Constitutive Association of Cell Surface CCR5 and CXCR4 in the Presence of CD4

Jinhai Wang,* Raymond Alvarez, Gregory Roderiquez, Ennan Guan, and Michael A. Norcross

Laboratory of Immunology, Division of Therapeutic Proteins, Office of Biotechnology Products, Center for Drug Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892

Abstract   Chemokine receptors CCR5 and CXCR4 are the major coreceptors of HIV-1 infection and also play fundamental roles in leukocyte trafficking, metastasis, angiogenesis, and embyogenesis. Here, we show that transfection of CCR5 into CXCR4 and CD4 expressing 3T3 cells enhances the cell surface level of CXCR4. In CCR5 high expressing cells, cell surface level of CXCR4 was incompletely modulated in the presence of the CXCR4 ligand CXCL12/SDF-1α. CCR5 was resistant to ligand-dependent modulation with the CCR5 ligand CCL5/RANTES. Confocal laser microscopy revealed that CCR5 was colocalized with CXCR4 on the cell surface. In CD4 expressing CCR5 and CXCR4 double positive NIH 3T3 cells, immunoprecipitation followed by Western blot analysis revealed that CCR5 was associated with CXCR4 and CD4. CXCR4 and CCR5 were not co-immunoprecipitated in cells expressing CCR5 and CXCR4 but without CD4 expression. Compared to NIH 3T3CD4 cells expressing CXCR4, the entry of an HIV-1 X4 isolate (HCF) into NIH 3T3CD4 expressing both CXCR4 and CCR5 was reduced. Our data indicate that chemokine receptors interact with each other, which may modulate chemokine–chemokine receptor interactions and HIV-1 coreceptor functions. J. Cell. Biochem. 93: 753–760, 2004.   Published 2004 Wiley-Liss, Inc.[†]

Key words: chemokine receptor; chemokine; HIV-1; viral entry

HIV-1 isolates are classified as R5, X4, or R5X4 viruses depending on whether CCR5, CXCR4, or both CCR5 and CXCR4 are used for entry. R5 HIV-1 are the dominant isolates replicating in vivo at the time of seroconversion [Cheng-Mayer et al., 1988; Tersmette et al., 1989; Schuitemaker et al., 1992]. In 40–50% of HIV-1 infected individuals, X4 isolates emerge and replace R5 isolates as the major viral population [Cheng-Mayer et al., 1988; Tersmette et al., 1989; Schuitemaker et al., 1992; Kinter et al., 1998]. It is unclear why R5 HIV-1 is the primary viruses transmitted sexually. CXCR4 has been characterized as the major coreceptor for T tropic (X4) isolates [Feng et al., 1996] and will associate with CD4 in the presence of gp120 [Lapham et al., 1996]. As a chemokine receptor, CXCR4 is functional in monocytes/macrophages as assessed by receptor downmodulation, chemotaxis, calcium mobilization [Wang et al., 2001a].

Chemokines/chemokine receptors play fundamental roles in leukocyte trafficking, metastasis, angiogenesis, and embyogenesis [Berger et al., 1999; Murphy, 2001]. CC and CXC chemokines are two distinct groups of chemokines. Chemokines, especially CC chemokines, may interact with multiple chemokine receptors, and these cross interactions are strictly limited within either CC or CXC subfamily. In response to ligand some GPCRs undergo homodimerization and ligand dependent endocytosis. Cytokines can also regulate receptor endocytosis through ligand-independent endocytosis as we and others have reported for the CCR5 and CXCR4 receptors [Wang et al., 1998, 2001a; Lee et al., 1999a; Garzino-Demo et al., 2000]. Recently heterodimerization of GPCR was found between GBRl and GBR2, and between opioid receptors κ and δ [Kaupmann et al., 1998; White et al., 1998; Jordan and Devi, 1999]. Heterooligomers with enhanced functional activity were formed in receptors for dopamine and somatostatin [Rocheville et al., 2000a,b].

Chemokine receptor heterodimerization of CXCR4 or CCR5 with CCR2V64I was suggested to be the mechanism by which individuals with the CCR2V64I allele show delayed AIDS progression [Mellado et al., 1999]. However, the mechanism of heterodimerization of GPCRs or chemokine receptors has yet to be determined. It has been shown previously that CCR5 constitutively associates with CD4 on cell surface [Xiao et al., 1999]. Here, we show that CCR5 increases CXCR4 expression, modifies ligand-mediated endocytosis of CXCR4, and interferes with X4 virus infection. CXCR4 was found to be stably associated with CCR5 and CD4, suggesting that alterations of CXCR4 function by CCR5 may resulted from close association of these receptors in multicomponent complexes with CD4.

MATERIALS AND METHODS

Cells and Reagent

NIH 3T3 was purchased from ATCC (Manassas, VA). NIH 3T3CD4, NIH 3T3CD4CCR5, and NIH 3T3CD4CXCR4 were generated by Dr. Littman et al. [Deng et al., 1997] and received from NIH AIDS Reagent Program. NIH 3T3CD4CXCR4CCR5 cell line was generated in our laboratory by stable transfection of NIH 3T3CD4CXCR4 cells with pSynCCR5. The sequence encoding CCR5 in pSynCCR5 was optimized for mammalian cell codon usage. A sequence encoding a single glycine residue followed by the bovine rhodopsin C9 peptide tag (TETSQVAPA) was introduced immediately 5′ to the natural stop codon of CCR5 [Mirzabekov et al., 1999]. Cells were cultured in DMEM containing 10% fetal bovine serum (HyClone, Logan, UT), penicillin (100 U/ml), and streptomycin (100 mg/ml). Cell surface CD4 and CCR5 levels are similar on each respective cell lines whereas CXCR4 is higher on NIH 3T3CD4CCR5CXCR4 cell line than on NIH 3T3CD4CXCR4 cell line as assessed by FACS. SDF-1α, CCL3/MIP-1α, and RANTES were purchased from PepTech (Rocky Hill, NJ). R5 isolate Ba-L, X4 isolate HCF, R5X4 isolate RTTKN were described as before [Bou-Habib et al., 1994; Wang et al., 2001].

Immunofluorescence Staining and Flow Cytometry

Cells were stained with FITC-labeled monoclonal antibody to CCR5 (2D7) and PE-labeled monoclonal antibody to CXCR4 (12G5) and then analyzed by flow cytometry acquisition and analysis using CellQuest software as described previously [Wang et al., 1999]. PE- or FITC-labeled isotype antibodies were used as controls.

Confocal Laser Microscopy

Cells cultured on glass slides were fixed and stained with PE or FITC-labeled mouse monoclonal antibodies to CXCR4 (12G5) and CCR5 (2D7). Cell surface fluorescence was visualized with a LSM5 confocal laser-scanning microscope (Carl Zeiss, Thornword, NY). Emission from FITC (515–540 nm) and PE (560 nm) was detected after excitation at 488 and 533 nm, respectively. Nonspecific background fluorescence was determined by staining with labeled isotype control antibodies. Prolong antifade mounting reagent (Molecular Probe, Eugene, OR) was applied to stained samples on slides.

Immunoprecipitation and Immunoblot Analysis

Cells (1×10^7) were washed, scraped into 0.8 ml of lysis buffer [20 mM Tris-HC1 (pH 8.3), 150 mM NaCl, 20 mM EDTA, 1% Brij97, pepstatin A (1 μg/ml), aprotinin (10 μg/ml) and leupeptin (10 μg/m1)](19), incubated for 1 h at 4°C, and then centrifuged at 8,000g for 15 min. For immunoprecipitation, the resulting supernatants were pretreated for 2 h at 4°C with protein G-Sepharose (Amersham Biosciences, Uppsala, Sweden) before incubation overnight with primary monoclonal antibody 1D4 (National Cell Culture Center) specific for the bovine rhodopsin C9 peptide tag (TETSQVAPA) of CCR5 or with anti-tubulin monoclonal antibody H300 and protein G-Sepharose. For immunoblot analysis, immunoprecipitates were heated at 80°C for 10 min and resolved by SDS–PAGE on 4–12% gradient gels, and the separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was exposed for 1 h to PBS containing 3% BSA and then probed for 1 h with goat polyclonal antibodies to the COOH-teminal region of CXCR4 (C-20, 1:1,000 dilution), CCR5 (C-20), CD4 (C18), or Tubulin (H300) (all from Santa Cruz Biotechnology, Inc, Santa Cruz, CA). After three washes with PBS containing 1% BSA, the membranes were exposed to horseradish peroxidase-conjugated swine anti-goat IgG (1:1,000 dilution). Immune complexes were detected with a chemiluminescent substrate (Santa Cruz Biotechnology).

Detection of Viral DNA

Viral DNA was isolated from NIH 3T3CD4 cells and its derivatives after exposure to HIV-1 isolates overnight and then amplified by the polymerase chain reaction with primers targeted to the gag region (SK38: 5′-ATAATCCACC TA TCCCAGTAG GAGAAAT-3′ and SK39: TTT-GGTCCTT GTCTTATGTC CAGAATGC-3′) as described previously [Wang et al., 2001].

RESULTS

Impact of CCR5 on Ligand-Mediated Downmodulation of CXCR4

In order to study cross-talk in ligand mediated endocytosis of HIV-1 coreceptors, we stably transfected NIH 3T3CD4CXCR4 cell lines with pSynCCR5. The resulting CCR5-CXCR4 double positive cell line expressed high level of cell surface CCR5, slightly increased CD4 and higher level of CXCR4 than the parent NTH 3T3CD4CXCR4 cell line (Fig. 1a). The increase in CXCR4 cell surface level was also observed in five other CCR5-CXCR4 double positive NIH 3T3CD4 clones (Fig. 1b). The resulting NIH 3T3CD4CXCR4CCR5 cell line was tested for sensitivity to ligand dependent modulation using ligands specific for CCR5 or CXCR4. We found that CCR5 in these cells was resistant to downmodulation by CCL5/RANTES (Fig. 1c) and CCL3/MIP-1α (data not shown) similar to resistance reported in HOS CD4CCR5 cells [Brandt et al., 2002]. In contrast, CXCR4 modulation by CXCL12/SDF-lα varied depending on cell surface level of CCR5. Downmodulation of CXCR4 by SDF-lα was evident in CCR5 low cells, while incomplete modulation of CXCR4 was seen in CCR5 high cells. In the NIH 3T3CD4CXCR4 cell line, CXCR4 was sensitive to SDF-1 downmodulation (data not shown). The apparent cell surface level of CXCR4 was not affected by treatment with a ligand dissociating solution of 0.05 M glycine HC1 with 0.1 M NaC1, indicating that SDF-1 does not interfere with the interaction of the 12G5 mAb with CXCR4 on the cell surface. This result indicates that the presence of CCR5 affects cell surface level of CXCR4 and ligand-mediated down-modulation of CXCR4.

Colocalization of CCR5 With CXCR4

To study the mechanism by which CCR5 effects CXCR4 expression, NIH 3T3CD4CXC-R4CCR5 cells were grown on glass slides and double stained with FITC-labeled monoclonal anti-CCR5 antibody 2D7 and PE-labeled monoclonal anti-CXCR4 antibody 12G5 and then observed using confocal laser microscopy. As shown in Figure 2, CCR5 is colocalized with CXCR4 on the cell surface. Cells stained with isotype control antibodies were negative (not shown).

Association of CCR5 With CXCR4

It has been shown previously that CCR5 and CD4 are associated constitutively on the cell surface [Xiao et al., 1999; Hernanz-Falcón et al., 2004]. To investigate whether CCR5 is constitutively asssociated with CXCR4, NIH 3T3CD4CXCR4CCR5 cells were lysed and immunoprecipitated with monoclonal antibody (1D4) against the bovine rhodopsin C9 peptide tag (TETSQVAPA) or with monoclonal antibody to human tubulin, followed by blotting with anti-CCR5 (C20), anti-CXCR4 (C20), anti-CD4 (C18), or anti-tubulin antibody. As shown in Figure 3, CXCR4 and CD4, but not tubulin were coprecipitated with CCR5, while neither CXCR4, CD4, nor CCR5 were coprecipitated with tubulin. CCR5 was not precipitated from NIH 3T3CD4CXCR4 cells (not shown). Co-immunoprecipitation of CXCR4 and CCR5 was not observed in cells that do not express CD4 (data not shown).

Cell Surface Level of CCR5 Interferes With CXCR4 Mediated HIV-1 Entry

To test the impact of CCR5 on CXCR4-mediated viral entry or CXCR4 on CCR5-mediated entry, NIH 3T3CD4, NIH 3T3CD4CXCR4, NIH 3T3CD4CCR5, and NIH 3T3CD4CXCR4CCR5 cells were exposed to X4 isolate HCF, R5 isolate Ba-L, or R5X4 isolate RTTKN overnight, and then viral entry was assessed using semi-quantitative PCR. As shown in Figure 4, no entry occurred in NIH 3T3CD4 cells. Viral DNA was detected in HCF infected NIH 3T3CD4CXCR4 cells (lane 2, HCF), weak in NIH 3T3CD4CCR5 cells (lane 3, HCF). Less viral DNA was detected in HCF exposed NIH 3T3CD4CXCR4CCR5 cells (lane 4, HCF) than in HCF infected NIH 3T3-CD4CXCR4 cells (lane 2, HCF). Viral DNA was also detected in Ba-L infected NIH 3T3CD4CCR5 cells (lane 3, Ba-L), but not NIH 3T3CD4CXCR4 cells (lane 2, Ba-L). Compared to Ba-L exposed NIH 3T3CD4CCR5 cells,

slightly less viral DNA was detected in Ba-L exposed NIH 3T3CD4CXCR4CCR5 cells (lane 4, Ba-L). The entry of RTTKN into NIH 3T3CD4CCR5 cells (lane 3, RTTKN) was the highest, followed by infection of NIH 3T3-CD4CCR5CXCR4 cells (lane 4, RTTKN) and NIH 3T3CD4CXCR4 cells (lane 2, RTTKN). The results indicate that CCR5 interfered with CXCR4 mediated X4 viral entry, whereas the impact of CXCR4 on R5 viral entry was less.
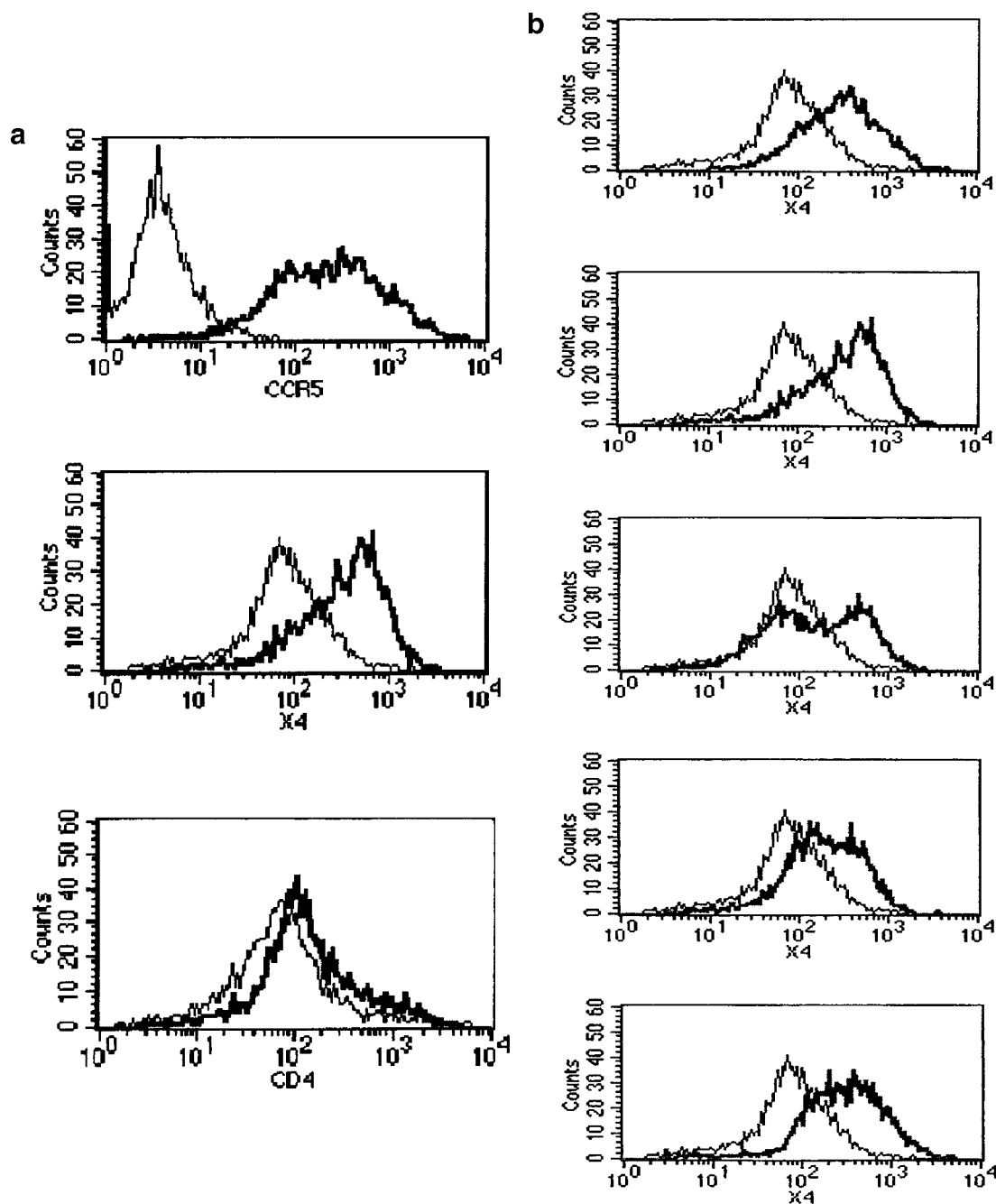
Fig. 1. Role of CCR5 in cell surface CXCR4 expression and SDF-la mediated downmodulation of CXCR4. **a**: The expression of CCR5, CXCR4, and CD4 on NIH 3T3CD4CXCR4 (thin line) and on NIH 3T3CD4CXCR4CCR5 (thick lane). **b**: The expression of CXCR4 on NIH 3T3CD4CXCR4 (thin line) and on five different NIH 3T3CD4CXCR4CCR5 clones (thick lane). **c**: NIH 3T3CD4CCRXXCR4 cells were treated with 1 μg of SDF-lα or RANTES at 37°C for 5 min, stained with PE-labeled anti-CXCR4 antibody and FITC-labeled anti-CCR5 antibody, then analyzed on FACS. PE-or FITC-labeled isotype antibodies were used as control. Data shown are representative of three similar experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
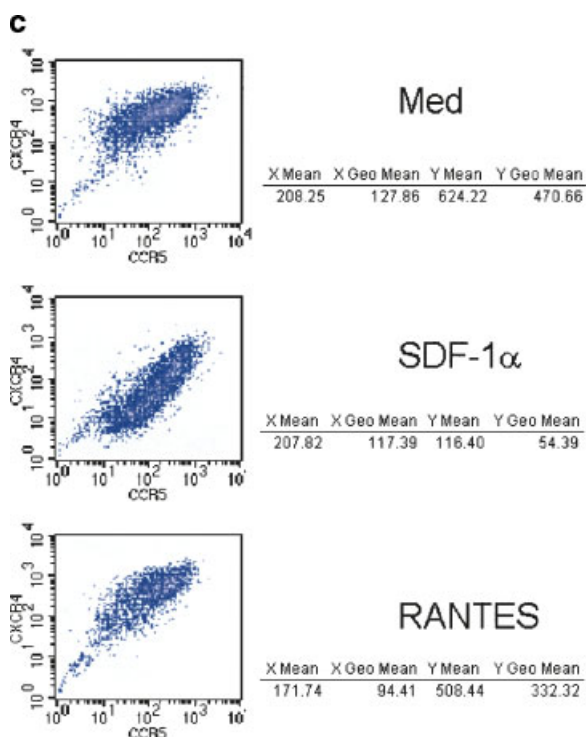
Fig. 1. (*Continued*)

DISCUSSION

In this study, we demonstrate that cell surface CCR5 modulates cell surface CXCR4 level and function by forming CCR5–CXCR4 complexes and that this interaction may control CXCR4 mediated X4 virus entry.

It has been shown previously that CD4 constitutively associates with CCR5, but not with CXCR4 [Xiao et al., 1999]. In the presence of gp120 and high levels of CD4, CXCR4 was detected in CD4 immunoprecipitates. In the presence of low level of CD4, only minimal amounts of CXCR4 were detected in CD4 immunoprecipitates [Lapham et al., 1996; Dimitrov et al., 1999; Xiao et al., 1999; Lee et al., 2000]. In T cells, high levels of CD4 are sufficient for complex formation between CD4 and CCR5 during R5 HIV-1 infection, and sufficient to form complexes between CD4 and CXCR4 in the presence of gp120. However, in monocytes/macrophages, the expression of CD4 is limited and may preferentially associate with CCR5, thereby limiting access of CXCR4 to CD4 in the presence of gp120. Our results suggest an alternative possibility that a direct interaction
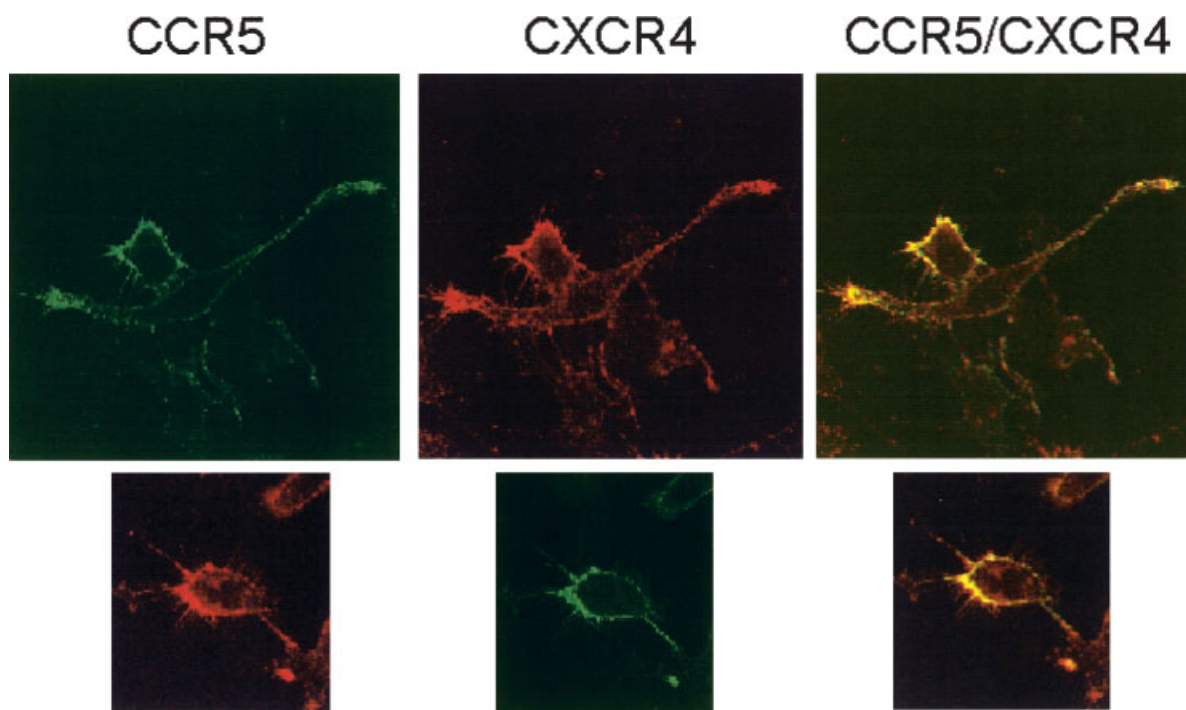

Fig. 2. Confocal analysis of cell surface CCR5 and CXCR4. NIH 3T3CD4CCR5CXCR4 cells were plated on glass slides and cultured overnight. Cells were blocked with cold 10% normal rabbit serum in PBS for 30 min at 4°C, then stained with mixture of equal volume of PE-labeled anti-CXCR4 antibody and FITC-labeled anti-CCR5 antibody at 4°C for 30 min.
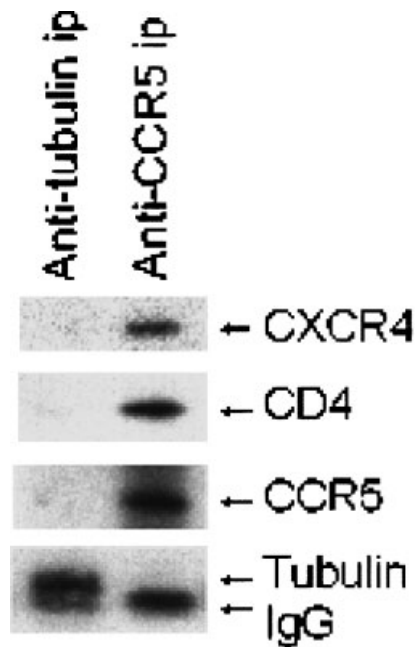
Fig. 3. Co-immunoprecipitation of CXCR4 and CD4 with CCR5. NIH 3T3CD4CCR5CXCR4 cells were lysed and processed as described in ''materials and methods.'' Data are one representative of three similar experiments.

between CXCR4 and CCR5 may contribute to viral resistance.

It has been previously demonstrated that transfection of feline CCR5 into CXCR4 expres-
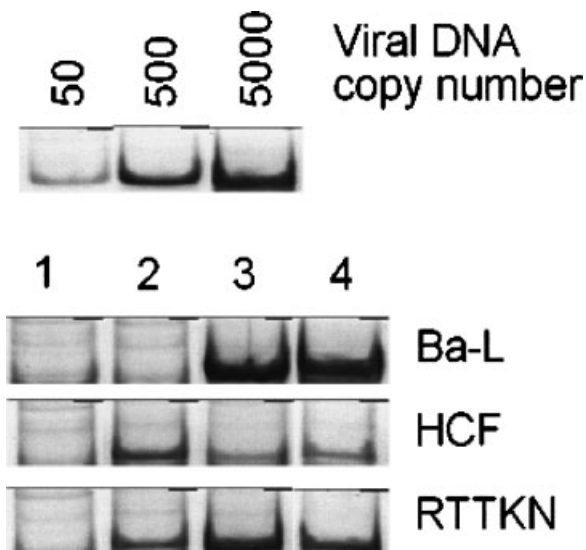
Fig. 4. Impact of co-expressed CCR5 and CXCR4 on viral entry. NIH 3T3CD4 cells and its derivatives (1, NM3T3CD4; 2, NIH 3T3CD4CCXCR4; 3, NIH 3T3CD4CCR5; 4, NIH 3T3CD4C-XCR4CCR5) are exposed to X4 virus HCF, R5 virus Ba-L, or R5X4 virus RTTKN overnight, washed with PBS extensively, then lysed and subject to semi-quantitative PCR analysis. Data shown are representative of three separate experiments.

sing cells increased the percent of cells expressing CXCR4, while not enhancing the amount of receptor per cell. Here, we show that transfection of CCR5 into CD4 and CXCR4 expressing 3T3 cells increased the cell surface level of CXCR4. The mechanism for the enhancement is not clear. It is possible that through complex formation between these receptors, CCR5 prevents or reduces spontaneous endocytosis of CXCR4, thereby increasing CXCR4 density on cell surface.

The resistance of CCR5 to ligand-mediated downmodulation has been reported previously [Brandt et al., 2002]. The mechanism of this resistance is also not clear, although it has been suggested that the endocytic machinery for CCR5 is different from that of CXCR4. We found that in the presence of SDF-1α, CCR5 high cells retained medium to high levels of CXCR4, whereas in CCR5 low cells, CXCR4 was more completely modulated. Our data suggest that one possible mechanism for the reduction in modulation is via the formation of complexes between that CCR5 and CXCR4 that may retain CXCR4 on the cell surface even in the presence of ligand.

The relationship among CD4, CCR5, and CXCR4 is complex. CCR5 and CXCR4 exhibit homogeneous microclusters with CD4 on T cells, respectively. CCR5 and CXCR4 immunogolds were not colocalized in human macrophages, which express low level of CD4 [Singer et al., 2001]. A recent article published by Babcock et al. [2003] indicates that CXCR4 constituitively forms dimers but is not associated with CCR5 in the absence of CD4. We have similar findings that CXCR4 was not co-immunoprecipitated with CCR5 in the absence of CD4. Collectively these results together with data presented in this study suggest that the interaction of CXCR4 with CCR5 is CD4 dependent. Consistent with this hypothesis is the finding of colocalisation of CCR5 with CXCR4 and CD4 on the surface of a transformed T cell line [Popik et al., 2002]. Recently, CCR5 mutant, CCR5Delta32 was found to directly interact with CXCR4 in the absence of CD4 [Agrawal et al., 2004], suggesting that conformational changes in the variant CCR5Delta32 may overcome the requirement for CD4 in heterodimer formation.

CCR5 and CXCR4 are co-expressed on many cell types, such as macrophages, CD4$^+$ T cells, CD8$^+$ T cells, dendritic cells, thymocytes,

hematopoietic cells, mast cells/basophiles, neurons, astrocytes, microglia, epithelium, endothelium, vascular smooth muscle, and fibroblasts [Deichmann et al., 1997; Rottman et al., 1997; Ruiz et al., 1998; Wang et al., 1998, 1999, 2001a,b; Zaitseva et al., 1998; Albright et al., 1999; Lee et al., 1999; Westmoreland et al., 2002]. Chemokine receptor heterocomplex formation may not only affect HIV-1 infection of T cells, but could have broad impact on many aspects of cell function, such as cell migration and ligand-mediated endocytosis. Combinatorial signaling through constitutive or chemokine induced chemokine receptor association may fundamentally differ between different pairs of chemokines and receptors. Heterologous receptor interactions should be considered when designing therapeutic interventions targeting chemokine–chemokine receptor function.

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