The \( \alpha \)-Chemokine, Stromal Cell-derived Factor-1\( \alpha \), Binds to the Transmembrane G-protein-coupled CXCR-4 Receptor and Activates Multiple Signal Transduction Pathways*

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The \( \alpha \)-chemokine stromal cell-derived factor (SDF)-1\( \alpha \) binds to the seven transmembrane G-protein-coupled CXCR-4 receptor and acts to modulate cell migration and proliferation. The signaling pathways that mediate the effects of SDF-1\( \alpha \) are not well characterized. We studied events following SDF-1\( \alpha \) binding to CXCR-4 in a model murine pre-B cell line transfected with human CXCR-4. There was enhanced tyrosine phosphorylation and association of components of focal adhesion complexes such as the related adhesion focal tyrosine kinase, paxillin, and Crk. We also observed activation of phosphatidylinositol 3-kinase. Wortmannin, a selective inhibitor of phosphatidylinositol 3-kinase, partially inhibited the SDF-1\( \alpha \)-induced migration and tyrosine phosphorylation of paxillin. SDF-1\( \alpha \) treatment selectively activated p44/42 mitogen-activated protein kinase (Erk 1 and Erk 2) and its upstream kinase mitogen-activated protein kinase kinase but not p38 mitogen-activated protein kinase, c-Jun amino-terminal kinase or mitogen activated protein kinase kinase. We also observed that SDF-1\( \alpha \) treatment increased NF-\( \kappa \)B activity in nuclear extracts from the CXCR-4 transfectants. Taken together, these studies revealed that SDF-1\( \alpha \) activates distinct signaling pathways that may mediate cell growth, migration, and transcriptional activation.

Chemokines and their receptors have recently received considerable attention because of their emerging role in immune and inflammatory responses, hematopoiesis, and HIV\( ^1 \) infection (1–7). Four classes of chemokines have been defined based on the arrangement of the conserved cysteine (C) residues of the mature proteins; the CXC or \( \alpha \)-chemokines; CC or \( \beta \)-chemokines; C or \( \gamma \)-chemokines; and CXXXC or \( \delta \)-chemokines (1, 2, 7).

The CXC chemokine, stromal cell-derived factor (SDF-1\( \alpha \)), was first cloned from mouse bone marrow and characterized as a pre-B cell growth-stimulating factor (8–13). Two isoforms, SDF-1\( \alpha \) and SDF-1\( \beta \), have been identified that are encoded by a single gene and arise by alternative splicing (9). SDF-1\( \alpha \) is widely expressed and, in addition to its effects on pre-B cells, is a potent chemotactic factor for monocytes, T-lymphocytes, and CD34\(^+\) human progenitor cells (8–13). Knock-out mice lacking SDF-1\( \alpha \) protein show abnormalities in B cell lymphopoiesis, bone marrow myelopoiesis, and also have nonfatal ventricular septal defects (11).

SDF-1\( \alpha \) was recently shown to be a ligand for the chemokine receptor CXCR-4 (14–18). CXCR-4 is a seven transmembrane G-protein-coupled receptor (19, 20). Recently, it has been shown that CXCR-4 expression can be regulated by receptor phosphorylation-dependent and -independent mechanisms (19). A diversity of white cells including peripheral blood lymphocytes, monocytes, thymocytes, pre-B cells, and dendritic and endothelial cells express the CXCR-4 receptor (14, 21–26). CXCR-4 has been shown to act as a co-receptor for the binding of T cell tropic HIV-1 strains (6, 15–17). SDF-1\( \alpha \) and its various analogues can inhibit CXCR-4-mediated HIV-1 infection in vitro (27, 28).

Despite the increasingly prominent role of SDF-1\( \alpha \) and its receptor CXCR-4 in the regulation of cell proliferation, migration, and HIV infection, relatively little is known about the signaling pathways that may mediate these effects (19, 20). In this study, we show that SDF-1\( \alpha \) stimulation in CXCR-4 transfectants results in the increased phosphorylation of focal adhesion components, including the related adhesion focal tyrosine kinase (RAFTK/Pyk2), Crk, and paxillin. SDF-1\( \alpha \) treatment activated the p44/42 MAP kinases (Erk 1 and 2), PI-3 kinase, and NF-\( \kappa \)B. These studies indicate that activation of CXCR-4 results in modulation of signaling molecules and transcription factors that mediate changes in the cytoskeletal apparatus and also regulate cell growth.

EXPERIMENTAL PROCEDURES

Reagents and Materials—RAFTK antibodies were generated using C domain glutathione S-transferase fusion proteins as described previously (29). Serum R-4250 was chosen for further studies based on its titer in enzyme-linked immunosorbent assay. This antisera does not cross-react with FAK and recognizes both human and murine forms of RAFTK. Monoclonal anti-phosphotyrosine antibody (4G10) was a generous gift from Dr. Brian Druker (Oregon Health Sciences University, Portland, OR). Purified antibodies to JNK, p38 MAP kinase, p44/42 MAPK, and recombinant GST-c-Jun amino-terminal proteins (1–79 amino acids) were obtained from Santa Cruz Laboratories (Santa Cruz, CA). Antibodies to paxillin and Crk were obtained from Transduction Laboratories, Inc. (Lexington, KY). Monoclonal antibodies to CXCR-4 and the isotype control were from Pharmingen (San Diego, CA). Electrophoresis reagents were obtained from Bio-Rad. The protease inhibitors leupeptin and a\(_2\)-antitrypsin as well as all other reagents were obtained from Sigma. Wortmannin was obtained from Calbiochem, and...
CXCR-4-mediated Signal Transduction Pathways

**Fig. 1. Expression of CXCR-4.** L1.2 or CXCR-4 L1.2 cells (1 × 10⁶) were stained with anti-PE-coupled CXCR-4 antibody and analyzed by FACS. Class-matched PE-coupled mouse IgG was used as a negative control.

**Fig. 2. Activation of RAFTK upon SDF-1α stimulation of CXCR-4 L1.2 cells.** Cell lysates (1 mg) from unstimulated cells (0) or cells stimulated with SDF-1α (100 ng/ml) for the indicated times were immunoprecipitated with RAFTK antibody or normal rabbit serum as the control. A, the immune complexes were resolved on 7.5% SDS-PAGE gels and subjected to serial immunoblotting with anti-phosphotyrosine antibody (top panel) and RAFTK antibody (bottom panel). B, the immunoprecipitates were subjected to autokinase assay. C, the immune complexes were subjected to in vitro kinase assays using poly(Glu:Tyr), 4:1, as a substrate. The [γ-³²P]ATP incorporated proteins were resolved on 10% SDS-PAGE, followed by autoradiography. C, control.

The nitrocellulose membrane was from Bio-Rad. Indo-1 acetoxymethyl ester (Indo-1 AM) was purchased from Molecular Probes (Eugene, OR).

**Construction of CXCR-4 Stable Transfectants—** We used a murine pre-B lymphoma cell line, L1.2, for the transfection studies. CXCR-4 cDNA, tagged at the amino terminus with a Flag epitope (Asp-Tyr-Lys), was subcloned into the pcDNAIII expression vector. The DNA was stably transfected into the L1.2 cells as described (30–32), and G418-selective medium was used to select for transfectants. Cell-surface expression of CXCR-4 on the transfectants was confirmed by FACS analysis.

**Cell Culture—** The L1.2 cells were grown at 37 °C in 5% CO₂ in RPMI 1640 with 10% fetal calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate, 50 µg/ml penicillin, 50 µg/ml streptomycin, and 0.8 mg/ml Geneticin (G418) (Life Technologies, Inc.).

**FACS Analysis—** The CXCR-4 L1.2 transfectants (1 × 10⁶) were washed twice with ice-cold PBS, 5% FCS, and then analyzed by flow cytometry to determine the levels of surface expression of these receptors.

**Calcium Flux Assay—** The CXCR-4 transfectants were washed with RPMI 1640 and resuspended at 10 × 10⁶ cells/ml in the RPMI medium. The cells were loaded with Indo-1 AM (Molecular Probes) by adding 5 µl of Indo-1 solution to the 10 × 10⁶ cells that were suspended in 1 ml of RPMI solution and incubated for 45 min at 37 °C. Cells were diluted to 1 × 10⁶/ml, treated with SDF-1α, and analyzed for calcium mobilization by flow cytometry (Coulter Electronics, Hialeah, FL) as described (33). Calcium flux assays and all other subsequent signaling assays were repeated at least three times.

**Stimulation of Cells—** Cells were washed twice with RPMI 1640 (Life Technologies, Inc.) and resuspended at 10 × 10⁶ cells/ml in the same medium. Cells were starved for 4 h at 37 °C and then stimulated with different concentrations of SDF-1α at 37 °C for various periods. After stimulation, cells were lysed in modified RIPA buffer (50 µl Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, leupeptin, and pepstatin, 10 mM sodium vanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate). Total cell lysates (TCL) were clarified by centrifugation at 10,000 × g for 10 min. Protein concentrations were determined by protein assay (Bio-Rad). Cell lysis, immunoprecipitation, immunoblotting, kinase assays, and autophosphorylation assays were carried out as described below.

**Immunoprecipitation and Western Blot Analysis—** For the immunoprecipitation studies, identical amounts of protein from each sample were clarified by incubation with protein A-Sepharose CL-4B or Gammabind plus Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C. The beads were removed by brief centrifugation, and the solution was incubated with different primary antibodies for each experiment for 4 h or overnight at 4 °C. Antibody-antigen complexes were immunoprecipitated by incubation with 50 µl of protein A-Sepharose or Gammabind Sepharose (10% suspension) for 4 h at 4 °C. The Sepharose beads were washed three times with modified RIPA buffer and one time with PBS to remove the nonspecifically bound proteins. Bound proteins were solubilized in 40 µl of 2× Laemmli buffer and further analyzed by immunoblotting. The samples were separated on SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were blocked and probed with primary antibody for 3 h at room temperature (RT) or 4 °C overnight. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescent (ECL) system (Amersham Pharmacia Biotech). Monoclonal antibody (4G10, IgG2a) was used for Western blot analysis of the phosphotyrosine protein.

**RAFTK Kinase Assays—** In vitro kinase assays were performed as described earlier (34). The cell lysates immunoprecipitated with RAFTK antisera were washed twice with RIPA buffer and once in kinase buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 5 mM MgCl₂, 5 mM MnCl₂, 100 mM Na₃VO₄). For the in vitro kinase assays, the immune complex was incubated in kinase buffer containing 25 µg of poly(Glu:Tyr): 4:1, 20–50 kDa (Sigma), and 5 µCi of [γ-³²P]ATP at RT for 30 min. The reaction was stopped by adding 2× SDS sample buffer and boiling the sample for 5 min at 100 °C. Proteins were then separated on 10% SDS-PAGE and detected by autoradiography. Normal rabbit serum was used as a negative control. The autophosphorylation assay was carried out by incubating the immune complex in kinase buffer containing 5 µCi of [γ-³²P]ATP at RT for 30 min. The reaction was stopped by adding 4× SDS sample buffer and boiling the sample for 5 min. Proteins were then separated on SDS-PAGE and detected by autoradiography.

**JNK, p44/42 MAP Kinase, and p38 MAP Kinase Assays—** The JNK assay was performed as described earlier (35). Briefly, cell lysates were immunoprecipitated with JNK antibody (Santa Cruz Biotechnology).
RESULTS

SDF-1α Treatment Induces Ca²⁺ Flux in CXCR-4 L1.2 Transfectants—
Human CXCR-4 cDNA was stably transfected into the murine pre-B lymphoma cell line, L1.2. Untransfected and transfected cell lines were analyzed for CXCR-4 expression. As shown in Fig. 1, CXCR-4 transfectants expressed high levels of the receptor in these transfected cells. Signal transduction by the binding of ligands to their cognate chemokine receptors involves characteristic calcium fluxes. To confirm that the CXCR-4 L1.2 cells expressing functional human CXCR-4 receptors retained this fundamental signaling property, the cells were treated with SDF-1α, and calcium fluxes were monitored by FACS analysis. SDF-1α treatment induced characteristic calcium fluxes in the CXCR-4-L1.2 cells (data not shown).

SDF-1α Treatment Activates RAFTK—
RAFTK, a recently identified member of the focal adhesion kinase family, has been shown to be activated by various growth factors and chemokines (34, 35–40). We therefore investigated whether SDF-1α activates RAFTK in L1.2 transfectants. We observed rapid phosphorylation of endogenous murine RAFTK in the transfected L1.2 cells upon SDF-1α stimulation (Fig. 2A). We also observed an increase in the intrinsic tyrosine kinase activity of RAFTK following SDF-1α treatment, as determined by an autophosphorylation assay and in vitro kinase assay in which poly(Glu:Tyr) (4:1) was used as an exogenous substrate (Fig. 2, B and C).

SDF-1α Treatment Induces Tyrosine Phosphorylation and Association of Focal Adhesion Components—
Paxillin and Crk, which are components of focal adhesions, have been shown to play an important role in cell migration and adhesion (41–43). Thus, we sought to investigate whether SDF-1α treatment of CXCR-4-L1.2 cells would result in changes in the phosphoryl-

**RESULTS**

**SDF-1α Treatment Induces Ca²⁺ Flux in CXCR-4 L1.2 Transfectants—**Human CXCR-4 cDNA was stably transfected into the murine pre-B lymphoma cell line, L1.2. Untransfected and transfected cell lines were analyzed for CXCR-4 expression. As shown in Fig. 1, CXCR-4 transfectants expressed high levels of the receptor in these transfected cells. Signal transduction by the binding of ligands to their cognate chemokine receptors involves characteristic calcium fluxes. To confirm that the CXCR-4 L1.2 cells expressing functional human CXCR-4 receptors retained this fundamental signaling property, the cells were treated with SDF-1α, and calcium fluxes were monitored by FACS analysis. SDF-1α treatment induced characteristic calcium fluxes in the CXCR-4-L1.2 cells (data not shown).

**SDF-1α Treatment Activates RAFTK—**RAFTK, a recently identified member of the focal adhesion kinase family, has been shown to be activated by various growth factors and chemokines (34, 35–40). We therefore investigated whether SDF-1α activates RAFTK in L1.2 transfectants. We observed rapid phosphorylation of endogenous murine RAFTK in the transfected L1.2 cells upon SDF-1α stimulation (Fig. 2A). We also observed an increase in the intrinsic tyrosine kinase activity of RAFTK following SDF-1α treatment, as determined by an autophosphorylation assay and in vitro kinase assay in which poly(Glu:Tyr) (4:1) was used as an exogenous substrate (Fig. 2, B and C).
The state of these proteins. As shown in Fig. 3, A and B, SDF-1α stimulation resulted in enhanced tyrosine phosphorylation of paxillin and Crk. Equivalent amounts of these proteins were present in each lane (bottom panels).

It has been shown that upon activation by cytokines the adaptor molecule Crk associates with other components of focal adhesions to enhance signaling (41–43). We therefore investigated whether SDF-1α treatment results in changes in the association of Crk with paxillin and RAFTK. As shown in Fig. 4, A and B, Crk associates with paxillin and RAFTK, and this association was enhanced upon SDF-1α treatment.

**SDF-1α Activation Stimulates PI-3 Kinase Activity and Its Inhibition Reduces the Tyrosine Phosphorylation of Paxillin—**We investigated the effect of SDF-1α on PI-3 kinase activity. PI-3 kinase is an important mediator of chemotaxis in certain cell types (36, 44–46). As shown in Fig. 5A, SDF-1α treatment increased the PI-3 kinase activity of CXCR-4 L1.2 transfectants. The role of PI-3 kinase in mediating SDF-1α-induced migration was further examined using the selective PI-3 kinase inhibitor, wortmannin. As shown in Fig. 5B, SDF-1α-induced migration of CXCR-4 L1.2 transfectants, and pretreatment with wortmannin inhibited the SDF-1α-induced migration of cells (Fig. 5C). Further examination revealed that wortmannin treatment also partially inhibited the SDF-1α-induced tyrosine phosphorylation of paxillin (Fig. 5D). Equal amounts of paxillin were present in each lane (Fig. 5D, bottom panel).

**SDF-1α Activation Stimulates the MAP Kinase Pathway—**It has previously been shown that RAFTK acts upstream of MAP kinase and the JNK pathway (39, 47). Recently, we have also shown that the β-chemokine, MIP-1β, stimulated JNK kinase in human CCR5 L1.2 transfectants (33). We further showed that RAFTK mediates activation of JNK in these cells. Fig. 6A shows that SDF-1α treatment of CXCR-4 L1.2 cells resulted in
complexes were subjected to in vitro kinase reactions in the presence of the respective substrates for each kinase as indicated. The reaction products were separated on 15% SDS-PAGE and detected by autoradiography. Normal rabbit IgG immunoprecipitate was used as a negative control. C, control.

A

Time (min) 0 0.5 2 5 10 30 60 C

MBP

MAP kinase

B

Time (min) 0 0.5 2 5 10 30 60 C

GST c-Jun

p38 kinase

C

Time (min) 0 0.5 2 5 10 30 60 C

MBP

JNK kinase

FIG. 6. SDF-1α treatment selectively activates p42/42 MAP kinase (Erk 1 and 2) but not JNK or p38 MAP kinase. CXCR-4 L1.2 cell lysates (500 μg) unstimulated (0) or stimulated with SDF-1α (100 ng/ml) for the indicated times were immunoprecipitated with either anti-p44/42 MAP kinase (Erk 1 and 2) (A), anti-JNK kinase (B), or anti-p38 MAP kinase (C). The immunocomplexes were subjected to in vitro kinase reactions in the presence of the respective substrates for each kinase as indicated. The reaction products were separated on 15% SDS-PAGE and detected by autoradiography. Normal rabbit IgG immunoprecipitate was used as a negative control. C, control.

A

Time (min) 0 0.5 2 5 10 30 60 C

p42

M KK-1

B

Time (min) 0 0.5 2 5 10 30 60 C

JNK

M KK-4

FIG. 7. SDF-1α treatment activates M KK-1 but not M KK-4. CXCR-4 L1.2 cell lysates (500 μg) unstimulated (0) or stimulated with SDF-1α (100 ng/ml) for the indicated times were immunoprecipitated with either M KK-1 antibody (A) or M KK-4 antibody (B). The immunocomplexes were subjected to in vitro kinase reaction in the presence of specific substrates for each kinase as indicated. The reaction products were separated on 15% SDS-PAGE and detected by autoradiography. Immunoprecipitate with purified IgG was used as a negative control. C, control.

The rapid activation of p44/42 MAP kinase. However, no significant effect on JNK or p38 MAP kinase was observed (Fig. 6, B and C). Furthermore M KK-1, which acts upstream of p44/42 MAP kinase, was activated whereas M KK-4, which acts upstream of JNK, was not altered in response to SDF-1α treatment (Fig. 7, A and B). Wortmannin had no effect on SDF-1α-mediated p44/42 MAP kinase activation (data not shown).

SDF-1α Modulates NF-κB in CXCR-4 L1.2 Transfectants—Various inflammatory and growth-promoting cytokines activate NF-κB activity, which is known to regulate transcription of growth-promoting host genes as well as HIV proviral genes via the long terminal repeat element (48, 49). To determine whether chemokines may modulate its activity, the binding activity of the NF-κB target sequence was tested with electrophoretic mobility shift assay using nuclear proteins prepared from SDF-1α-treated cells. As shown in Fig. 8, SDF-1α treat-


teins included RAFTK, Crk, and paxillin. RAFTK, also known as Pyk2 or Cak-β, has been shown to play important roles in various signal transduction pathways (39–43). RAFTK has been shown to be phosphorylated by α- and β-chemokines and HIV-1 envelope glycoproteins from T-tropic and macrophage-tropic strains (20). Paxillin has also been demonstrated to be phosphorylated by the β-chemokines, MIP-1β and RANTES, and to participate in integrin-mediated signal transduction pathways (33, 51). Crk is a docking protein and plays an important role in assembling signaling complexes (41–43). We have observed enhanced association of Crk with paxillin and RAFTK upon SDF-1α stimulation. Phosphorylation of the focal adhesion components RAFTK, paxillin, and Crk and their association with each other may result in the formation of signaling complexes inducing changes in the cytoskeleton that mediate SDF1-α-triggered chemotaxis.

Prior studies have demonstrated that PI-3 kinase and its metabolic products play an important role in signaling pathways related to chemotaxis (46). We observed an increase in PI-3 kinase activity after SDF-1α treatment. Inhibition of PI-3 kinase activity by wortmannin reduced SDF-1α-induced cell migration and phosphorylation of paxillin. These results suggest that PI-3 kinase and paxillin phosphorylation play important roles in the SDF-1α-induced cell migration. The data also suggest that PI-3 kinase acts upstream in the signal transduction pathway leading to the tyrosine phosphorylation of paxillin. PI-3 kinase activity has previously been shown to be important for the tyrosine phosphorylation of paxillin mediated by platelet-derived growth factor (52). However, integrin-induced tyrosine phosphorylation of paxillin does not appear to require PI-3 kinase activity (53).

We also investigated the effects of SDF-1α on the downstream pathways that are known to mediate transcriptional activation. We observed that SDF-1α selectively activated p44/42 MAP kinase (Erk 1 and 2), but not p38 MAPK or JNK. Interleukin 8, another member of the CXC chemokine family, has been shown to activate both p44/42 and p38 MAPK but not JNK (54, 55). Interleukin 8 differs from SDF-1α by having the characteristic ELR motif (glutamic acid-leucine-arginine) immediately preceding the first cysteine residue near the amino terminus. β-Chemokines activate p38 MAPK and JNK (33). This suggests selective regulation of MAP kinase pathways by different types of α- and β-chemokines. The p44/42 and p38 MAP kinases and JNK kinases are regulated by dual specificity kinases which are specific for each MAPK subgroup, thus allowing for their independent regulation. We found that SDF-1α activates MKK-1 (also known as MEK-1), which selectively activates p44/42 (Erk) subgroups (56). However, M KK-4 (also known as SEK-1 or JNKK), which activates both p38 MAPK and JNK but does not activate p44/42 (Erk) subgroups (57, 58), was not activated by SDF-1α. These data provide further new information on the specificity of chemokine signaling effects.

Our studies suggest that SDF-1α can lead to the activation of NF-κB, a nuclear transcriptional factor. NF-κB activation has been extensively studied in inflammatory and immunoregulatory cells and has been shown to regulate gene expression in lymphocytes in response to antigen and cytokine stimulation (48). NF-κB is also an important transcription factor in HIV proviral gene expression (49). Activated NF-κB has also been shown to regulate the expression of various chemokines by binding to their promoter regions (59). Recently, NF-κB was shown to have anti-cell death functions. NF-κB activation by tumor necrosis factor-α or ionizing radiation suppresses the signal for apoptosis (60, 61). SDF-1α induction of NF-κB activity could result in enhanced cell proliferation, expression of other chemokine genes, as well as enhanced transcription of HIV gene products. This last point may be relevant to the current consideration of therapeutically using chemokines like SDF-1α to inhibit HIV infection.

Our results provide new information on the signal transduction pathways utilized by the α-chemokine receptor CXCR-4 and show how SDF-1α may act on a molecular level to regulate cell migration and growth. We have shown that SDF-1α stimulation induces the tyrosine phosphorylation and association of focal adhesion components RAFTK, paxillin, and Crk which may result in the formation of signaling complexes. It appears that SDF-1α stimulation of PI-3 kinase activity is essential for its chemotactic effects. Furthermore, SDF-1α selectively activates the p44/42 MAP kinase (Erk) but not the p38 MAP kinase or JNK. These results suggest that specific functions of various chemokines may be regulated by different members of the MAP kinase family.

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