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Constitutive cell surface association between CD4 and CCR5

(HIV/AIDS/receptors)

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ABSTRACT **HIV-1 entry into cells involves formation of a complex between gp120 of the viral envelope glycoprotein (Env), a receptor (CD4), and a coreceptor. For most strains of HIV, this coreceptor is CCR5. Here, we provide evidence that CD4 is specifically associated with CCR5 in the absence of gp120 or any other receptor-specific ligand. The amount of CD4 coimmunoprecipitated with CCR5 was significantly higher than that with the other major HIV coreceptor, CXCR4, and in contrast to CXCR4 the CD4–CCR5 coimmunoprecipitation was not significantly increased by gp120. The CD4–CCR5 interaction probably takes place via the second extracellular loop of CCR5 and the first two domains of CD4. It can be inhibited by CCR5- and CD4-specific antibodies that interfere with HIV-1 infection, indicating a possible role in virus entry. These findings suggest a possible pathway of HIV-1 evolution and development of immunopathogenicity, a potential new target for antiretroviral drugs and a tool for development of vaccines based on Env–CD4–CCR5 complexes. The constitutive association of a seven-transmembrane-domain G protein-coupled receptor with another receptor also indicates new possibilities for cross-talk between cell surface receptors.**

It has been known for many years that, in addition to the primary receptor CD4, HIV-1 requires cofactor molecules to enter cells (reviewed in ref. 1). It was hypothesized (2, 3) that the entry cofactors may directly associate with the complex between CD4 and the HIV-1 envelope glycoprotein (Env; gp120-gp41) and therefore serve as coreceptors. The identification of the entry cofactors as chemokine receptors (4–9) not only solved a long-standing puzzle about HIV tropism and pathogenesis but also provided new tools for understanding the mechanism of HIV entry. It was later demonstrated that gp120, CD4, and the HIV-1 coreceptor CXCR4 can be coimmunoprecipitated, suggesting that the complex between these three molecules plays a critical role in the initial stages of the entry process (10). By using a displacement assay, it was shown that in the presence of CD4, gp120 associates with the other major HIV-1 coreceptor, CCR5 (11, 12). It was also found that gp120 induces CD4–CXCR4 membrane colocalization (13, 14), suggesting the formation of higher order molecular complexes.

Several previous observations hinted that CD4 could interact with coreceptor molecules even in the absence of gp120, but convincing evidence for the existence of such an interaction between cell surface-associated molecules was lacking (10, 11, 13, 15). Here, we demonstrate that cell surface CD4 associates with CCR5 in the absence of gp120 or other chemokine-receptor- or CD4-specific ligands, we partially characterize regions of the two molecules that are involved in this interaction, and we show a functional correlation between this association and HIV-1 Env-mediated fusion. We propose that the CD4–CCR5 interaction plays an important role in the viral entry process and in HIV-1 evolution and immunopathogenesis and could be a new target for antiviral drugs and a tool for vaccine design. We also suggest that the association of a seven-transmembrane-domain G protein-coupled receptor (CCR5) with another cell surface receptor (CD4) provides a potential pathway for cross-talk between cell surface receptor molecules.

MATERIALS AND METHODS

Cells, Viruses, Chemokines, Soluble CD4 Fragments, and mAbs. 3T3 cells expressing CD4, CD4 and CCR5, or CXCR4 (6) and A2.01.T4.T8 cells expressing hybrid CD4–CD8 molecules (see, e.g., ref. 16) were provided by D. Littman (New York University). Primary human CD4 T cells were purified from an apheresis of a healthy donor by negative selection to >95% purity as described (17). Purified cells were cultured at 10^6 cells per ml in RPMI with 10% FCS (HyClone), 2 mM glutamine, and 50 mg/ml gentamicin (Biofluids, Rockville, MD). These cells were stimulated with either 5 mg/ml Con A (Calbiochem) and 100 units/ml IL-2 (Boehringer Mannheim) (+ cells) or 5×10^5 cells per ml L cells stably transfected with the human CD32 gene (a gift of G. Delespesse, University of Montreal, Canada) and 200 ng/ml anti-CD3 antibody UCHT1 (18) (++ cells). After 3 days of culture, CD4 T cells were removed from the L cells, and 100 units/ml IL-2 was added. Human macrophages and monocytes were obtained from peripheral blood. Monocytes were allowed to differentiate for 14 days in the presence of human macrophage colony-stimulating factor. Recombinant vaccinia viruses used for the reporter gene fusion assay (19) and for expression of the R5 HIV Env (Bal31, JRFL) (4, 20) and CD4 (21) were described previously. Vaccinia virus containing the gene for CCR5 (vvCCR5–1107) was developed by using the CCR5 cDNA from pCDNA3 (provided by M. Parmentier, Universite Libre de Bruxelles, Belgium), which was subcloned into the *Sma*I site of pMC1107 (22) by *Bam*HI–*Xba*I restriction and blunt-end cloning into the *Sma*I site. The recombinant vaccinia virus was then obtained by using standard techniques employing *Ecogpt* selection (23) The ^{125}I-labeled human MIP-1α was purchased from DuPont/NEN, and unlabeled chemokines were from R&D systems. The soluble D1D2CD4 fragment was a gift from J. Sodroski (Harvard Medical School, Boston), the anti-CD4 mAbs (CG7, CG1) and a control mAb (CG1) were a gift from J. Gershoni (University of Tel Aviv) and G. Denisova (Food and Drug Administration, Bethesda, MD), and the anti-CD4 mAb OKT4 (ascites fluid) were a gift from H. Golding. The anti-CD4

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polyclonal antibody T4-4 was obtained through the AIDS Research and Reference Reagent Program from R. Sweet (SmithKline Beechman). The anti-CCR5 mAbs m180, m181, m182, and m183 were purchased from R&D Systems, 2D7 and 5C7 have been described (24). The goat polyclonal anti-CCR5 antibody CKR5(C20) was purchased from Santa Cruz Biotechnology.

Immunoprecipitation. Cells (typically 5–10 $\times$ 10^6 per sample) were washed once with PBS, labeled with biotin if needed, and then resuspended in cold (4°C) PBS at a final density of 10^7 cells per ml. Immunoprecipitating antibodies at the required concentration, typically 1.5–3 μg/ml, were added to the cell suspension and incubated with gentle mixing for 4 hr at 4°C or at 37°C for 30 min with similar results. Cells were then pelleted by centrifuging and resuspended in lysis buffer [1% Brij97, 5 mM iodoacetamide, added immediately before use, 150 mM NaCl, 20 mM Tris (pH 8.2), 20 mM EDTA, and protease inhibitors] at 4°C for 40 min with gentle mixing. In an alternative protocol, the cells were first lysed and then incubated with the immunoprecipitating antibody with essentially the same results. The nuclei were pelleted by centrifuging at 17,000 $\times$ *g* for 25 min in a refrigerated Eppendorf centrifuge. Protein G-Sepharose beads (Sigma) prewashed with PBS were added to the samples and incubated at 4°C for 14 hr. The beads were then washed four times with 1 ml of ice-cold lysis buffer. Samples were then eluted by adding 4$\times$ sample buffer for SDS/PAGE and boiled for 5 min or kept overnight at 37°C with essentially the same results. They were run on SDS/10% PAGE and were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 20 mM Tris·HCl (pH 7.6) buffer containing 140 mM NaCl, 0.1% Tween-20, and 5% nonfat powdered milk. For Western blotting, these membranes were incubated with the respective antibodies, then washed and incubated with horseradish peroxidase-conjugated secondary antibodies. For detection of cell surface biotinylated proteins, the nitrocellulose membranes were incubated with streptavidin-conjugated horseradish peroxidase. In both cases they were developed by using the supersignal chemiluminescent substrate from Pierce. The images were acquired by using a Bio-Rad phosphoimager at the highest resolution (0.1 mm) or by using sensitive film, and printed by a laser printer (Lexmark Optra S 1650) at the highest resolution (1,200 $\times$ 1,200). Silver staining was performed by using the Silver Stain Plus kit following the company protocol (Bio-Rad).

MIP-1α Displacement Assay. Chemokine binding was carried out as reported (11).

Confocal Laser Scanning Microscopy. HeLa CD4 cells (25) maintained in DMEM supplemented with 10% fetal calf serum and 500 μg/ml G418 (GIBCO/BRL) were transiently transfected with an expression vector containing myc-tagged CCR5 as described (13). Transfected cells were evaluated for expression of CCR5 at the cell surface by using indirect immunofluorescent staining with the anti-myc mAb 9E10 (26) followed by anti-Mophycoerythrin and flow cytometric analysis as described (13). Confocal laser scanning microscopy was carried out as described (13) by using a Leica TCS 4D instrument (Leica, Heidelberg, Germany) interfaced with an argon/krypton ion laser and with fluorescence filters and detectors allowing detection of FITC and Texas Red markers. To identify fluorescence colocalization, correlation maps were calculated by using a local statistical method as described (28).

Cell–Cell Fusion Assay. The cell–cell fusion assay was previously described (19).

RESULTS

Specific Coimmunoprecipitation of CD4 and CCR5 from Cell Lines. It was previously found that the soluble D1D2 fragment, but not the entire extracellular portion of CD4, interferes with the chemokine MIP-1α for binding to CCR5, indicating possible interactions between CD4 and CCR5 (11). Although the differences between the two-domain and the four-domain fragments of CD4 could be attributed to a better exposure of conformational epitopes in the two-domain CD4 fragment, an alternative possibility is that the CD4D1D2–CCR5 interaction does not reflect the properties of the wild-type (membrane-associated) CD4 binding to CCR5. Therefore, we used an immunoprecipitation assay, optimized for high efficiency and reproducibility, to directly detect the interactions between native membrane-associated CD4 and CCR5.

To demonstrate coimmunoprecipitation of cell surface-associated CD4 and CCR5, we used 3T3 cell lines transfected to express CD4 (3T3.CD4), CD4 and CXCR4 (3T3.CD4.CXCR4) or CD4 and CCR5 (3T3.CD4.CCR5). The CD4 concentrations at the surfaces of these cells were very similar: their ratios were 1.26:1.2:1, respectively, as measured by using flow cytometry (data not shown; see also Fig. 1*A*, lane II). The surface concentrations of CCR5 and CXCR4 were also very similar (flow cytometry data not shown). Because CD4 is easier to detect by Western blotting and biotinylation than CCR5, in most cases we used anti-CCR5 antibodies to coimmunoprecipitate CD4. We found that the N terminus-specific mAb 5C7 (29) was the most efficient antibody in immunoprecipitating CCR5 compared with a battery of other anti-CCR5 antibodies (data not shown). This mAb coimmunoprecipitated surface-associated CD4 in 3T3 cell lines coexpressing CD4 and CCR5 (Fig. 1*A*, lane IV). The band was CD4 because it aligns with the CD4 band obtained by direct immunoprecipitation using an anti-CD4 mAb (OKT4) (Fig. 1*A*, lane II), has the expected molecular weight (55 kDa), and reacts specifically in the Western blot assay (Fig. 1*A*, lane V). CD4 was highly and specifically enriched in the coimmunoprecipitates as was observed by comparison of cell-surface biotinylated lysates that were not subjected to immunoprecipitation (Fig. 1*A*, lane I) with the coimmunoprecipitates (Fig. 1*A*, lane IV). In these experiments, CCR5 was not detected because of the low efficiency of biotinylation; however, it was observed by using Western blotting (Fig. 1*A*, lane VI). Similar results were obtained for a number of cell lines, including PM1 and L1.2 transfectants, that coexpress CCR5 and CD4 (data not shown).

The specificity of the CD4 coimmunoprecipitation was demonstrated through several control experiments. The anti-CCR5 mAb 5C7 did not coimmunoprecipitate CD4 from 3T3.CD4 cells that do not express CCR5 (Fig. 1*A*, lanes IV and V). In another experiment, an antibody (4G10) to a related chemokine receptor (CXCR4) was used. This antibody is able to efficiently immunoprecipitate CXCR4 and coimmunoprecipitate CD4 and CXCR4 in cells expressing these two molecules (Fig. 3*A*) but did not coimmunoprecipitate CD4 in cells expressing CD4 and CCR5 (Fig. 1*A*, lane III). In addition, the amount of coimmunoprecipitated CD4 was proportional to the amount of immunoprecipitated CCR5, demonstrated by using two anti-CCR5 mAbs with different immunoprecipitating activities (Fig. 1*B*), further suggesting a specific CD4–CCR5 interaction. We coimmunoprecipitated CCR5 with an anti-CD4 antibody (OKT4) (Fig. 1*C*, lane 2), but not with a control antibody (CG10), which does not bind to CD4 (lane 4), in cells coexpressing these two molecules (lane 2), but not in CCR5 negative cells (lane 3).

To further evaluate the specificity of the CD4–CCR5 interaction and to address the question of whether other proteins interact with CCR5 and potentially influence the CD4–CCR5 association, we used silver staining of proteins immunoprecipitated by the anti-CCR5 mAb 5C7 in parallel with biotinylation (some proteins, particularly chemokine receptors, are poorly labeled by biotin and at low concentrations may not be detected). Apart from the bands corresponding to CCR5 and CD4, the only other major bands are those representing the heavy and light chains of the precipitating mAb 5C7 and bands that are apparently not specific to CCR5, because they were immunoprecipitated in CCR5-negative cells (Fig. 1*D*). These data not only imply that the interaction between CD4 and CCR5 is not mediated by another molecule but also indicate that the coimmunoprecipitation of CD4 and CCR5 is unlikely to be due to compartmentalization of these two molecules within defined membrane microdomains,

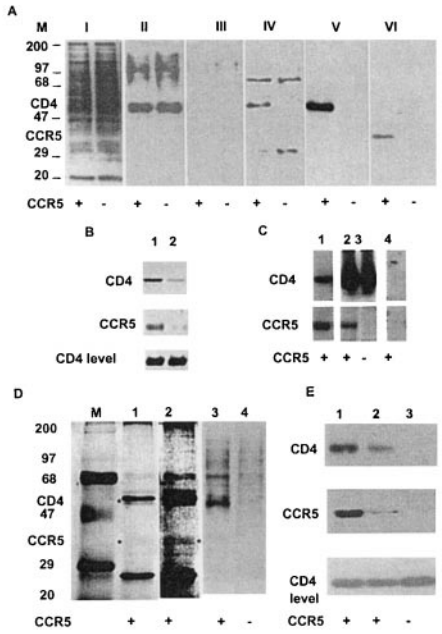

FIG. 1. Specific coimmunoprecipitation of cell surface-associated CD4 and CCR5 from 3T3 cells coexpressing these two molecules. (*A*) Equal numbers of 3T3 cells expressing CD4 and CCR5 (+) or CD4 only (−) were biotinylated, processed as described in *Materials and Methods*, and either used as a whole-cell lysate (0.25% of total, gel I) or immunoprecipitated with an anti-CD4 mAb (OKT4) (gel II), anti-CXCR4 mAb (4G10) (gel III), or anti-CCR5 mAb 5C7 (gels IV–VI). The biotinylated proteins were detected by using streptavidin–horseradish peroxidase (gels I-IV). CD4 and CCR5 were detected in an aliquot of the same samples as in gel IV by using Western blotting with an anti-CD4 polyclonal antibody (T4-4) (gel V) or an anti-CCR5 polyclonal antibody [CKR5(C20)] (gel VI). (M denotes molecular markers, and the numbers are in kDa). (*B*) Coimmunoprecipitation of CD4 by the anti-CCR5 mAbs m180 (lane 1) and m181 (lane 2). The coimmunoprecipitated CD4 and the immunoprecipitated CCR5 were detected by sing Western blotting as in *A*. (*C*) Coimmunoprecipitation of CCR5 from 3T3.CD4.CCR5 cells with anti-CD4 antibodies. 3T3.CD4.CCR5 cells (lanes 1, 2, and 4) or 3T3.CD4 cells (lane 3) were used for immunoprecipitation by OKT4 (lanes 2 and 3) or by a control antibody (CG10) (lane 4). Lane 1 shows for comparison immunoprecipitation with the anti-CCR5 mAb 5C7. CD4 and CCR5 were detected by using Western blotting as in *A*. (*D*) Cell lysates were immunoprecipitated by the anti-CCR5 mAb 5C7, the immunoprecipitation product was analyzed by using a silver stain kit (lanes 1 and 2) and compared with proteins detected by streptavidin–horseradish peroxidase in biotinylated lysates (lanes 3 and 4). M denotes molecular weight marker, and + and − denote 3T3.CD4.CCR5 or 3T3.CD4 cells, respectively. ∗, bands caused by CD4 and CCR5. The two bands above and below CD4 are caused by the 5C7 mAb heavy and light chain, respectively. Lane 2 represents lane 1 at higher sensitivity, where CCR5 is clearly seen. (*E*) CD4–CCR5 coimmunoprecipitation is not significantly affected by cholesterol depletion. 3T3.CD4.CCR5 cells were treated with 10 mM methyl-β-cyclodextrin for 1 hr at 37°C (which caused significant cytotoxicity) and used for immunoprecipitation by the anti-CCR5 antibody 5C7 (lane 2), and compared with untreated cells (lane 1) and 3T3.CD4 cells (lane 3). CD4 and CCR5 were detected by using Western blotting as in *A*. *Bottom* shows Western blotting of CD4 from whole-cell lysates.

which would lead to coimmunoprecipitation of a greater number of proteins. Another experiment supporting this notion shows that cell lysis with two different detergents (Brij97 and NP40) did not disrupt the CD4–CCR5 interaction (data not shown). Furthermore, depletion of cholesterol from the cell membrane with methyl-β-cyclodextrin, a procedure that was shown to disrupt microdomain structure (see, e.g., ref. 30), did not block the coimmunoprecipitation of CD4 and CCR5 (Fig. 1*E*).

Coimmunoprecipitation of CD4 and CCR5 in Primary CD4$^+$ T Cells, Macrophages, and Monocytes. Results similar to those described above for cell lines were also obtained with primary human cells susceptible to HIV-1 entry. Our initial attempts to coimmunoprecipitate CD4 and CCR5 from the surface of primary T lymphocytes resulted in very weak bands that were at the limit of assay sensitivity, because of the low level expression of CCR5 at the surface of these cells and the relatively small percentage of cells expressing it as evaluated by flow cytometry (data not shown). By using two alternative procedures for activating the CD4$^+$ T cells, as described in *Materials and Methods*, expression of CCR5 was significantly increased to high (+) and very high (++) levels, corresponding on average to ≈ 2–4×10^3 and 3–5 $\times$ 10^4 molecules, respectively, as estimated by using quantitative flow cytometry. In these cells, CD4 levels were similar, and the amount of CD4 coimmunoprecipitated with the anti-CCR5 mAb 5C7 correlated with their cell fusion efficiency (Fig. 2*A*). The amount of coimmunoprecipitated CD4 in human macrophages and monocytes also correlated with the efficiency of their fusion with cells expressing the HIV-1 JRFL Env (Fig. 2*B*) but not with the surface concentration of CD4. Indeed, the monocytes expressed similar or higher levels of CD4, but the amount of coimmunoprecipitated CD4 was undetectable or barely detectable in our assay (Fig. 2*B*). The larger amount of coimmunoprecipitated CD4 in macrophages is likely related to the higher levels of CCR5 compared with monocytes—on average ≈ 5–10×10^3 vs. $< 2 \times 10^3$ molecules per cell as estimated by using quantitative flow cytometry. However, the CCR5 levels in macrophages were lower compared with the ++ CD4$^+$ T cells, and we were not able to detect the immunoprecipitated CCR5 by Western blotting (data not shown).

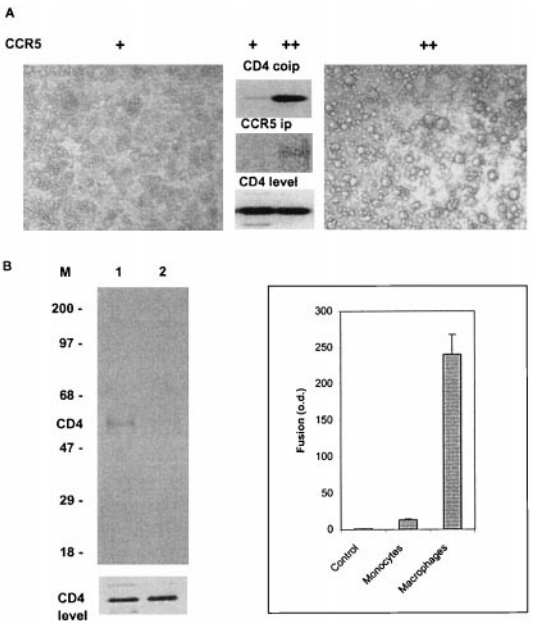

FIG. 2. Coimmunoprecipitation of CD4 and CCR5 in primary cells. (*A*) Human CD4 T cells expressing high (+) and very high (++) amounts of CCR5 were used for immunoprecipitation with the anti-CCR5 mAb 5C7 (*Center*) or fusion with HeLa cells expressing the HIV-1 JRFL Env (*Left* and *Right*). The coimmunoprecipitated CD4 (*Center Top*) and immunoprecipitated CCR5 (*Center Middle*) were detected by using Western blotting as in Fig. 1*A*. The CD4 Western blotting of whole-cell lysates is shown (*Bottom*) as a measure of the level of CD4. The average number of syncytia for ++ cells was 92 ± 10.5, for + cells was 29 ± 7, and for control HeLa cells was 6 ± 3. The average diameter of syncytia from the ++ cells was about 4-fold larger than that for the + cells. (*B*) Human macrophages (*Left*, lane 1) and monocytes (*Left*, lane 2) were used for CCR5 immunoprecipitation. CD4 coimmunoprecipitation was detected as in *A*. The CD4 Western blotting of whole-cell lysates is shown (*Bottom*) as a measure of the level of CD4. (*Right*) Fusion of these cells with HeLa cells expressing the HIV-1 JRFL Env as quantitated by the β-galactosidase assay. The control represents HeLa cells that do not express HIV-1 Env.

CD4 Interaction with CCR5 Is Stronger than That with CXCR4 and Is Not Increased in the Presence of gp120. We previously found that CD4 could associate weakly with CXCR4 even in the absence of gp120 (10). However, the results varied in a cell line- and assay condition-dependent manner. To evaluate the strength of the CD4–CCR5 association relative to the CD4–CXCR4 interaction, 3T3 cell lines expressing CD4 and either CCR5 or CXCR4 at approximately the same surface concentrations were used. In the 3T3.CD4.CXCR4 cells, CD4 associated weakly with CXCR4, but the association was dramatically increased by addition of X4 HIV-1 Env gp120 (IIIB) (Fig. 3*A*). In contrast, the amount of CD4 coimmunoprecipitated by the anti-CCR5 mAb 5C7 in the 3T3.CD4.CCR5 cells was high even in the absence of gp120, and the addition of X4R5 (89.6) or R5 (JRFL) HIV-1 Env gp120 did not significantly increase the CD4–CCR5 coimmunoprecipitation (Fig. 3 *B* and *C*). The quantity of CD4 coimmunoprecipitated by anti-CCR5 mAbs in the absence of gp120 was about the same as the quantity of CD4 coimmunoprecipitated by anti-CXCR4 mAbs in the presence of gp120. These results indicate that the CD4–CXCR4 association is weaker than the CD4–CCR5 interaction and that the preformed complexes between CD4 and CCR5 are close to a saturation level, where the addition of gp120 cannot further increase their complex formation.

The First Two Domains of CD4 and the Second Extracellular Loop of CCR5 Are Probably Involved in the Formation of the CD4–CCR5 Complex. To identify possible regions of CD4 that are responsible for the interaction with CCR5, we used a cell line (A2.01.T4.T8) expressing a hybrid CD4–CD8 molecule containing the first two domains of CD4, which was previously shown to support HIV-1 Env-mediated fusion (31) although at a lower rate than the wild-type CD4 (16). The A2.01.T4.T8 cells were induced to express CCR5 with a recombinant vaccinia virus encoding the CCR5 gene. These cells, coexpressing CCR5 and the CD4-CD8 hybrid molecule, fused with cells expressing the R5 HIV-1 Env Bal, although at somewhat lower efficiency compared with cells expressing wild-type CD4 (data not shown). These results are analogous to our previously reported observations of fusion between the A2.01.T4.T8 cells and cells expressing the X4 HIV-1 Env IIIB (16). The CD4–CD8 molecules were coimmunoprecipitated by an anti-CCR5 mAb from the A2.01.T4.T8 cells expressing CCR5 but not from those infected with control wild-type vaccinia virus (Fig. 4*A*). The surface levels of CD4–CD8 molecules in the cells infected with the CCR5 and wild-type vaccinia viruses were not significantly different as quantified by flow cytometry (data not shown) and Western blotting (Fig. 4*B*). To establish that the CD8 portion of the CD4–CD8 hybrid molecule was not involved in the interaction with CCR5, we used HeLa cells expressing either CD4 or CD8. CCR5 was again expressed in these cells by recombinant vaccinia virus. In those cells only CD4 but not CD8 was coimmunoprecipitated with an anti-CCR5 mAb (5C7), demonstrating that the CD8 portion of the hybrid CD4–CD8 molecule was unlikely to be involved in the interaction with CCR5 (data not shown). Together, these results suggest that the first two domains of CD4 associate with CCR5.

We also confirmed the previous observations that a soluble fragment of CD4 consisting of the first two domains (sCD4D1D2) competes with macrophage inflammatory protein (MIP)1-α for CCR5 (11). Interestingly, we found that the HIV-1 infection inhibiting mAb CG7 at 60 nM almost completely blocked the ability of sCD4D1D2 to displace the chemokine MIP-1α (Table 1). The same antibody significantly inhibited the coimmunoprecipitation of CCR5 by the anti-CD4 mAb OKT4 (Fig. 4*C*). Unlike CG7, a second anti-CD4 mAb (CG1) and a control mAb (CG10) were not effective in preventing the displacement of MIP1-α by sCD4D1D2 (Table 1). Although we do not know whether sCD4D1D2 represents a good model for native CD4 with respect to CCR5 binding, these results do suggest that specific regions of CD4, potentially those overlapping the epitope of CG7, probably located within the first CD4 domain (32), are involved in the association with CCR5.

To localize regions of CCR5 interacting with CD4, we used mixtures of mAbs recognizing the N terminus and the first (ecl-1) and second (ecl-2) extracellular loops of CCR5. Increasing the concentration of a mAb against the ecl-2 of CCR5 (2D7) in a mixture with the 5C7 reduced the total amount of coimmuno-

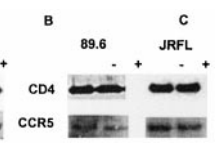

FIG. 3. The effect of gp120 on the CCR5–CD4 and CXCR4–CD4 association. (*A*) 3T3.CD4.CXCR4 cells were incubated with the anti-CXCR4 mAb 4G10 in the absence (−) or presence (+) (5 μg/ml) of HIV-1 IIIB gp120. The CD4 and CXCR4 were detected by using Western blotting with either the polyclonal anti-CD4 Ab T4–4 or 4G10. (*B*) 3T3.CD4.CCR5 cells were incubated with the anti-CCR5 mAb 5C7 in the absence or presence (5 μg/ml) of HIV-1 89.6 gp120. The CD4 and CCR5 were detected by using Western blotting as described in Fig. 1*A*. (*C*) The same as in *B* but gp120 from the R5 HIV-1 JRFL was used instead of the dual tropic 89.6.

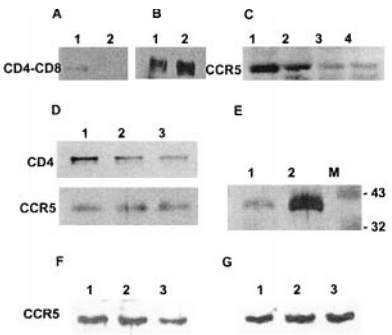

FIG. 4. Involvement of the first two domains of CD4 and the second extracellular loop of CCR5 in the CD4–CCR5 association. (*A*) Coimmunoprecipitation of CD4–CD8 hybrid molecules containing the first two domains of CD4 by an anti-CCR5 mAb. The A2.01.T4.T8 cells expressing the hybrid CD4–CD8 molecule were infected with a recombinant vaccinia virus (vvCCR5–1107) (lane 1), encoding the gene for CCR5, and a control wild-type (WR) vaccinia virus (lane 2). The anti-CCR5 mAb 5C7 was used to immunoprecipitate CCR5 and T4–4 for detection of CD4 by Western blotting. (*B*) The amount of CD4–CD8 molecules in A2.01.T4.T8 cells infected with the CCR5 (lane 1) or WR (lane 2) vaccinia virus was not significantly different as demonstrated by Western blot with an anti-CD4 Ab (T4–4). (*C*) Coimmunoprecipitation of CCR5 by an anti-CD4 mAb (OKT4) is inhibited in the presence of another anti-CD4 mAb (CG7). For comparison, lane 1 shows CCR5 immunoprecipitated by 5C7. Lanes 2, 3, and 4 represent the amount of CCR5 coimmunoprecipitated by a mixture of OKT4 (ascites fluid 3.5 μl/ml) and increasing concentrations of the anti-CD4 mAb CG7 (0, 5, and 10 μg/ml, respectively). (*D*) CD4 coimmunoprecipitation by 5C7 is inhibited in the presence of another anti-CCR5 mAb (2D7) (29) directed to the second extracellular loop. Equal amounts (3 μg/ml) of 5C7, which does not affect HIV entry, were mixed with increasing amounts (0, 3, and 6 μg/ml) (lanes 1, 2, and 3, respectively) of the HIV-1-blocking mAb 2D7 and used for immunoprecipitation. The sample obtained from 9×10^6 3T3.CD4.CCR5 cells was divided into two portions, and the smaller one (1/6 of total) was used for Western blot of CD4 by T4–4, and the rest were used for Western blot of CCR5 by the CKR5(C20). (*E*) The CCR5-terminus-specific mAb 5C7 (lane 2) immunoprecipitates CCR5 much more efficiently than the CCR5 ecl-2-specific mAb 2D7 (lane 1). Equal amounts (4 μg/ml) of these two mAbs were used for immunoprecipitation of CCR5 in 3T3.CD4.CCR5 cells. The molecular markers are shown on the right side (lane M). (*F* and *G*) Differential inhibition by two anti-CCR5 mAbs, m182 and m183 (which do not immunoprecipitate CCR5 as measured by our assay) of the CCR5 coimmunoprecipitation by the anti-CD4 mAb OKT4. Lanes 1, 2, and 3 represent 1, 2, and 4 μg/ml of m182 and m183, respectively. CCR5 was detected by Western blotting with CKR5(C20).

Table 1. CG7 displacement inhibition of MIP-1α

mAb	Displacement, %
sCD4D1D2	53
MIP-1α	100
CG7 + sCD4D1D2	2.5
CG10 + sCD4D1D2	56
CG1 + sCD4D1D2	47

The anti-CD4 mAb CG7 inhibits the displacement of CCR5-bound MIP-1α by a soluble CD4 fragment containing its first two domains (sCD4D1D2). The antibodies were used at a concentration of 60 nM; the signal-to-noise ratio was greater than 10.

precipitated CD4 (Fig. 4*D*), whereas the amount of immunoprecipitated CCR5 was slightly increased (Fig. 4*D*), probably resulting from the additional, yet weak immunoprecipitation of CCR5 with 2D7 (Fig. 4*E*). Another HIV-1-blocking mAb specific for the CCR5 ecl-2 (m182; R&D Systems) showed similar although weaker inhibitory effects (Fig. 4*F*) in contrast to the non-HIV-1-blocking anti-CCR5 mAb m183, which did not interfere with the CD4–CCR5 coimmunoprecipitation (Fig. 4*G*). Other mAbs to the N terminus and mAbs to ecl-1 of CCR5 did not decrease the amount of coimmunoprecipitated CD4 when used in combination with 5C7 (data not shown). These results suggest that the ecl-2 of CCR5 is involved in the interaction with CD4. However, even at the highest concentration of mAb (2D7) used (50 μg/ml), the inhibition of the CD4–CCR5 coimmunoprecipitation was not complete, indicating that regions additional to the second extracellular loop of CCR5 are probably involved in the interaction with CD4.

Membrane-Associated CD4 Colocalizes with CCR5. We further examined whether the CD4 and CCR5 molecules associate by using confocal laser scanning microscopic analysis of fluorescently labeled molecules. Colocalization of the two molecules was observed as demonstrated by the yellow (red-green colocalization) staining, suggesting formation of large multimolecular complexes between these two molecules (Fig. 5). A correlation map was prepared by using software developed in this laboratory. The regions of true overlap of green and red staining were selected from the noncolocalized staining and the background fluorescence and represented as white dots. A relatively high degree of colocalization is evident from this analysis (Fig. 5 *Lower*). Less colocalization was observed between CD4 and CXCR4 (Fig. 5 *Left*) than with CCR5. It was previously shown that addition of X4 HIV-1 Env gp120 increases the colocalization of CD4 and CXCR4 (13). The extent of this increase appears to be comparable with the colocalization between CD4 and CCR5 in the absence of gp120 in a manner reminiscent of the immunoprecipitation data shown in Fig. 3. No significant colocalization was observed between CD45 and CCR5 or HLA class I and CCR5, suggesting specificity in the interaction between CD4 and CCR5 (data not shown). These results suggest that CCR5 (and to a lesser extent CXCR4) not only associates with native membrane-associated CD4 but that the two molecules form large multimolecular complexes possibly because of their dimeric structures (ref. 33, and data not shown).

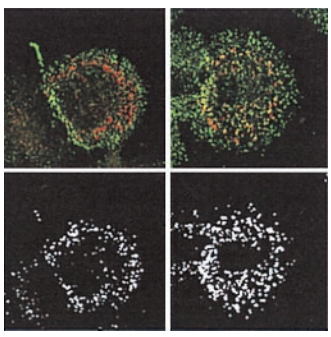

FIG. 5. Colocalization of CD4 and CXCR4 (*Left*) or CCR5 (*Right*). A CXCR4 (or CCR5)-myc tag-expressing HeLa cell, double stained for CD4 (green) and CXCR4 (or CCR5) (red) (*Upper*). Colocalization of the two molecules is demonstrated by the yellow (red-green colocalization) staining, suggesting their clusterization. Correlation maps of these images (*Bottom*) show the regions of true overlap of green and red staining selected from the noncolocalized staining and the background fluorescence, represented as white dots.

Inhibition of the CD4–CCR5 Interaction Correlates with the Inhibition of HIV-1 Env-Mediated Fusion. It has been demonstrated that the anti-CCR5 mAb 2D7 inhibits entry of R5 and R5X4 HIV-1 into U87MG-CD4 cells expressing transfected CCR5 (24). To investigate the possibility of a relationship between inhibition of the CCR5–CD4 interaction and HIV-1 Env-mediated fusion, we used a recombinant vaccinia virus-based reporter gene assay for quantitation of cell–cell fusion (19). The mAb 2D7 inhibited fusion between 3T3.CD4.CCR5 cells and cells expressing R5 (Bal and JRFL) and R5X4 (89.6) HIV Envs. The inhibition was concentration-dependent, and cell–cell fusion was significantly decreased at concentrations in the range of 0.5–50 μg/ml, a concentration range similar to that observed for inhibition of the CD4–CCR5 interaction (data not shown). For several other anti-CCR5 mAbs, there was correlation ($r = 0.98$, $P = 0.001$) between inhibition of fusion and inhibition of the CD4–CCR5 interaction (Table 2). These results indicate that the CD4–CCR5 interaction may play a role in membrane fusion mediated by the HIV-1 Env. However, as was similar to the inhibition of the CD4–CCR5 interaction, even at the highest mAb concentration used (50 μg/ml), the inhibition of fusion was not complete. The lack of complete inhibition of fusion suggests that multiple interactions, possibly both CD4–CCR5 and gp120–CCR5, and multiple interaction sites involving other extracellular regions of CCR5, are involved in the initial steps of HIV-1 entry.

DISCUSSION

The results of this study suggest that CD4 is constitutively associated with CCR5 in the plasma membrane of various cell types. This demonstration and characterization of a seven-transmembrane-domain G protein-coupled receptor association with another receptor at the cell surface in the absence of stimuli suggests new possibilities for cross-talk between plasma membrane receptors. The physiological role of such an association if any, given the apparent redundancy of CCR5 function (reviewed in ref. 1), is presently unknown.

Whatever the physiological role of the CD4–CCR5 interaction is, it may have played a critical role in the evolution of HIV-1 and the development of viral immunopathogenesis. As was originally suggested by R. Weiss (reviewed in refs. 1 and 34), based on the observation that some strains of HIV-2 use the HIV coreceptor CXCR4 as a primary receptor (35), one might speculate that CCR5 was initially used as a primary receptor by a predecessor of HIV. In support of this notion are the observations that a number of SIV strains can enter CD4-negative cells by using

Table 2. Correlation between inhibition of HIV-1 (Bal)-mediated fusion and CD4–CCR5 interactions by anti-CCR5 mAbs

mAb	Fusion inhibition, %	CD4–CCR5 inhibition, %
None	0	0
5C7	0	0
5C7 + 2D7	49	40
m180	0	0
m180 + m181	7	12
m180 + m182	16	10
m180 + m183	2	0

A mixture of the anti-CCR5 mAb 5C7 with 2D7 or m180 with m181, m182, or m183 (3 μg/ml for each antibody) was used. The signal intensity for each CD4 band is presented as a percentage of the signal produced in the absence of inhibiting antibody.

CCR5 (36) and that SIV gp120 can bind CCR5 directly (37). Our demonstration of a strong intrinsic association between CD4 and CCR5 suggests a possible evolutionary pathway in which virus variants using both CD4 and CCR5 arose. The close proximity of the CCR5 and CD4 molecules probably enhanced adaptation of the virus to the new receptor molecule CD4. HIV may have further evolved to use CD4 for attachment in some cell types (38) and later adapted to use CXCR4 as an alternative coreceptor. The adaptation to the CD4 molecule as a primary receptor was probably a critical event for development of the specific characteristics of HIV disease in humans that is characterized by depletion of $CD4^+$ T lymphocytes.

Previously, we speculated that the interaction of CD4 with CCR5 is stronger than with CXCR4, leading to larger quantities of CD4–CCR5 complexes compared with CD4–CXCR4 complexes, and that this may be important for the differential susceptibility of cells, including macrophages, to entry of X4 and R5 HIV isolates, especially at low levels of CD4 expression (1, 34, 39). The data presented here support our hypothesis about the relative strength of CXCR4 and CCR5 association with CD4 and provide some evidence for the correlation between HIV Env-mediated cell–cell fusion and the quantity of preformed CD4–CCR5 complexes, although they do not exclude the possibility for direct influence of the CCR5 surface concentration.

The possibility that the CD4–CCR5 interaction is potentially important for the mechanism of HIV entry is supported by the finding that a mAb to CCR5 (2D7) that inhibits fusion and infection mediated by R5 and R5X4 HIV Envs also inhibits the CD4–CCR5 interaction. It is tempting to speculate that the inhibition of fusion is due at least partially to interference with this association. Previous studies suggested that 2D7 competes with gp120 for binding to CCR5 and therefore its mechanism of fusion inhibition is caused by inhibition of the gp120–CCR5 interaction (29). Taken together, these results suggest that, although the CD4–CCR5 interaction could play a role in fusion mediated by HIV Env, multiple interactions are involved in the early stages of the fusion process. The current paradigm of the initial stages of HIV-1 entry proposes an initial high-affinity binding of gp120 to CD4, leading to conformational changes in both molecules, which results in an enhanced binding of gp120 to CCR5 and exposure of the fusion peptide (reviewed in refs. 1 and 34). The recent elucidation of the crystal structure of a CD4–gp120 complex supports this idea (40). Our findings that an inhibitory anti-CCR5 mAb also interferes with the CD4–CCR5 interaction at about the same concentration as that required for inhibition of HIV-1 Env-mediated fusion and that cell–cell fusion correlates with the surface concentration of CD4–CCR5 complexes suggest that the interaction of CD4 with CCR5 could also be important for the early events of the HIV-1 entry process.

Based on these data, we speculate that agents inhibiting the CD4–CCR5 interaction could interfere with HIV entry and HIV Env-mediate fusion by at least two possible mechanisms: (*i*) dissociation of CD4 and CCR5 may prevent the interaction between gp120 and CCR5, thus inhibiting the formation of a fusion-competent multimolecular complex, and (*ii*) the binding of CD4 to CCR5 induces conformational changes in either or both molecules that are needed for the subsequent stages of virus entry; inhibition of their interaction would prevent those conformational changes and affect entry. Therefore, the CD4–CCR5 interaction could serve as a target for development of anti-HIV-1 agents that may not be toxic because the physiological role, if any, of the CD4–CCR5 interaction may not be important for survival, as indicated by the existence of healthy people who are homozygous for the CCR5-deletion mutant (reviewed in ref. 1). Interestingly, the ecl-2 of CCR5, which we found to be potentially implicated in the interaction with CD4, is also critical for chemokine binding as demonstrated by using CCR2/CCR5 chimera (41) as well as competition with the anti-CCR5 mAb 2D7 (42). This result may indicate that this region is a potentially good target for generating therapeutic agents not only against HIV-1 but also against inflammatory responses.

The recent suggestion that intermediate fusion structures may elicit immune responses to conserved neoepitopes (43) has raised the hope that Env–CD4–CCR5 complexes could be used as vaccines, as previously suggested (3). The finding that CCR5 and CD4 strongly associate may help in the design and development of such vaccines.

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