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

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Ramesh K. Gajdusek, Stephanie A. Brubaker, Joshua Meyer, Parmesh Dutt, Yangming Yang, Shixin Qin, Walter Newman, and Jerome E. Groopman§

From the Divisions of Experimental Medicine, and Hematology/Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215 and ‡LeukoSite, Inc., Cambridge, Massachusetts 02142

The α-chemokine stromal cell-derived factor (SDF)-1α 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-1α are not well characterized. We studied events following SDF-1α 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-1α-induced migration and tyrosine phosphorylation of paxillin. SDF-1α 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-1α treatment increased NF-κB activity in nuclear extracts from the CXCR-4 transfectants. Taken together, these studies revealed that SDF-1α 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 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 α-chemokines; CC or β-chemokines; C or γ-chemokines; and CXXXC or δ-chemokines (1, 2, 7).

The CXC chemokine, stromal cell-derived factor (SDF-1α), was first cloned from mouse bone marrow and characterized as a pre-B cell growth-stimulating factor (8–13). Two isoforms, SDF-1α and SDF-1β, have been identified that are encoded by a single gene and arise by alternative splicing (9). SDF-1α 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-1α protein show abnormalities in B cell lymphopoiesis, bone marrow myelopoiesis, and also have nonfatal ventricular septal defects (11).

SDF-1α 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-1α and its various analogues can inhibit CXCR-4-mediated HIV-1 infection in vitro (27, 28).

Despite the increasingly prominent role of SDF-1α 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-1α 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-1α treatment activated the p44/42 MAP kinases (Erk 1 and 2), PI-3 kinase, and NF-κB. 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 assay 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 α-antitrypsin as well as all other reagents were obtained from Sigma. Wortmannin was obtained from Calbiochem, and...
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-Glu:Tyr), 4:1, as a substrate. The 32P-incorporated proteins were clarified by incubation with protein A-Sepharose CL-4B or GammaBind plus Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C. The cell lysates immunoprecipitated with RAFTK antibody or normal rabbit serum as a 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 immune precipitates were subjected to autophosphorylation assay. C, the immune complexes were subjected to in vitro kinase assays using poly(Glu:Tyr), 4:1, as a substrate. The 32P-incorporated proteins were resolved on 10% SDS-PAGE, followed by autoradiography. C, control.

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).
SDF-1α stimulation. CXCR-4 L1.2 transfectants were stimulated with SDF-1α (100 ng/ml) for different times. Lysates (1 mg) from un-treated or treated cells were immunoprecipitated with either anti-paxillin antibody (A) or anti-Crk antibody (B). Immunocomplexes were resolved on 7.5% or 12% SDS-PAGE, respectively, and subjected to autoradiography. Rabbits IgG was used as a negative control.

In Vitro PI-3 Kinase Assay—PI-3 kinase assays were performed as described (36). Briefly, equal amounts of protein from each sample were immunoprecipitated with either anti-phosphotyrosine antibody (4G10) or control IgG. Western blots were developed by washing the samples three times each with PBS containing 1% Nonidet P-40, followed by 0.5 mM Tris containing 0.5% lithium chloride and then by TE buffer. Samples were resuspended in 20 μl of TE buffer, 20 μl of phosphoinositol (10 μg, Avanti Polar Lipids, Alabaster, AL), and 10 μl of ATP mix (1 mM HEPES, 10 μM ATP, 1 mM MgCl₂, 5 μCi of [γ-32P]ATP) and incubated at RT for 10 min. The reaction was stopped by adding 60 μl of 2× sample buffer and 160 μl of chloroform:methanol (1:1, v/v). Lipids were separated on a preparative silica TLC plate using a solvent system of chloroform:methanol:water:ammonium hydroxide (20%) (35:35:5:5.7), followed by autoradiography at −80 °C.

Chemotaxis Assay—The chemotaxis assay was performed in 24-well plates containing 5-μm porosity inserts (Costar Corp., Kennebunk, ME). Cells grown in RPMI 1640 medium containing 10% FCS were washed twice and suspended as 10 × 10⁶ cells per ml in RPMI 1640 and H199 medium (1:1) containing 0.5% bovine serum albumin. Chemokines were then added to the bottom wells, and 100 μl (1 × 10⁶) of cells were loaded onto the inserts. Cells migrating to the bottom well were collected after 2–4 h and counted on a flow cytometer. To assess the effect of wortmannin on migration, the cells were resuspended in medium containing different concentrations of wortmannin, and the chemotaxis assays were done as described above.

Electrophoretic Mobility Shift Assay—Double-stranded oligonucleotides containing the consensus binding site for NF-κB (5′-AGT GAC TTT CCC AGG C-3′) were labeled with [γ-32P]ATP (3,000 Ci/mmol, NEN Life Science Products) using polynucleotide kinase (Promega, Madison, WI) according to established procedures. 10 μg of nuclear extract were incubated with labeled DNA (0.4 ng, 4,000 cpm) for 10 min at RT in the presence of DNA-binding buffer and 250 ng of poly(dI-dC) oligomer (Boehringer Mannheim) as described previously (37). The complexes were then separated on a 7.5% polyacrylamide gel and autoradiographed. The results shown are representative of findings from three independent experiments.

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 (94, 38–40). 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 phosphory-
vation 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 the 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).

**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
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 MKK-1, which acts upstream of p44/42 MAP kinase, was activated whereas MKK-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).

**DISCUSSION**

The chemokine receptor CXCR-4 and its cognate ligand SDF-1α have recently gained considerable interest because of their role in HIV pathogenesis and hematopoietic progenitor migration (11, 12, 15, 16). Furthermore, SDF-1α has been shown to be essential for B cell lymphocyte development, since SDF-1α null mice have a major defect in both fetal liver and bone marrow B cell lymphopoiesis (11). However, relatively little is known about the signaling pathways mediated by CXCR-4 upon binding of its ligand SDF-1α or of HIV envelope proteins (19, 20). In this study, we have used human CXCR-4-transfected murine pre-B lymphoma L1.2 cells as a model to investigate the signal transduction pathways mediated by the CXCR-4 receptor upon binding to its cognate ligand, SDF-1α.

Chemokines have been shown to affect chemotaxis which plays a key role in a variety of cell responses including development, wound repair, inflammation, and metastasis (1–7). Coordinated regulation of multiple steps including adhesion and cytoskeleton modification is required for these processes. Various cytokines that induce chemotaxis modulate the formation and function of focal adhesions (50). These adhesions are cytoskeletal structures that form adherent contacts with the extracellular matrix. Changes in the structure of the actin cytoskeleton have been shown to be associated with phosphorylation of focal adhesion components. In the present study, SDF-1α enhanced the phosphorylation and association of proteins involved in the formation of focal adhesions. These pro-
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 (20, 33, 34, 38–40). RAFTK has been shown to be phosphorylated by α- and β-chemokines and HIV-1 envelope glycoproteins from T- and macrophage-tropic strains (20). Paxillin has also been demonstrated to be phosphorylated by the β-chemokines, MIP-1α 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 SDF1α-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 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. Erk 1 and 2, also known as MAPK and JNK, are serine-threonine kinases. The p44/42 and p38 MAP kinases and JNK kinases are regulated by dual specificity protein kinases (54, 55). Interleukin 8 differs from SDF-1α and 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 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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REFERENCES

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