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Abstract

Members of the chemokine receptor family CCR5 and CXCR4 have recently been shown to be involved in the entry of human immunodeficiency virus (HIV) into target cells. Here, we investigated the regulation of CXCR4 in rat basophilic leukemia cells (RBL-2H3) stably transfected with wild type (Wt CXCR4) or a cytoplasmic tail deletion mutant (ΔCyto CXCR4) of CXCR4. The ligand, stromal cell derived factor-1 (SDF-1) stimulated higher G-protein activation, inositol phosphate generation, and a more sustained calcium elevation in cells expressing ΔCyto CXCR4 relative to Wt CXCR4. SDF-1 and phorbol 12-myristate 13-acetate (PMA), but not a membrane permeable cAMP analog induced rapid phosphorylation as well as desensitization of Wt CXCR4. Phosphorylation of ΔCyto CXCR4 was not detected under any of these conditions. Despite lack of receptor phosphorylation, calcium mobilization by SDF-1 in ΔCyto CXCR4 cells was partially desensitized by prior treatment with SDF-1. Of interest, the rapid release of calcium was inhibited without affecting the sustained calcium elevation, indicating independent regulatory pathways for these processes. PMA completely inhibited phosphoinositide hydrolysis and calcium mobilization in Wt CXCR4 but only partially inhibited these responses in ΔCyto CXCR4. cAMP also partially inhibited these responses in both Wt CXCR4 and ΔCyto CXCR4. SDF-1, PMA, and cAMP caused phosphorylation of phospholipaze Cβ3 in Wt and ΔCyto CXCR4 cells. Both SDF- 1 as well as PMA induced rapid internalization of Wt CXCR4. SDF-1 but not PMA induced internalization of ΔCyto CXCR4 albeit at reduced levels relative to Wt CXCR4. These results indicate that signaling and internalization of CXCR4 are regulated by receptor phosphorylation dependent and independent mechanisms. Desensitization of CXCR4 signaling, independent of receptor phosphorylation, appears to be a consequence of the phosphorylation of phospholipase Cβ3.

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Regulation of Human Chemokine Receptors CXCR4

ROLE OF PHOSPHORYLATION IN DESENSITIZATION AND INTERNALIZATION*

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Members of the chemokine receptor family CCR5 and CXCR4 have recently been shown to be involved in the entry of human immunodeficiency virus (HIV) into target cells. Here, we investigated the regulation of CXCR4 in rat basophilic leukemia cells (RBL-2H3) stably transfected with wild type (Wt CXCR4) or a cytoplasmic tail deletion mutant (ΔCyto CXCR4) of CXCR4. The ligand, stromal cell derived factor-1 (SDF-1) stimulated higher G-protein activation, inositol phosphate generation, and a more sustained calcium elevation in cells expressing ΔCyto CXCR4 relative to Wt CXCR4. SDF-1 and phorbol 12-myristate 13-acetate (PMA), but not a membrane permeable cAMP analog induced rapid phosphorylation as well as desensitization of Wt CXCR4. Phosphorylation of ΔCyto CXCR4 was not detected under any of these conditions. Despite lack of receptor phosphorylation, calcium mobilization by SDF-1 in ΔCyto CXCR4 cells was partially desensitized by prior treatment with SDF-1. Of interest, the rapid release of calcium was inhibited without affecting the sustained calcium elevation, indicating independent regulatory pathways for these processes. PMA completely inhibited phosphoinositide hydrolysis and calcium mobilization in Wt CXCR4 but only partially inhibited these responses in ΔCyto CXCR4. cAMP also partially inhibited these responses in both Wt CXCR4 and ΔCyto CXCR4. SDF-1, PMA, and cAMP caused phosphorylation of phospholipase Cβ3 in Wt and ΔCyto CXCR4 cells. Both SDF-1 as well as PMA induced rapid internalization of Wt CXCR4. SDF-1 but not PMA induced internalization of ΔCyto CXCR4 albeit at reduced levels relative to Wt CXCR4. These results indicate that signaling and internalization of CXCR4 are regulated by receptor phosphorylation dependent and independent mechanisms. Desensitization of CXCR4 signaling, independent of receptor phosphorylation, appears to be a consequence of the phosphorylation of phospholipase Cβ3.

Chemokines are a group of proteins that mediate directed migration and activation of leukocytes (1). These have been classified into two main families, CXC or CC-chemokines. Two newly identified proteins define additional groups of C and CX3C chemokines based on the position of conserved cysteines (2, 3). Both CC and CXC chemokines bind to seven transmembrane G-protein-coupled receptors which transduce signals through heterotrimeric G-proteins (4). Several recent studies showed that chemokines play a significant role in human immunodeficiency virus (HIV-1)[1] infection and that the chemokine receptors CCR5 and CXCR4 along with CD4 act as major co-receptors for the macrophage tropic and T-cell tropic HIV-1 strains entry, respectively, into target cells (5–10). While the C-C chemokines such as MIP1α, MIP1β, and regulated upon activation, normal T expressed and secreted can activate CCR5 the CXC chemokine SDF-1 is the ligand for CXCR4 (11–13). Recently, CD4 independent infection by HIV-2 was shown to be mediated by CXCR4 (14). HIV-1 envelope glycoproteins interact with chemokine receptors in a CD4-dependent and in one case independent manner (15–17). More recently, additional members of the chemokine receptors gene family, some of which act as HIV-1 coreceptors, were identified (18, 19).

Chemokine receptors couple to members of the G_i or G_q family of G-proteins (20–23). Other chemoattractant receptors have recently been found to be regulated by phosphorylation at the level of receptors and phospholipase C (24, 25). To understand chemokine receptor-activated signal transduction pathways and their down-regulation, the well established RBL-2H3 cell model (26–28) was adopted for the stable functional expression of native and mutated forms of CXCR4. Using this model, we demonstrate that CXCR4 signaling is regulated at the level of receptor as well as a downstream component.

EXPERIMENTAL PROCEDURES

Materials—[^{32}P]Orthophosphate (8500–9120 Ci/mmol), [^{125}I]iodine (17.0 Ci/mg), ^{125}I-label SDF-1 (2200 Ci/mmol), *myo*-[^{2}H]inositol (24.4 Ci/mmol), and [γ-^{32}P]GTP (6000 Ci/mmol) were purchased from NEN Life Science Products Inc. Monoclonal 12CA5 antibody was obtained from Boehringer Mannheim and 12G5 antibody was a gift from Dr. J. Hoxie and generously shared by Dr. Haynes. Affinity purified polyclonal antibodies against PLCβ3 were obtained from Santa Cruz Biotechnology. Recombinant as well as chemically synthesized SDF-1 were from R&D systems. Geniticin (G418) and all tissue culture reagents were purchased from Life Technologies, Inc. Protein G-agarose and protease inhibitors were purchased from Boehringer Manheim. Indo-1 acetoxymethyl ester and pluronic acid were purchased from Molecular Probes. GDP, GTP, and ATP were purchased from Sigma.

Construction of Epitope-tagged CXCR4 and Truncated CXCR4—Nucleotides encoding a 9-amino acid hemagglutinin-epitope (HA) se-

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[1] The abbreviations used are: HIV, human immunodeficiency virus; RBL, rat basophilic leukemia cells; ΔCyto, cytoplasmic tail deletion mutants; SDF-1, stromal cell derived factor-1; PMA, phorbol 12-myristate 13-acetate; MIP, macrophage inflammatory protein; FACS, fluorescence activated cell sorting; PKC, protein kinase C; PKA, protein kinase A; PLC, phospholipase C; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; Wt, wild type; DMEM, Dulbecco's modified Eagle's medium.

TABLE I

Amino acid sequences of the carboxyl-terminal tails of the wild type CXCR4 and the COOH-terminal truncation mutant of CXCR4

Potential phosphorylation sites in the wild type CXCR4 are indicated in bold.

	308 ↓	352 ↓
C-tail Wt CXCR4	KFK**T**SAQHAL**T**S**V**S**R**G**SS**LKIL**S**KGKRGGH**SS**V**ST**E**S**E**SSS**FH**SS**-COOH	
ΔCyto CXCR4	KFK**T**AAQHALA-COOH	

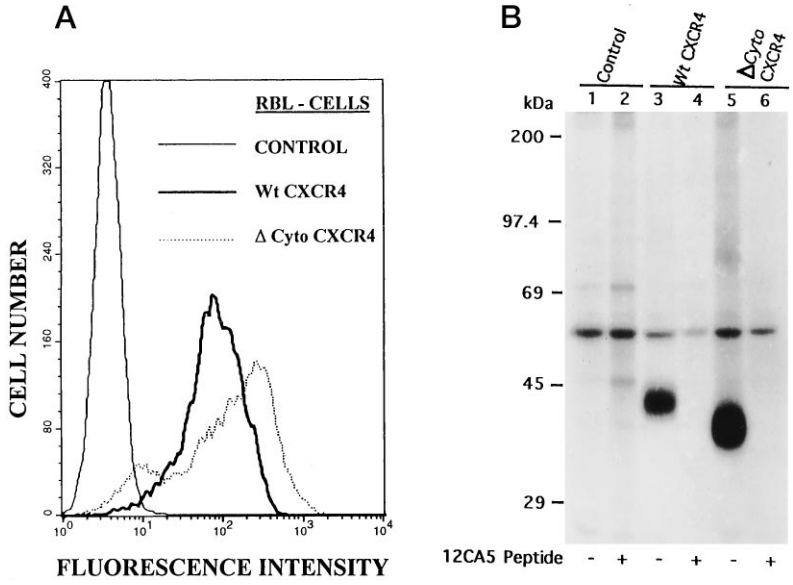

FIG. 1. **Expression of Wt CXCR4 and ΔCyto CXCR4 in RBL cells.** *A*, indirect immunofluorescence staining of native RBL cells (*thin line*), RBL cells stably expressing HA-tagged human CXCR4 (*thick line*), or the ΔCyto CXCR4 (*broken line*) with 12G5 monoclonal antibody and fluorescein isothiocyanate-conjugated goat anti-mouse IgG. *B*, SDS-PAGE analysis of immunoprecipitated Wt CXCR4 and ΔCyto CXCR4. Untransfected RBL cells (*Control, lanes 1* and *2*), cells expressing the Wt CXCR4 (*lanes 3* and *4*), or ΔCyto CXCR4 (*lanes 5* and *6*) were radioiodinated, solubilized and immunoprecipitated with anti-HA mAb 12CA5 in the presence (*lanes 2, 4,* and *6*) and absence (*lanes 1, 3,* and *5*) of excess (10 μg) 12CA5 peptide.

quence (YPYDVPDYA) was inserted between the NH_2-terminal initiator methionine and the second amino acid of human CXCR4 by polymerase chain reaction methods as described previously for other chemoattractant receptors (27, 28). The same epitope tag was also placed at the COOH-terminal end before the stop codon in some constructs. A COOH terminally truncated CXCR4 at the amino acid 318 by altering the codon 319 into a stop codon was also made using standard polymerase chain reaction methods. The integrity of the epitope tag as well as the rest of the molecule was confirmed by dideoxy sequencing after cloning into eukaryotic expression vectors pcDNA3.1 and pRK-5.

Cell Culture and Transfection—RBL-2H3 cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (27, 28). RBL cells (1×10^7 cells) were transfected by electroporation with pRK5 containing the receptor cDNAs (25 μg) along with pcDNA3 (5 μg) plasmid containing the geneticin-resistant marker and clones were isolated as described previously (27, 28).

Radioligand Binding Assays—For ligand binding assays, RBL cells (5×10^5) were distributed in Eppendorf tubes in a final volume 100 μl of binding medium (DMEM supplemented with 20 mM HEPES and 10 mg/ml bovine serum albumin) containing different amounts of unlabled SDF-1 and 0.2 nM ^{125}I-labeled SDF-1 (2200 Ci/mmol). After incubation at 4 °C for 2 h, the cells were centrifuged through a 10% sucrose gradient. The tips of tubes containing the pellets were cut and the radioactivity determined in a γ-counter. Nonspecific binding in the presence of 100 nM SDF-1 (25–40% of total binding) was subtracted to give specific binding (29).

GTPase Activity—RBL cells (5×10^7) were washed in phosphate-buffered saline, membranes were prepared and GTPase activity was determined in the absence and presence of various concentrations of SDF-1 as described previously (28, 30, 31).

Phosphoinositide Hydrolysis and Ca^{2+} Mobilization—RBL cells were subcultured overnight in 96-well culture plates (50,000 cells/well) in an inositol-free medium supplemented with 10% dialyzed fetal bovine serum and 1–2 μCi/ml [^{3}H]inositol. Cells were washed with HEPES-buffered saline containing 20 mM LiCl and 0.1% bovine serum albumin and incubated in the same buffer with and without SDF-1. Reactions were stopped by adding 200 μl of chloroform, methanol, 4 N HCl (100:200:2) and the generation of total [^{3}H]inositol phosphates was determined (27, 28). For Ca^{2+} mobilization, cells (3×10^6) were washed in HEPES-buffered saline and loaded with 1 μM Indo I-AM in the presence of 1 μM pluronic acid for 30 min at room temperature. The cells were washed and resuspended in 1.5 ml of buffer and intracellular Ca^{2+} mobilization was measured as described (30).

Immunoprecipitations—RBL-2H3 cells (5–10 × 10^6) were surface labeled with ^{125}I, lysed, immunoprecipitated with 12CA5 antibody, resolved by SDS-PAGE, and visualized by autoradiography as described (27, 28). Phosphorylation of CXCR4 was performed essentially as described for other chemoattractant receptors (27, 28). Briefly, RBL-2H3 cells (2.5×10^6) were subcultured overnight in 60-mm tissue culture dishes. The following day, cells were rinsed twice with 5 ml of phosphate-free DMEM and incubated in the same medium supplemented with [^{32}P]orthophosphate (150 μCi/dish) for 90 min to metabolically label the intracellular ATP pool. Labeled cells were stimulated and epitope-tagged CXCR4 were immunoprecipitated from lysates with 12CA5 (10 μg) monoclonal antibody and analyzed by SDS-PAGE and visualized by autoradiography (27, 28). Phosphorylation of PLCβ3 was performed essentially by the same method as described above except 1-1.5μg of PLCβ3 antibody was used in place of 12CA5 antibody.

CXCR4 Internalization—Stably transfected RBL cells were incubated for 30 min at 37 °C with 100 nM SDF-1 or 100 nM PMA in HEPES (20 mM)-buffered DMEM. Cells were washed with ice-cold medium and incubated with 12G5 antibody for 60 min at 4 °C. The cells were again washed in cold medium and incubated with fluorescein isothiocyanate-labeled goat anti-mouse IgG. Single color immunofluorescence analysis of 10,000 cells was performed on a FACScan flow cytometer. Percent receptor internalization was calculated from the mean channel fluorescence values of cells treated with buffer *versus* ligand or PMA.

RESULTS

Functional Expression of CXCR4 in RBL-2H3 Cells—To study the regulation of the chemokine receptor CXCR4, stably transfected clonal lines of RBL cells expressing epitope-tagged wild type (Wt CXCR4) or a COOH-terminal truncation mutant at amino acid 318 of CXCR4 (ΔCyto CXCR4, Table I) were generated. Fig. 1*A* shows FACS analysis with the CXCR4-specific 12G5 antibody (14). The epitope tag-specific 12CA5 antibody was used to immunoprecipitate the Wt CXCR4 (~44 kDa) and ΔCyto CXCR4 (~41 kDa) proteins from surface-iodinated RBL cells (Fig. 1*B, lanes 3* and *5*). No ^{125}I-labeled proteins in this size range were detected in untransfected RBL cells. The epitope tag peptide (YPYDVPDYA) inhibited the immunoprecipitation of these proteins (Fig. 1*B, lanes 4* and *6*).

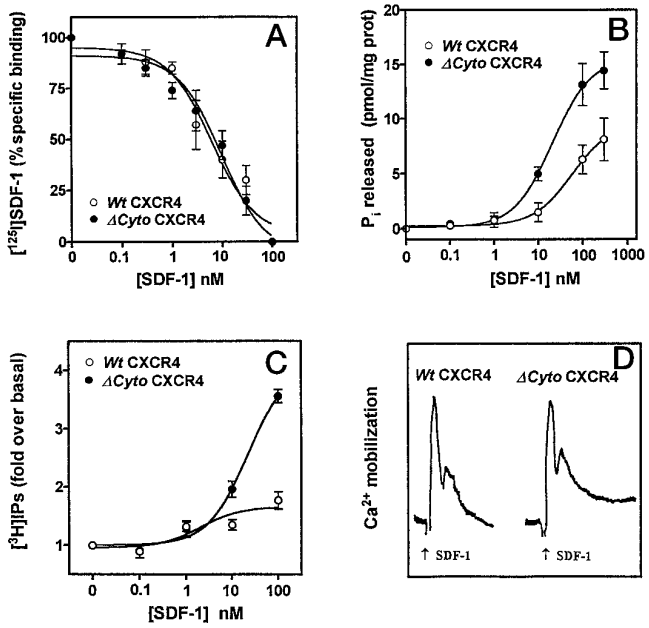

FIG. 2. **Characterization of Wt CXCR4 and ΔCyto CXCR4 in RBL cells.** *A*, displacement of radiolabled SDF-1 binding. Wt CXCR4 and ΔCyto CXCR4 RBL cells (5×10^5) were incubated with 0.2 nM ^{125}I-labeled SDF-1 (2200 Ci/mmol) at 4 °C for 2 h in the presence of the indicated concentrations of unlabeled SDF-1. Percent specific binding was calculated for each concentration of unlabled SDF-1. The data presented are average of four independent determinations each for both Wt CXCR4 and ΔCyto CXCR4 cells. *B,* for GTPase activity, membranes were prepared from RBL cells expressing either Wt CXCR4 or ΔCyto CXCR4 and assayed at different concentrations of SDF-1. Data shown are average from three independent experiments each performed in triplicate. *C,* generation of total inositol phosphates by treatment of Wt CXCR4 and ΔCyto CXCR4 RBL cells with different concentrations of SDF-1 were determined as described under "Experimental Procedures." Data are presented as fold-stimulation over basal, which was 217 ± 21 cpm for Wt CXCR4 and 237 ± 34 cpm for ΔCyto CXCR4. The experiment was repeated three times with similar results. *D,* RBL cells were loaded with Indo-1, and SDF-1 (10 nM) induced Ca^{2+} mobilization was measured.

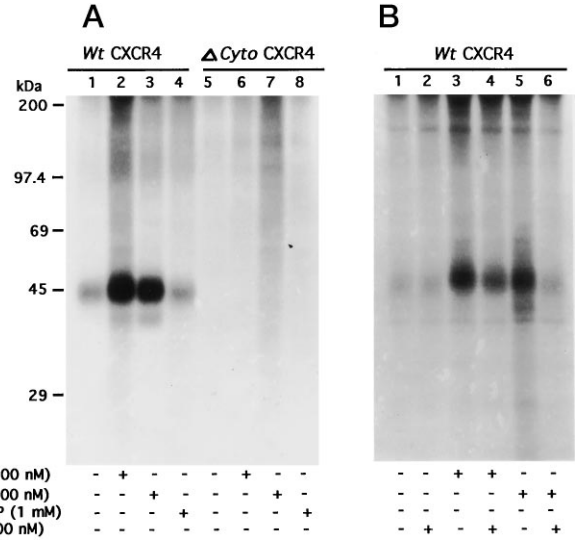

FIG. 3. **Phosphorylation of CXCR4.** *A,* ^{32}P-labeled Wt CXCR4 or ΔCyto CXCR4 RBL cells (3×10^6 cells/60-mm dish) were incubated for 5 min with SDF-1 (*lanes 2* and *6*), PMA (*lanes 3* and *7*), or 8-(4-chlorophenylthio)-cAMP (*cpt-cAMP*) (*lanes 4* and *8*) as indicated. Cells were lysed and immunoprecipitated with 12CA5 antibody, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. This experiment was repeated three times with similar results. *B,* effect of staurosporine on SDF-1 and PMA-induced CXCR4 phosphorylation. RBL cells expressing Wt CXCR4 were preincubated for 5 min with (*lanes 2, 4,* and *6*) or without (*lanes 1, 3*, and *5*) staurosporine (100 nM) and stimulated with SDF-1 (*lanes 3* and *4*) or PMA (*lanes 5* and *6*) for 5 min. Cells were lysed and immunoprecipitated with 12CA5 antibody, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

The epitope-tagged Wt and ΔCyto CXCR4 expressed in RBL cells bound SDF-1 with similar apparent affinity (Wt CXCR4 K_d = 6.1 ± 1.5 nM; ΔCyto CXCR4 K_d = 9.6 ± 1.4 nM, Fig. 2*A*). The native receptors expressed in neuronal cells had a K_d = 54 ± 6.4 nM. (17). Based on the maximum specific binding of SDF-1, a similar number of Wt CXCR4 (9800 ± 5200) or ΔCyto CXCR4 (7200 ± 1800) receptors were expressed in these cells. The receptors expressed in RBL cells were functionally active and induced SDF-1 dose-dependent GTPase activity in membranes, inositol phosphate production and calcium mobilization in whole cells (Fig. 2, *B-D*). The ΔCyto CXCR4 was more active relative to Wt CXCR4 in stimulating GTPase activity (Fig. 2*B*) and phosphoinositide hydrolysis (Fig. 2*C*). ΔCyto CXCR4 resulted in a ~4-fold increase in total inositol phosphates upon SDF-1 treatment as compared with the ~1.6-fold increase observed with the Wt CXCR4 (Fig. 2*C*). While the peak calcium mobilization by the Wt CXCR4 or ΔCyto CXCR4 were comparable (250–300 nM) the calcium transients induced by the ΔCyto CXCR4 contained a prolonged phase of elevated intracellular calcium (Fig. 2*D*).

Phosphorylation of CXCR4—SDF-1 and PMA, but not a membrane permeable analog of cAMP, stimulated by several-fold, the phosphorylation of the 44-kDa form of Wt CXCR4 (Fig. 3*A, lanes 1–4*). None of these stimulated detectable phosphorylation of the ΔCyto CXCR4 even after prolonged exposures (Fig. 3*A, lanes 5–8*). Staurosporine, a PKC inhibitor, completely inhibited PMA-induced phosphorylation (Fig. 3*B, lanes 5* and *6*) but partially inhibited the SDF-1 induced phosphorylation (Fig. 3*B, lanes 3* and *4*).

Desensitization of CXCR4 Signaling—Calcium mobilization in response to SDF-1 was completely desensitized in cells expressing Wt CXCR4 by prior treatment with SDF-1 or PMA both of which phosphorylate the receptor. Interestingly, cAMP which did not cause phosphorylation of the Wt CXCR4 also desensitized the calcium mobilization by ~70% (Fig. 4*A*), suggesting a distal site of inhibition. Desensitization of calcium responses in ΔCyto CXCR4 showed that prior treatment with SDF-1 resulted in almost complete inhibition of the initial calcium peak with substantial maintenance of the sustained response (Fig. 4*A*). The sustained response was not seen in the presence of EGTA, indicating the second phase results from calcium influx (data not shown). Treatment with pertussis toxin resulted in complete inhibition of both rapid and sustained increases in calcium in ΔCyto CXCR4 cells (data not shown). PMA and cAMP also resulted in inhibition of the calcium responses in ΔCyto CXCR4 (Fig. 4*A*). The effect of PKC and PKA activation on the generation of inositol phosphates by the Wt and ΔCyto CXCR4 cells was determined. PKC activation resulted in complete inhibition of inositol phosphate generation in Wt CXCR4 cells and partial inhibition in ΔCyto CXCR4 cells (Fig. 4*B*). cAMP resulted in partial inhibition of inositol phosphates both in Wt and ΔCyto CXCR4 cells (Fig. 4*B*). Among the known phospholipase Cβ isoforms, only PLCβ3 is expressed in RBL cells (25). The effects of SDF-1, PMA, and cAMP on the phosphorylation of PLCβ3 are shown in Fig. 5. cAMP resulted in ~2-fold increase in PLCβ3 basal phosphorylation where as SDF-1 and PMA resulted in ~3-fold increases in PLCβ3 phosphorylation.

Role of Phosphorylation in CXCR4 Internalization—Internalization was studied in RBL cells using the 12G5 antibody. Fig. 6*A* is a typical FACS analysis of Wt CXCR4. Data from several independent experiments on internalization of CXCR4

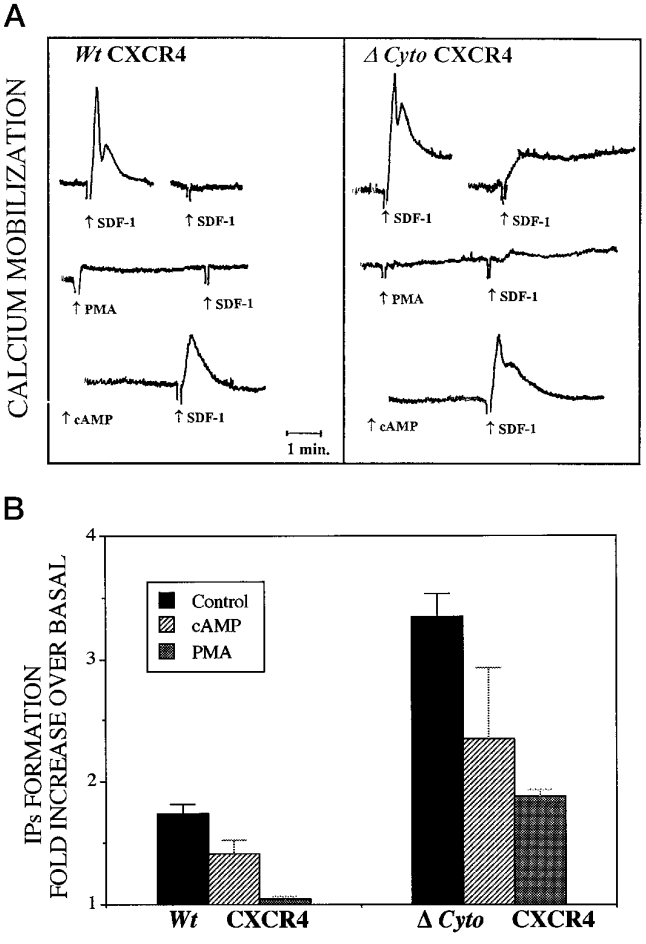

FIG. 4. **Desensitization of CXCR4.** *A,* RBL cells expressing Wt CXCR4 or ΔCyto CXCR4 (3×10^6 cells/assay) loaded with Indo-1 were stimulated with SDF-1 (10 nM) and calcium transients shown. For homologous desensitization, SDF-1 (10 nM) stimulated cells were washed and 3 min later were restimulated with SDF-1 (10 nM) and the calcium trace was recorded for 2–3 min. Whenever complete inhibition of calcium release was observed, thrombin (1.0 units/ml), which causes calcium mobilization in RBL cells, was used to verify normal Indo-1 loading of the cells (not shown). For determining the effects of PMA and cAMP, cells loaded with Indo-1 were treated with PMA (100 nM) or 8-(4-chlorophenylthio)-cAMP (1.0 mM) and 3 min later were stimulated with SDF-1 (10 nM). The data shown for each of the conditions was representative of a minimum of three to five independent measurements. *B,* effect of cAMP and PMA on generation of inositol phosphates. Cells were labeled with *myo*-[^{3}H]inositol and preincubated for 10 min in buffer (*Control*), PMA (100 nM), or 8-(4-chlorophenylthio)-cAMP (1.0 mM) and total inositol phosphates (*IPs*) released by stimulation with 100 nM SDF-1 was determined as described under "Experimental Procedures." Data are presented as fold stimulation over basal inositol phosphates from three independent experiments of the average of triplicate measurements in each experiment.

and ΔCyto CXCR4 by SDF-1 or PMA treatment are shown in Fig. 6*B*. Both SDF-1 and PMA induced internalization of the Wt CXCR4. The ΔCyto CXCR4 was also internalized by SDF-1 treatment, although consistently lower than the Wt CXCR4. PMA did not induce internalization of ΔCyto CXCR4 (Fig. 6*B*).

DISCUSSION

In this study, a well established model system was utilized to investigate the regulation of CXCR4, a chemokine receptor of considerable biological interest because of its role as a co-receptor for HIV-1 infection. The results suggest at least two distinct mechanisms for regulation of signal transduction, one at the level of receptor phosphorylation and the other at the level of phospholipase C activation. Functional expression of the native and the cytoplasmic tail deletion mutant of CXCR4

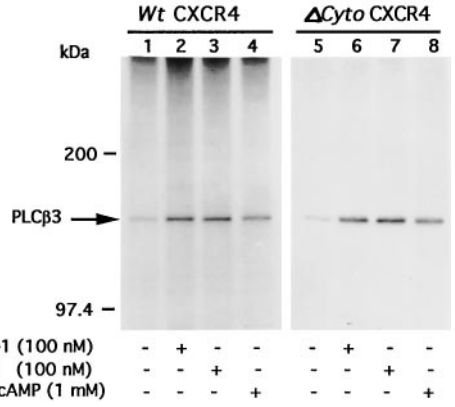

FIG. 5. **Phosphorylation of PLCβ3.** RBL cells expressing Wt CXCR4 or ΔCyto CXCR4 (3×10^6 cells/60-mm dish) were ^{32}P-labeled and incubated for 5 min with SDF-1 (*lanes 2* and *6*), PMA (*lanes 3* and *7*), or 8-(4-chlorophenylthio)-cAMP (*cpt-cAMP*) (*lanes 4* and *8*) as indicated. Cells were lysed, immunoprecipitated with PLCβ3 antibody, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. This experiment was repeated twice with similar results.

allowed for clear distinctions to be made of relative contributions of multiple mechanisms in the overall regulation of these receptors.

The higher activity of ΔCyto CXCR4 in inositol phosphates formation and in the induction of sustained calcium fluxes relative to Wt CXCR4 receptors suggests loss of some down-regulatory control of the function of the mutant receptors. While comparable number of receptors with similar affinity were expressed in Wt CXCR4 and ΔCyto CXCR4 transfectants, cells expressing the latter receptor displayed a more potent ligand-induced GTPase activity (Fig. 2*B*). Such enhanced G-protein activation was previously noted for other phosphorylation-deficient mutants of G-protein-coupled receptors, including the chemoattractant receptors for platelet activating factor (32, 33). This is likely due to the lack of desensitization in G-protein coupling (34). Of interest, activation of ΔCyto CXCR4 by SDF-1 resulted in a biphasic calcium transient (Figs. 2*C* and 4*A*). Desensitization of calcium mobilization revealed unexpected complexity in the regulation of these receptors. While the calcium mobilization in Wt CXCR4 was completely desensitized by prior treatment with SDF-1, only the rapid release but not the sustained increase was inhibited in ΔCyto CXCR4. Selective desensitization of the first calcium peak suggests that calcium influx, as opposed to mobilization of intracellular calcium, is independently regulated, perhaps by distinct pertussis toxin-sensitive G-proteins or G-protein independent effectors. This remains to be explored.

The cytoplasmic tail of CXCR4 contains 18 serine/threonine residues which are likely targets for phosphorylation by G-protein-coupled receptor kinases and second messenger-activated protein kinases (35). Like other CXC chemokine receptors (28, 36), CXCR4 is also phosphorylated by PKC activation by PMA (Fig. 3). The complete inhibition of PMA-induced phosphorylation and partial inhibition of SDF-1-induced phosphorylation by staurosporine, a PKC inhibitor, suggests that agonist-induced phosphorylation of CXCR4 has two components, one represented by the activation of PKC and another due to a staurosporine-insensitive G-protein-coupled receptor kinase (GRK) that phosphorylates the agonist occupied form of the receptors. Multiple G-protein-coupled receptor kinases were previously identified in phagocytic leukocytes (37).

Receptor phosphorylation is an important mechanism for the homologous and heterologous desensitization of chemoattractant receptors (27, 28, 38). However, signals other than receptor phosphorylation were reported to be responsible for a phe-

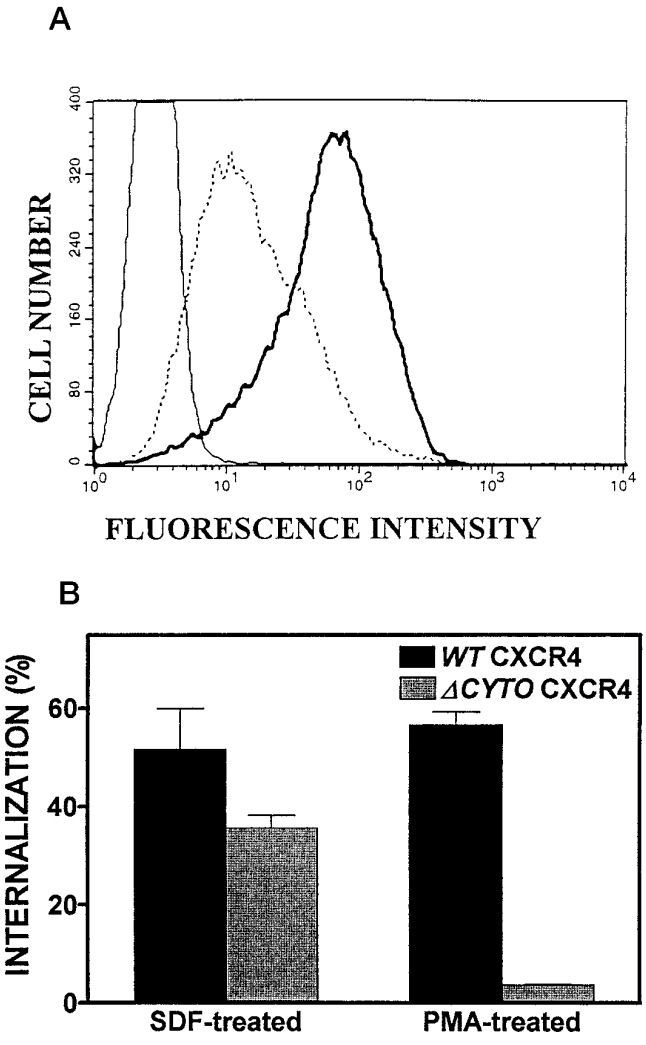

FIG. 6. **Internalization of CXCR4.** *A,* FACS analysis of surface expression of CXCR4. RBL cells (5×10^5/sample) expressing the Wt CXCR4 were incubated in HEPES (20 mM)-buffered DMEM for 30 min at 37 °C in the presence and absence of SDF-1 (100 nM) and washed in ice-cold medium and incubated with 12G5 antibody for 1 h followed by the fluorescein isothiocyanate-labeled goat anti-mouse IgG for 30 min. Cells were washed and fixed in 2% formaldehyde in phosphate-buffered saline and analyzed in FACScan flow cytometer. The staining of cells in the absence of primary antibody 12G5 (*thin line*) or with 12G5 antibody in cells incubated with (*broken line*) and without SDF-1 (*thick line*) treatment are shown. *B,* effect of SDF-1 and PMA on internalization of Wt CXCR4 and ΔCyto CXCR4. The effect of PMA and SDF-1 treatment on the surface expression of Wt CXCR4 and ΔCyto CXCR4 was measured as described above. Mean fluorescence values without any treatment were taken as 100%, and based on the mean fluorescence after treatment, the percent receptors lost from the surface were calculated. Data shown are average of three independent experiments and in each experiment duplicate samples were measured.

nomenon termed class desensitization (38, 39). Recently, it was shown that PLCβ3 was phosphorylated both by PKC and PKA mediated pathways (25) and PLCβ2 was phosphorylated by PKA (24). Previous Western blot experiments using isoform-specific antibodies have shown that PLCβ3 is the only known PLCβ isoform expressed in RBL cells. Recent experiments using reverse transcriptase-polymerase chain reaction also indicate that PLCβ3 is the only PLCβ isoform expressed in these cells.[2] The data presented herein with CXCR4 and ΔCyto CXCR4 indicates that both PKC and PKA activation negatively regulates the signaling of CXCR4 independent of receptor phosphorylation. In addition, PKC also has a receptor phosphorylation-dependent action on the function of CXCR4. Demonstration of PLCβ3 phosphorylation by SDF-1, PMA, and cAMP in cells expressing ΔCyto CXCR4 suggest that phosphorylation of this enzyme may be responsible for attenuation of signal transduction through phospholipase C (Fig. 5).

Ligand binding is known to cause rapid internalization of many G-protein-coupled receptors, including chemokine receptors CXCR1 and CXCR2 (36, 40–42). SDF-1 binding results in the internalization of CXCR4 and phosphorylation facilitates this process but is not absolutely required. In the case of β2-adrenergic receptors, phosphorylation by receptor kinases was shown to enhance the arrestin-dependent internalization (43). PKC activation by PMA also induced sequestration of CXCR4 which was completely dependent on the presence of cytoplasmic tail. Phosphorylation of the cytoplasmic tail of CXCR4 is likely responsible for the down-regulation of CXCR4, but this contention will have to be confirmed by substitution mutations of the PKC sites on the receptor. PMA-induced down-regulation of CD4 in the presence of GP120 required an accessory protein and it was recently shown that this accessory protein is likely CXCR4 (44, 45). The results presented here suggest that this down-regulation may involve phosphorylation on the cytoplasmic tail of CXCR4.

The phosphorylation independent reduction in surface expression of ΔCyto CXCR4 suggests an additional motif regulating internalization. Multiple mechanisms for internalization of surface proteins were described (40, 46, 47). Recent studies have demonstrated that phosphorylation at either one of the two independent clusters of phosphorylation sites in m2-muscarinic receptors is sufficient for agonist-induced internalization of the receptor, whereas mutation of both clusters severely impaired internalization (48). Internalization of type A cholecystokinin receptor was unaffected by C-terminal truncation, whereas internalization of type B cholecystokinin receptor was significantly reduced in a C-terminal truncation mutant (49). While arrestins and dynamin appear to play an important role in phosphorylation-dependent sequestration of G-protein-coupled receptors (43, 46), the molecular mechanisms of phosphorylation-independent sequestration observed here with CXCR4 and previously with other G-protein-coupled receptors (49) remain to be determined.

It is, at present, not known whether internalization of receptors has any direct relevance to HIV-1 infection. While signal transduction through G-proteins is not required for the usage of CCR5 as co-receptor for HIV-1L, it is not known whether internalization defective mutants will act as co-receptors (50, 51). The rapid ligand-induced internalization will have the effect of reducing surface expression and as a consequence availability of the co-receptor. In addition, PMA induced desensitization as well as down-regulation of CXCR4 suggests that activation of other receptors that enhance PKC activity are likely to have an effect on signal transduction and surface expression of this receptor.

In summary, we have established a model for stable functional expression of CXCR4 and shown that receptor activity is regulated at multiple levels by receptor phosphorylation dependent and independent mechanisms. The ability to express mutant receptors should allow for the analysis and functional consequences of interactions of HIV-1 GP120 and CD4 with CXCR4.

[2] B. Haribabu, S. Sozzani, H. Ali, and R. Snyderman, unpublished results.

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