Chemokines and their receptors play an important role in immune surveillance and inflammatory response. These molecules have also been implicated in regulating the immune response by regulating the localization and activation of leukocytes. The chemokine receptors CCR5 and CXCR4, which act as co-receptors along with CD4 for HIV docking and entry, are down-modulated by their respective ligands, MIP-1α/SDF-1α or by the HIV envelope protein, gp120. We have studied the role of the proteasome pathway in the down-regulation of these receptors. Using the yeast and mammalian two-hybrid systems, we observed that the CCR5 receptor is constitutively associated with the ζ subunit of proteasome. Immunoprecipitation studies in CCR5 L1.2 cells revealed that this association was increased with MIP-1β stimulation. The proteasome inhibitors, lactacystin and epoxomicin, attenuated MIP-1β-induced CCR5 down-modulation as detected by fluorescence-activated cell sorter analysis and confocal microscopy. The proteasome inhibitors also inhibited the SDF-1α and gp120 protein-induced down-modulation of the CXCR4 receptor in Jurkat cells. However, the inhibitors had no significant effect on the gp120-induced internalization of the CD4 receptor. These inhibitors also blocked cognate ligand-mediated chemotaxis but had no effect on the HIV-1α-induced p44/42 MAP kinase or MIP-1β-induced p38 kinase activities, thus indicating differential effects of the inhibitors on signaling mediated by these receptors. These results indicate that the CCR5 and CXCR4 receptor down-modulation mechanisms and chemotaxis mediated by these receptors are dependent upon proteasome activity.

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Chemokines and their receptors play a critical role in host immune surveillance and important mediators of human immunodeficiency virus (HIV) pathogenesis and inflammatory response. The chemokine receptors CCR5 and CXCR4, which act as co-receptors along with CD4 for HIV docking and entry, are down-modulated by their respective ligands, MIP-1α/SDF-1α or by the HIV envelope protein, gp120. We have studied the role of the proteasome pathway in the down-regulation of these receptors. Using the yeast and mammalian two-hybrid systems, we observed that the CCR5 receptor is constitutively associated with the ζ subunit of proteasome. Immunoprecipitation studies in CCR5 L1.2 cells revealed that this association was increased with MIP-1β stimulation. The proteasome inhibitors, lactacystin and epoxomicin, attenuated MIP-1β-induced CCR5 down-modulation as detected by fluorescence-activated cell sorter analysis and confocal microscopy. The proteasome inhibitors also inhibited the SDF-1α and gp120 protein-induced down-modulation of the CXCR4 receptor in Jurkat cells. However, the inhibitors had no significant effect on the gp120-induced internalization of the CD4 receptor. These inhibitors also blocked cognate ligand-mediated chemotaxis but had no effect on the HIV-1α-induced p44/42 MAP kinase or MIP-1β-induced p38 kinase activities, thus indicating differential effects of the inhibitors on signaling mediated by these receptors. These results indicate that the CCR5 and CXCR4 receptor down-modulation mechanism and chemotaxis mediated by these receptors are dependent upon proteasome activity.

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AIDS Research (to R. K. G.). The costs of publication of this article were shown to inhibit HIV infection in vitro (13–16). Subsequently, stromal-derived factor-1α (SDF-1α/CXCL12), the cognate ligand for the CXCR4 receptor, was also shown to inhibit infection by T-tropic viruses (17–19). It has been demonstrated that the ligand-induced endocytosis of CCR5 and CXCR4 plays an important role in inhibiting HIV entry into the cells (20, 21). Furthermore, effective anti-HIV activity of the chemically modified form of the CC chemokines correlates with the ability of these ligands to induce irreversible and efficient down-regulation of CCR5 (20). Recently, Brandt et al. (50) have shown that the ability of chemokines to block the infection of HIV depends upon the efficiency of the ligand to internalize the CCR5 receptor.

Although the trafficking properties of chemokine receptors are important in HIV infection and immune regulation, the downstream events occurring after internalization of these receptors are not well defined. Receptor phosphorylation-dependent and -independent mechanisms have been shown to regulate CXCR4 receptor internalization (22–25). The cytoplasmic tail of CCR5 has been shown to play a major role in receptor internalization and signaling. Recently, a degradation motif was identified in the C-terminal domain of CXCR4 (26). Moreover, the agonist-mediated ubiquitination of the CXCR4 receptor was observed to be blocked when the lysine residues in this degradation motif were mutated. In the present studies, we further delineate the signaling pathway that regulates CXCR4 and CCR5 down-modulation induced by cognate ligands and the HIV envelope protein gp120. We have observed that the proteasome pathway plays a major role in the down-modulation of these receptors.

Proteasomes have been shown to play an important role in regulating the levels of several cell surface receptors including the interleukin-2 receptor and platelet-derived growth factor receptor (27–29). Proteasomes are also essential for the production of peptides for MHC (major histocompatibility complex) class I antigen presentation (30). In addition, proteasomes have also been implicated as controlling the level of certain transcriptional factors and cell cycle regulatory proteins (31, 32). It has been shown that proteasome inhibitors blocked interference with HIV gag polypeptide processing and decreased the infectivity and release of secreted virions (33). HIV-1 encoded Env and Vpu protein-mediated degradation of the CD4 receptor are also dependent on proteasomes (34, 35).

Because proteasomes play a major role in viral processing, we thought it interesting to determine whether the proteasome pathway also regulates HIV co-receptor expression. Moreover, it was of interest to find out whether the proteasome pathway

mal T cell expressed and secreted; HIV, human immunodeficiency virus; MAP, mitogen-activated protein; CAT, chloramphenicol acetyltransferase; SDF, stromal-derived factor; PBL, peripheral blood lymphocytes.
also regulates chemotaxis mediated by these receptors. Our present work suggests that chemokine CXCR4 and CCR5 receptor down-modulations are indeed regulated by the protosomal pathway. Furthermore, our studies have also shown that the proteasome pathway also regulates CXCR4 and CCR5-mediated chemotaxis but has no effect on MAP kinases induced by these receptors.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The C-terminal cytosolic tail of the CCR5 receptor was amplified using primers with flanking EcoRI and SaI sites and inserted into the C-terminal cytosolic tail of the CCR5 (36). The sense primer was 5'-CCCGAATTCCCATCATGCCTTTG-3' and the antisense primer was 5'-TCTGTACGTGCGGGCGTTAACGGCCACAGATTTC-3'. For the yeast two-hybrid screening, the PCR fragment was ligated to the EcoRI and XhoI sites of pLEXA-BD (CLONTECH), a 2-his 3-plasmid, to generate a CCR5-LexA fusion. The sequence of the construct was confirmed by dideoxy sequencing. For the mammalian two-hybrid interaction assay, the PCR fragment was ligated to the EcoRI and SaI sites of pM (CLONTECH) to generate a fusion protein of the CCR5 cytosolic tail and DNA-binding domain of GAL4. The positive clones obtained from the yeast two-hybrid screening were excised from pB2AD (CLONTECH) using the EcoRI and XhoI sites and ligated to the pVP16 vector (CLONTECH), generating a fusion protein with a VP16 activating domain.

**Yeast Two-hybrid Interaction Assay**—The yeast two-hybrid assay was performed according to the instruction manual (CLONTECH). Briefly, EGY48-p80 yeast cells with the lacZ gene were transformed with the pLexA-CCR5 cytosolic tail and the Jurkat cell cDNA library in pB2AD using the lithium acetate method. The transformants were selected on galactose/raffinoseuriaHisTrpLeu-plates. The positive clones were plated on galactose/raffinoseuriaHisTrpLeu-5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal). Blue colonies were used as putative positive interacting clones. The plasmids from the positive clones were rescued in Escherichia coli KC8, and the clones were identified by sequencing and homology searches using BLAST.

**Mammalian Two-hybrid Interaction Assay**—For interaction studies in mammalian cells, the CLONTECH Matchmaker™ mammalian two-hybrid assay system and 293T cells were used. The pM-CCR5 cytosolic tail fused to the GAL4 DNA binding domain and the pVP16 plasmid containing the positive clones from the yeast two-hybrid system fused to the VP16 transactivation domain were constructed. The reporter gene assay was performed using the pG5CAT plasmid (CLONTECH). 293T cells were transfected with the pM and pVP16 constructs along with the reporter constructs pG5CAT and pCMV-β-Gal. For measurement of CAT activity, the proteins were normalized against the specific activity of β-galactosidase. CAT activity was determined by measuring the incorporation of [14C]chloramphenicol (PerkinElmer Life Sciences) into butyryl-CoA (CLONTECH) and analyzed by thin layer chromatography.

**Isolation of Cells**—Stimulation of cells was carried out as described earlier (36–39). Briefly, CCR5 L1.2 or Jurkat cells were washed twice with Hanks' buffered salt solution (Cellgro) and then resuspended in the Hanks' buffered salt solution with a density of 10⁷ cells/ml. The cells were subsequently serum-starved for 1 h at 37°C. Serum-starved cells were stimulated with 100 ng/ml MIP-1β (PeproTech, Inc.) or 1.2 μg/ml gp120 (Protein Sciences Corp.) at 37°C for various time periods. At the end of the stimulation, the cells were harvested by centrifugation and lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 mM chymostatin, 100 μg/ml trypsin inhibitor, 10 μg/ml pepstatin, 10 mM sodium vanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate). The cell lysates were clarified by centrifugation at 10,000 x g for 10 min. Protein concentrations were determined by the Bio-Rad detergent-compatible protein assay.

**Immunoprecipitation and Western Blot Analysis**—Equal amounts of protein from the stimulated time points were clarified by incubation with protein A-Sepharose CL-4B or GammaBind™ Sepharose beads (both from Amersham Biosciences) for 1 h at 4°C. The Sepharose beads were washed twice by brief centrifugation in a total volume of 50 μl of lysis buffer with different primary antibodies for 2 h at 4°C. Immunoprecipitation of the antibody-antigen complexes was performed by incubation at 4°C overnight with 50 μl of protein A-Sepharose or GammaBind™ Sepharose (50% suspension). Non-specific interacting proteins were removed by washing the beads thrice with modified radioimmunoprecipitation assay buffer and once with phosphate-buffered saline. Immune complexes were solubilized in 50 μl of 2× Laemmli buffer, boiled, and subjected to SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membranes. The membranes were then blocked in 5% nonfat milk protein for 2 h at 37°C or overnight at 4°C and probed with primary antibody for 1 h at room temperature or at 4°C overnight. Immunoreactive bands were visualized with horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence system (ECL, Amersham Biosciences).

**Flow Cytometry**—Jurkat or CCR5 L1.2 cells were preincubated with or without the proteasome inhibitors lactacystin (25 μM) or epoxomycin (25 μM) in RPMI 1640 containing 10% fetal bovine serum for 2 h at 3°C and then stimulated with 1.2 μg/ml gp120 or 1 μg/ml MIP-1β for 30 min and washed with ice-cold phosphate-buffered saline followed by fixing in 2% formaldehyde for 15 min at room temperature. The CCRX4 receptor on the Jurkat cells or the CCR5 receptor on the CCR5 L1.2 cells was stained with phycoerythrin-coupled anti-CXCR4 or anti-CCR5 antibody, respectively, for 1 h at 4°C. The cells were then washed with phosphate-buffered saline, suspended in 1% formaldehyde in phosphate-buffered saline, and subjected to flow cytometric analysis.

**Confocal Microscopy**—Jurkat or CCR5 L1.2 cells were cytographed on slides. The cells were preincubated with or without the proteasome inhibitors lactacystin (25 μM) or epoxomycin (25 μM) in RPMI 1640 containing 10% fetal bovine serum for 1 h at 3°C. The cells were then stimulated with 1.2 μg/ml gp120 or 1 μg/ml MIP-1β for 30 min. The stimulation was arrested by washing the cells in ice-cold phosphate-buffered saline followed by fixing in 4% paraformaldehyde for 10 min at room temperature. The cells were then permeabilized with 0.2% Triton X-100 followed by blocking with 5% bovine serum albumin for 30 min at 4°C. The CCRX4 receptor on the Jurkat cells was stained with anti-CXCR4 antibody coupled to phycoerythrin (PharMingen) for 1 h. The CCR5 receptor on the CCR5 L1.2 cells was stained with goat anti-CCR5 antibody (Santa Cruz Biotechnology) overnight at 4°C followed by Texas-red labeled anti-goat IgG (Vector). Slides were examined using a Leica TCS confocal microscope.

**Isolation of Peripheral Blood Lymphocytes**—Lymphocytes from peripheral blood (PBLs) were isolated as described previously (37). Briefly, PBLs were isolated from heparinized venous blood collected from healthy donors by Ficoll-Hypaque density gradient centrifugation at 3000 rpm for 25 min. The cells were suspended in RPMI containing 10% fetal calf serum, 2 mM glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin. Monocytes were depleted by two rounds of adherence to plastic. Nonadherent cells were stimulated with phytohemagglutinin (5 μg/ml) for 3 days. Cells were removed to fresh medium supplemented with recombinant human interleukin-2 (Advanced Biotechnologies, Columbia, MD). Two days later, the stimulated cells were used as target cells in migration assays. To determine whether any of the added agents were toxic, the viability of the cells following various treatments was monitored by trypan blue uptake. No significant toxicity was observed.

**Migration Assays**—Migration assays were performed according to the procedures described previously (37). Briefly, CCR5 L1.2 cells/Jurkat cells/PBLs were washed twice and suspended at 10⁶ cells/ml in RPMI containing 10% fetal calf serum. A 24-well plate containing 5 μm porous inserts (Costar Corp., Kennebunk, ME) was used for this experiment. Before performing the migration assay, cells were treated with different concentrations of the proteasome inhibitors epoxomycin and lactacystin or the appropriate control solvent (Me2SO) for 60 min. 100 μl (1 × 10⁶ cells) from each sample was loaded onto the upper well. 0.6 ml of medium containing SDF-1α (50 ng/ml) for the Jurkat cells/PBLs or MIP-1β (100 ng/ml) for CCR5 L1.2 cells was used. The plates were incubated for 3 h at 37°C in 5% CO₂. After incubation, the porous inserts were removed carefully, and the viable cells were counted on a microscope using a hemocytometer. No toxic effects on the cells were observed. The results are expressed as the percent of migrated cells as compared with the control (untreated cells). Each experiment was performed three or four times in triplicate.

**p38 MAP Kinase Assay**—CCR5 L1.2 cells were stimulated with 200 ng/ml MIP-1β as described above. After the protein concentration was normalized, the cell lysates were immunoprecipitated with p38 antibody (Santa Cruz Biotechnology). The immune complexes were washed twice with radioimmunoprecipitation assay buffer and twice with kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl₂, 20 μM ATP). Finally, the immune complexes were centrifuged in a total volume of 50 μl of kinase buffer containing 7 μg of myelin basic protein (Upstate Biotechnology, Lake Placid, NY) and 5 μCi of [γ-³²P]ATP for 20 min at 30°C. The proteins were separated on 15% SDS-PAGE, and bands were detected by autoradiography.

**Statistical Analysis**—The results are expressed as the mean ± S.D. of data obtained from three or four experiments performed in duplicate.
or triplicate. The statistical significance was determined using the Student’s t test.

RESULTS

The Cytosolic Tail of CCR5 Associates with the Proteasome ζ Subunit—To understand the signaling events mediated by the CCR5 receptor, we performed yeast two-hybrid screens using the C-terminal cytosolic tail of the CCR5 receptor as bait. In recent years, the yeast two-hybrid system has been used as a powerful genetic tool to rapidly select previously uncharacterized proteins and to identify novel components of signaling networks. We used the Lex-A-dependent yeast two-hybrid system to examine the potential interaction between the cytoplasmic tail of the CCR5 receptor and other signaling molecules. Several positive clones that interacted with the CCR5 cytoplasmic tail were isolated and sequenced. Some of these clones represented unknown proteins. One of the proteins interacting with the CCR5 cytoplasmic tail was shown to be ζ, an α-subtype of the 20 S catalytic particle of the 26 S proteasome. This association appeared to be specific, as we did not observe association of this gene with the cytoplasmic tail of the Kaposi sarcoma-associated herpesvirus-encoded G-protein-coupled receptor under similar conditions.

The interaction was further confirmed in 293T cells using the mammalian two-hybrid system. The CCR5 receptor protein was expressed as a fusion to the Gal4 DNA-binding domain, and the proteasome ζ subunit was expressed as a fusion to the herpes simplex virus VP16 protein activation domain. The vectors were cotransfected into the 293T mammalian cell line along with a reporter plasmid harboring the chloramphenicol acetyltransferase (CAT) gene. The reporter plasmid contains a CAT gene under the control of five consensus Gal4 binding sites. The transfection was carried out along with the β-galactosidase gene to analyze transfection efficiency. Interaction between the two fusion proteins leads to increased expression of the CAT reporter gene. CAT enzyme activity was assayed from the transfected cell lysates and normalized against β-galactosidase specific activity. The ζ subunit showed a marked increase in the level of CAT activity as compared with the controls, indicating a possible interaction with the CCR5 receptor (Fig. 1A).

To further characterize the interaction between CCR5 and the proteasome ζ, co-immunoprecipitation assays were performed in L1.2 cells transfected with the CCR5 receptor. The endogenous proteasome ζ was observed to be associated with the CCR5 receptor in unstimulated cells (Fig. 1B, lane 2). Furthermore, upon stimulation with MIP-1β, the cognate ligand of the CCR5 receptor, the interaction between the CCR5 receptor and proteasome ζ subunit increased (Fig. 1). These results confirm the two-hybrid assay results regarding interaction between the CCR5 receptor and the ζ subunit.

Ligand-induced CCR5 Receptor Internalization Is Regulated by Proteasomes—Because the cytoplasmic tail of the CCR5 receptor was shown to interact with the ζ subunit of proteasome, we next explored the role of this proteasome in ligand-induced proteasome down-modulation. Earlier studies have shown that proteasomes regulate internalization of several cell surface receptors (27–29). As shown in Fig. 2, A and B, pretreatment of cells with the specific proteasome inhibitors lactacystin and epoxomicin completely inhibited MIP-1β-induced internalization of the CCR5 receptor. Both of these inhibitors attenuated ligand-induced internalization of the CCR5 receptor in a dose-dependent manner (Fig. 2, C and D).

These observations were further confirmed by confocal microscopy. Cells stimulated with MIP-1β in the presence or absence of the proteasome inhibitors were analyzed for CCR5 receptor expression. As shown in Fig. 2E, in the unstimulated cells, most of the receptor was observed to be localized on the cell surface. Upon stimulation with MIP-1β, the surface expression of the CCR5 receptor was reduced. However, the internalization of the receptor was blocked by pretreatment of cells with the proteasome inhibitors. These results further confirm that proteasomes play an important role in CCR5 receptor expression and internalization.

SDF-1α and gp120-induced CXCR4 Internalization Is Also Dependent on Proteasome Function—We have also analyzed the effect of the proteasome inhibitors on HIV gp120- and SDF-1α-induced internalization of another coreceptor of HIV, CXCR4. Earlier studies have shown that the CXCR4 receptor undergoes rapid internalization upon stimulation with the viral envelope protein gp120 or its cognate ligand, SDF-1α. As shown in Fig. 3, pretreatment of cells with the specific proteasome inhibitors lactacystin and epoxomicin blocked down-modulation of the CXCR4 expression induced by SDF-1α or HIV gp120. Dose-dependent inhibition of CXCR4 receptor down-modulation by HIV gp120 (Fig. 4, A and B) or SDF-1α (Fig. 4, C and D) was observed. Maximum inhibition of down-modulation was observed when cells were pretreated with 25 μM lactacystin or epoxomicin. No effect on cell viability was observed at these concentrations (data not shown).

These results were further confirmed by confocal microscopy. Pretreatment of cells with proteasome inhibitors followed by stimulation with SDF-1α or gp120 did not alter the cell surface expression of the CXCR4 receptor (Fig. 4E).

The Proteasome Inhibitor Lactacystin Does Not Significantly Block CD4 Internalization—Earlier studies have shown that gp120 treatment also induces down-modulation of the CD4 receptor (40). We also studied the involvement of the protea-
some pathway in CD4 receptor down-modulation. As shown in Fig. 5A, pretreatment of cells with the proteasome inhibitor lactacystin did not significantly block gp120-induced CD4 receptor down-modulation. We have further shown that gp120-induced CXCR4 receptor internalization was dependent on the presence of CD4 because gp120-treated cells lacking the CD4 receptor did not show any down-modulation of the receptor. However, internalization of the CXCR4 receptor in CD4-negative cells was observed upon stimulation with SDF-1α (Fig. 5B).

**FIG. 2.** CCR5 receptor internalization is regulated by proteasomes. CCR5 L1.2 cells were preincubated with either dimethyl sulfoxide (DMSO) as control or the proteasome inhibitors lactacystin (25 μM; A) or epoxomicin (25 μM; B) in RPMI 1640 containing 10% fetal bovine serum for 1 h at 37 °C followed by stimulation with MIP-1β. The CCR5 receptor on the cells was stained with anti-CCR5 antibody coupled to phycoerythrin and subjected to flow cytometric analysis. Panels C and D represent the dose-dependent effect of the proteasome inhibitors upon internalization of the receptor. Data are representative of three separate experiments. E, the CCR5 L1.2 cells stimulated with MIP-1β in the presence of lactacystin (25 μM) or epoxomicin (25 μM) were cytofuged on slides and processed for the levels of the CCR5 receptor by confocal microscopy as described under “Experimental Procedures.” Lac, lactacystin; Epox, epoxomicin.

**FIG. 3.** SDF-1α and gp120-induced CXCR4 receptor internalization is regulated by proteasomes. Jurkat cells were preincubated with or without the proteasome inhibitors lactacystin (25 μM) or epoxomicin (25 μM) in RPMI 1640 containing 10% fetal bovine serum for 1 h at 37 °C. The cells were stimulated with 1 μg/ml SDF-1α or 1.2 μg/ml gp120 for different time periods and then subjected to flow cytometric analysis as described under “Experimental Procedures.” SDF-1α stimulation in the absence (■) or presence of lactacystin (○) or epoxomicin (▲) or cells stimulated with gp120 in the absence (●) or presence (▲) of lactacystin or epoxomicin (×) were analyzed. For controls, the cells were incubated with lactacystin (♦) or epoxomicin (□) alone. Data are representative of three separate experiments. Lac, lactacystin; Epox, epoxomicin.

The Proteasome Pathway May Also Modulate CXCR4 and CCR5 Receptor-mediated Chemotaxis—We also studied the effect of proteasome inhibitors on CCR5 and CXCR4-mediated chemotactic function. Pretreatment of CCR5 L1.2 cells with proteasome inhibitors attenuated MIP-1β induced chemotaxis in a dose-dependent manner. As shown in Fig. 6A, a nearly 80% decrease in chemotaxis was observed with the proteasome inhibitor epoxomicin (50 μM), whereas only a 20% inhibition was shown with lactacystin (50 μM) (Fig. 6B). Higher concentrations of lactacystin were required for inhibition of chemotaxis. These inhibitors were also shown to block 40–50% of the SDF-1α-induced chemotaxis of Jurkat cells and nearly 30% of that in PBLs with epoxomicin treatment (Fig. 7). We also determined the effect on the viability of the cells of treatment with the proteasome inhibitors at concentrations that inhibited chemotaxis. No effect on cell viability using the proteasome inhibitors was observed (data not shown).

**FIG. 4.** CCR5 and CXCR4 Receptor-mediated MAP Kinase Pathways—Because proteasome inhibitors blocked CXCR4 and CCR5-mediated chemotactic functions, we studied the effect of these compounds on MAP kinases activated by these receptors. In our previous studies, we have shown that CCR5 activation mediates p38 kinase, whereas CXCR4 activation mediates p42/44 MAP kinase activities (37, 40, 41). As shown in Fig. 8, A (upper panel) and B, epoxomicin pretreatment (50 μM) had no significant effect on SDF-1α-induced p42/44 phosphorylation in Jurkat cells. Equivalent amounts of p42/44 proteins were present in each lane (Fig. 8A, lower panel). Similarly, pretreatment of CCR5 L1.2 cells with epoxomicin (500 μM) had no effect on MIP-1β-induced p38 kinase activity (Fig. 8, C and D).

**DISCUSSION**

CCR5 and CXCR4 receptor interaction with the HIV-1 surface envelope glycoprotein gp120 triggers molecular events...
that eventually result in HIV infection. It has been shown that gp120 can induce rapid internalization of these receptors (40, 42). Furthermore, the cognate ligands of CCR5 (MIP-1α or RANTES) and of CXCR4 (SDF-1α) can efficiently block HIV-1 infection mediated by CCR5 or CXCR4 (13–16). Moreover, these compounds have also been known to induce the down-regulation of CCR5 and CXCR4 at the cell surface. Therefore,

![Diagram](https://example.com/diagram.png)

**Fig. 4.** Effect of proteasome inhibitors on gp120- and SDF-mediated CXCR4 internalization. Jurkat cells were preincubated with varying concentrations of proteasome inhibitors for 1 h at 37 °C. Following preincubation, the cells were stimulated with gp120 (A and B) or SDF-1α (C and D). The cells were analyzed by flow cytometry using anti-CXCR4 antibodies coupled to phycoerythrin as described under “Experimental Procedures.” Data are representative of three separate experiments. E, Jurkat cells were also analyzed for the levels of the CXCR4 receptor by confocal microscopy as described under “Experimental Procedures.” Lac, lactacystin; Epox, epoxomicin.

**Fig. 5.** Effect of proteasome inhibitor on gp120-mediated CD4 internalization and the importance of CD4 for CXCR4 internalization. A, Jurkat cells preincubated with or without lactacystin (Lac, 50 μM) were analyzed for the expression of CD4 upon treatment with HIV gp120 by using flow cytometry. B, graph represents the level of CXCR4 receptor in a variant of Jurkat cells (CD4+/CXCR4+) or Jurkat cells (CD4+/CXCR4−) unstimulated (UN) or stimulated with either SDF-1α (SDF) or gp120.

**Fig. 6.** Proteasome inhibitors block the MIP-1α-induced migration of CCR5 L1.2 cells. Cells pretreated with the proteasome inhibitors epoxomicin (A) or lactacystin (B) for 60 min were subjected to chemotactic assay in the presence of MIP-1α (100 ng/ml) as described under “Experimental Procedures.” *, p < 0.05, n = 3. DMSO, dimethyl sulfoxide.
information about the mechanism of CCR5 and CXCR4 receptor internalization by HIV gp120 or upon stimulation with chemokine ligands is important for the development of antiviral strategies. In this study, we have shown that the proteasome pathway plays an important role in ligand- and gp120-mediated CCR5 and CXCR4 receptor internalization.

Recent studies have shown that the cytoplasmic tail of the CCR5 receptor is involved in receptor trafficking (43). Shoida et al. (43) showed that a natural variant of CCR5 that lacked the C-terminal was impaired for surface expression. Similarly, complete removal of the cytoplasmic tail of CCR5 almost completely abolished trafficking to the cell surface. The naturally occurring CCR5 mutant, CCR5Δ32, which has a 32-bp deletion in the second extracellular loop, reduced the cell surface expression of CCR5 in a gene dose-dependent manner (44). The C-terminal tail of the CCR5 receptor harbors a highly basic domain and a cysteine cluster. This motif was observed to be critical for the cell surface expression of CCR5 (45). To further analyze the role of this domain for ligand- and gp120-mediated signal transduction and receptor internalization, we used the yeast two-hybrid approach to identify the proteins that interact with the CCR5 cytoplasmic tail. We observed a physical interaction between the CCR5 cytoplasmic tail and ζ, an α subtype of the 20 S catalytic particle of the 26 S proteasome. We further confirmed this association by using the mammalian two-hybrid system and under in vitro conditions by co-immunoprecipitation. The observed association was enhanced by MIP-1β stimulation. Recently, the ζ subunit of proteasome was shown to associate with presenilin-1 (PSEN1) in the cells (46). Presenilin-1 has been shown to be degraded via the proteasome pathway during the development of Alzheimer’s disease.

Because 26 S proteasome is involved in the turnover of various cellular proteins (47, 48), we anticipated that the proteasome pathway might regulate CCR5 receptor trafficking. In the present studies, we used the specific proteasome inhibitors lactacystin and epoxomicin to explore the potential role of proteasomes in the endocytosis of the CCR5 receptor. Pretreatment of cells with these inhibitors completely prevented endocytosis of the CCR5 receptor. Further studies indicated that the proteasome pathway also regulates endocytosis of another coreceptor of HIV-1, CXCR4. Both ligand- and HIV gp120-induced down-modulation of the CXCR4 receptor was completely dependent on the proteasome pathway. However, gp120-induced endocytosis of the HIV receptor, CD4, was not significantly inhibited by the proteasome inhibitor pretreatments. Interestingly, the gp120-mediated down-modulation of the CXCR4 receptor was observed to be dependent on the CD4 receptor. Absence of the CD4 receptor prevented down-modulation of the CXCR4 receptor upon gp120 stimulation but not upon SDF-1α stimulation. These studies suggest that gp120-induced down-modulation of the CXCR4 and CD4 receptors may involve different mechanisms but that CXCR4 down-modulation induced by gp120 requires CD4. The proteasome pathway has previously been shown to play a major role in the ligand-mediated endocytosis of a variety of receptors (27–29).
Recently, it was shown that the Ubiquitin/proteasome system also regulates HIV gag polypeptide processing and HIV-1-encoded Vpu protein-mediated degradation of the CD4 receptor (34, 35). It has also been implicated as playing a role in CXCR4 lysosomal sorting and degradation (26). Furthermore, the CCR532 mutation has been shown to result in a product that never reaches the cell surface (44, 45). It has been postulated that this could be the result of misfolding and consequent proteolytic degradation, perhaps by the proteasome.

We also investigated the effect of proteasome inhibitors on the downstream functional effects mediated by the CCR5 and CXCR4 receptors. We observed a reduction of about 80% in CCR5 and about 40–50% in CXCR4-mediated chemotaxis upon pretreatment of cells with the proteasome inhibitors. However, these compounds had no effect on CCR5-mediated p38 kinase and CXCR4-mediated MAP kinase activities. Proteasomes have been shown to regulate the activity of transcription factor NF-xB by degrading phosphorylated IkB (31). IkB acts as an inhibitor for the translocation of NF-xB to the nucleus. In our previous studies, we have shown that nitric oxide plays an important role in SDF-1a-induced and CXCR4-mediated chemotactic activity (36). Furthermore, our studies have also shown that SDF-1a-induced MAP kinase activation is not dependent on the nitric oxide and NF-xB pathways. Proteasomes have also been shown to regulate chemotaxis mediated by tumor necrosis factor x (TNF-a) in oral squamous carcinoma cells (49). Moreover, the degradation of IkB was also suppressed by proteasomes in tumor necrosis factor x-inhibited cells.

These studies provide new information about the role of the proteasome pathway in regulating CCR5 and CXCR4 down-modulation induced by cognate ligands and HIV gp120. Proteasomes may also partially regulate CCR5- and CXCR4-mediated chemotaxis. Both of these events are important in HIV infection and in other physiological and pathological processes.

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REFERENCES

1. Mackay, C. R. (2001) Nat. Immunol. 2, 95–101
2. Christopherson, K. R., and Hromas, R. (2001) Stem Cells 19, 388–396
3. De Groot, C. J., and Woodruff, M. N. (2001) Prog. Brain Res. 132, 533–544
4. Bromley, H. E. (2001) Int. J. Hematol. 74, 9–17
5. Moser, B., and Loetscher, P. (2001) Nat. Immunol. 2, 123–128
6. Haruk, R. (2001) Cytokine Growth Factor Rev. 12, 313–335
7. Ansel, K. M., and Cyster, J. G. (2001) J. Leukocyte Biol. 69, 123–129
8. Knobel, M., and Clynes, R. A. (2001) Nature 41, 50–56
9. Stantchev, T. S., and Broder, C. C. (2001) Cytokine Growth Factor Rev. 12, 219–243
10. Murakami, T., and Yamamoto, N. (2000) Int. J. Hematol. 72, 412–417
11. Kindler, A., Arthos, J., Cirala, C., and Fuci, A. S. (2000) Immunol. Rev. 177, 88–98
12. Broder, C. C., and Collman, R. G. (1997) J. Leukocyte Biol. 62, 20–29
13. Townsend, J. R., Graham, G. J., Landau, N. R., Rasala, B., and Nibbs, R. J. (2000) J. Biol. Chem. 275, 39254–39261
14. Jiang, Y., and Jolly, P. E. (1999) J. Hum. Virol. 2, 123–132
15. Simmons, G., Clapham, P. R., Picard, L., Offord, R. E., Rosenkilde, M. M., Schwartz, T. W., Buser, R., Wells, T. N., and Proffout, A. E. (1997) Science 276, 276–279
16. Simmons, G., Reeves, J. D., Hobbitts, S., Stine, J. T., Gray, P. W., Proffout, A. E., and Clapham, P. R. (2000) Immunol. Rev. 177, 112–126
17. Valenzuela-Fernandez, A., Palanche, T., Amara, A., Magerus, A., Altmeyer, R., Delaunay, T., Virelizier, J. L., Baleyx, F., Galizi, J. L., and Arentzana-Seisdedos, F. (2001) J. Biol. Chem. 276, 26590–26598
18. He, J., Chen, Y., Farzan, M., Cho, H., Ohagen, A., Gurtser, A., Ruscigli, Y., Yang, X., Hofmann, W., Newman, W., Mackay, C. R., Sodroski, J., and Gabuzda, D. (1997) Nature 385, 645–649
19. Oberlin, E., Amara, A., Bachelier, F., Bessia, C., Virelizier, J. L., Arentzana-Seisdedos, F., Schwartz, O. H., Heard, J. M., Clark-Lewis, I., Legler, D. F., Loetscher, M., Baggilomi, M., and Moser, B. (1996) Nature 382, 833–835
20. Mack, M., Luckow, B., Nelson, P. J., Cihak, J., Simmons, G., Clapham, P. R., Signoret, N., Marsh, M., Stangassinger, M., Borlait, F., Wells, T. N., Schlondorf, D., and Proffout, A. E. (1998) J. Exp. Med. 187, 1215–1224
21. Signoret, N., Oldridge, J., Pelchen-Matthews, A., Kasse, A., Tran, T., Grass, L. F., Rosenkilde, M. M., Schwartz, T. W., Holmes, W., Luther, M. A., Wells, T. N., Hezek, J. A., and Marsh, M. (1997) J. Cell Biol. 138, 651–664
22. Su, S. B., Gong, W., Grum, M., Utsumomiyi, I., Sargeant, R., Oppenheim, J. J., and Wang, J. M. (1999) J. Immunol. 162, 7126–7132
23. Wang, J., Guan, E., Rodriguez, G., Calvert, V., Alvarez, R., and Noceres, M. A. (2001) J. Biol. Chem. 276, 49236–49243
24. Haribabu, B., Richardon, R. M., Fisher, I., Sozanni, S., Peiper, S. C., Horuk, R., Ali, H., and Snyderman, R. (1997) J. Biol. Chem. 272, 26726–26731
25. Van Drenth, C., Jenkins, A., Ledwich, L., Ryan, T. C., Mashikian, M. V., Brazer, W., Center, D. M., and Cruikshank, W. W. (2000) J. Immunol. 165, 6536–6535
26. Marchese, A., and Benovic, J. L. (2001) J. Biol. Chem. 276, 45059–45152
27. Yu, A., and Malek, T. R. (2001) J. Biol. Chem. 276, 381–385
28. van Kerkhof, P., Govers, R., Alves dos Santos, C. M., and Strous, G. J. (2000) J. Biol. Chem. 275, 1575–1539
29. Barou, V., and Schwartz, M. W. (2000) J. Biol. Chem. 275, 39138–39323
30. Andreolivice, M. J. (2001) Curr. Opin. Hematol. 8, 12–16
31. Gallo, J. V., Rado, O. J., Goldberg, A. L., and Maniatis, T. (1994) Cell 78, 773–785
32. Dirico, J. (1994) Histol. Histopathol. 9, 197–202
33. Ikeno, T., Takeuchi, H., Jimi, E., Beppu, M., Shinohora, M., and Shirasuna, K. (1998) Int. J. Cancer 77, 578–585
34. Bradfield, S. M., Mariani, R. H., Holland, A. U., Hope, T. J., and Landau, N. R. (2002) J. Biol. Chem. 277, 12197–12199
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