparin and to activate cells. The secretion of full-length SDF-1 α and β by cells and the subsequent processing of SDF-1 α and β by serum to generate SDF-1 species of differing specific activities has not been previously reported. In addition, the existence of a serum protease, which cleaves SDF-1α at COOH terminus and reduces its biological activities, has not been previously suspected. Unlike other chemokines whose expression is induced by specific signals, SDF-1 is constitutively expressed. Based on the new
information presented here, regulated degradation is likely to play a critical role in the control of SDF-1 function.

Previous studies have recognized the potential importance of NH$_2$-terminal proteolytic processing as a modification of functional importance for SDF-1 $\alpha$. Synthetic forms of SDF-1 $\alpha$ lacking the NH$_2$ terminal residues KP had no ability to activate the CXCR4 receptor. $^{18}$ Several metalloproteinases, were reported to cleave four or five, NH$_2$ terminal amino acids from SDF-1. $^{27,28}$ Leukocyte elastase, a protease which removes three amino-terminal residues from SDF-1 $\alpha$, $^{29}$ was proposed to contribute to the release of hematopoietic stem cells from the bone marrow to the peripheral circulation by inactivating SDF-1. $^{23}$ CD26/dipeptidyl peptidase IV was reported to remove the amino-terminal two amino acids from SDF-1 $\alpha$ to generate an inactive chemokine. $^{26}$ However, the physiological significance of CD26/dipeptidyl peptidase processing of SDF-1$\alpha$ had not been previously demonstrated. Based upon the nature of the SDF-1 cleavage products, blocking experiments with a specific inhibitor, and the fact that CD26/dipeptidyl peptidase is present in serum in catalytically active soluble form $^{40}$ and at sufficiently high concentrations, $^{26}$ we conclude that this enzyme is responsible for the NH$_2$-terminal processing and inactivation of SDF-1$\alpha$ and $\beta$ in serum. Since CD26/dipeptidyl peptidase is present on cells of many lineages, it could also regulate SDF-1 biological activity as a cell-bound enzyme. $^{41}$

Prior to the current studies, there was little information on COOH-terminal cleavage of SDF-1$\alpha$. A form of SDF-1$\alpha$ consisting residues 1-67, lacking the COOH-terminal lysine, was purified from the culture supernatant of MS-5 stromal cells, $^{4}$ but the origin and significance of this isoform were not explored. Since this cleaved form of
SDF-1α was biologically active, subsequent studies have utilized the SDF-1α 1-67 variant. Even the crystal and NMR spectroscopy structure of SDF-1α were derived from this variant form of SDF-1α. 18,19 Proteolytic processing at the carboxy-terminus, although uncommon among CXC chemokines, is not unique to SDF-1α. 34,42-45 Noteworthy, the pattern of SDF-1α cleavage at the COOH terminus and metal dependency of the reaction bears similarity to the characteristics of *Armillaria mellea* protease, a metalloenzyme that can work close to the termini of polypeptide chains and displays primary specificity towards peptide bonds where a lysine residue contributes an α-amino-group. 46,47

Studies of the structural basis for SDF-1α biological activity have failed to reveal a role for the COOH domain. However, it is worth noting that such studies have utilized the SDF-1α 1-67 variant, which lacks the carboxy-terminal lysine. Interestingly, it was observed that SDF-1β, which is identical to SDF-1α, except for four additional amino acid residues present at the carboxy terminus, was about two-fold more potent than SDF-1α 1-67. 18 This difference was not explained. We confirm here this difference in activity, and further show that it is attributable to the lack of the carboxy terminal lysine in SDF-1α, which reduces the affinity of SDF-1α for heparin. Earlier experiments showed that the combined substitution of the basic amino acids Lys 24, His 25 and Lys27 by serine generated a variant SDF-1α molecule that had lost heparin-binding capacity. 22 The same cluster of basic amino acids was proposed to contribute to electrostatic interactions with the negatively charged extracellular domain of CXCR4. 19 Based on our results, we suggest that the carboxy terminal lysine (Lys 68), perhaps in conjunction with adjacent lysines at positions 64, 56 and 54, contributes to heparan sulfate binding.
The binding of SDF-1 to glycosaminoglycans, both on the cell surface and bound to the extra-cellular matrix, is believed to be important for chemokine function, presumably because it provides a means for achieving enhanced concentrations of immobilized SDF-1 available for binding to CXCR4. Recently, cell surface-bound SDF-1 was found to mediate lymphocyte attachment and transmigration under physiologic flow conditions. Here, we further show that heparin binding protects SDF-1 from cleavage at the carboxy-terminus, and thus serves to preserve SDF-1 activity in local sites.

Since the COOH-terminal cleavage reduces the heparin-binding affinity and the biological activity of SDF-1 α, such processing would be expected to have a regulatory role in vivo. In particular, this would add another tool to precisely regulate the biological activity of the chemokine by effecting changes in the specific activity of the ligand.

SDF-1 has emerged as a critical regulator for normal development of the nervous, hematopoietic and cardiovascular systems. After birth, the chemokine is constitutively expressed in virtually all tissues, and regulates hematopoiesis, lymphocyte homing and angiogenesis. It is not surprising that in the absence of transcriptional regulation, evolution has devised a complex and hierarchical pathway of degradation.

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Figure Legends

**Figure 1.** Detection of SDF-1α and β in MS-5 cells and in serum.  
A. Culture supernatants from MS-5 cells produced in serum-free medium (8 ml), control serum-free medium (8 ml), cell lysates of MS-5 cells (1x10^6 cell equivalents), recombinant human SDF-1α (20 ng) and SDF-1 β (20 ng) were subjected to immunoprecipitation with anti-SDF-1α + β antibodies, and immunoblotted with antibodies that recognize both SDF-1α and SDF-1 β or antibodies that preferentially recognize SDF-1 β.  
B. Aliquots of recombinant SDF-1α and SDF-1 β and serum from two normal individuals (A and B; pre-cleared with Protein-G) were subjected to immunoprecipitation with anti-SDF-1 α + β antibodies and the immunoprecipitates immunoblotted with antibodies that recognize both SDF-1α and SDF-1 β.

**Figure 2.** Demonstration of SDF-1α and SDF-1 β cleavage by human serum.  
Recombinant SDF-1α and SDF-1 β were incubated for 20 h at room temperature alone or with 10% normal human serum.  
A. Mass spectrometry of recombinant SDF-1α after 20 h incubation at room temperature alone or with 10% normal human serum.  
B. Mass spectrometry of recombinant SDF-1β after 20 h incubation at room temperature alone or with 10% normal human serum.  The average molecular mass after deconvolution of the indicated ion peaks (on the left) is shown on the right.  Representative experiments.

**Figure 3.** Kinetic analysis of SDF-1α cleavage by human serum.  
Recombinant SDF-1α (1-68) was incubated at room temperature for the indicated times (0-20 h) with 10% human serum and analyzed by mass spectrometry.  The average molecular mass after
deconvolution of the indicated ion peaks (on the left) is shown on the right.

Representative experiment.

**Figure 4.** Characterization of SDF-1α cleavage by serum evaluated by mass spectrometry.  
**A.** Fresh human serum was pre-incubated for 15 min at 37°C with or without the CD26/dipeptidyl-peptidase inhibitor AB192 (50 µM), added (10% final dilution) to recombinant SDF-1α, and then incubated at room temperature for 20 h.  
**B.** Fresh human serum was heated at 56°C for 30 min or left at 4°C for 30 min, added (10% final dilution) to recombinant SDF-1 α, and then incubated at room temperature for 20 h.  
**C.** Fresh human serum was pre-incubated at room temperature for 15 min with or without 20 mM EDTA, added (10% final dilution) to recombinant SDF-1α, and then incubated at room temperature for 10 min.

**Figure 5.** SDF-1 affinity for heparin and cell surface proteoglycans.  
**A.** Recombinant full-length SDF-1β (1-72) and SDF-1α (1-68), and synthetic SDF-1α 1-67 and 3-67 were injected at various concentrations (200, 100, 50, 25 and 12.5 nM) over a BIAcore sensorchip containing streptavidin plus biotinylated heparin. The signal (measured in RU) was recorded over 120 sec association phase and 120 sec dissociation phase. The dissociation equilibrium constants (K_d) are shown for each set of data. Representative results from 3 independent experiments performed.  
**B.** SDF-1α binding to HUVEC evaluated by FACS analysis. Recombinant full-length SDF-1α (1-68) and synthetic SDF-1α 1-67 were added at various concentrations (2000, 1000 and 500 ng) to HUVEC (1x10⁶ cells). After incubation (4°C, 30 min), the cells were stained for surface SDF-1.
Representative experiment of 3 performed. C. Recombinant SDF-1α (50 ng) was incubated for 10 min in buffer alone or with heparin (50 or 1000 µg/ml) with or without addition of fresh human serum. Lane 1: SDF-1α alone; lane 2: SDF-1α plus 10% human serum; lane 3: SDF-1α plus heparin 50 µg/ml; lane 4: SDF-1α plus heparin 1000 µg/ml; lane 5: SDF-1α plus heparin 50 µg/ml plus 10% human serum; lane 6: SDF-1α plus heparin 1000 µg/ml plus 10% human serum. Samples were immunoblotted with rabbit (top panel) or goat (bottom panel) antibodies against SDF-1α and SDF-1β.

Figure 6. Receptor engagement, stimulation of cell proliferation, and chemotaxis by SDF-1 species.

A. Calcium fluxes in Fura-2 loaded Jurkat cells in response to varying concentrations of recombinant SDF-1α (1-68) and SDF-1β (1-72), and synthetic SDF-1α (1-67) and SDF-1α 3-67. The results reflect fluorescence measurements as a ratio of excitation at 340 and 380 nm. B. DW-34 pre-B cell proliferation in response to varying concentrations (1.9-240 ng/ml) recombinant full-length SDF-1β (1–72), SDF-1α (1-68), SDF-1α 1-67 (generated by 10 min incubation with 10% fresh human serum at room temperature), and SDF-1α 3-67 (generated by 20 h incubation with fresh serum). The results reflect the mean cpm/culture (±SEM) of triplicate determinations; representative experiment of 5 performed. C. DW-34 pre-B cell proliferation in response to varying concentrations (12.5-100 ng/ml) recombinant full-length SDF-1α (1-68), synthetic SDF-1α 1-67, and synthetic SDF-1α 3-67. The results reflect the mean cpm/culture (±SEM) of triplicate determinations; representative experiment. D. BL-41 B-cell chemotaxic response to
recombinant full-length SDF-1α (1-68) and synthetic SDF-1α (1-67) at varying concentrations (5-625 ng/ml). The results reflect the mean (±SEM) number of cells that have migrated to the lower chamber in 5 experiments performed.
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Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Differential processing of stromal-derived factor-1 α and β explains functional diversity

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