HIV Coreceptor Downregulation as Antiviral Principle: SDF-1\(\alpha\)-dependent Internalization of the Chemokine Receptor CXCR4 Contributes to Inhibition of HIV Replication

By Ali Amara,* Sylvie Le Gall,‡ Olivier Schwartz,‡ Jean Salamero,¶ Monica Montes,i Pius Loetscher,¶ Marco Baggiolini,¶ Jean-Louis Virelizier,* and Fernando Arenzana-Seisdedos*

From the *Unité d’Immunologie Virale and ‡Laboratoire Rétrovirus et Transfert Générique, Institut Pasteur, 75724 Paris Cedex 15, France; †Laboratoire Molécules Mammifères du Transport Intracellulaire, C entre N ational de la Recherche Scientifique UMR 144, Institut Curie, 75248 Paris Cedex 05, France; ‡Laboratoire d’Immunologie Cellulaire, C entre N ational de la Recherche Scientifique URA 625, C ERI, 75013 Paris, France; and †Laboratoire K ocher Institut, Université de Bern, C H-3000 Bern 9, Switzerland

Summary

Ligation of CCR5 by the CC chemokines RANTES, MIP-1\(\alpha\) or MIP-1\(\beta\), and of CXCR4 by the CXC chemokine SDF-1\(\alpha\), profoundly inhibits the replication of HIV strains that use these coreceptors for entry into CD4\(^+\) T lymphocytes. The mechanism of entry inhibition is not known. We found a rapid and extensive downregulation of CXCR4 by SDF-1\(\alpha\) and of CCR5 by RANTES or the antagonist RANTES(9-68). Confocal laser scanning microscopy showed that CCR5 and CXCR4, after binding to their ligands, are internalized into vesicles that qualify as early endosomes as indicated by colocalization with transferrin receptors. Internalization was not affected by treatment with Bordetella pertussis toxin, showing that it is independent of signaling via G\(_i\)-proteins. Removal of SDF-1\(\alpha\) led to rapid, but incomplete surface reexpression of CXCR4, a process that was not inhibited by cycloheximide, suggesting that the coreceptor is recycling from the internalization pool. Deletion of the COOH-terminal cytoplasmic domain of CXCR4 did not affect HIV entry, but prevented SDF-1\(\alpha\)-induced receptor downregulation and decreased the potency of SDF-1\(\alpha\) as inhibitor of HIV replication. Our results indicate that the ability of the coreceptor to internalize is not required for HIV entry, but contributes to the HIV suppressive effect of CXC and CC chemokines.

Expression of CD4 is necessary but not sufficient for productive infection of human cells with HIV (1, 2). The existence of an additional recognition site was postulated several years ago (3–4), and it was recently shown that some chemokine receptors fulfill such a function (5–10). CXCR4 and CCR5 are the major HIV coreceptors (5–7, 10), although similar functions were also reported for CCR2b and CCR3 (8, 9). It has been shown that the CC chemokines, RANTES, MIP-1\(\alpha\), and MIP-1\(\beta\), which are agonists for CCR5, inhibit entry of primary, non-syncytium-inducing (NSI) strains that are preferentially isolated at early stages of the infection (11). The CXC chemokine, SDF-1\(\alpha\), the ligand of CXCR4, inhibits cell fusion and infection by HIV strains of the syncytium-inducing (SI) phenotype that are usually isolated at late, symptomatic stages of the disease (12, 13).

Chemokines act via seven-transmembrane domain receptors that couple to heterotrimeric G\(_i\)-proteins. Their antiviral activity is thought to depend on competition for the binding of the HIV envelope (Env) glycoprotein gp120 to chemokine receptors (14, 15). It has been reported that mere occupancy of HIV coreceptors by chemokines, in the absence of G\(_i\)-protein-mediated signaling, is sufficient for inhibition of HIV infection. In fact, RANTES inhibits HIV infection of cells treated with B. pertussis toxin, and a CCR5 antagonist, RANTES(9-68), was shown to prevent infection by primary NSI isolates (14, 16). The chemokines, however, could also inhibit viral entry by downregulating the expression of their receptors which may be endocytosed upon ligand binding, as previously shown for the IL-8 and MCP-1 receptors (17, 18). Therefore, we have studied this process and the effect of receptor uptake on the HIV suppressive activity of chemokines. In this paper we describe a rapid, profound downregulation of CXCR4 by SDF-1\(\alpha\)
and of CCR5 by RANTES and RANTES(9-68) in different cells, and show that the HIV suppressive effect of chemokines is markedly reduced when receptor endocytosis does not occur.

Materials and Methods

DNA Expression Vectors, Cells, and Chemokines. The CXCR4 WT expression vector contains the LESTR cDNA (19) cloned in a pC DNA3 plasmid (Invitrogen, The Netherlands). The CXCR4 ΔCyt vector was prepared by a PCR-based strategy, by deleting the last 41 amino acids that correspond to the COOH-terminal intracytoplasmic domain of CXCR4. A PCR-synthesized CCR5 DNA insert deleted of the stop codon was fused to a red-shifted variant of the wild-type Green Fluorescent Protein (GFP) in a pEGFP plasmid (Clontech, CA). All the PCR products were sequenced by the dyeodeoxy method. HeLa is a human epithelial cell line. U373-C4D4 LTR lacZ cell clone derived from the human glioblastoma cell line U373-MG (20), was transduced with human CD4 and carries an integrated E. coli β-galactosidase gene driven by a HIV-1 LTR. U373-MG cells, contrary to CEM and HeLa cells, do not express CXCR4 constitutively (21). HeLa-CCR5-GFP cells (clone P4-C5) were derived from HeLa-CD4 LTR lacZ (clone P4-2) (22) cotransfected with the CCR5-GFP vector and a plasmid carrying a hygromycin-resistance cassette. CHO is a Chinese hamster epithelial cell line. The CHO-CCR5-GFP cell clone was established by transfecting the CCR5-GFP vector that encodes a neomycin resistance gene. The CCR5-GFP receptor proved to be fully competent to support viral entry of a pcDNA3 plasmid (Invitrogen, The Netherlands). The CXCR4 gene driven by a HIV-1 LTR. U373-CD4-LTR lacZ (clone P4-C5) were derived from HeLa-CD4 LTR lacZ (clone P4-2) (22) cotransfected with the CCR5-GFP vector and a plasmid carrying a hygromycin-resistance cassette. CHO is a Chinese hamster epithelial cell line. The CHO-CCR5-GFP cell clone was established by transfecting the CCR5-GFP vector that encodes a neomycin resistance gene. The CCR5-GFP receptor proved to be fully competent to support viral entry

Indirect Immunofluorescence Staining. CHO- and HeLa-CCR5-GFP cells were cultured on glass coverslips in 24-well plates. CEM cells were seeded on coverslips coated with poly-l-lysine. The cells were treated for 30 min at 37°C with 200 μM SDF-1α, RANTES, or RANTES(9-68) (23). After incubation with ligands, the cells were washed and fixed for 20 min in 3.7% paraformaldehyde-PBS, washed again in PBS, mounted in 133 μg/ml Mowiol (HOECHST), 33% glycerol, 133 mM Tris-HCl, pH 8.5, and analyzed by confocal microscopy. After fixation, CEM cells were incubated for 15 min in PBS and 0.1 M glycine, then permeabilized with 0.05% saponin in PBS supplemented with 0.2% BSA for 15 min. The cells were then incubated for 45 min at room temperature with the anti-CXCR4 monoclonal antibody 12GS (24). A kind gift of Dr. J. Hoxie (University of Pennsylvania Medical Center, Philadelphia, PA), and labeled with a secondary, fluorescein-conjugated (FITC) goat anti-mouse IgG antibody. For staining with iron-loaded human transferrin coupled to rhodamine (Tf-RITC), cells were deprived of transferrin for 30 min at 37°C in serum-free medium, and then incubated for 30 min at 37°C with Tf-RITC along with the appropriate chemokine or antagonist. Confocal laser scanning microscopy and double-fluorescence analysis were performed with a TCS4D confocal microscope (Leica, Nussloch, Germany) interfaced with Argon/Krypton lasers. Simultaneous double-fluorescence acquisitions were performed using the 488- and the 568-nm laser lines to excite FITC and RITC dyes using a 100× oil immersion Plan APO objective (NA = 1.4). The fluorescence was detected with the appropriate double-fluorescence dichroic mirror and band pass filters, and measured with blue-green and red side sensitive one photomultipliers.

Cell Transfection. Flow Cytometry Analysis and Recording of Intracellular Ca2+ ([Ca2+]i) Changes. Cells (8 × 104) were resuspended in Dulbecco's modified Eagle's medium, supplemented with 10% FCS, 1-4 μg of the appropriate DNA expression vectors, and 12 μg of a non-coding carrier DNA plasmid. Electroporation was performed in 4-mm cuvettes at 220 V, 960 μF in a Bio-Rad Gene Pulser. For flow cytometry, cells (5 × 106) were incubated for 1 h with anti-CXCR4 and subsequently labeled with a secondary, PE-conjugated goat anti-mouse IgG antibody (Southern Biotechnology, Birmingham, AL). After staining, cells were fixed in 1% paraformaldehyde-PBS containing 0.2% BSA and CXCR4 expression was analyzed with a FACScan® cytofluorimeter (Becton Dickinson, Mountain View, CA). CXCR4 expression in monocyte-depleted PBMC was studied on gated CD4+ cells. The cells were incubated with anti-CXCR4 and labeled with the PE-conjugated secondary antibody followed by a FITC-conjugated anti-CD4 antibody (Leu3a, Becton Dickinson). Real-time recordings of [Ca2+] were performed with an IMSTAR imaging system in Fura-2 loaded cells as described previously (25). To remove cell-bound SDF-1α, the cells were exposed for 1 min at 37°C to an acid buffer, pH 3.0, consisting of 50 mM glycine and 100 mM NaCl buffer (106 cells per ml), and then centrifuged and resuspended into the appropriate medium (17).

Viral Infections. U373-C4D4-LTR lacZ cells transfected with either the CXCR4 WT or CXCR4 ΔCyt vectors were seeded in microplate wells (104 cells per well) in a final volume of 200 μl. 24 h after transfection the cells were incubated with infectious supernatants obtained from MT4 cell cultures infected with the HIV-1 molecular clone NL4-3 (HIV-1NL4-3) (40 ng of HIV-1 p24 per well). SDF-1α or RANTES were added to the cells 20 min before infection and were present during the whole culture period. Cultures were carried out in triplicate. Infected cells were identified by staining for β-galactosidase. HIV-1(ΔEnv)G is a pseudotyped HIV-1 NL4-3 variant with a replacement of the Env protein with the Env (G) glycoprotein of the vesicular stomatitis virus (VSV). To generate infectious supernatants of HIV-1(ΔEnv)G, plasmids encoding the HIV-1 NL4-3 proviral DNA deleted of the env gene and the Env (G) glycoprotein of VSV were cotransfected in HeLa cells. U373-C4-D4 LTR lacZ cells were infected with HIV-1(ΔEnv)G infectious supernatants (4 ng of HIV-1 p24 per well).

Results and Discussion

Chemokine Receptor Internalization. After incubation with SDF-1α, surface expression of CXCR4 in CEM cells (Fig. 1a), monocyte-depleted PBMC (Fig. 1b), or HeLa cells (Fig. 1c), was analyzed by flow cytometry using anti-CXCR4. Before analysis, SDF-1α-treated cells were washed in an acidic glycine buffer, a procedure previously adopted to dissociate IL-8 from its receptors (17). The washing removed receptor-bound SDF-1α that could interfere with the binding of anti-CXCR4 (data not shown). As shown in Fig. 1a–c, SDF-1α induced a profound decrease of cell surface receptors in all three cell types. The downregulation of CXCR4 was specific for SDF-1α and was not ob-
CEM cells treated with SDF-1α decrease of the number of surface-detectable receptors. Of chemokine up to 60 min did not lead to an additional regulation of CXCR4 was not dependent on signaling by expression (Fig. 1). Pretreatment with SDF-1α (data not shown) did not prevent the decrease of cell surface receptor expression (Fig. 1d). T is indicates that the observed downregulation of CXCR4 was not dependent on signaling by G proteins. T he downregulation was rapid. It was already detectable 5 min after addition of SDF-1α and progressed for ~20 min (Fig. 1e). Further incubation in the presence of chemokine up to 60 min did not lead to an additional decrease of the number of surface-detectable receptors.

T o investigate the fate of CXCR4 after downregulation, CEM cells treated with SDF-1α and washed with the acidic buffer were further incubated at 37°C in the absence of the chemokine. Surface re-expression of CXCR4 was detected within the first 15 min (Fig. 1f), but no further increase in receptor density was observed for up to 60 min. R e-expression was not dependent on de novo protein synthesis since it was not affected by the presence of cycloheximide. T hese results suggest that after binding of SDF-1α CXCR4 is internalized and re-expressed at the cell surface by a recycling mechanism. R ecycling is likely to account for the fact that the expression of CXCR4 never decreased below 10–25% even at high SDF-1α concentrations and prolonged incubation times.

Ligand-induced endocytosis of CXCR4 was assessed in CEM cells by confocal laser scanning microscopy. In the absence of chemokine, CXCR4 was mainly detected at the cell surface. Exposure to SDF-1α induced a dramatic redistribution of the staining that is consistent with the intracellular accumulation of the receptor. T his effect was specific for SDF-1α and was not observed when the cells were exposed to RANTES (Fig. 2a, CXCR4). T he subcellular distribution of internalized CXCR4 was studied by simultaneous labeling with anti-CXCR4 (green) and human transferrin coupled to rhodamine (Tf-RITC, red) which is taken up into early endosomes. As shown in Fig. 2a (CXCR4 + Tf-RITC), both markers were largely colocalized, as revealed by the yellow spots, indicating a significant accumulation of internalized CXCR4 in early endosomes. Similar experiments were performed to study the ligand-induced endocytosis of CCR5. S ince anti-CCR5 antibodies for immunodetection were not available, we used cell clones permanently expressing CCR5 fused to the fluorescence marker GFP (CHO-CCR5-GFP and HeLa-CCR5-GFP). Addiction of RANTES to either clone induced receptor endocytosis (Figs. 2b, b and c, CCR5). Colocalization with Tf-RITC, in analogy to the above experiments, shows that CCR5-GFP accumulates preferentially in early endosomes, as indicated by the clusters of yellow spots in the juxtanu-
Figure 2. Internalization of CXCR4 (a) and CCR5 (b and c) were analyzed by confocal laser scanning microscopy in CEM (a), CHO-CCR5-GFP (b), and HeLa-CCR5-GFP (c) cells after exposure to 200 nM SDF-1α, RANTES or RANTES(9-68) for 30 min at 37°C. –, untreated cells; CXCR4, cells labeled with anti-CXCR4 and a FITC-conjugated secondary antibody; CCR5, autofluorescence of CCR5-GFP; CXCR4+Tf-RITC and CCR5+Tf-RITC, simultaneous detection of Tf-RITC (red) and either CXCR4 or CCR5-GFP (green). Yellow spots indicate colocalization of chemokine receptor and Tf-RITC.
clear region (Fig. 2 b, CCR5 + Tf-RITC). The occurrence of internalized CXCR4 or CCR5 that do not colocalize with Tf-RITC (green spots) might reflect the transfer of receptors to late endosomes. The CCR5 antagonist RANTES (9-68), which was previously shown to block infection by monocytotropic HIV isolates despite its inability to elicit Ca\textsuperscript{2+} mobilization and chemotaxis (16), was as effective as RANTES as inducer of CCR5-GFP internalization (Fig. 2, b and c). This is in agreement with the observation that the SDF-1α-dependent internalization of CXCR4 was not affected by pertussis toxin and confirms that chemokine receptor endocytosis does not require signaling via Gi-proteins.

**Figure 3.** Effect of deletion of the COOH-terminal cytoplasmic domain of CXCR4. (a) HeLa cells were transiently transfected with the CXCR4 WT or the CXCR4 ΔCyt expression vector, along with a plasmid encoding the reporter gene GFP (pEGFP). 24 h later, the cells were incubated for 45 min at 37°C in the presence or absence of 300 nM SDF-1α, labeled with anti-CXCR4, and analyzed by flow cytometry. Expression of GFP allows to distinguish transfected (GFP+) and nontransfected (GFP-) cells. After transfection with CXCR4 WT or CXCR4 ΔCyt, SDF-1α-dependent down-regulation of the endogenous and the transfected receptor were monitored in GFP+ and GFP- cells, respectively. (–) HeLa cells were transiently transfected with the CXCR4 WT or CXCR4 ΔCyt expression vector, along with the pEFGP plasmid. 24 h later, the cells were incubated for 45 min at 37°C with 300 nM SDF-1α or with 20 ng/ml PMA, labeled with anti-CXCR4 and surface expression of CXCR4 was analyzed by flow cytometry in GFP+ cells. (c) CHO cells were transfected with either the CXCR4 WT or the CXCR4 ΔCyt expression vectors and were loaded 48 h later with Fura-2. CHO control cells were transfected with vector DNA alone (pcDNA3). Recordings of [Ca\textsuperscript{2+}]\textsubscript{i} changes after stimulation with 200 nM of SDF-1α are shown.

COOH-terminally truncated receptor. It was previously shown that endocytosis of IL-8 receptors requires an intact COOH-terminal cytoplasmic domain (26). The role of the corresponding domain of CXCR4 was, therefore, investigated using HeLa cells that were transiently transfected with a vector expressing a CXCR4 cDNA deleted of the last 41 amino acids (CXCR4 ΔCyt). HeLa cells were chosen because they constitutively express CXCR4, and allow to perform simultaneous analysis of SDF-1α effects on endogenous and transfected CXCR4. Cotransfection of either CXCR4 WT or CXCR4 ΔCyt with a GFP reporter vector permitted to distinguish transfected from nontransfected
cells. Incubation with SDF-1α induced a profound down-regulation of CXCR4 WT, but did not affect the surface expression of the COOH-terminally truncated receptor, CXCR4 ΔCyt (Fig. 3, a and b). As expected the endogenous CXCR4, in nontransfected cells which are identified by the lack of GFP fluorescence, was also markedly down-regulated by SDF-1α (Fig. 3 a). PMA had a similar effect. It downregulated CXCR4 WT (Fig. 3 b) as well as the endogenous CXCR4 (not shown), but not CXCR4 ΔCyt (Fig. 3 b). These results are in agreement with a previous report showing downregulation of CXCR4 by PMA in human T lymphocytes (27), and suggest that phosphorylation of serines and threonines in the COOH-terminal region are involved in internalization. It has been shown that phosphorylation of the COOH-terminal domain is essential for arrestin-mediated uptake of seven-transmembrane receptors via clathrin-coated pits (28, 29).

CHO cells transiently expressing either CXCR4 WT or CXCR4 ΔCyt were used to assess receptor signaling. As shown by the changes in the cytosolic free Ca²⁺ concentration in response to SDF-1α, the wild-type and the COOH-terminally truncated receptor were equally capable of eliciting a response indicating that the COOH-terminal domain of CXCR4 is not required for Gα-protein coupling (Fig. 3 d). This result is in agreement with a previous report indicating that deletion of the COOH-terminal domain of the IL-8 receptors did not affect signal transduction (26).

HIV Entry. The capacity of the CXCR4 ΔCyt molecule to act as coreceptor for HIV-1 entry was studied in the human, U373-CD4 LTRlacZ astrocytoma cell clone which does not express endogenous CXCR4 and carries an integrated β-galactosidase reporter gene driven by the HIV-1 LTR. Both CXCR4 ΔCyt and the wild-type receptor could be expressed with similar efficiency, as assessed by FACS analysis and by scoring of HIV-1 infected cells. It was, therefore, possible to study the role of receptor occupancy and receptor internalization as mechanisms for the HIV suppressive activity of SDF-1α, using U373-CD4 LTRlacZ cells that were transiently transfected with CXCR4 ΔCyt or CXCR4 WT. HIV infection was inhibited by SDF-1α in a concentration-dependent manner in cells expressing the truncated or the wild-type receptor, confirming that both bind the chemokine and can act as HIV coreceptors. However, the efficacy of SDF-1α as an inhibitor of infection was markedly lower in cells expressing CXCR4 ΔCyt (Fig. 4). In cells with the wild-type receptor SDF-1α has two effects that decrease infectability, competition for HIV binding and a drastic reduction of receptor numbers by rapid endocytosis. Both mechanisms operate at the same time suggesting that the full antiviral effects of SDF-1α is a combination of competition for receptor binding by the virus and receptor endocytosis. In cells bearing the COOH-terminally truncated receptor downregulation cannot occur, and protection by SDF-1α is less efficient because receptor density remains high. Findings similar to those in the U373-CD4 LTRlacZ glioblastoma cells were obtained in HElA-CD4 LTRlacZ cells, where SDF-1α was less efficient as an inhibitor of HIV replication when CXCR4 ΔCyt was expressed (data not shown). The selectivity of SDF-1α as an inhibitor of CXCR4-dependent infection is indicated by two lines of evidence. RANTES was unable to block entry of the CXCR4-dependent HIVNL4-3 viral clone in U373-CD4 LTRlacZ cells, and SDF-1α did not inhibit infection by an Env-deleted HIVNL4-3 clone pseudotyped with the Env (G) protein of VSV (Fig. 4).

We have shown that CXCR4 and CCR5 are rapidly down-regulated by endocytosis when the cells are exposed to the appropriate chemokine ligand. The mechanism of receptor internalization was ligand dependent, but was clearly dissociable from chemokine-induced Gα-protein signaling. In fact, CXCR4 internalization was not affected by Gα-protein inactivation with pertussis toxin, and CCR5 internalization was induced by the antagonist RANTES (9-68) which binds to CCR5 but does not activate Gα-proteins. On the other hand, no internalization was observed in cells expressing CXCR4 deleted of its COOH-terminal, cytoplasmic domain, although Gα-protein-dependent [Ca²⁺]i changes were not impaired.

Deletion of the COOH-terminal domain does not affect the ability of CXCR4 to act as a coreceptor for HIV entry. If receptor endocytosis is prevented by truncation, however, SDF-1α is clearly less efficient as an inhibitor of HIV
replication. Our present results show that the ability of the receptor to internalize is not required for HIV entry, but may contribute to the HIV suppressive effect of chemokine ligands by reducing the density of coreceptors. Viral entry is a complex phenomenon in which gp120 attachment to CD4 creates a high-affinity binding site for the coreceptor, leading to membrane fusion (30, 31). Chemokines thus appear to exert two types of anti-HIV activities, competition for HIV-1 binding, and downregulation of coreceptor surface expression. Both processes are functionally linked since receptor occupancy triggers internalization, and are likely to synergize with each other.

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Address correspondence to Fernando Arenzana-Seisdedos, Unité d’Immunologie Virale, Institut Pasteur, 75724 Paris, Cedex 15, France.

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