Phorbol Esters and SDF-1 Induce Rapid Endocytosis and Down Modulation of the Chemokine Receptor CXCR4

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Abstract. The chemokine receptor CXCR4 is required, together with CD4, for entry by some isolates of HIV-1, particularly those that emerge late in infection. The use of CXCR4 by these viruses likely has profound effects on viral host range and correlates with the evolution of immunodeficiency. Stromal cell-derived factor-1 (SDF-1), the ligand for CXCR4, can inhibit infection by CXCR4-dependent viruses. To understand the mechanism of this inhibition, we used a monoclonal antibody that is specific for CXCR4 to analyze the effects of phorbol esters and SDF-1 on surface expression of CXCR4. On human T cell lines SupT1 and BC7, CXCR4 undergoes slow constitutive internalization (1.0% of the cell surface pool/min). Addition of phorbol esters increased this endocytosis rate >6-fold and reduced cell surface CXCR4 expression by 60 to 90% over 120 min. CXCR4 was internalized through coated pits and coated vesicles and subsequently localized in endosomal compartments from where it could recycle to the cell surface after removal of the phorbol ester. SDF-1 also induced the rapid down modulation (half time ∼5 min) of CXCR4. Using mink lung epithelial cells expressing CXCR4 and a COOH-terminal deletion mutant of CXCR4, we found that an intact cytoplasmic COOH-terminal domain was required for both PMA and ligand-induced CXCR4 endocytosis. However, experiments using inhibitors of protein kinase C indicated that SDF-1 and phorbol esters trigger down modulation through different cellular mechanisms.

SDF-1 inhibited HIV-1 infection of mink cells expressing CD4 and CXCR4. The inhibition of infection was less efficient for CXCR4 lacking the COOH-terminal domain, suggesting at least in part that SDF-1 inhibition of virus infection was mediated through ligand-induced internalization of CXCR4. Significantly, ligand induced internalization of CXCR4 but not CD4, suggesting that CXCR4 and CD4 do not normally physically interact on the cell surface. Together these studies indicate that endocytosis can regulate the cell-surface expression of CXCR4 and that SDF-1–mediated down regulation of cell-surface coreceptor expression contributes to chemokine-mediated inhibition of HIV infection.

S EVERAL members of the family of leukocyte chemokine receptors have been implicated in the fusion and entry of human and simian immunodeficiency viruses. Chemokine receptors are members of the superfamily of seven transmembrane domain, G protein-coupled receptors that bind small peptides of the so-called CXC (α) and CC (β) families of inflammatory chemokines (for review see 42, 52, 54). Initially, the CXC chemokine receptor CXCR4 (previously termed LESTR, HUMSTER, and Fusin [21, 37]) was identified as a coreceptor, together with CD4, for the entry of T cell line–adapted human immunodeficiency virus (HIV)[1]-1 viruses (6, 21). Subsequently, the CC chemokine receptor CCR5 was found to

1. *Abbreviations used in this paper*: HA, hemagglutinin; HIV, human immunodeficiency virus; MIP, macrophage inflammatory peptide; PDB, phorbol dibutyrate; SDF, stromal cell–derived factor.

be required for the entry of macrophage tropic viruses (10, 14, 18). Other chemokine receptors (CCR3, CCR2b, and CCR1) have been implicated in the entry of dual (10, 17) and neurotropic viruses (28), while CXCR4, CCR3, and an orphan receptor VT28 can mediate the entry of CD4-independent strains of HIV-2 (20, 55; for an extensive review of HIV coreceptor usage see 40). The use of particular chemokine receptors by HIV-1 may have important biological consequences not only for the viral host range, but also for pathogenesis, since viruses isolated in the initial stages of infection primarily use CCR5, while those isolated from patients with advanced immunodeficiency may use CXCR4 in addition to, or in place of, CCR5 (13).

The precise role of chemokine receptors in virus entry is unclear. The initial interaction of the viral envelope protein (Env) with CD4 is believed to induce conformational changes in Env (19, 39, 57) that facilitate an interaction with the chemokine receptor (62, 64) and assembly of a tri-molecular complex of CD4, chemokine receptor, and Env (36). The interaction of Env with both CD4 and CXCR4 appears to be crucial for the events that lead to viral fusion and entry into the cell. Significantly, the CC chemokines, macrophage inflammatory polypeptide (MIP)-1α, MIP1β, and RANTES (regulated on activation normal T cell expressed and secreted) can inhibit the entry of macrophage tropic HIV-1 isolates into CCR5-positive target cells (12) and stromal cell–derived factor (SDF)-1, the ligand for CXCR4, can inhibit infection of at least some T cell line-adapted viruses (7, 45). The mechanism through which these agents inhibit infection is unclear. The chemokines could inhibit viral entry by blocking the interaction of the Env with the chemokine receptor (62, 64). Alternatively, as observed with other G protein–coupled receptors (33, 56, 61, 63), the ligand may induce internalization, thereby preventing assembly of the fusion complex.

We previously described a murine monoclonal antibody, 12G5, that is specific for CXCR4 (20). Among a panel of CHO cell lines that stably expressed CXC (CXCR1, CXCR2, and CXCR4) and CC (CCR1-5) receptors, 12G5 reacted only with cells that expressed CXCR4. Subsequent studies have mapped the 12G5-binding site to a conformational epitope that includes the second extracellular loop of CXCR4 (Hoxie, J.A., unpublished results). In this study, we have used 12G5 to evaluate the effects of phorbol esters and SDF-1 on CXCR4 expression and the extent to which surface levels of CXCR4 are regulated by endocytosis.

Materials and Methods

Reagents

All tissue culture reagents were from GIBCO BRL, Ltd. (Paisley, Scotland), and other chemicals were from Sigma Chemical Co. (Poole, England), unless otherwise indicated. Tissue culture plastic was from Nunc (Roskilde, Denmark), and radioactive reagents were from Amersham International plc (Little Chalfont, England). Recombinant SDF-1α was purified from *E. coli*. This SDF contained an additional NH$_2$-terminal methionine. However, the protein was biologically active as demonstrated by (*a*) Ca^{2+} flux assays on Fura-2–loaded SupT1 cells and CHO-CXCR4 cells; (*b*) potent activity (10–100 pM) in a CXCR4-transfected melanophore assay; (*c*) inhibition of HIV-1 entry, and (*d*) ligand-induced receptor down modulation (see text). In addition, chemically synthesised SDF-1 (7, 45) was kindly provided by Dr. Ian Clark-Lewis (University of British Columbia, Vancouver, Canada).

Cells

The CD4-positive human T cell line SupT1, and a CD4^{-ve} derivative of SupT1 called BC7 (20), were maintained in RPMI-1640 containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (PenStrep). CHO-K1 were maintained in DMEM F12 containing 10% FCS, glutamine, and PenStrep as above. The rhabdomyosarcoma cell line RD was obtained from P. Clapham (Institute of Cancer Research, London, UK) and maintained in DME containing 5% FCS, glutamine, and PenStrep as above. Mv-1-Lu mink cells stably expressing human CD4 (Mv-1-Lu-CD4) were obtained from the Medical Research Council AIDS Reagents Programme (NIBSC, Potters Bar, UK) and maintained in DME containing 10% FCS, glutamine, and PenStrep as above and 1 mg/ml G418.

CHO-K1 cells stably expressing human CXCR4, or either CXCR4 or CCR4 tagged at the NH$_2$ terminus with an epitope (YPYDVPYASLRS) from the influenza virus haemagglutinin (HA), have been described (20). Mv-1-Lu-CD4 cells were transfected by electroporation with human CXCR4 in the mammalian expression vector pTEJ8 (34), together with pBABE-hygro (41). Clones were selected in medium containing 500 μg/ml hygromycin and screened for CXCR4 expression by immunofluorescence using 12G5. Mv-1-Lu-CD4 cells expressing a CXCR4ΔCyt protein lacking 42 amino acids from the COOH-terminal cytoplasmic domain were generated using a human CXCR4 construct in which the threonine 311 codon was replaced with a stop codon by site-directed mutagenesis.

Antibodies

The anti-CXCR4 mAb 12G5 (IgG$_{2a}$) and the anti-CD4 mAb Q4120 (IgG$_1$) were described previously (20, 29). FITC-conjugated L120 (anti-CD4) was purchased from Becton Dickinson UK Ltd. (Oxford, UK), and rabbit antibodies against human LAMP1 were kindly provided by Dr. Sven Carlsson (University of Umeå, Umeå, Sweden).

12G5 and Q4120 were ^{125}I labeled using Bolton and Hunter reagent. Briefly, Bolton and Hunter reagent (0.5 mCi at ∼2,000 Ci/mmol) was dried onto the sides of a 1.5-ml microcentrifuge tube. Antibody (∼650 pmol) in 50 μl 0.1 M borate buffer, pH 8.5, was added, the tube vortexed, and the reaction incubated at room temperature for 20 min. The reaction was stopped by addition of 0.2 M glycine in borate buffer and the iodinated protein separated from the reagents by gel filtration over an Econo-pac 10DG column (Bio Rad, Hemel Hempstead, UK) eluted with PBS containing 0.25% gelatine and 0.02% NaN$_3$. Specific activities of 303 to 391 Ci/mmol were obtained for different iodinations. Radioiodinated proteins were stored in small aliquots at −20°C and were stable for up to 4 mo.

Binding Assays

Antibody binding on live cells was carried out at 4°C. Adherent cells, usually in 16-mm wells, were incubated with radiolabeled antibody in binding medium (BM: RPMI-1640 without bicarbonate, containing 0.2% BSA and 10 mM Hepes, and adjusted to pH 7.4, unless indicated otherwise) for 1 to 5 h at the indicated temperatures. Subsequently, the label was removed and the cells washed 4 times with cold BM and twice with cold PBS. The cells were then drained, collected in 400 μl 0.2 M NaOH, and each well rinsed with 400 μl H$_2$O. The cells and washings were transferred to tubes for γ counting. Fixed cells were used for binding analysis at ambient temperature (20–22°C) or 37°C. For these experiments, the cells were washed in PBS and fixed in 3% paraformaldehyde (PFA) in PBS for 10 min at room temperature. Subsequently, the cells were washed 4 times with PBS and free aldehyde groups quenched with 50 mM NH$_4$Cl in PBS. The cells were again washed with PBS and then incubated with labeled antibody as above. Protein concentrations were determined using bicinchoninic acid (Pierce, Chester, UK).

Phorbol Ester and SDF-1 Mediated Down Modulation

Cells were incubated in BM or in BM containing phorbol ester or SDF-1 as indicated in the text. For some experiments the cells were treated with 0.5 μM staurosporin or with 1 μM calphostin C (LC Laboratories Europe, Alexis Corporation Ltd, Nottingham, UK) for 30 min before addition of an equal volume of medium containing SDF-1 or phorbol ester. Cells treated with calphostin C were incubated under a fluorescent strip light for 3 min at room temperature before incubation at 37°C (9). After treatment, the cells were placed on ice and cooled by addition of 10 ml of ice-cold BM. T cells were then centrifuged (1,500 rpm for 5 min), washed

once in PBS, and fixed with PFA as described above. After quenching and washing with BM the cells were labeled with 0.5 nM ^{125}I-12G5 antibody for 2 h at room temperature. Subsequently, the cells were washed twice in BM and once in PBS, resuspended in 3 ml of PBS, layered onto a 1-ml cushion of 5% BSA in PBS, centrifuged (1,800 rpm for 5 min), and the cell pellets recovered for γ counting. For adherent cells 12G5 binding was as described above.

Endocytosis Assays

Endocytosis assays on adherent or suspension cells were performed essentially as described (48, 49). Suspension cells were harvested by centrifugation, washed twice, and resuspended at 5×10^6 cells/ml in 4°C BM containing 1 nM ^{125}I-12G5. The cells were placed on a rotator and antibody bound for 2 h at 4°C. Subsequently, the cells were washed twice in BM to remove free antibody, and resuspended in 37°C BM with or without PMA. At the indicated times, duplicate 1-ml aliquots were removed, placed into 5 ml ice-cold BM, and the cells collected by centrifugation (1,500 rpm for 5 min). One aliquot for each pair was incubated for 5 min in cold BM adjusted to pH 2.0, to elute cell surface-bound antibody, and the other was washed in BM. Subsequently, the cells were layered onto a 5% BSA cushion, centrifuged, and recovered for γ counting as described above.

Adherent cells were seeded in 16-mm diameter wells in either 4- or 24-well plates and grown for 2 d to a final density of 1 to 2×10^5 cells/well. The cells were cooled on ice, washed with BM, and incubated with 300 µl BM containing 1 nM ^{125}I-12G5 for 2 h on ice. Subsequently, the free antibody was washed away and the cells warmed by addition of 1 ml 37°C BM. At the indicated times the cells were returned to 4°C, the media collected, and the cells washed with cold BM. For each time point at least four wells were used. For half of the wells, the cells were collected directly in 400 µl 0.2 M NaOH and transferred to tubes for γ counting (total cell-associated activity). To determine the intracellular activity, the remaining wells were rinsed twice with 0.5 ml of 4°C BM adjusted to pH 2.0, and then incubated twice for 3 min with 1 ml of the same medium to remove cell-surface antibody. The cells were harvested in NaOH as above. The proportion of internalized activity for each time point was determined by dividing the acid-resistant activity by the total cell-associated activity, and endocytic rates were calculated by analysis of the data from the first 5 min of warm up.

Immunofluorescence Microscopy

Method One. T cells were immobilized on 13-mm poly-D-lysine–coated glass coverslips, fixed and quenched as described above, and stained intact or after treatment with 0.05% saponin for 10 min at room temperature. All solutions were made in PBS. Antibodies were diluted in PBS containing 0.2% gelatine and, for permeabilized cells, 0.05% saponin. Cells were incubated for 1 h with primary antibodies. Fluorescent second layer antibodies were all diluted 1:2,000 in 0.2% gelatine, and incubations were for 1 h.

Method Two. T cells were washed twice in cold BM and labeled with 7 µg/ml (50 nM) 12G5 for 2 h at 4°C. Some samples were also labeled with FITC-conjugated L120. The cells were washed twice in cold BM. One aliquot of cells was kept at 4°C, while the others were incubated at 37°C in BM with or without PMA. At the indicated times, 1 ml of cell suspension was transferred to cold BM, centrifuged, and washed in cold BM. The cells were fixed in 3% PFA, washed twice in PBS, and free aldehyde groups quenched using 50 mM NH_4Cl. The cells were attached to poly-D-lysine coated coverslips, washed in PBS containing 0.2% gelatine and 0.05% saponin for 15 min, and incubated for 30 min with anti–mouse-Biotin (Amersham Intl. plc) and, where indicated, antibodies against LAMP1. The cells were washed again and stained with streptavidin conjugated to FITC or Texas red, or Rhodamine-conjugated goat anti–rabbit antibodies (Pierce) as appropriate. Subsequently the coverslips were mounted in Moviol and examined using a microscope (Optiphot-2; Nikon, Melville, NY) equipped with a laser scanner (MRC 1024; Bio Rad). The images were assembled in and printed directly from Adobe Photoshop.

Flow Cytometry

Expression of cell surface antigens was analyzed using a FACSCalibur® flow cytometer (Becton Dickinson). Briefly, cells were centrifuged (2,000 RPM for 5 min), resuspended in ice cold PBS containing 0.1% BSA and 0.02% NaN_3, and incubated with 50 nM 12G5 for 30 min at 4°C. Subsequently, the cells were incubated with FITC- or PE-conjugated F(ab)′$_2$ goat anti–mouse IgG (Tago Laboratories, Burlingame, CA).

Electron Microscopy

T cells were collected by centrifugation (1,500 rpm for 5 min), washed once in BM containing 1% BSA, and labeled in 50 nM 12G5 for 2 h at 4°C. To determine nonspecific binding, some cells were incubated in medium without the primary antibody. After three washes in BM/1% BSA, cells were labeled with 10-nm protein A-gold particles (British Biocell International, Cardiff, UK) for an additional 2 h at 4°C. Cells were washed once in BM/1% BSA and twice in BM containing 0.1% BSA and divided into three aliquots. One sample was kept on ice. The other two were warmed to 37°C for 2 min by resuspension in 37°C BM/0.1% BSA with or without PMA. Subsequently, the cells were returned to 4°C by dilution with 10 ml of ice-cold BM/0.1% BSA, harvested by centrifugation, and fixed in 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.2, containing 50 mM KCl and 2.5 mM $MgCl_2$. Alternatively, fresh cells were prefixed by mixing cell suspensions in an equal volume of double-strength fixative (4% PFA/0.4% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4). After 10 min at room temperature, the cells were collected and resuspended gently in 2% PFA/0.2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for an additional 80 min. Cells were washed and quenched overnight in PBS containing 1% BSA and 50 mM glycine before staining with 50 nM 12G5 for 2 h at room temperature, followed by protein A-gold for 2 h. After postfixation in osmium tetroxide, cells were stained in Kellenberger's uranyl acetate, dehydrated, and embedded in Epon. Ultrathin sections were examined using a transmission electron microscope (EM 400; Phillips, Eindhoven, The Netherlands).

HIV-1 Infection

For HIV-1 infection, semi-confluent Mv-1-Lu-CD4/CXCR4 and -CD4/CXCR4ΔCyt cells were trypsinized, washed, and 2×10^4 cells seeded in each well of a 96-well plate in 200 µl DME with 10% FCS and cultured for 2 d before infection. The cultures were treated with SDF-1 or RANTES at twice the indicated concentration for 30 min at 37°C. An equal volume of HIV_{IIIB} diluted from a stock virus (10^3 focus forming units) was added and the cells cultured for 14 h before the virus and chemokine were removed. The cells were cultured for an additional 2 d and then fixed in methanol/acetone. They were then stained with the anti–HIV-1 Gag mAbs E67.1 and 38:96K for 1 h and subsequently with a goat anti–mouse IgG conjugated to β galactosidase (Seralab, Crawley Down, UK) for 1 h. β Galactosidase activity was detected by incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and foci of blue cells were viewed by light microscopy and counted.

Results

12G5 Binding

To use 12G5 as a probe for CXCR4 trafficking we initially characterized the binding properties of this antibody on human RD and SupT1 cells constitutively expressing CXCR4 and on CHO cells expressing recombinant human CXCR4 with or without an NH_2-terminal HA tag. In preliminary experiments we found that on cells labeled at 4°C and on cells fixed with 3% PFA and labeled at room temperature or 37°C, antibody binding at $\sim K_d$ concentration was slow. At 4°C it took 5 h to reach near saturation ($t_{1/2}$ maximum binding, 1 nM = 80 min), while at both room temperature and at 37°C, binding was slightly faster ($t_{1/2}$ maximum binding = 55 min) but still required >5 h to reach steady state (not shown). Similar amounts of antibody were bound after 5 h at all temperatures, suggesting that the 12G5 epitope was not significantly affected by aldehyde fixation. No binding was seen on CHO cells expressing HA-tagged CCR4 under the same conditions. Although binding was slow, the dissociation rate for bound antibody was also slow. On CHO-CXCR4-HA <12% of the bound antibody dissociated from the cells over 2 h at 37°C (not shown), while on BC7 cells, >80% remained cell bound after 1 h (see Fig. 3 A).

To determine the level of CXCR4 expression we incubated cells with increasing concentrations of ^{125}I-12G5 for 5 h. Even at the highest ^{125}I-12G5 concentration used, binding on CXCR4-HA cells was not fully saturated (Fig. 1 *A*), probably because these cells express relatively high levels of the CXCR4-HA protein. Scatchard analysis of the data indicated $\sim 1.3 \times 10^6$ antibody binding sites per cell with a K_d of 4 nM (Fig. 1 *B*). Similar analysis of BC7 cells indicated 50,000–100,000 binding sites with a K_d of 2 to 4 nM (not shown). Analysis of the RD cell line indicated $\sim 380,000$ 12G5 binding sites per cell with a K_d of 2.2 nM (Fig. 1 *B*). HeLa cells that are permissive for T cell line-adapted HIV-1 viruses, when transfected with CD4 (21), expressed <60,000 12G5 binding sites (not shown). In alternative assays using unlabeled 12G5 to compete ^{125}I-12G5 (1 nM), binding on CHO-CXCR4-HA cells was saturated at 20 to 30 nM 12G5 with an IC_{50} of 3.3 nM.
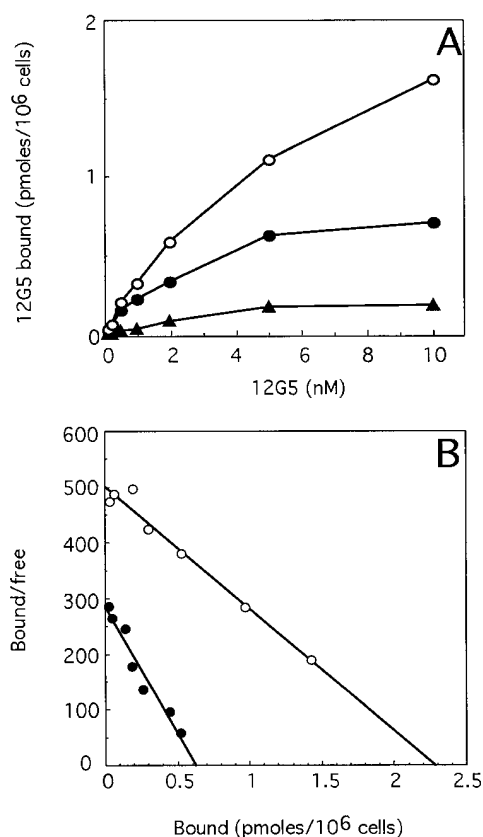

Figure 1. Binding properties of 12G5 on CXCR4-expressing cells. (*A*) Concentration dependence of 12G5 binding. CHO cells expressing CXCR4-HA (○) or CCR4-HA (▲) and RD cells (●) were incubated with increasing concentrations of ^{125}I-12G5 (up to 10 nM) for 5 h at 4°C. Aliquots of the unbound label (*free*) were taken for counting and the cells washed and harvested. The protein per well was determined and used to calculate the amount of antibody bound per 10^6 cells. The binding recorded on CCR4-HA cells was taken as background and was deducted from the other cell lines to generate the binding data used in the Scatchard analysis illustrated in *B*. (*B*) Scatchard analysis of 12G5 binding. The bound and free 12G5 activities derived from the experiment illustrated in *A* were used for Scatchard-type analysis of 12G5 binding to native CXCR4 expressed on RD cells (●) and CXCR4-HA expressed on CHO cells (○).

Scatchard analysis of this data indicated a single class of binding site with a K_d of 1 nM and $\sim 10^6$ binding sites per cell (not shown).

Together the K_ds for 12G5 binding on different cell lines fell within the range 1–5 nM, and the iodinated antibody and native protein had similar affinities for antigen. For subsequent biochemical experiments, ^{125}I-12G5 was used at 1 nM, unless indicated otherwise, while higher concentrations of unlabeled mAb were used for morphological assays.

Down Modulation of Cell Surface CXCR4

Previous studies have indicated that phorbol esters can modulate the cell surface expression of CD4 (31, 51) and may also influence CXCR4 expression (2, 25, 26, 36). To examine the effect of phorbol ester on CXCR4 expression directly, we incubated SupT1 cells at 37°C in the presence or absence of 100 ng/ml PMA. After 60 min the cells were fixed and stained with 12G5 either intact or after permeabilization with saponin. In the absence of PMA, clear cell surface CXCR4 staining was seen on intact SupT1 cells (Fig. 2 *A*, *A*). When untreated cells were permeabilized before incubation with antibody, much of the cell surface staining was lost, suggesting that the 12G5 epitope on CXCR4 was sensitive to detergent. Nevertheless, some internal punctate staining was observed (Fig. 2 *A*, *B*). After phorbol ester treatment, the cell surface staining on intact cells was significantly decreased (Fig. 2 *A*, *C*); concomitantly, the intracellular fluorescence increased markedly (Fig. 2 *A*, *D*), suggesting that phorbol esters induced endocytosis of cell surface CXCR4.

To determine the extent, concentration dependence, and kinetics of phorbol ester-induced down modulation, SupT1 and BC7 cells were incubated for 2 h in the absence or presence of phorbol ester. The cells were then cooled, labeled at 4°C with 12G5 and goat anti–mouse-FITC, and analyzed by FACS. Fig. 2 *B* indicates that the expression of CXCR4 was reduced by 65 and 95% on the PMA-treated SupT1 and BC7 cells, respectively. Control experiments on SupT1 cells indicated that CD4 underwent similar down modulation in the presence of PMA, but that MHC class 1 antigens did not (data not shown). Using a similar assay we determined that the 100 nM (60 ng/ml) PMA gave maximal CXCR4 down modulation on SupT1 (Fig. 2 *C*). Routinely, 100 ng/ml PMA was used for subsequent experiments. The kinetics of phorbol ester-induced down modulation showed that cell surface CXCR4 levels decreased with a half time of ~ 15 min for SupT1 cells (see Fig. 8 *A*).

Endocytosis of CXCR4 in T Cells

The appearance of increased intracellular CXCR4 staining after phorbol ester treatment (Fig. 2 *A*) suggested that PMA-induced CXCR4 down modulation occurred through endocytosis of CXCR4, rather than by mechanisms that altered the conformation of CXCR4 at the cell surface. To measure endocytosis directly we used ^{125}I-12G5 in assays previously established for CD4 (48, 49, 51). BC7 cells were labeled with ^{125}I-12G5 for 2 h at 4°C (conditions that inhibit endocytosis), washed, and then warmed to 37°C in the presence or absence of PMA. Antibody remaining at
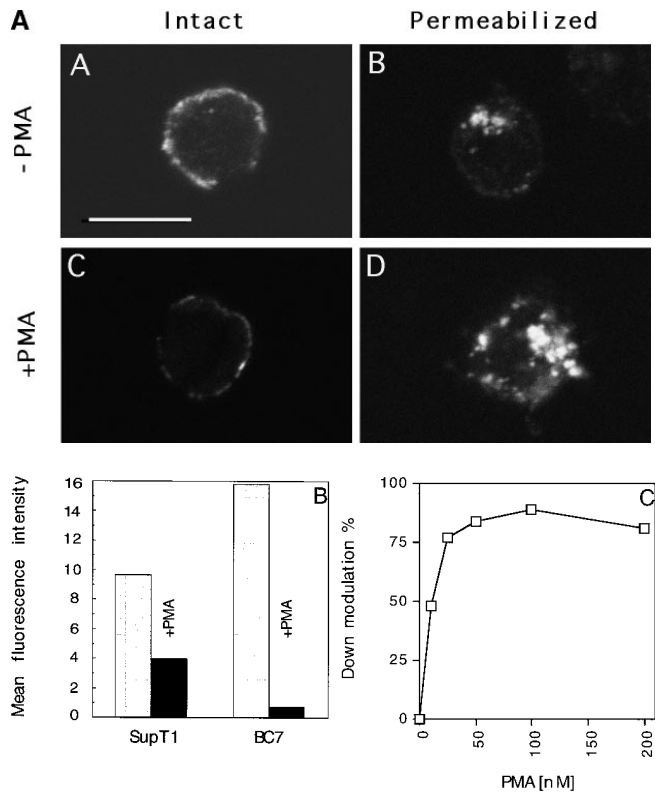
Figure 2. Phorbol ester-induced down modulation of CXCR4. (*A*) Immunofluorescence analysis of CXCR4 down modulation on SupT1 cells. Cells were incubated in medium with (*A, C* and *D*) or without (*A, A* and *B*) 100 ng/ml PMA for 60 min at 37°C. The cells were then fixed and stained with 12G5 either intact (*A, A* and *C*) or after permeabilization (*A, B* and *D*) with saponin. (*B*) SupT1 and BC7 cells were incubated in medium with (■) or without (□) 100 ng/ml PMA for 120 min at 37°C. Subsequently the cells were fixed and stained first with 12G5 and then with a FITC-conjugated anti–mouse reagent. The stained cells were analysed by FACScan® and the mean fluorescence intensity determined for each sample. (*C*) The dose dependence for PMA-induced down modulation of CXCR4 was determined on SupT1 cells. Cells were incubated in medium containing the indicated concentration of PMA for 60 min at 37°C. The cells were then fixed, stained as described for *B*, and analyzed by FACScan®. Down modulation was calculated from the mean fluorescence intensity for each sample and compared to cells stained without primary antibody. Bar, 25 μm.

the cell surface after the 37°C incubation was removed by briefly incubating the cells in 4°C media adjusted to pH 2.0. Control experiments indicated that >95% of the cell surface mAb was eluted under these conditions (not shown). The remaining acid-resistant, cell-associated activity was intracellular.

In BC7 cells, bound ^{125}I-12G5 was observed to undergo slow (∼1% of the cell surface pool/min) constitutive endocytosis (Fig. 3 *A*). Uptake reached steady state 30–60 min after warm up, when ∼18% of the initial cell surface pool of antibody was inside the cells. These rates were similar to the bulk flow internalization of cytoplasmic domain-deleted forms of CD4 measured previously in T cell lines (48) and suggest that CXCR4 undergoes slow ligand-independent internalization and recycling on these cells. When cells were warmed in the presence of PMA, the rate
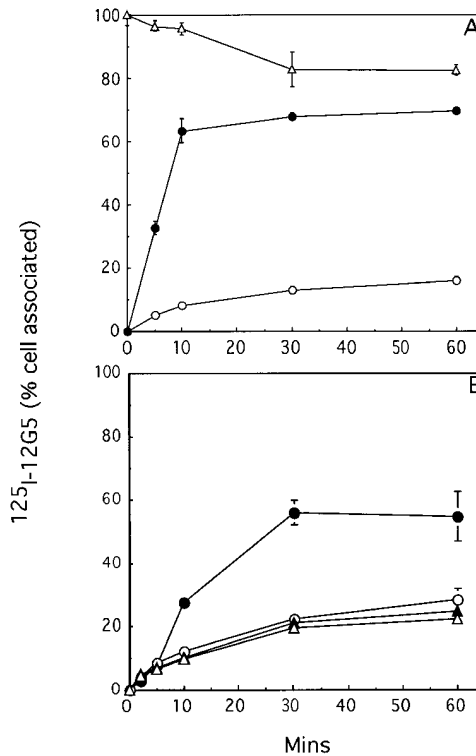

Figure 3. Endocytosis of CXCR4 in BC7 and Mv-1-Lu cells. BC7 cells in suspension (*A*) or confluent cultures of Mv-1-Lu-CD4/CXCR4 cells (*B*) were labeled at 0 to 4°C with ^{125}I-12G5, washed, and warmed to allow endocytosis of the ligand. The amount of internalized antibody was determined by acid washing as described in Materials and Methods. The plots show the acid-resistant activity as a proportion of the total cell-associated counts for cells warmed in the absence (○) or presence of PMA (●). In *A* the total cell-associated activity is shown for the course of the experiment (△). *B* (△ and ▲) shows the endocytosis kinetics of CXCR4ΔCyt in the absence and presence of PMA, respectively. All data points show means and standard deviations for triplicate samples of representative experiments.

of uptake was increased by >6-fold and reached steady state between 15 and 30 min, when ∼80% of the cell-associated radioactivity was intracellular. The PMA-induced increase in CXCR4 endocytosis was again similar to the phorbol ester-induced endocytosis and down modulation of CD4 (31, 32, 51) and suggests that the activation of protein kinase C can induce the exposure of one or more endocytosis signals in CXCR4. Very similar data were obtained with SupT1 cells (not shown).

Endocytosis of CXCR4 in Transfected CHO and Mink Cells

We also examined the properties of human CXCR4 expressed in transfected cell lines. Initially we used stable CHO cell lines but found that these cells show fast constitutive endocytosis of CXCR4 (∼2.5% per min) in the absence of phorbol ester and ligand. In these cells the uptake of radiolabeled antibody reached a peak after 30 min of endocytosis, when 60% of the initial cell surface pool was inside the cells (not shown). Furthermore, the uptake of ^{125}I-12G5 was only slightly enhanced by PMA and cell sur-

face CXCR4 expression was not significantly down modulated by PMA (data not shown).

As CHO cells did not reflect the trafficking of CXCR4 observed on T cell lines, we investigated alternative cells to analyze CXCR4 trafficking. CXCR4-transfected Mv-1-Lu-CD4 cells, a nonpolarized mink epithelial cell line, showed properties similar to those of T cells. When CXCR4 was stably expressed in these cells, we observed slow constitutive endocytosis of CXCR4 at $\sim$1% of the cell surface pool per minute. When phorbol ester was added to the medium, the rate of endocytosis increased 3–4-fold (Fig. 3 B). At steady state, $\sim$60% of the initial cell surface pool of ^{125}I-12G5 was intracellular. In these cells we examined whether a mutant CXCR4 construct containing an early stop codon that prevents synthesis of the last 42 amino acids of the 45 amino acid COOH-terminal cytoplasmic domain of the molecule was able to undergo endocytosis. CXCR4ΔCyt was expressed on the cell surface at levels similar to CXCR4 but, in contrast to the full length constructs, CXCR4ΔCyt was internalized slowly (Fig. 3 B) at rates and to levels comparable to the basal endocytosis of CXCR4.

In addition, we analyzed the endocytosis of CD4 in Mv-1-Lu-CD4 cells expressing CXCR4 or CXCR4ΔCyt. In the absence of phorbol ester, CD4 was internalized slowly ($\sim$1% per min) on both CXCR4- and CXCR4ΔCyt-expressing cells, as reported for other transfected cells (48, 49). Addition of phorbol ester induced rapid endocytosis of CD4 on both CXCR4 and CXCR4ΔCyt cells (data not shown), indicating that both were capable of responding to phorbol ester and mediating endocytosis. Thus the lack of phorbol ester-induced endocytosis of CXCR4ΔCyt was due to the loss of the CXCR4 COOH-terminal domain and not defects in the ability of these cells to mediate endocytosis.

Together these data indicate that the trafficking properties of CXCR4 can be variable in different cellular backgrounds. However, for CXCR4 trafficking, transfected mink cells showed properties similar to those of T cells. In mink cells, CXCR4 undergoes slow constitutive endocytosis and recycling that is markedly enhanced by phorbol ester and likely to involve one or more endocytosis signals associated with the COOH-terminal domain of the molecule.

Constitutive and Phorbol Ester-induced Endocytosis of CXCR4 Occurs through Clathrin-coated Pits

To determine the route of CXCR4 endocytosis we examined the distribution of CXCR4 by immunoelectron microscopy. SupT1 and BC7 cells were labeled on ice with 12G5 and protein A-gold and either fixed directly or after warming to 37°C for 2 min in the presence or absence of PMA. The 2 min time point was selected as the time at which we expected to see the highest numbers of gold particles undergoing endocytosis. Thin sections were examined in the electron microscope and the distribution of gold particles on the cell surface scored.

As indicated in Table I, some background labeling was seen on cells labeled with protein A-gold alone. However, particle counts on cells incubated with 12G5 and protein A-gold indicated that the specific labeling was at least sevenfold above background. On cells labeled with 12G5-protein A-gold and maintained at 4°C, 2–3% of the gold particles
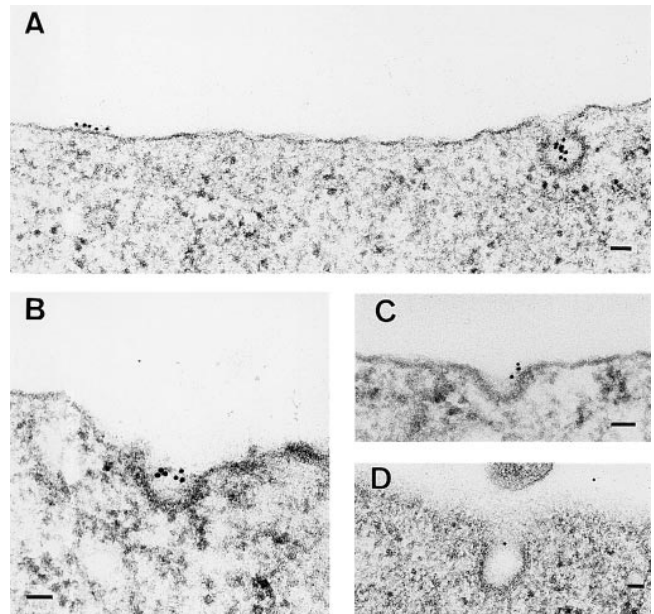

Figure 4. EM immunolocalization of CXCR4 on the surface of SupT1 and BC7 cells. SupT1 (*A* and *B*) and BC7 (*C* and *D*) cells were labeled at 4°C with 12G5 followed by protein A-gold (PAG$_{10}$) and either fixed directly (*A*) or warmed to 37°C for 2 min in medium containing PMA (100 ng/ml; *B* and *C*). Alternatively, cells were fixed in 2% paraformaldehyde/0.2% glutaraldehyde before labeling with 12G5 and PAG$_{10}$ (*D*). Bars, 50 nm.

were located over invaginations of the plasma membrane with cytoplasmic coats characteristic of clathrin (Fig. 4). On cells warmed to 37°C for 2 min, similar levels of labeling in coated pits and coated vesicles were detected (1.4 and 2.0% of particles for BC7 and SupT1 cells, respectively). After warm up in media containing phorbol ester, the number of particles associated with coated pits and coated vesicles was increased 3–4-fold (Table I). This increase was consistent with the phorbol ester-induced increase in the rate of CXCR4 endocytosis determined biochemically (see above), and suggested that the coated pit pathway is responsible

Table I. Cell Surface Distribution of 12G5-Protein A-Gold–labelled CXCR4

12G5-Protein A-gold	Particles counted	Cell profiles	Particles in coated pits
	BC7 cells		%
No 1°	50	30	0
Prefix	558	52	1.3
4°C	366	30	2.5
37°C 2 mins	587	50	1.4
37°C 2 mins PMA	749	67	4.0
	SupT1 cells		
No 1°	16	33	0
4°C	207	20	2.8
37°C 2 mins	759	46	2.0
37°C 2 mins PMA	297	19	6.4

SupT1 and BC7 cells were labeled with 12G5 and protein A-gold either before or after fixation (*Prefix*), as described in Materials and Methods. Cells labeled before fixation were kept on ice or were warmed to 37°C in the presence or absence of PMA. Subsequently all cells were fixed and processed for electron microscopy. Individual gold particles were scored at the microscope and their association with coated pits recorded.

for the majority, if not all, of the constitutive and phorbol ester-induced endocytosis of CXCR4 in these cells. Human lymphoid cells do not express Vip21/Caveolin (47), and no indication of particle association with noncoated invaginations of the plasma membrane was seen. After warm up, gold particles were also occasionally observed in larger vesicular structures resembling endosomes (not shown).

We observed some clustering of gold particles when cells were stained with 12G5 and protein A-gold, although this was variable with cell type and protein A-gold preparation. Notably, the clustering was similar in cells labeled on ice or warmed in the presence or absence of PMA, suggesting that it did not contribute to the enhanced localization in coated pits seen with PMA. Furthermore, when BC7 cells were fixed in PFA/glutaraldehyde before staining with 12G5 and protein A-gold, only single scattered gold particles were observed. Of these, 1.3% were found in coated pits, a figure similar to that found on cells stained on ice (see above).

Phorbol Ester Down Modulated CXCR4 Is Located in Endosomes

To determine where the antibody, and hence CXCR4, was located, we visualized 12G5 in SupT1 cells using indirect immunofluorescence and confocal laser scanning microscopy. Cells were labeled with 12G5 in the cold and warmed to 37°C in the presence or absence of 100 ng/ml PMA. Subsequently the cells were returned to 4°C, fixed, permeabilized, and stained with anti–mouse Ig secondary reagents. For some experiments, cells were also labeled with antibodies against CD4, or after fixation and permeabilization, with antibodies directed against the lysosomal membrane glycoprotein LAMP1.

Fig. 5 shows that when SupT1 cells were labeled at 4°C with antibodies to CD4 (*green*) and CXCR4 (*red*), both labels were seen at the cell surface with overlapping, though not completely colocalized, distributions. After warm up to 37°C in the presence of PMA, the cell surface staining for both labels was rapidly (within 5 min) reduced, and both labels were relocated into intracellular organelles located in the peripheral cytoplasm and in a cluster on one side of the nucleus. Frequently these organelles, which we presumed to be early endosomes, were labeled for both CXCR4 and CD4 (Fig. 5, indicated by the *yellow/orange* color). After 30 min of warm up, the staining for both CD4 and CXCR4 remained overlapped, but by 120 min the degree of overlap appeared to decrease, suggesting that the CD4 and CXCR4 molecules were segregated. The majority of vesicles containing 12G5 internalized for 60 min were accessible to a pulse of FITC-dextran applied during the final 15 min of 12G5 uptake, suggesting that CXCR4 was localized primarily to an endosome compartment (not shown). In the absence of PMA, some punctate intracellular staining for 12G5 appeared, presumably as a consequence of the constitutive endocytosis of CXCR4, but the cell surface labeling remained prominent (not shown). Little internalization of CD4 was observed in the absence of PMA, in keeping with the low level of constitutive uptake of this molecule in T cells (48, 50).

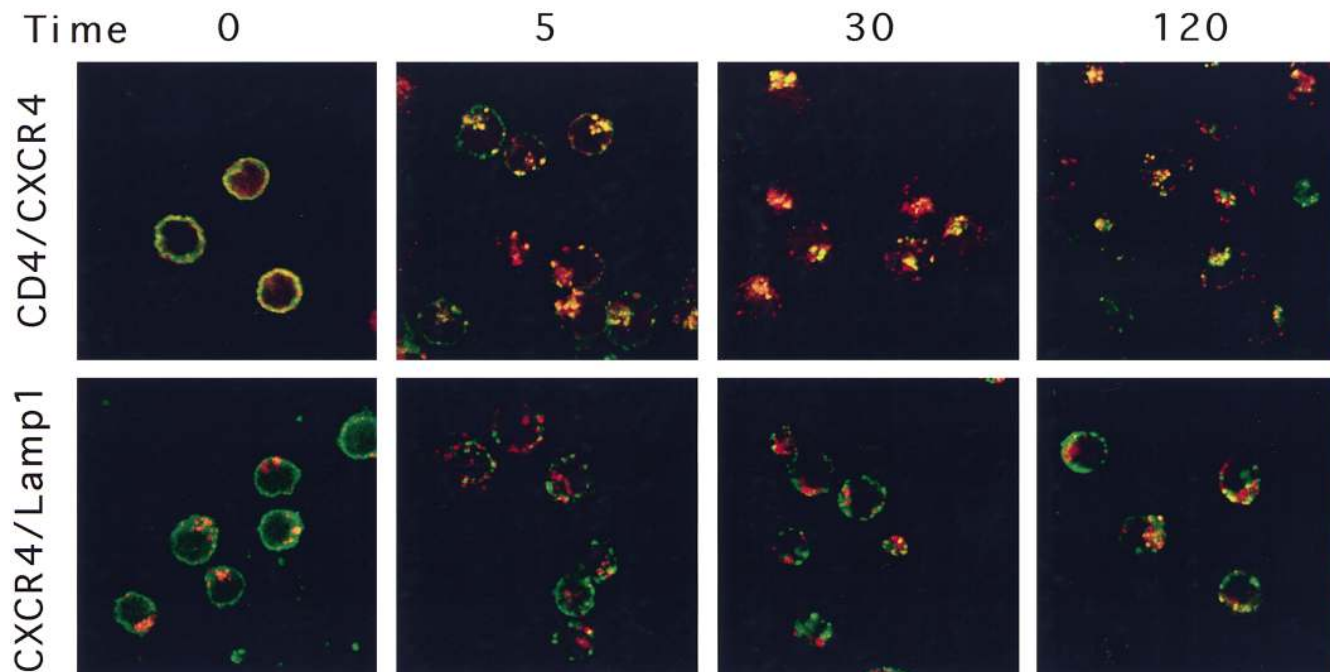The location of intracellular CXCR4 was compared to the distribution of LAMP1, an integral membrane glyco-

Figure 5. Immunofluorescence analysis of CXCR4 and CD4 internalization on SupT1 cells. Top figures (*CD4-green/CXCR4-red*): SupT1 cells were labeled with 12G5 and FITC-conjugated L120 (anti-CD4) at 4°C before warming to 37°C in the medium containing 100 ng/ml PMA. At the indicated times the cells were cooled, fixed, permeabilized, and stained with a biotin-conjugated isotype-specific anti–mouse reagent to detect 12G5, followed by streptavidin-Texas red. Lower figures (*CXCR4-green/LAMP1-red*): SupT1 cells were initially labeled with 12G5 alone. After fixation and permeabilization the cells were stained with anti-LAMP1 and Rhodamine-conjugated anti–rabbit antibodies and with a biotin-conjugated anti–mouse reagent and streptavidin-FITC to detect 12G5.
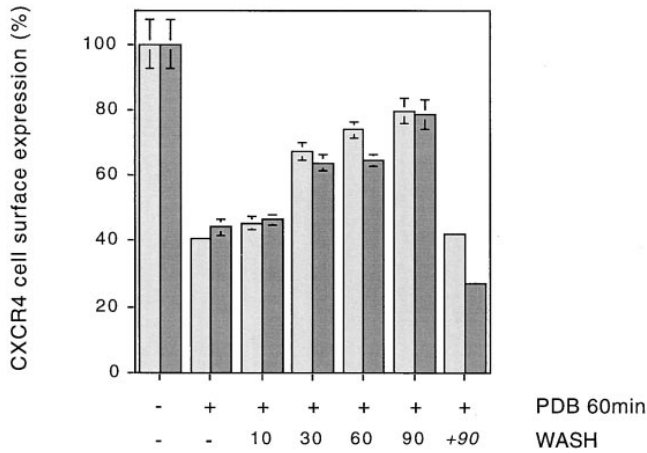
Figure 6. Recycling of internalized CXCR4. SupT1 cells were either left untreated or incubated in medium containing 100 ng/ml PDB for 60 min at 37°C. Aliquots of the treated cells were placed on ice or left in PDB for a further 90 min (*+90*). The remaining cells were washed three times with media to remove the PDB and incubated in fresh 37°C medium for the indicated time periods (☐). Subsequently, all cells were cooled to 4°C and incubated with ^{125}I-12G5 to determine cell surface CXCR4 levels. In parallel, a duplicate set of cells was treated in the same way with 100 µg/ml cycloheximide present throughout (☐). The data show the means and standard deviations from triplicate samples.

Figure 7. 12G5 binding to CXCR4 in the presence of SDF-1. (*A*) SupT1 cells were washed, fixed (3% PFA, 15 min), or cooled on ice and incubated in twofold dilutions of SDF-1 for 2 h at room temperature (☒) or 3 h at 4°C (☐), respectively. The cells incubated at 4°C were then fixed, and all the cells were labeled with 0.5 nM ^{125}I-12G5 for 2 h at room temperature. Subsequently, the cells were washed and the amount of bound antibody determined. Antibody bound in the presence of SDF-1 is expressed as a percentage of antibody bound in the absence of ligand and represents the means and SD for triplicate samples. (*B*) SDF-1 inhibition of HIV-1 infection. Mv-1-Lu-CD4/CXCR4 (■) and Mv-1-Lu-CD4/CXCR4ΔCyt (☐) were cultured in 96-well plates. The cells were incubated with twice the indicated concentration of SDF-1 (GlaxoWellcome) for 30 min before the addition of HIV-1$_{IIIB}$. After 14 h the virus and chemokines were removed and the cells incubated for a further 2 d. Finally, the cells were fixed and stained for infected cell foci and each focus scored as a single infection event. The number of focus-forming units per 100 µl of virus innoculum is plotted on the y axis. The bars show the means of two wells; the error bars are one standard deviation.

protein of late endosomes and lysosomes. After 5 min of warm up, both in the presence or absence (not shown) of PMA, punctate 12G5 labeling (*green*) was again seen in the periphery of the cell and in a more perinuclear location as described above. This staining appeared distinct from that of LAMP1 (*red*) and for the most part remained separate through the course of the experiment. Some overlap appeared after 120 min of labeling, but it is unclear at present whether this represents colocalization of CXCR4 and LAMP1 or our inability to resolve spatially close but distinct LAMP1- and CXCR4-containing organelles.

In addition to the fluorescence staining, we also determined whether internalized ^{125}I-12G5 was degraded. Analysis of TCA-soluble counts appearing in the medium indicated that <10% of the antibody initially bound to cells was degraded after 2 h incubation at 37°C (not shown). Similar levels of TCA-soluble activity were released from cells treated with PMA even though the intracellular level of ^{125}I-12G5 was increased 3–4-fold (Fig. 3). Together these results indicated that both CD4 and CXCR4 were internalized into endocytic organelles after phorbol ester treatment, and that little of the internalized 12G5 antibody was delivered to lysosomes within the time courses of these experiments.

Internalized CXCR4 Recycles to the Cell Surface

To determine whether CXCR4 internalized in the presence of phorbol ester can recycle to the cell surface, we incubated SupT1 cells at 37°C in phorbol dibutyrate (PDB), a phorbol ester that can be washed out of cells. The cells were incubated with PDB for 60 min and then either left with PDB for a further period, or washed and returned to
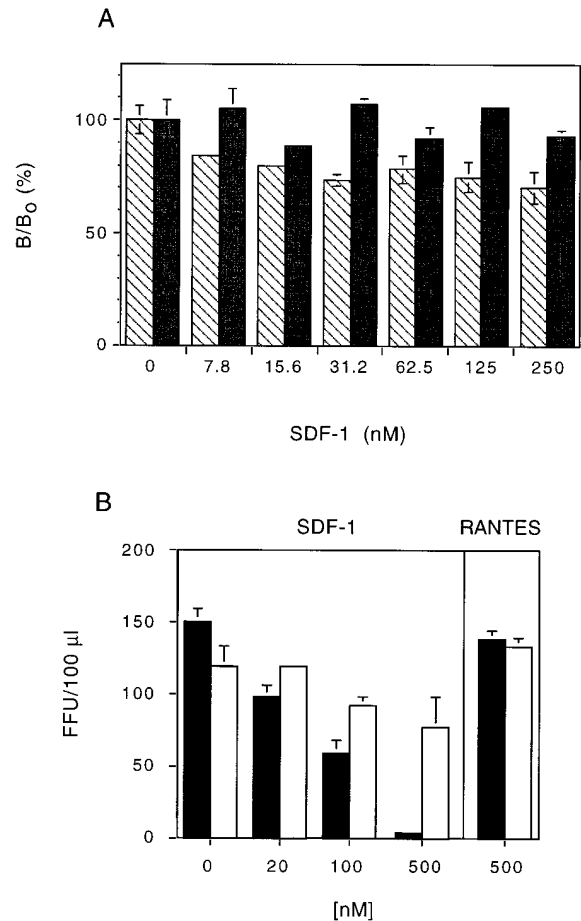
normal media for the indicated time periods. A second set of cells was treated similarly, but cycloheximide was included in the medium. Fig. 6 shows that incubation in PDB down regulated 60% of CXCR4 from the cell surface, and that CXCR4 expression remained low when the cells were maintained in PDB for a further 90 min. However, when the PDB-treated cells were returned to normal medium, cell surface CXCR4 levels recovered to ~80% of the initial levels over the subsequent 90 min (Fig. 6). Treatment with cycloheximide had no apparent effect on the reap-

pearance of CXCR4 on the cell surface, suggesting that re-expression occurred through recycling rather than delivery of newly synthesised CXCR4 to the plasma membrane. This conclusion was supported by experiments in which ^{125}I-12G5 internalized during incubation in medium containing PDB was observed to recycle to the cell surface when the PDB was removed (not shown). Together these experiments indicated that internalized CXCR4 can recycle to the cell surface.

SDF-1 Does Not Compete for 12G5 Binding on T Cells

The CXC chemokine SDF-1 was recently identified as the native ligand for CXCR4 (7, 45). To determine whether SDF-1 competes with ^{125}I-12G5 for binding, SupT1 cells were pre-incubated with SDF-1 either at 4°C or, after fixation, at room temperature and then incubated with ^{125}I-12G5 in the presence of SDF-1. At SDF-1 concentrations up to 2 μg/ml (250 nM), there was a slight (<30%) reduction of 12G5 binding on fixed cells incubated at room temperature but <10% reduction on cells incubated at 4°C (Fig. 7 A). 12G5 can partially inhibit SDF-1–mediated chemotactic responses, SDF-1–induced modulation of intracellular calcium, and SDF-1 binding to CXCR4 (8, 30). However, on SupT1 cells at least, SDF-1 did not efficiently compete for 12G5 binding.

The preparations of SDF-1 used for these experiments were tested for their abilities to elicit a Ca^{2+} flux in Fura-2–loaded SupT1 and CHO-CXCR4 cells. Both preparations of SDF-1 produced a rapid, transient increase in cytosolic free calcium, at 4 μg/ml (500 nM) as previously reported (7, 45; data not shown). We also examined the ability of SDF-1 to inhibit HIV-1 infection of Mv-1-Lu-CD4 cells expressing either CXCR4 or CXCR4ΔCyt. These cells can be infected with HIV-1 when induced to express both CD4 and an appropriate chemokine coreceptor (21). Before infection the cells were treated for 30 min with SDF-1 as indicated (Fig. 7 B) and then challenged with infectious HIV-1$_{IIIB}$. The cells were cocultured with virus overnight, washed, and then incubated for a further 2 d. Subsequently, the cells were fixed and stained for HIV-1 Gag. Fig. 7 B shows that HIV-1 infection of Mv-1-Lu-CD4/CXCR4 cells, as indicated by the numbers of stained foci, was virtually completely blocked in the presence of 500 nM SDF-1. Less effective inhibition was seen with lower SDF-1 concentrations. The β chemokine Rantes, which does not bind CXCR4, had no effect on infection. The CXCR4 ΔCyt molecule was also able to support HIV-1 infection in these cells (Fig. 7 B). However, infection of Mv-1-Lu-CD4/CXCR4ΔCyt cells was only inhibited ~60% at 500 nM SDF-1 (Fig. 7 B).

SDF-1 Down Modulates CXCR4

For chemokine receptors CXCR1, CXCR2, and CCR1, the presence of ligand initiates both signaling responses and rapid internalization of the cell surface receptor (11, 61). To determine whether SDF-1 induced endocytosis of its receptor, SupT1 cells were incubated in 500 nM SDF-1 for up to 60 min at 37°C. At the indicated times the cells were transferred to ice, fixed, and labeled with ^{125}I-12G5 to determine the level of cell surface CXCR4. Fig. 8 A shows that incubation in SDF-1 induced a rapid (50% in 5 min) down modulation of cell surface CXCR4, with only

20% of the initial cell surface levels remaining by 30 min of treatment. Although, we found that SDF-1 did not significantly inhibit 12G5 binding (Fig. 7 A), we repeated these experiments using the acid wash protocol (described above) to remove the surface-bound SDF-1 and obtained the same result (see below).

The concentration dependence of SDF-1–mediated CXCR4 down modulation was determined by incubating SupT1 cells in increasing dilutions of SDF-1 for 30 min. The cells were then cooled on ice, fixed, and labeled with ^{125}I-12G5. Fig. 8 B shows that maximum down modulation was induced at SDF-1 concentrations >125 nM. Partial down modulation was seen with lower concentrations. Similar binding was seen whether or not the cells were acid stripped before antibody labeling (Fig. 8 B).

To compare the down modulation induced by SDF-1 and phorbol ester, we treated SupT1 cells with 100 ng/ml PDB in parallel to cells treated with SDF-1. As indicated in Fig. 8 A, PDB induced down modulation of cell surface CXCR4 expression, though the time course (50% in ~15 min) was slower than that seen with chemokine. Recently, there has been interest in the notion that CXCR4 and CD4 associate on the cell surface and that the HIV-1 Env is able to interact with complexes of these molecules (36). In the phorbol ester experiments described above we observed that CD4 and CXCR4 were co-internalized into common early endosomes (Fig. 5). To determine whether SDF-1 could induce co-internalization of CD4, we induced CXCR4 internalization with SDF-1 for 1 h at 37°C, and subsequently the cells were labeled either with ^{125}I-12G5 or with an anti-CD4 monoclonal antibody ^{125}I-Q4120. Fig. 8 C shows that although SDF-1 could down modulate CXCR4, it did not induce CD4 internalization.

SDF-1 also induced down modulation of CXCR4 expressed in mink cells (Fig. 9). Mv-1-Lu-CD4/CXCR4 cells were treated with SDF-1 for periods up to 1 h. At the end of the incubation time the cells were washed with acid medium and the cell surface CXCR4 levels measured using ^{125}I-12G5. SDF-1 induced rapid down modulation of CXCR4 on these cells (Fig. 9) with a very similar time course to that seen in T cells (Fig. 8 A). More than 50% of the cell surface CXCR4 was removed within 10 min of addition of SDF-1 and ~90% down modulated by 60 min. In contrast, SDF-1 did not down modulate CXCR4ΔCyt expressed in Mv-1-Lu cells (Fig. 9), indicating that the COOH-terminal cytoplasmic domain was crucial for ligand and phorbol ester-induced down modulation.

SDF-1 and Phorbol Ester-induced Down Modulation of CXCR4 Involve Different Pathways

To determine whether similar mechanisms were involved in ligand- and phorbol ester-mediated internalization of CXCR4, we investigated the effect of PKC inhibitors on down modulation. SupT1 cells were treated with either staurosporin or calphostin C for 30 min at 37°C and then challenged with SDF-1 or PDB for 30 min at 37°C. Subsequently, cell surface CXCR4 and CD4 levels were determined and compared to those of untreated cells and cells treated with ligand or phorbol ester but not inhibitor. We found that both staurosporin and calphostin C inhibited PDB-induced down modulation of CXCR4 and CD4 (Fig.
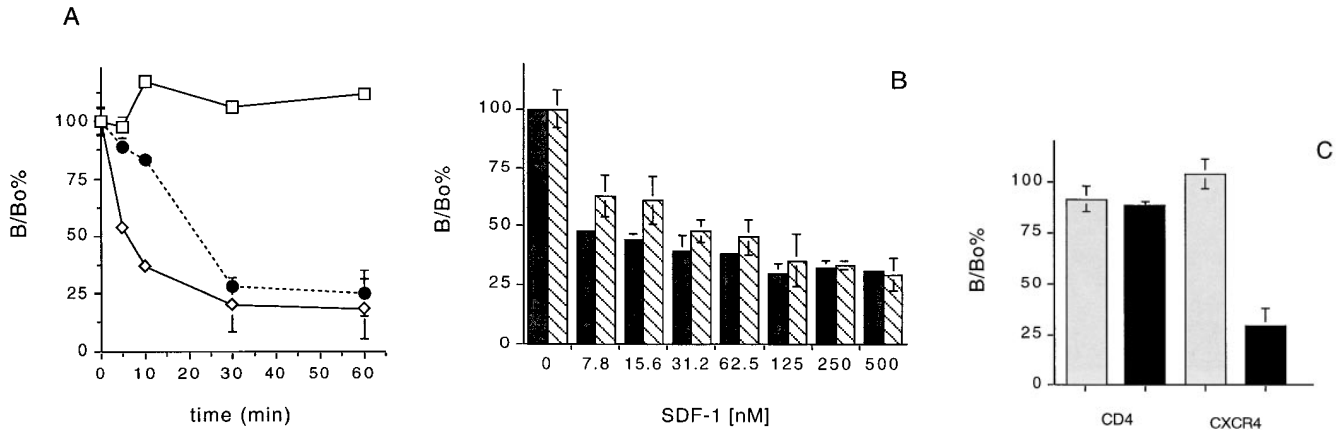
Figure 8. SDF-1- and PDB-induced down modulation of CXCR4 on SupT1 cells. (*A*) SupT1 cells were incubated in medium (□), medium containing 100 ng/ml PDB (●), or medium containing 500 nM SDF-1 (◇) for up to 60 min at 37°C. At the indicated time points, aliquots of cells were placed on ice, washed with cold binding medium, fixed, and incubated with 0.5 nM ^{125}I-12G5 for 2 h at room temperature. The values indicate the means and standard deviations for triplicate samples from a representative experiment. (*B*) SupT1 cells were incubated in twofold dilutions of SDF-1 for 30 min at 37°C. The cells were then cooled to 4°C and either fixed and labeled as in *A* with ^{125}I-12G5 for 2 h (■), or briefly incubated in low pH medium before fixation and labeling (▨). (*C*) SupT1 cells were incubated with (■) or without (□) 125 nM SDF-1 for 60 min at 37°C. The cells were then cooled to 4°C, fixed, and labeled as in *A* with ^{125}I-12G5 or 0.3 nM ^{125}I-Q4120 to detect cell surface CXCR4 and CD4, respectively.

10). However, neither inhibitor blocked SDF-1–induced down modulation of CXCR4. These data suggest that the phorbol ester-induced down modulation of CXCR4 and CD4 involves the action of PKC. In contrast, SDF-1–mediated CXCR4 down modulation appears to be independent of PKC activation.

Discussion

The subfamily of 7TM G protein-coupled receptors for chemokines has been implicated in the entry of human and simian immunodeficiency viruses into cells (for review see 40). In humans, the chemokine receptor family currently contains 13 members with known chemokine-binding activities. These include the receptors for the CC chemokines (CCR1–8), the receptors for the CXC chemokines (CXCR1–4), and the Duffy antigen that binds both CC and CXC chemokines (52, 54). In addition, a number of other CCR and CXCR family members have been identi-

fied, both from cellular sources (44, 65) and in viral genomes (see for example 4), for which the ligand-binding specificities are less well characterized or unknown. Of the well characterised receptors, CCR5 in conjunction with CD4 appears to be the principal coreceptor for macrophage tropic isolates of HIV-1 (40), whereas CXCR4 is used by T cell tropic and T cell line-adapted strains of HIV-1 (7, 21) and either in conjunction with, or independently of, CD4 by some strains of HIV-2 (20, 55). Other family members may also mediate entry for particular isolates of HIV-1, HIV-2, and SIV (40). At present, the exact role of the chemokine receptors in viral entry remains unclear. However, it appears likely that in conjunction with CD4 they facilitate conformational changes in the viral envelope glycoprotein that leads to fusion of the viral membrane with the plasma membrane of the target cell (40).

Chemokine receptors are expressed widely on lymphoid cells and have been implicated in the chemotactic recruitment of lymphocytes, neutrophils, and other leukocytes to
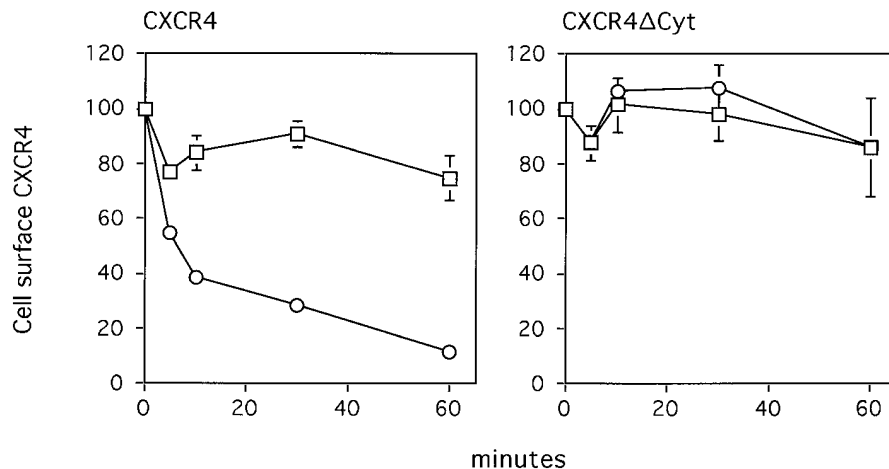

Figure 9. The COOH-terminal cytoplasmic domain is required for SDF-1 down regulation of CXCR4. Mv-1-Lu-CD4/CXCR4 and Mv-1-Lu-CD4/CXCR4ΔCyt were incubated in BM (□) or BM containing 500 nM SDF-1 (○) at 37°C. At the indicated times the cells were cooled on ice, washed with BM, and then incubated in BM adjusted to pH 2.0 (acid medium) for 10 min. The cells were then returned to BM (pH 7.4) at 4°C and cell surface CXCR4 determined using 0.5 nM ^{125}I-12G5. Each point shows the mean and SD of triplicate samples from a representative experiment.
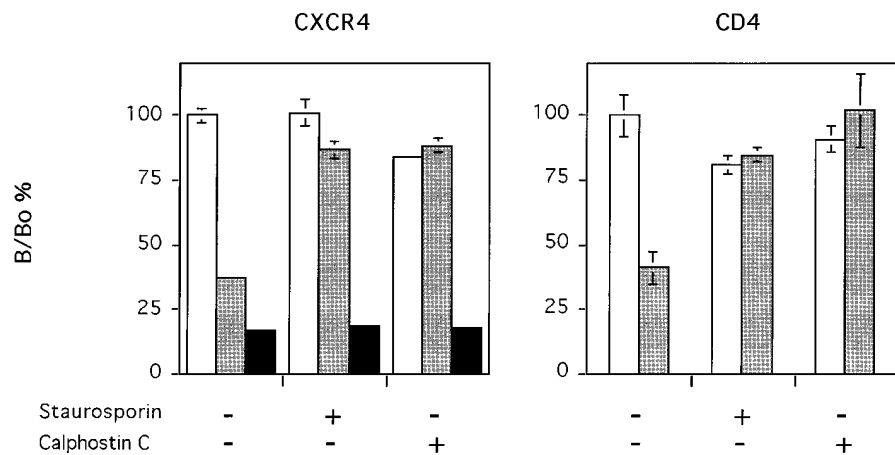
Figure 10. Effect of PKC inhibitors on SDF-1- and phorbol ester-induced down modulation of CXCR4 and CD4. SupT1 cells were washed twice by centrifugation and resuspended in 6 ml of BM, alone or with 0.5 μM staurosporin or 1 μM calphostin C, and incubated for 30 min at 37°C. For each condition 6×1 ml of cell suspension ($\sim 3.25 \times 10^6$ cells/ml) were diluted 1:1 with BM or BM containing 200 ng/ml PDB or 250 nM SDF-1 at 37°C for 30 min. The cells were then rapidly cooled on ice by dilution with 10 ml of cold PBS, centrifuged, and washed once in cold PBS. They were then fixed for 15 min in 3% PFA, washed, and quenched in 50 mM NH$_4$Cl and labeled with 0.5 nM [125]I-12G5 (for CXCR4) or 0.3 nM ^{125}I-Q4120 (for CD4) for 2 h at room temperature. The cell-associated activity was determined as described in Materials and Methods. Binding medium alone (□), 100 ng/ml PDB (▨), and 125 nM SDF-1 (■).

sites of inflammation (54). Significantly, the chemokine ligands for CCR5 (MIP-1α, MIP-1β, and Rantes), CCR3 (Eotaxin), and CXCR4 (SDF-1), as well as some receptor antagonists, can inhibit infection of cells by HIV viruses that use these receptors (3, 60). Studies with other 7TM proteins, and with IL-8 receptors (CXCR1 and 2) in particular, have indicated that ligand binding can induce rapid internalization and down modulation of the receptors from the cell surface (11, 22, 56, 61). Therefore, chemokines could exert their antiviral effects by sterically blocking binding of viral Env or by inducing internalization of the chemokine receptor. To understand the cellular mechanisms that regulate the surface expression of CXCR4, and thus its ability to function as an HIV coreceptor, we used the CXCR4-specific monoclonal antibody 12G5 (20) to evaluate CXCR4 endocytosis in response to phorbol ester and its natural ligand, SDF-1.

We found that CXCR4 on T cell lines undergoes slow constitutive endocytosis and recycling. The rate of internalization was ~1% of the cell surface pool per minute and reached steady state in 30 to 60 min, when ~20% of the initial surface pool was intracellular. EM analysis of cells labeled with 12G5 and protein A-gold indicated that ~1–2% of cell surface CXCR4 was associated with coated pits under these conditions. When SupT1 or BC7 cells were treated with phorbol esters, the cell surface expression of CXCR4 decreased. The possibility that the change in expression was due to phorbol ester-induced conformational changes in the protein that disrupted the 12G5 binding site was ruled out by the demonstration that CXCR4 with associated antibody was internalized. The biochemical experiments using [125]I-12G5 indicated that phorbol esters rapidly induced a sixfold increase in the rate of endocytosis of CXCR4 from the cell surface, resulting in >80% of the cell-associated radioactivity being located in intracellular compartments within 30 min. Furthermore, electron microscopy of immunogold-labeled PMA-treated cells indicated that the increased endocytosis occurred through enhanced interaction of CXCR4 with coated pits. The intracellular accumulation of CXCR4 may occur through increased endocytosis; in addition, inhibition of recycling may also contribute to down modulation. We have been unable to measure the rates of recycling in the continued presence of ligand or phorbol ester directly and cannot at present rule out effects on recycling. However, modelling calculations (51) and the observation that down modulation does not go to completion suggest that the increased rate of endocytosis is primarily responsible for the observed down modulation.

We previously demonstrated that CD4 endocytosis is also modulated by phorbol esters (32, 51). CD4 internalization is regulated through its association with the Src family kinase p56^{lck} and by the presence of an endocytosis signal in the cytoplasmic domain of CD4. This signal is dependent on phosphorylation of critical serine residues (38) and facilitates CD4 association with coated pits leading to endocytosis (51). The CD4 endocytosis signal involves serine 408 and a pair of leucine residues at positions 413 and 414 (58, 59; Pitcher, C., and M. Marsh, unpublished results). This motif (SQIKRLL in CD4, where S lies within a PKC phosphorylation site) is representative of a group of regulated endocytosis and trafficking signals that are active when the serine is phosphorylated but inactive when it is not (see for example 15, 16). A similar motif (SSLKIL; Ile can replace Leu in these signals) is present in the COOH-terminal cytoplasmic domain of CXCR4. Whether this sequence is involved in phorbol ester-induced endocytosis and down modulation of CXCR4 remains to be established. However, initial experiments indicate that CCR5, which lacks this motif, is not down modulated by phorbol esters (Hoxie, J.A., and M. Marsh, unpublished results). In addition, in keeping with results from the IL-8 receptor CXCR2 (53), our experiments indicate that the COOH terminus is required for rapid phorbol ester-induced CXCR4 endocytosis.

To examine the domains of CXCR4 involved in trafficking requires an appropriate cell line for transfection. In our initial experiments we used CHO-K1 cells stably expressing CXCR4 and HA-tagged CXCR4 molecules. However, we found that CXCR4 expressed in these cells underwent relatively fast constitutive endocytosis and that CXCR4 cell surface levels and endocytic rates were not

significantly modified by phorbol ester. We have previously found that the constitutive endocytosis of CD4 stably expressed in CHO cells is faster than that seen in other cell types (M. Marsh, unpublished results). As an alternative to CHO cells we analyzed the trafficking of CXCR4 expressed in mink lung cells Mv-1-Lu (these cells are used in our laboratory as an indicator cell line for HIV-2 infection studies as they lack 7TM coreceptors for HIV-2). In these cells the constitutive phorbol ester and ligand-induced trafficking of CXCR4 showed properties very similar to those observed in T cells. However, it is perhaps important to stress that the anomalous results we observed with CHO cells indicate that the analysis of the trafficking properties of these receptors in transfected cells should be approached with caution.

The CXC chemokine SDF-1 has been identified as a ligand for CXCR4. For a number of 7TM proteins, binding of ligand induces rapid internalization of the receptor through both clathrin-dependent and clathrin-independent pathways (11, 23, 24, 27, 33, 56, 61, 63, 66), though in some cases ligand binding does not induce internalization (35, 46). As shown here SDF-1 can induce rapid down modulation of CXCR4 in both transformed T cell lines and transfected mink cells. Furthermore, this down modulation does not require activation of PKC. The mechanism through which CXCR4 endocytosis occurs is unclear. Experiments with the β_2 adrenergic receptor suggest that ligand binding stimulates phosphorylation of the receptor by β adrenergic receptor-kinases (G protein-coupled receptor kinases) and interaction with β arrestins that can act as adaptors for clathrin-coated pits (27, 66). It is at present unclear whether similar mechanisms operate for CXCR4. However, our results indicate that phorbol esters and SDF-1 operate through different intracellular signaling pathways to induce CXCR4 internalization. The mechanism of phorbol ester-induced CXCR4 internalization may well involve PKC-mediated phosphorylation of a COOH-terminal domain signal similar to the endocytosis signal in CD4. Although the COOH-terminal is also required for SDF-1–induced down modulation, PKC activation is not required for ligand-induced down modulation. It remains to be determined whether CXCR4 contains multiple independent endocytosis signals or whether different signaling pathways can activate the same endocytosis signal.

Although there have been many studies on the trafficking of other 7TM G protein-coupled receptors, there is currently little data on the trafficking of the CXC and CC chemokine receptors. Here we have shown that CXCR4 is able to undergo efficient endocytosis after treatment of cells with phorbol esters, and that the receptor is also down modulated by SDF-1. In mice, SDF-1 is required for B cell lymphopoiesis, bone-marrow myelopoiesis, and for correct development of the heart (43). CXCR4 is known to be expressed on various lymphocytes (8) and other leukocytes (40), and SDF-1 is a potent chemokine for $CD34^{+ve}$ hematopoietic progenitor cells (1). In addition, the protein is also expressed on endothelial (Hoxie, J.A., and L.F. Brass, unpublished observations) and neuronal cells (30), though it is unclear whether it has similar functions on these cells. For T cells at least, it is perhaps significant that CXCR4 appears to respond to similar modulatory signals as CD4.

CD4 down modulation can be induced by antigen and other stimuli and involves the activation of PKC (38). Comodulation of CXCR4 together with CD4 may play a role in regulating the activity of CD4 positive T cells. By immunofluorescence we observed that CD4 and CXCR4 were initially co-internalized into the same endosomal organelles. However, we have no indication that the two molecules interact. The different constitutive endocytosis rates of CD4 and CXCR4 expressed on SupT1 cells (48), the absence of comodulation of CXCR4ΔCyt with CD4 on phorbol ester-treated Mv-1-Lu cells, and the selective SDF-1–induced down modulation of CXCR4 but not CD4, suggest these two proteins do not normally form stable associations. Thus the comodulation of CD4 and full length CXCR4 seen after phorbol ester treatment most likely occurs as a consequence of the two molecules containing similar trafficking signals. Moreover, the complex of CD4, CXCR4, and HIV-1 gp120 that has been proposed as an intermediate in viral fusion (36, 62, 64) is likely to be induced by the presence of the viral gp120 protein.

SDF-1 inhibits the entry of some T cell line-adapted HIV viruses into cells (7, 45). Although there is currently little data, initial studies have suggested that antagonists of chemokine receptors that block virus entry do not induce chemokine receptor internalization (60, 61), indicating that the chemokines may be able to interfere with Env binding. However, our findings, and similar data reported by Amara et al. (2), indicate that SDF-1 can induce rapid endocytosis of CXCR4, and that this chemokine is a more effective inhibitor of HIV-1 infection on cells expressing endocytosis-competent CXCR4 than on cells expressing CXCR4 ΔCyt, suggest that endocytosis may make a significant contribution to chemokine protection. The potential to down regulate the cell surface expression of the coreceptor molecules by ligand-dependent or -independent means may provide novel strategies for limiting HIV infection.

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