Spontaneous and Ligand-induced Trafficking of CXC-Chemokine Receptor 4*

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A chimeric protein consisting of CXC-chemokine receptor 4 (CXCR4) and the green fluorescent protein (GFP) was used for studying receptor localization and trafficking in real time in stably transduced HeLa, U-937, CEM, and NIH/3T3 cells. CXCR4-GFP was fully active as a co-receptor in mediating human immunodeficiency virus (HIV) entry. Both CXCR4 and CXCR4-GFP were found to undergo significant spontaneous endocytosis. Only 51.5 ± 7.8% of receptor molecules were found on the plasma membrane in CD4-positive cells, 43.9 ± 8.5% were found in CD4-negative HeLa cells, 75.6 ± 9.7% were found in U-937 cells, 72.5 ± 7.9% were found in CEM cells, and almost none were found in NIH/3T3 cells. Stromal cell-derived factor-1α induced rapid endocytosis of cell surface receptor molecules. A significant part of CXCR4 was targeted to lysosomes upon binding of the ligands, and recycling of internalized CXCR4 was not efficient. Only about 30% of receptor molecules recycled back to the cell surface in HeLa cells, 5% recycled in U937, and 10% recycled in CEM cells, suggesting that the protective effect of chemokines against HIV infection can be attributed not only to competition for binding but also to depletion of the co-receptor molecules from the cell surface. Envelope glycoprotein gp120 of syncytia-inducing lymphoctic tropic HIV-1 strains induced rapid internalization of CXCR4 in both CD4-negative and CD4-positive cells, suggesting that gp120 is a high affinity ligand of CXCR4.

Chemokine receptor CXCR41 functions as a co-receptor for T-cell tropic/syncytia-inducing HIV-1 and HIV-2 strains (1–7). CXCR4 is selective for a single chemokine, stromal cell-derived factor-1 (2), which is a potent lymphocyte chemoattractant (8). The receptor belongs to a family of G-protein-coupled receptors that are involved in regulation of numerous biological processes. G-protein-coupled receptor function is significantly regulated by receptor trafficking within the cell (9). CXCR4 was shown to be expressed in a variety of cells, but the activity of SDF-1α on various cells varies significantly (8, 10). We have shown previously that another GPCR, cholecystokinin receptor type A, undergoes spontaneous endocytosis that is cell type-dependent and may account for attenuation of receptor function in different cell populations (11). Spontaneous and ligand-induced internalization controls the number of functionally active GPCR on the plasma membrane. Although the antiviral activity of chemokines is clearly linked to their ability to bind to the HIV-1 co-receptors, the specific mechanisms of this effect are poorly characterized. It was recently shown that CXCR4 undergoes SDF-1α-induced internalization in CEM cells (12), but localization and spontaneous trafficking of the receptor in different cell types were not addressed, and the mechanisms of receptor internalization and recycling were not characterized. Here we describe CXCR4 localization and trafficking in live U-937, CEM, HeLa, and NIH/3T3 cells, studied with the help of CXCR4-GFP fusion protein. Attachment of GFP did not influence the ability of CXCR4 to mediate HIV entry. CXCR4-GFP allowed for the first time the characterization of receptor localization and distribution inside the living cells, thereby avoiding possible artifacts caused by fixation and cell permeabilization. The present study provides evidence that the viral envelope glycoprotein gp120 is a high affinity ligand of CXCR4 and that SDF-1α and gp120 induce endocytosis of CXCR4 that is significantly irreversible.

EXPERIMENTAL PROCEDURES

Materials

Rhodamine red concanavalin A (ConA), tetramethylrhodamine-transferrin, hexyl ester of rhodamine 6G, MitoTracker Red CMXRos, and LysoTracker Red dye were purchased from Molecular Probes Inc. (Eugene, OR). SDF-1α was a kind gift from Dr. J. Oppenheim and Dr. A. Z. Howard (National Cancer Institute). The following reagents were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, National Institutes of Health): gp120 MN and LAV (MicroGeneSys and Immunodiagnostics Inc.), a molecular clone of HIV-1 pYK-JRCSF (13), and monoclonal antibodies to human CXCR4 12G5 (7), gp120 MN and LAV were also purchased from Protein Sciences, Inc. (Meriden, CT).

Methods

Construction of the CXCR4-GFP cDNA—The full-length cDNA encoding CXCR4 was synthesized by reverse transcription-polymerase chain reaction using total RNA isolated from human peripheral blood mononuclear cells and primers based on the published sequence (14). The polymerase chain reaction product was subcloned into pcMVGF-Pg525 (11) to generate p25CXR4-GFP expressing CXCR4-GFP. The 1823-base pair fragment of CXCR4-GFP pg525 was excised by ScaI/EcoRI, blunt-ended with T4 DNA polymerase, and subcloned into XhoI and BsiWI-digested pHIT111 (15).

Expression of CXCR4-GFP Using Retroviral Vectors—The generation of high titer retroviral stocks for cell transduction was carried out as described by Yee et al. (16). A three-plasmid expression system was used to generate murine leukemia virus-derived retroviral vector particles by transient co-transfection in 293T human kidney cells by calcium phosphate method as reported (17). Approximately 1 × 10⁶ of adherent cells or 1 × 10⁶ cells in suspension were transduced with concentrated pseudotyped retroviral vector in 1 ml of culture medium. 2 h later, fresh medium was added, and the cells
were further incubated for 48 h. Subsequently, cells were maintained in complete medium containing 500 μg/ml of G418. The selection of stably transduced cell lines was performed by fluorescence-activated cell sorting as described (11).

**Pseudotyped Virus Infection Assay**—Luciferase reporter viruses were prepared in 293T cells as described (17) using pNL4-Luc-R-E vector (18) and Env expression plasmid. The HIV-1 JR-CSF envelope expression plasmid was generated by excision of Env open reading frame from pYK-JR-CSF (13) and subcloned into the expression plasmid pCMV. The HIV-1 pNL-3 or JR-CSF envelope expression plasmids were used in co-transfection with pNL4-Luc-R-E vector to generate luciferase-reporter viral particles. Chinese hamster ovary cells, transiently co-transfected with pCMV CD4 and either pCMV CXCR4 or pCMV CXCR4-GFPsg25, were transduced with the pseudotyped viruses. After 96 h, cytoplasmic extracts were prepared as described (19). The luciferase activity in 25 μl of lysate was assayed in 125L Luminometer (BioOrbit).

**Confocal Laser Scanning Microscopy**—Cells were grown in coated 50-mm cover glass bottom dishes (MatTek, MA) in medium without phenol red and observed on a Zeiss inverted LSM 410 laser scanning confocal microscope. Fluorescence of GFP and fluorescein was excited using a 488-nm argon/krypton laser, and emitted fluorescence was detected with 515–540 nm band pass filter. For LysoTracker Red, MitoTracker Red CMXRos, rhodamine red, and tetramethylrhodamine, a 568-nm helium/neon laser was used for excitation, and fluorescence was detected with a 590–640 nm band pass filter. Treatment of the cells with organelle markers was performed as described previously (11).

**Determination of the Relative Number of the CXCR4-GFP on the Cell Surface Using CLSM**—The cells were rinsed with cold PBS and treated with rhodamine red ConA (10 μg/ml in PBS) for 2 min at 4 °C and fixed with 4% formaldehyde in PBS for 3 min. After a final rinse with cold PBS, the cells were observed with the confocal microscope using a 63× oil immersion lens, a pinhole of 25, and an electronic zoom of 3 to yield a final magnification of 1000× of the stored images. Fluorescence pixels of the green fluorescent and of the co-localization mask of green and red fluorescence were counted with Zeiss LSM software. The percentage of the receptors on the cell surface was determined as the ratio of the co-localization mask pixels and total green fluorescence.

**Indirect Immunofluorescence**—HeLa cells were incubated with tetramethylrhodamine-transferrin as described (11), fixed with 4% formaldehyde in PBS for 20 min, and permeabilized with PBS containing 0.1% Nonidet P-40 for 10 min. Staining with monoclonal antibodies to CXCR4 was performed for 1 h at 37 °C. Fluorescein-conjugated anti-mouse antibodies were added for 1 h at 37 °C, and cells were analyzed by CLSM. Extensive washes with PBS were performed after each step throughout the procedure. The controls for specificity included incubation with nonimmune serum, omission of the first antibodies, and staining of nonpermeabilized cells.

**RESULTS**

**Construction and Characterization of CXCR4-GFP Fusion Protein**—For microscopic visualization of CXCR4 molecules, we have constructed a chimeric molecule consisting of the human CXCR4 protein fused to the complete GFPsg25 protein at its C terminus. To test the functionality of the CXCR4-GFP fusion in mediating HIV-1 infection, we used a virus entry assay based on single cycle infection by an Env-deficient virus (17). The pCXCR4-GFPsg25 or the pCXCR4 expression vectors were transiently transfected into Chinese hamster ovary cells together with human CD4. Transfected cells were infected with a HIV-1-based luciferase reporter virus (18) pseudotyped with Env glycoprotein from either HIV-1 molecular clone NL4–3 or JR-CSF. The level of HIV infection was evaluated by measurement of luciferase activity. Similar levels of luciferase activity were detected in cells expressing either wild-type CXCR4 or the chimeric protein CXCR4-GFP (data not shown), demonstrating that the fusion protein was fully functional in mediating viral entry. The levels of luciferase activity were about 30 times higher in cells infected with a T-cell tropic virus than in the control experiment using envelope-deficient particles or particles pseudotyped with CCR5-dependent JRCSF envelope.

**Localization of CXCR4-GFP in Different Cell Types in the Absence of Ligands**—A significant part of the green fluorescence corresponding to CXCR4 was observed on the cell membrane in both CD4+ and CD4-HeLa cells and in U-937 and CEM cells stably expressing CXCR4-GFP (Fig. 1, A, C, and D). The receptor molecules appeared to be evenly distributed on the plasma membrane. Faint labeling of endoplasmic reticulum was observed in all cell lines (data not shown). A large portion of the receptor molecules had intracellular localization and co-localized with the endosomal marker, tetramethyl rhodamine transferrin, in all cells (Fig. 2A). Relative distribution of the fluorescent CXCR4-GFP was determined as described previously (11). In the absence of the ligands, CD4-negative HeLa cells had 43.9 ± 8.5% of the receptors on the cell surface, CD4- HeLa cells had 51.5 ± 7.8%, U-937 had 75.6 ± 9.7%, CEM cells had 72.5 ± 7.9%, and NIH/3T3 had almost none. No receptor molecules could be detected in lysosomes (Fig. 2C) or in mitochondria (data not shown). To verify that attachment of GFP did not influence localization and trafficking of the receptor, we have performed immunolocalization of endogenous CXCR4 in HeLa cells using monoclonal antibodies to the receptor. The staining pattern was identical to CXCR4-GFP distribution, and intense staining of endosomal compartments was observed in the absence of the ligands (Fig. 2B).

**Ligand-induced Translocation of CXCR4-GFP**—In all stably transduced cell lines, addition of SDF-1a caused translocation of CXCR4-GFP from the cell surface into intracellular vesicles (Figs. 2B, 3, and 4). gp120 of the T-cell tropic viral strains MN and LAV caused rapid endocytosis of cell surface CXCR4-GFP in both CD4-positive and CD4-negative HeLa cells (Fig. 4). gp120 MN was as potent as SDF-1a in inducing CXCR4-GFP internalization, and a concentration of less than 10 nM was sufficient to achieve the maximal effect. However, at least 100 nM of gp120 LAV was needed to reach the same efficiency. We have found that the activity of gp120 preparations differed significantly depending on the source, with preparations obtained from MicroGeneSys being the most potent. Thus, care should be taken in the interpretation of results obtained by using different sources of gp120. 10 nM SDF-1α induced almost complete depletion of the receptor molecules from the cell mem-

![Fig. 1. CLSM images of cells expressing CXCR4-GFP (in green).](image-url)
brane in HeLa and U937 cells, whereas about 40% of cell surface receptor molecules remained on the cell surface in CEM cells even in the presence of 100 nM ligand. Ligand-induced endocytosis could be detected as early as 10 min after addition of the ligands and was maximal after 30 min. Co-localization of CXCR4-GFP with a marker for endosomal compartments, tetramethylrhodamine-conjugated transferrin, increased in a time-dependent manner after exposure of the cells to either gp120 or SDF-1α (Fig. 4B), suggesting that internalization occurred predominantly through the clathrin-dependent pathway. Unlike 

CXCR4, a significant portion of the receptor molecules was localized to lysosomes after addition of the ligands (Fig. 2D).

**Receptor Recycling**—The relative amount of CXCR4-GFP on the cell surface was determined after exposure to the ligands as described (11). CXCR4-GFP expressing cells were pretreated with cycloheximide to inhibit de novo synthesis of receptor molecules and incubated with the ligands (10 nM gp120 MN or 50 nM SDF-1α) for 30 min at 37°C. The cells were rinsed with medium containing cycloheximide, left in the incubator for varying time intervals, and then briefly exposed to ice-cold rhodamine B ConA and observed by CLSM. In the control experiment, untreated cells were left in the incubator in the presence of cycloheximide. The number of receptor molecules on the cell surface was determined by co-localization with ConA (11). It did not change in the untreated cells during the time of the experiment but decreased significantly upon addition of SDF-1α in all tested cell lines (Fig. 3). After removal of the ligand, cell surface CXCR4-GFP increased about 30% in HeLa cells (Fig. 3A) and did not change significantly in U937 and CEM cells (Fig. 3, B and C). Thus, recycling of CXCR4 is notably less efficient than that of CCKAR, and a significant portion of receptor molecules is targeted to the degradative pathway.

**DISCUSSION**

Intracellular trafficking of GPCR is an important cellular mechanism of receptor activity regulation because it determines the number of receptor molecules present on the plasma membrane. Structurally homologous receptors may differ markedly in the pathways of trafficking (20), and trafficking of the same GPCR can be significantly different in different cell types (11).

CXCR4 is the major co-receptor for the entry of T-cell tropic/syncytia-inducing strains of HIV-1 (1–6) and HIV-2 (7). We have undertaken a detailed study of trafficking of CXCR4 in cells of different types and in the cells exposed to chemokine and viral ligands to assess the role of receptor pathways in receptor function and inhibition of HIV infection.

Fusing the C terminus of GFP was found previously to produce fully functional cholecystokinin (CCKAR) (11) and β2-adrenergic receptors (21). This approach allowed for a study of receptor endocytosis, sorting from the ligand and recycling in real time in live cells (11). Similarly, CXCR4-GFP was found fully functional in mediating HIV entry into the cells.

Like CCKAR, CXCR4-GFP was distributed evenly along the cell membrane, suggesting that there is no preassociation of the receptor molecules with certain parts of membranes. The small number of receptors found in the endoplasmic reticulum most probably represents the pool of newly synthesized mol-
ecules that were not yet transported to the plasma membrane. Overexpression of CCKAR-GFP led to saturation of all intracellular membranes with receptor molecules. Thus, the absence of CXCR4-GFP in mitochondria is indicative of a lack of receptor overexpression in the studied cell lines.

CXCR4 was found to undergo significant spontaneous endocytosis in all the cell lines examined, and the process was more important than what had been observed for the cholecystokinin receptor (11) and another HIV-1 co-receptor CCR5 in the same cell lines. Spontaneous endocytosis was independent of the level of receptor expression. The degree of spontaneous endocytosis varied significantly in different cell types, being highest for all three receptors (CXCR4, CCR5, and CCKAR) in NIH/3T3 cells, where almost no CXCR4 molecules could be detected on the cellular membrane. Thus, spontaneous endocytosis may account for very low or no cell surface expression of CXCR4 in some cell lines. CXCR4 mRNA has been reported to be widely expressed in leukocytes and related cell lines, yet SDF-1α acts only on certain leukocyte subsets (8, 10). One can speculate that cell surface receptors in certain cell types may be down-regulated by spontaneous endocytosis to the levels that do not allow detectable cellular responses.

Like many other GPCRs, cell surface CXCR4 undergoes ligand-induced internalization. We found that endocytosis was the predominant mechanism of CXCR4 internalization, although we cannot exclude the possibility that other mechanisms, such as internalization through noncoated pits, exist as minor components.

We have found previously that the cholecystokinin receptors sort very efficiently from the ligands in endosomes, recycle with almost 100% efficiency, and could not be detected in lysosomes (11). Similarly, the CXCR4 receptor was suggested previously to recycle to the plasma membrane in CEM cells after removal of the ligand from the incubation medium (12). Here we demonstrate that recycling of CXCR4 is not efficient, and, unlike CCKAR, a significant portion of CXCR4 molecules could be detected in lysosomes after addition of the ligand. Interestingly, no CXCR4 could be detected in lysosomes in the absence of the ligands, suggesting that spontaneous endocytosis is not accompanied by receptor degradation, whereas ligand-induced endocytosis led to depletion of the cell surface of receptor molecules, and the process was irreversible in the presence of a protein synthesis inhibitor. There was a slight variation in the degree of recycling in different cell lines, which was highest in HeLa cells. However, even in this cell line, not more than 30% of internalized receptor molecules made it back to the cell surface. Several other GPCR were reported to undergo partial recycling. hCGR/LH (22), thrombin (23), and thyrotrpin-releasing hormone (24) receptors also appeared to be targeted to the degradative pathway.

Targeting of CXCR4 to lysosomes and only partial recycling was observed in all of the cell lines examined, suggesting that the protective effect of the SDF-1α against HIV infection can be attributed not only to competition for binding to CXCR4 but also to depletion of the cell surface of the co-receptor molecules.

The viral envelope glycoproteins gp120 and gp160 were reported previously to bind to CXCR4 and compete with SDF-1α interaction (25, 26). Our data demonstrate that gp120 is a high affinity ligand of CXCR4 and can be as potent as SDF-1α in inducing rapid endocytosis of cell surface CXCR4 both in the presence and in the absence of CD4. Down-regulation of the co-receptor by envelope protein that is shedded from the surface of infected cells can thus contribute significantly to the kinetics of infection and also to the selection of virus mutants with certain receptor and cell tropism. In addition, we have found recently that gp120 MN acts as an agonist in inducing CXCR4-mediated signal transduction, whereas gp120 LAV is an antagonist and inhibits SDF-1α-induced intracellular Ca2+ release. This observation suggests that the action of the viral envelope proteins on the cells can go beyond down-regulation of the chemokine receptors. A study of biological effects of different gp120 strains on different cell types is currently ongoing in our laboratory.

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