CD4 and CCR5 Constitutively Interact at the Plasma Membrane of Living Cells

A CONFOCAL FLUORESCENCE RESONANCE ENERGY TRANSFER-BASED APPROACH

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Human immunodeficiency virus entry into target cells requires sequential interactions of the viral glycoprotein envelope gp120 with CD4 and chemokine receptors CCR5 or CXCR4. CD4 interaction with the chemokine receptor is suggested to play a critical role in this process but to what extent such a mechanism takes place at the surface of target cells remains elusive. To address this issue, we used a confocal microspectrofluorimetric approach to monitor fluorescence resonance energy transfer at the cell plasma membrane between enhanced blue and green fluorescent proteins fused to CD4 and CCR5 receptors. We developed an efficient fluorescence resonance energy transfer analysis from experiments carried out on individual cells, revealing that receptors constitutively interact at the plasma membrane. Binding of R5-tropic HIV gp120 stabilizes these associations thus highlighting that ternary complexes between CD4, gp120, and CCR5 occur before the fusion process starts. Furthermore, the ability of CD4 truncated mutants and CCR5 ligands to prevent association of CD4 with CCR5 reveals that this interaction notably engages extracellular parts of receptors. Finally, we provide evidence that this interaction takes place outside raft domains of the plasma membrane.

Entry of human immunodeficiency virus (HIV) into target cells relies on sequential interactions between gp120, the surface subunit of the viral glycoprotein envelope (Env), with cell surface CD4 and the G-protein-coupled chemokine receptors CXCR4 or CCR5 that act as co-receptors (1). Following binding to the co-receptor, conformational changes in Env are thought to expose its transmembrane subunit gp41, which inserts into the host cell plasma membrane and to initiate fusion and the infection processes (2).

Triggering of an efficient fusion between viral and host cell membranes is thought to be a cooperative process that requires multiple engagements between Env and its receptors (3–5). Virus entry thus depends on membrane density of CD4 and chemokine receptors, which is expected to be influenced by their sequestration into delimited membrane domains. In support of this view, high-resolution electron microscopy-based approaches have demonstrated that CCR5, CXCR4, and CD4 form homogeneous microclusters on cell surface microvilli in primary macrophages and T cells (6). The requirement of cholesterol for chemokine receptor functions and HIV entry (7–9) led to the hypothesis that cholesterol- and sphingolipid-enriched raft membrane domains represent privileged sites in which receptors localize. Nonetheless, the observations that co-receptors barely associate with rafts (8, 10, 11) and that CD4 mutants localizing to non-raft domains are fully competent for HIV entry (8, 12) challenged this view.

Clustering within domains is also likely to favor interaction between receptors, which is consistent with the proposed existence and functioning of CCR5 and CXCR4 as oligomers (13–16), a current view that also prevails for other classes of G-protein-coupled receptors (17, 18). In early co-immunoprecipitation studies performed in primary T cells and macrophages, Xiao et al. (19) also proposed that CD4 and CCR5 were sufficiently close enough to oligomerize and interact constitutively. Such interactions are likely to favor the association rate of gp120 with CCR5, recently proposed as being strictly dependent upon a close vicinity of CD4 and CCR5 in living cells (20). Furthermore, the observation that CD4 poorly co-immunoprecipitated with CXCR4 (19, 21, 22) supports the attractive hypothesis that preferential interactions between CCR5 and CD4 may be relevant to the predominance of R5-tropic HIV isolates in the course of viral infection. Nevertheless, this possibility remains debated, as it was recently inferred using co-precipitation, high-resolution deconvolution microscopy and resonance energy transfer techniques, that CD4 and CCR5 do not exist in a stable complex at the cell membrane (23, 24). Although the reasons for these differences are not yet clear, it is noteworthy that in primary cells, the amount of CD4 that co-immunoprecipitated with CCR5 correlated with the efficiency of fusion with cells expressing R5-tropic HIV Env (19). This
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suggests that part of CD4 and co-receptors associate at the plasma membrane of target cells, which may be of prime importance in the course of viral entry.

To gain further insight into this issue, we set up a non-invasive approach to investigate interactions between CD4 and CCR5 selectively at the plasma membrane of living cells. We used a confocal microspectrofluorimeter to detect fluorescence resonance energy transfer (FRET) at a single cell level between enhanced blue and green fluorescent proteins (eBFP and eGFP) fused to CD4 and CCR5 receptors. We developed an effective method for fluorescence spectrum analysis that reveals constitutive associations between the two tagged receptors at the surface of cells. We found that binding of R5-tropic HIV gp120 stabilized associations between receptors, which makes it likely that ternary complexes form between Env, CD4, and co-receptors before the virus-to-cell fusion process begins, as previously proposed (25). In contrast, the CCR5 ligands CCL4 and TAK779, which inhibit virus entry, displaced the co-receptor from its association with CD4. Finally, based on experiments using CD4 mutants, we propose that these associations engage extracellular parts of the receptors, and take place in non-raft domains of the plasma membrane.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, Chemicals, and Reagents—The HEK 293T cell line was cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (Sigma) at 37 °C in 5% CO2. Stable expression of tagged receptors in these cells was achieved using a previously described lentivirus-based strategy, which permits efficient and long term transgene expression without clone selection (26). For the transient transfections performed in this study, cells were plated onto serum-coated 35-mm circular microscope coverslips and transfected 1 day later using the calcium phosphate-DNA co-precipitation method at DNA concentrations ranging from 0.25 to 2 μg. The chemokine CCL4/MIP-1β was provided by Dr. F. Baleux (Institut Pasteur, Paris). TAK779 and gp120 from the R5-tropic BaL and X4-tropic LAI HIV-1 strains were obtained from the AIDS Research and Reference Reagent Program catalog of National Institute of Health (Bethesda, MD).

CD4 and CCR5 Constructs—Briefly, fragments corresponding to the CCR5 and CD4 open reading frames lacking the initiation and stop codons were inserted upstream of the eGFP or eBFP cDNAs and downstream of an epitope tag from the bacteriophage T7 fused to the cDNA encoding for the cleavable α7 subunit of acetylcholine-nicotinic signal peptide (PS) (27) to target the chimeric receptor to the plasma membrane. The fragments, named PS-T7-CCR5-eGFP and PS-T7-CD4-eBFP, were then cloned into Prc/cMV (Invitrogen) or HIV-1-based lentiviral pTRIP vectors (a gift from Dr. P. Charneau, Institut Pasteur, Paris). CD4-derived mutants were cloned into pcDNA3. CD4Δ2 and CD4Δ3,1– expression vectors were previously described (8). Briefly, mutagenesis by PCR of the wild-type CD4 cDNA was performed to substitute Cys residues at positions 419, 422, 445, and 447 with Ala residues using overlap extension with T7, Sp6, and primers containing the mutations. The CD4 deletion mutants, CD4Δ2 and CD4Δ3,1–, were obtained using a PCR strategy. Both were deleted in the first 125 residues, corresponding to the first extracellular domain D1. The CD4Δ3,1– mutant was also deleted of the last 40 amino acids that correspond to the C-terminal intracytoplasmic domain. cDNAs were then inserted in-frame into pcDNA3 vectors (Invitrogen) downstream of the rhodopsin C9 tag (28) fused to the mouse Igc-chain signal peptide.

Flow Cytometry Analysis—Cell surface expression of receptors was determined as described previously (29) using a BD Biosciences FACS-Calibur. Staining of receptors was performed using the phycoerythrin (PE)-conjugated anti-CCR5 2D7 or anti-CD4 SK3 mAbs (BD Biosciences). Sorting of polyclonal cell populations homogeneously expressing tagged receptors was performed using a FACSTARPLUS cytometer (BD Biosciences).

Fluorescence Imaging—he HEK 293T cells (105) expressing CD4 or its derivative mutants were plated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum on polyllysine-coated glass coverslips overnight at 37 °C in 5% CO2, and then fixed in phosphate-buffered saline, 4% paraformaldehyde at room temperature for 10 min. Cells were stained for 30 min at room temperature with a fluorescein isothiocyanate-conjugated anti-CD4 mAb targeting the D4 domain (CD4v4, BD Biosciences) in phosphate-buffered saline containing 0.2% bovine serum albumin. After 3 washes with phosphate-buffered saline, 0.2% bovine serum albumin, cells were mounted in Vectashield medium containing 4’,6-diamidino-2-phenylindole (Vector Laboratories). Plated cells expressing either CCR5- or CD4-GFP were mounted in Vectashield medium. Imaging was performed on a Zeiss microscope (Oberkochen, Germany) using a Plan Apochromat ×63/1.4-oil immersion objective. Images were collected with a cooled CCD camera (Axioam MRm), using Axiovision imaging software (Zeiss). Optical sectioning was performed according to the structured illumination principle using the ApoTome system (Zeiss).

Functional Analysis of Chimeric Receptors—for [35S]GTPγS binding experiments, crude membranes from wt- or GFP-CCR5-expressing HEK 293T cells were prepared as previously reported (29). Membranes (10 μg of protein) were incubated in 96-well microplates for 15 min at 30 °C in assay buffer (20 mM Hapes, pH 7.4, containing 100 mM NaCl, 10 μg/ml saponin, 3 mM MgCl2, 1 μM GDP), in the presence or absence (basal [35S]GTPγS binding) of CCL4 at the indicated concentrations. [35S]GTPγS (Amersham Biosciences) at 0.1 nM was subsequently added to membranes, which were further incubated for 30 min at 30 °C. Incubation was stopped by centrifugation (800 × g for 10 min) at 4 °C, and removal of supernatants. Microplates were counted in a Wallac 1450 Microbeta Trilux and data were analyzed with the GraphPad Prism software. For assessment of cell surface expression of receptor variants, saturation binding experiments on intact cells using [35S]labeled gp120 from the Bx08 R5-tropic HIV-1 strain were carried out as described previously (29). HIV-1 infections of cells expressing the chimeric receptors were carried out using pseudotyped cell-free virions that were generated as follows. HEK 293T cells were transiently co-transfected with an HIV-1_pNL4-3_Luc_envelope missing deficent (Env(−)), proviral DNA carrying the luc reporter gene in place of the HIV-1 nef gene and the R5-HIV-1-BaL Env...
expressing vector. Viruses were harvested at 48 h after infection and quantitated by HIV-1 Gag P24 enzyme-linked immunosorbent assay (PerkinElmer Life Sciences). For infection, cells (2 × 10^5/well) in 24-well plates were incubated for 4 h at 37 °C with viruses at the indicated Gag P24 concentrations, washed, and cultured for 2 days. Luciferase activity in cell lysates was determined as previously described (8).

**Immunoblotting**—For immunodetection we used antibodies against CD4 (16, Novocastra) and CD28 (C-20, Santa-Cruz). Immobilized antigen-antibody complexes were detected with secondary horseradish peroxidase-conjugated anti-species IgG (Amersham Biosciences), developed by enhanced chemiluminescence (ECL Plus, Amersham Biosciences), and quantified using a LAS-1000 CCD camera (Image Gauge 3.4 software, Fuji Photo Film Co., Tokyo, Japan).

**Fluorescence Spectroscopy**—48 h after transfection, cells were washed in assay buffer (137.5 mM NaCl, 1.25 mM MgCl₂, 1.25 mM CaCl₂, 6 mM KCl, 5.6 mM glucose, 10 mM Hepes, 0.4 mM NaH₂PO₄, pH 7.4). Cells were incubated at 25 °C for 1 h in assay buffer with or without the ligands sCD4, CCL4, or gp120 at the indicated concentrations of CCL4. Results were representative of two independent experiments performed in triplicate. E, cell surface expression of wild-type CD4 (CD4wt, red line) and CD4-BFP (blue line) were determined using the PE-conjugated anti-CD4 mAb 2D7 by flow cytometry. The filled peak represents the isotype control mAb (PE-conjugated IgG2a). D, CCL4-induced [35S]GTP-S binding to crude membranes derived from HEK 293T cells stably expressing CCR5wt (solid circles) or CCR5-GFP (open circles) at similar levels. Membranes were incubated in assay buffer containing 0.1 mM [35S]GTP-S, 1 μM GDP, and 3 mM MgCl₂ at the indicated concentrations of CCL4. Results were representative of two independent experiments performed in triplicate. E, cell surface expression of wild-type CD4 (CD4wt, red line) and CD4-BFP (blue line) were determined using the PE-conjugated anti-CD4 mAb SK3 by flow cytometry. The filled peaks represent the isotype control mAb (PE-conjugated IgG1). F, HEK 293T cells (10⁵ cells) stably expressing tagged receptors with or without their wild-type counterpart were subjected to infection with R5-HIV-1-Bal (7.2 ng of Gag P24) in 24-well plates for 4 h. Luciferase activity was analyzed 48 h after infection and normalized for protein content assessed by the bicinchoninic acid protein assay reagent. The inhibitory effect of the CCR5 antagonist TAK779 at 1 μM on infection of cells co-expressing CD4-BFP and CCR5-GFP is also shown. RLU, relative light unit.

**RESULTS AND DISCUSSION**

**Functional Characterization of the Tagged Receptors**—For FRET experiments to visualize interactions between CD4 and CCR5, N-terminal-tagged receptors with the T7-tag epitope were fused in-frame at their C-tail with GFP derivatives (eBFP or eGFP, Fig. 1A). To assess whether the chimeric receptors were properly addressed to the cell surface, we stably expressed eGFP-labeled CCR5 or CD4 in HEK 293T cells using a lentiviral-based strategy, which relies on integration of the transgene into the host DNA, thereby resulting in a polyclonal cell population. Imaging of these cells revealed that tagged CCR5 (Fig. 2A) and CD4 (Fig. 2B) localize similarly to its eGFP-labeled counterpart (Fig. 2B). We then

Figure 1. Schematic representation of the receptor chimeras and mutants used in this study. A, enhanced blue (eBFP) and green (eGFP) fluorescent proteins were fused to the C-tail of CD4 and CCR5, respectively. Solid boxes represent a cleavable signal peptide derived from the nicotinic receptor α7-subunit and hatched boxes the T7 tag epitope. B, wild-type and mutant CD4 (CD4wt), CD4401 and CD4445 receptors contain a deletion of the D1 domain, with CD4445 also lacking the C-tail. The cysteine residues 419, 422, 445, and 447 were replaced with alanine in the CD4445 mutant. Solid and hatched boxes represent signal peptides and the C9 tag, respectively.

Figure 2. Cell surface expression and functional properties of fluorescent receptors. A and B, fluorescence microscopy imaging of HEK 293T cells stably expressing eGFP-tagged CCR5 (A) or CD4 (B). C, cell surface expressions of wild-type (wt) CCR5 (CCR5wt, red line) and CCR5-GFP (blue line) were determined using the PE-conjugated anti-CCR5 mAb 2D7 by flow cytometry. The filled peak represents an isotype control mAb (PE-conjugated IgG2a). D, CCL4-induced [35S]GTP-S binding to crude membranes derived from HEK 293T cells stably expressing CCR5wt (solid circles) or CCR5-GFP (open circles) at similar levels. Membranes were incubated in assay buffer containing 0.1 mM [35S]GTP-S, 1 μM GDP, and 3 mM MgCl₂ at the indicated concentrations of CCL4. Results were representative of two independent experiments performed in triplicate. E, cell surface expression of wild-type CD4 (CD4wt, red line) and CD4-BFP (blue line) were determined using the PE-conjugated anti-CD4 mAb SK3 by flow cytometry. The filled peaks represent the isotype control mAb (PE-conjugated IgG1). F, HEK 293T cells (10⁵ cells) stably expressing tagged receptors with or without their wild-type counterpart were subjected to infection with R5-HIV-1-Bal (7.2 ng of Gag P24) in 24-well plates for 4 h. Luciferase activity was analyzed 48 h after infection and normalized for protein content assessed by the bicinchoninic acid protein assay reagent. The inhibitory effect of the CCR5 antagonist TAK779 at 1 μM on infection of cells co-expressing CD4-BFP and CCR5-GFP is also shown. RLU, relative light unit.
used populations of HEK 293T cells stably expressing either the tagged receptors or their wild-type counterparts for subsequent comparative studies of their functional properties. As a common hallmark of chemokine receptor function is to mediate G-protein-mediated signaling, we first assessed the ability of CCR5-GFP to activate G-proteins in response to chemokines using a \[^{[35S]}\text{GTP}\text{S}\] binding-based assay (33). When expressed at similar levels in HEK 293T cells (Fig. 2C), the fluorescent receptor was as efficient as the wild-type receptor in activating G-proteins in response to the CC-chemokine CCL4 (Fig. 2D). This indicates that CCR5-GFP preserves its ability to interact with, and to signal to, its natural ligands. In contrast, membranes from untransfected parental cells were consistently unresponsive to CCL4 stimulation (data not shown and Ref. 29). Dose-response curves of chemokine-induced \[^{[35S]}\text{GTP}\text{S}\] binding revealed, however, that the fluorescent receptor displayed a slightly decreased potency to activate G-proteins (EC\(_{50}\) = 9.6 ± 1.9 nM), as compared with the wild-type receptor (EC\(_{50}\) = 2.3 ± 0.6 nM) (Fig. 2D). In line with this, we have previously described that expression of wild-type CCR5 in HEK 293T cells resulted in a fraction of receptors spontaneously activating G-proteins (29), a process that we show here to be somewhat reduced for CCR5-GFP (Fig. 2D). CCR5-GFP or the wild-type receptor-expressing HEK 293T cells were then transiently transfected with either CD4 or its eBFP-tagged version (Fig. 2E) and assessed for infection by R5-tropic strains. Fig. 2E shows the results from inoculations of cells with pseudotyped cell-free virions generated by trans-complementation of a luciferase reporter HIV-1 provirus (Env\(^\text{−}\)) with an R5-HIV-1 BaL Env. As compared with the wild-type receptor-expressing cells, roughly similar yields of viral replication were measured in cells with the fluorescent receptors. This indicates that N- and C-terminal tagging of CCR5 and CD4 with the T7-tag epitope and fluorescent tagging of CCR5 and CD4 with the T7-tag epitope and fluorescent acceptor eGFP is marginal when the donor eBFP is simultaneously expressed in the same cell population of HEK 293T cells stably co-expressing CD4-BFP and CCR5-GFP acquired in a typical FRET experiment. It is apparent that fluorescence spectra greatly differ from one cell to another. This likely arises from different levels of receptor expression as well as the contribution of cell autofluorescence, and renders collective analysis from cell suspensions not relevant.

Measurement of FRET between CD4-BFP and CCR5-GFP—For the subsequent FRET experiments, we selected a polyclonal population of HEK 293T cells stably co-expressing CD4-BFP and CCR5-GFP (at levels equal to 4 and 1.5 \(\times\) 10\(^{5}\) receptors/cell, respectively, as deduced from saturation binding experiments of \[^{[35S]}\text{S}\] labeled Env to CCR5-GFP-expressing cells occurred with an affinity (K\(_{d}\) = 18 nm) similar to that described for the wild-type receptor (data not shown and Ref. 34).

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We assessed whether the TCC values deduced from cells expressing CD4-BFP and CCR5-GFP resulted from FRET as a consequence of specific interactions between both chimeric receptors. It has been reported previously that a soluble form of human CD4 (sCD4) coated onto enzyme-linked immunosorbent assay plates can associate with detergent-solubilized, purified CCR5 (39) whereby suggesting binding of the extracellular part of CD4 to the chemokine receptor. Accordingly, we assessed the possibility that in our assay system recombinant sCD4, which contains the four Ig-like domains of CD4, displaces CD4-BFP from specific interactions with CCR5-GFP.

We observed that adding sCD4 at a saturating concentration (100 nM) to cells expressing both tagged receptors resulted in a shift of the blue TCC to higher values (Fig. 3C), with a parallel decrease of the overall green TCC values (Fig. 3D, where green TCC are expressed as a function of blue TCC, see below). This result indicates that the TCC values from cells expressing CD4-BFP and CCR5-GFP is not solely the consequence of independent spectral contributions of donors and acceptors. It rather implies that these TCC values reflect resonance energy transfer as a consequence of interactions between both tagged receptors, which in turn are decreased upon sCD4 addition. This conclusion was drawn from the following observations. It is apparent from Fig. 3, B and C, that, in contrast to the blue and green TCC, the red TCC values vary only slightly both from one cell to another and upon sCD4 binding to cells. Thus, according to Equation 7, representing the green TCC as a function of blue TCC, see below). This result indicates that the TCC values from cells expressing CD4-BFP and CCR5-GFP resembles those deduced from cells expressing CD4-BFP alone (dark blue curve), with the lowest values of blue TCC being the most right-shifted and the highest ones being less affected, thereby indicating that the effects of sCD4 were more efficient in cells where the blue TCC is low. As the lowering of eBFP fluorescence in these cells results mainly from a resonance energy transfer to the acceptor eGFP, this result strongly suggests that scD4 selectively modifies TCC in cells where FRET between CD4-BFP and CCR5-GFP occurred between CD4-BFP and CCR5-GFP. As an additional evidence, we confirmed that sCD4 does not affect blue TCC values deduced from cells expressing CD4-BFP alone (light blue curve). Quantifying the influence of scD4 on CD4-BFP- and CCR5-GFP-expressing cells was carried out by numbering the.

Co-expression of both tagged receptors resulted in TCC values that differ clearly from those obtained with untransfected cells or cells expressing either CCR5-GFP or CD4-BFP alone, thus indicating distinct spectral properties. Notably, blue TCC from cells expressing both receptors were broadly lower than the values from cells with CD4-BFP alone (Fig. 3, B and E).

**Constitutive Interactions between CD4-BFP and CCR5-GFP**—We assessed whether the TCC values deduced from cells expressing CD4-BFP and CCR5-GFP resulted from FRET as a consequence of specific interactions between both chimeric receptors. It has been reported previously that a soluble form of human CD4 (sCD4) coated onto enzyme-linked immunosorbent assay plates can associate with detergent-solubilized, purified CCR5 (39) whereby suggesting binding of the extracellular part of CD4 to the chemokine receptor. Accordingly, we assessed the possibility that in our assay system recombinant sCD4, which contains the four Ig-like domains of CD4, displaces CD4-BFP from specific interactions with CCR5-GFP. We observed that adding sCD4 at a saturating concentration (100 nM) to cells expressing both tagged receptors resulted in a shift of the blue TCC to higher values (Fig. 3C), with a parallel decrease of the overall green TCC values (Fig. 3D, where green TCC are expressed as a function of blue TCC, see below). This result indicates that the TCC values from cells expressing CD4-BFP and CCR5-GFP is not solely the consequence of independent spectral contributions of donors and acceptors. It rather implies that these TCC values reflect resonance energy transfer as a consequence of interactions between both tagged receptors, which in turn are decreased upon sCD4 addition. This conclusion was drawn from the following observations. It is apparent from Fig. 3, B and C, that, in contrast to the blue and green TCC, the red TCC values vary only slightly both from one cell to another and upon sCD4 binding to cells. Thus, according to Equation 7, representing the green TCC as a function of blue TCC, see below). This result indicates that the TCC values from cells expressing CD4-BFP and CCR5-GFP resembles those deduced from cells expressing CD4-BFP alone (dark blue curve), with the lowest values of blue TCC being the most right-shifted and the highest ones being less affected, thereby indicating that the effects of sCD4 were more efficient in cells where the blue TCC is low. As the lowering of eBFP fluorescence in these cells results mainly from a resonance energy transfer to the acceptor eGFP, this result strongly suggests that scD4 selectively modifies TCC in cells where FRET between CD4-BFP and CCR5-GFP occurred between CD4-BFP and CCR5-GFP. As an additional evidence, we confirmed that sCD4 does not affect blue TCC values deduced from cells expressing CD4-BFP alone (light blue curve). Quantifying the influence of scD4 on CD4-BFP- and CCR5-GFP-expressing cells was carried out by numbering the.

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FIGURE 4. Effects of ligands on FRET between tagged receptors. The effects of the CCR5 antagonist TAK779 at 10 or 100 nM (black bars), the gp120 subunit from the R5-tropic HIV-1 strain BaL at 10 or 100 nM (gray bars), or the X4-tropic HIV-1 LAI at 100 nM (hatched bars) on FRET between CD4-BFP/CCR5-GFP stably expressed in HEK 293T cells are shown. Incubations were carried out at room temperature for 1 h in isotonic buffer before acquisition of fluorescence spectra. Values are given as the mean ± S.E. of three to six independent experiments with 52 acquisitions each. Positive values upon addition of gp120 represent an increase of FRET between CD4-BFP and CCR5-GFP. The effects of sCD4 at 100 nM shown in Fig. 3 are shown for comparison.

cells with a blue TCC value being right-shifted beyond the threshold value of mean + σ deduced from the basal state (red curve). Using this approach, we found that sCD4 diminishes FRET between CD4-BFP and CCR5-GFP in a dose-dependent manner, with 39 and 47% of cells expressing the tagged receptors displaying a significantly increased value of blue TCC in the presence of 10 and 100 nM sCD4, respectively, as compared with the basal state (Fig. 3F). Our results (Fig. 3F) show that wild-type CD4 expression also results in an increase in blue TCC values. This confirms that the FRET arises from specific constitutive interactions between CD4-BFP and CCR5-GFP rather than from nonspecific encounters due to high receptor expression levels. In keeping with this, transient expression of another T cell antigen, the surface glycoprotein CD28, in these HEK 293T cells, was found to have only marginal effects on the expression levels by Western blot analysis).

Effect of Ligands on FRET between CD4-BFP and CCR5-GFP—The diminished FRET between CD4-BFP and CCR5-GFP we observed upon sCD4 addition strongly suggested that both receptors engage their extracellular parts to associate. To investigate this possibility, we assessed whether binding of the CC-chemokine CCL4/MIP-1β to CCR5 displaces the receptor from interaction with CD4. Following the FRET analysis described above, we show here that similarly to sCD4, CCL4 at a saturating concentration (2 nM) significantly increased the blue TCC value of almost 40% of cells (Fig. 4). This means that fluorescence of CD4-BFP in these cells was enhanced upon CCL4 binding to CCR5-GFP, thus suggesting that the chemokine decreases interactions between tagged receptors. Experiments were carried out at room temperature, which makes it unlikely that the lowering of FRET between CD4-BFP and CCR5-GFP arises from CCL4-induced endocytosis of tagged CCR5. One can rather speculate that the molecular determinants in the receptor that have been reported to be required for chemokine binding, i.e. the second extracellular loop and the N-terminal domain (31, 40), are also required for association with CD4. As a consequence, binding of the chemokine would hinder CD4 interaction with CCR5 by a competitive mechanism. It is interesting to note that the CXC chemokine SDF-1/CXCL12, which also binds to the extracellular regions of CXCR4 (41, 42), has recently been reported from another FRET-based analysis to have no effect on interactions between CD4 and CXCR4 (43). This strongly suggests that the two major HIV co-receptors, CCR5 and CXCR4, display distinct structural requirements for association with CD4. Alternatively, we cannot rule out the possibility that the conformational changes in CCR5 triggered by CCL4 have an influence on receptor association with CD4, and this assumption is indeed in agreement with previous works showing that soluble forms of CD4 allosterically modulate CCR5 and decrease the binding affinity of CCL4 (39, 44). As CCR5 exists in distinct conformational states at the cell surface (31), it results from the latter hypothesis that they may exhibit different abilities to interact with CD4, which could be of relevance for the HIV entry process.

In line with this assumption, TAK779, a quaternary ammonium ion that inhibits R5-tropic virus entry into cells by binding to CCR5 (see Fig. 2F), impairs CCR5-GFP association with CD4-BFP (Fig. 4). In contrast to CCR5 agonists, TAK779 interacts with residues located within the transmembrane domain of the receptor (45, 46), and is believed to prevent interaction of gp120 with CCR5 by an allosteric mechanism (2). In fact, TAK779 has a noncompetitive antagonistic effect on chemokine binding to CCR5 (47) and we have previously reported that it precludes spontaneous coupling of the receptor to heterotrimeric G-proteins (29), both of these observations highlighting that TAK779 modifies the conformation of CCR5. Our present data thus open the challenging possibility that displacement of CCR5 from interacting with CD4 as a result of co-receptor conformational changes contribute to the TAK779 antiviral properties.

Finally, we found that addition of gp120 from the R5-tropic HIV-1 strain BaL to cells expressing CD4-BFP and CCR5-GFP resulted in an increase of FRET, because 26 and 31% of cells displayed a significantly diminished blue TCC value compared with the basal state in the presence of the viral glycoprotein at 10 and 100 nM, respectively (Fig. 4). In contrast, we observed that gp120 from the X4-tropic strain LAI used at similar concentrations consistently failed to increase FRET between tagged receptors (Fig. 4), thus indicating that these effects of BaL gp120 specifically rely on its binding to CCR5-GFP. This result is in agreement with a recent FRET analysis showing that attachment of effector cells expressing R5-tropic Env to target cells stabilizes CD4-CCR5 interactions (23), and gives further support to the proposal that ternary complexes between Env, CD4, and chemokine receptor form before the fusion process commences (25). It contrasts, however, with previous co-immunoprecipitation approaches showing that associations between CD4 with CCR5 occur readily and are not further increased upon addition of gp120 (19). However, it is likely that this discrepancy results from the different experimental
approaches used to demonstrate CD4 associations with co-receptors. Indeed, immunoprecipitation studies refer to cell populations where it is not possible to determine subcellular sites at which associations occur, so that intracellular associations may mask discrete gp120-induced associations of CD4 with co-receptors at the cell surface. Our confocal measurements of FRET applied to single-cell analysis permit us to overcome this limitation by measuring signals from plasma membranes, and as such will be useful to delineate the interesting possibility that differential associations between CD4 and the co-receptors CCR5 and CXCR4 underlie the different susceptibilities of cells to entry of X4- and R5-tropic HIV isolates (19, 21, 48).

The Structural Determinants of CD4 Involved in CD4/CCR5 Interactions—To delineate regions of CD4 that govern the association with CCR5, we generated CD4 mutants with amino acid substitutions or sequence deletions (see Fig. 1B) and then assessed their ability to disrupt FRET following transient transfection in HEK 293T cells expressing CD4-BFP and CCR5-GFP (Fig. 5C). Fig. 5C presents evidence that deleting the D1 domain of CD4 (CD4ΔD1 mutant) resulted in a receptor that is impaired in its ability to compete for interactions between targeted receptors. Indeed, expression of CD4ΔD1 in cells expressing CD4-BFP and CCR5-GFP led to less than 20% of these cells that displayed a decrease of FRET, as compared with more than 30% for the wild-type receptor. Nevertheless, although impaired in this process, CD4ΔD1 was consistently able to reduce FRET to some extent, as compared with non-relevant receptors (i.e. CD28), thus suggesting that other molecular determinants are required for interaction of CD4 with CCR5. In support of this possibility, previous co-immunoprecipitation observations suggested that together with D1, the primary binding site for HIV-1 gp120, the second extracellular domain of CD4 also associates with CCR5 (19). We next investigated whether the cytoplasmic C-tail of CD4, which clusters the molecular determinants controlling localization of the receptor into raft membrane domains, might also contribute to interaction with CCR5 (8, 12, 49). The C-tail of CD4 was deleted in addition to the D1 domain and we then evaluated the ability of the resulting mutant (CD4ΔCc) to compete with CD4-BFP for association with CCR5-GFP. However, in contrast to CD4ΔD1 that localizes at the plasma membrane (as revealed by Western blot analysis and immunofluorescence, Fig. 5, A and B), albeit to a slightly lesser extent than wild-type CD4, CD4ΔCc accumulates intracellularly and no longer displaces interactions between the tagged receptors at the cell surface (Fig. 5C), thus precluding further investigations. We used the CD4P-L- mutant that lacks the cysteine residues within the C-tail of the receptor, which are required for the receptor palmitoylation and interaction with the tyrosine kinase p56lck. In previous reports, we and others demonstrated that mutation of these residues dramatically prevents the association of CD4 with lipid raft domains (8, 50). As shown in Fig. 5C, we found that CD4P-L- is as efficient as its wild-type counterpart in displacing CD4-BFP/CCR5-GFP interactions. This data, together with the fact that CCR5-GFP localizes outside lipid rafts (data not shown), as we previously reported for endogenous CCR5 in primary cells (8), support the hypothesis that CD4/CCR5 interactions take place in non-raft domains of the membrane.
**CD4/CCR5 Interactions in Living Cells**

**Concluding Remarks**—In this study, CD4/CCR5 interactions have been analyzed by FRET measurements at a single-cell level using a previously described confocal microspectrofluorimeter (30). Our approach of FRET quantification and analysis shows that specific constitutive associations occur between CD4 and CCR5 at the plasma membrane. Interactions between CD4 and co-receptors at the cell surface of target cells have been postulated to influence susceptibility to HIV entry (19, 48, 51). For example, competition between CCR5 and CXCR4 for association with the primary receptor CD4 may contribute to cell tropism, *i.e.* the susceptibility of cells to infection by either R5- or X4-tropic strains of HIV (48). In this regard, the possibility that CD4 and CCR5 interact preferentially is interesting as this could explain the predominance of R5 viruses in the early stages of infection (19, 52). Comparative studies aimed at investigating the respective abilities of CCR5 and CXCR4 to interact with CD4 at the plasma membrane of individual cells will help to clarify this issue. The requirement for close proximity between CD4 and CCR5 for firm attachment of gp120 to target cells has been recently assessed at a single molecule level in living cells (20). It is proposed that after association with CD4, gp120 needs to search for CCR5 to create a new bond of higher stability, and as the interaction between gp120 and CD4 is weak and of short duration, it is proposed that receptors need to be close enough for this bond transfer to occur (20). It has been argued that this new bond forms when gp120 is still attached to CD4 (20), which is in agreement with our results showing increased interactions between CD4 and CCR5 upon addition of gp120 and the formation of ternary complexes of gp120, CD4, and chemokine receptor as intermediates of the fusion process (25).

We also found that interactions between CD4 and CCR5 notably engage extracellular parts of receptors. CD4 or co-receptors have been described to reside at the plasma membrane in distinct conformational and oligomeric states (13, 14, 53), but to what extent these parameters also affect CD4/co-receptor associations is poorly documented and even controversial. The degree to which HIV receptors interact is also expected to depend on their concentration and distribution at the surface of target cells and their recruitment to specific membrane domains. Based on observations that HIV infection needs plasma membrane cholesterol, it has been speculated that viruses use cholesterol-rich lipid rafts for entry into cells (7, 49, 54). Nevertheless, manifold observations argue against such an hypothesis. First, the targeting of CD4 to non-raft membrane domains is not detrimental to productive virus entry (8, 12). Second, peptides derived from the N-terminal region of gp41 ectodomain promote fusion when inserted into liquid disordered non-raft membranes (55). Finally, co-receptors localize to non-raft membrane domains in immortalized as well as in primary cells (8, 10, 11) despite the fact that their activities have been shown to be modulated by membrane cholesterol (56, 57). Extending these evidences, we show here that interactions of CD4 with CCR5 probably take place outside rafts. It is likely that cholesterol outside rafts modulates the CD4 to CCR5 interactions we demonstrate in this work. Indeed, it was recently reported that cholesterol controls lateral distribution of CD4 and co-receptors at the plasma membranes of host cells, which in turn may be of importance for HIV entry (9). Current evidence shows that biological membranes are composed of a great diversity of domains (58, 59), so that the composition and features of those where CD4 and co-receptors interact and segregate for HIV entry are far from being fully characterized. Delineating this issue using, for example, tracking dynamical parameters of receptors on intact cells (60, 61) is a challenging avenue that will help in understanding HIV pathogenesis.

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**REFERENCES**

1. Berger, E. A., Murphy, P. M., and Farber, J. M. (1999) *Annu. Rev. Immunol.* **17**, 657–700
2. Doms, R. W., and Trono, D. (2000) *Genes Dev.* **14**, 2677–2688
3. Kuhmann, S. E., Platt, E. J., Kozak, S. L., and Kabat, D. (2000) *J. Virol.* **74**, 7005–7015
4. Platt, E. J., Durnin, J. P., and Kabat, D. (2005) *J. Virol.* **79**, 4347–4356
5. Platt, E. J., Wehrly, K., Kuhmann, S. E., Chesebro, B., and Kabat, D. (1998) *J. Virol.* **72**, 2855–2864
6. Singer, I. I., Scott, S., Kawka, D. W., Chin, J., Daugherty, B. L., DeMartino, J. A., DiSalvo, J., Gould, S. L., Lineberger, J. E., Malkowitz, L., Miller, M. D., Mintau, L., Siciliano, S. J., Staruch, M. J., Williams, H. R., Zweierink, H. J., and Springer, M. S. (2001) *J. Virol.* **75**, 3779–3790
7. Manes, S., del Real, G., Lalcace, R. A., Lucas, P., Gomez-Mouton, C., Sanchez-Palomino, S., Delgado, R., Alcamí, J., Mira, E., and Martinez, A. C. (2003) *EMBO Rep.* **4**, 190–196
8. Percherancier, Y., Lagane, B., Planchenault, T., Staropoli, I., Altmeyer, R., Virelizier, J. L., Arenzana-Seisdedos, F., Hoeussli, D. C., and Bacherie, F. (2003) *J. Biol. Chem.* **278**, 3133–3161
9. Viard, M., Parolini, I., Sargiacomo, M., Fecchi, K., Ramoni, C., Ablan, S., Russetti, F. W., Wang, J. M., and Blumenthal, R. (2002) *J. Virol.* **76**, 11584–11595
10. Finnegan, C. M., and Blumenthal, R. (2006) *Antiviral Res.* **69**, 116–123
11. Kozak, S. L., Heard, J. M., and Kabat, D. (2002) *J. Virol.* **76**, 1802–1815
12. Popik, W., and Alce, T. M. (2004) *J. Biol. Chem.* **279**, 704–712
13. Babcock, G. J., Farzan, M., and Sodroski, J. (2003) *J. Biol. Chem.* **278**, 3378–3385
14. Issafras, H., Angers, S., Bulenger, S., Blanpain, C., Parmentier, M., Labbe-Jullie, C., Bouvier, M., and Marullo, S. (2002) *J. Biol. Chem.* **277**, 34666–34673
15. Lemay, J., Marullo, S., Jockers, R., Alizon, M., and Brelot, A. (2005) *Nat. Immunol.* **6**, 535–536
16. Percherancier, Y., Berchiche, Y. A., Slight, I., Volkmer-Engert, R., Tama-mura, H., Fujii, N., Bouvier, M., and Heverek, N. (2005) *J. Biol. Chem.* **280**, 9895–9903
17. Stanasila, L., Perez, J. B., Vogel, H., and Coteccchia, S. (2003) *J. Biol. Chem.* **278**, 40239–40251
18. Terrillon, S., and Bouvier, M. (2004) *EMBO Rep.* **5**, 30–34
19. Xiao, X., Wu, L., Stantchev, T. S., Feng, Y. R., Ugolini, S., Chen, H., Shen, Z., Riley, J. L., Broder, C. C., Sattentau, Q. J., and Dimitrov, D. S. (1999) *Proc. Natl. Acad. Sci. U S A.* **96**, 7496–7501
20. Chang, M. I., Panorchan, P., Dobrowsky, T. M., Tseng, Y., and Wirtz, D. (2005) *J. Virol.* **79**, 14748–14755
21. Lapham, C. K., Zaitseva, M. B., Lee, S., Romanstseva, T., and Golding, H. (1999) *Nat. Med.* **5**, 303–308
22. Basmaciogullari, S., Pacheco, B., Bour, S., and Sodroski, J. (2006) *Virology* **353**, 52–67
23. Furuta, R. A., Nishikawa, M., and Fujisawa, J. (2006) *Microbes Infect.* **8**, 520–532
24. Steffens, C. M., and Hope, T. J. (2003) *J. Virol.* **77**, 4985–4991
25. Mkrichyan, S. R., Markosyan, R. M., Eadon, M. T., Melikyan, G. B., and Cohen, F. S. (2005) *J. Virol.* **79**, 11161–11169
26. Amara, A., Vidy, A., Bouilla, G., Mollier, K., Garcia-Perez, J., Alcami, J., Blanpain, C., Parmentier, M., Virelizier, J. L., Charneau, P., and Arenzana-
Seisdedos, F. (2003) J. Virol. 77, 2550–2558
27. Peng, X., Katz, M., Gerzanich, V., Anand, R., and Lindstrom, J. (1994) Mol. Pharmacol. 45, 546–554
28. MacKenzie, D., Arendt, A., Hargrave, P., McDowell, J. H., and Molday, R. S. (1984) Biochemistry 23, 6544–6549
29. Lagane, B., Ballet, S., Planchenault, T., Balabanian, K., Le Poul, E., Blanpain, C., Percherancier, Y., Staropoli, I., Vassart, G., Oppermann, M., Parmentier, M., and Bachelerie, F. (2005) Mol. Pharmacol. 67, 1966–1976
30. Mazères, S., Rodriguez, F., Tocanne, J. F., and Lopez, A. (1996) Analusis 24, 20–22
31. Lee, B., Sharron, M., Blanpain, C., Doranz, B. J., Vakili, J., Setoh, P., Berg, E., Liu, G., Guy, H. R., Durell, S. R., Parmentier, M., Chang, C. N., Price, K., Tsang, M., and Doms, R. W. (1999) J. Biol. Chem. 274, 9617–9626
32. Martin-Garcia, J., Cocklin, S., Chaiken, I. M., and Gonzalez-Scarano, F. (2005) J. Immunol. 179, 6703–6713
33. Wieland, T., and Jakobs, K. H. (1994) Methods Enzymol. 237, 3–13
34. Doranz, B. J., Baik, S. S., and Doms, R. W. (1999) J. Virol. 73, 10346–10358
35. Pollok, B. A., and Heim, R. (1999) Trends Cell Biol. 9, 57–60
36. Berney, C., and Danuser, G. (2003) Biophys. J. 84, 3992–4010
37. Baranger, R., Martinez, L., Pittion, J. L., and Pouleau, J. (1991) Org. Geochem. 17, 467–475
38. Wright, W. D. (1928) Trans. Optical Soc. 30, 141–164
39. Staudinger, R., Phogat, S. K., Xiao, X., Wang, X., Dimitrov, D. S., and Zolla-Pazner, S. (2003) J. Biol. Chem. 278, 10389–10392
40. Blanpain, C., Doranz, B. J., Vakili, J., Rucker, J., Govaerts, C., Baik, S. S., Lorthioir, O., Migeotte, I., Libert, F., Baleux, F., Vassart, G., Doms, R. W., and Parmentier, M. (1999) J. Biol. Chem. 274, 34719–34727
41. Brelot, A., Heveker, N., Montes, M., and Alizon, M. (2000) J. Biol. Chem. 275, 23736–23744
42. Crump, M. P., Gong, J. H., Loetscher, P., Rajarathnam, K., Amara, A., Arenzana-Seisdedos, F., Virelizier, J. L., Baggiolini, M., Sykes, B. D., and Clark-Lewis, I. (1997) EMBO J. 16, 6996–7007
43. Toth, P. T., Ren, D., and Miller, R. J. (2004) J. Pharmacol. Exp. Ther. 310, 8–17
44. Wang, X., and Staudinger, R. (2003) Biochem. Biophys. Res. Commun. 307, 1066–1069
45. Dragic, T., Trkola, A., Thompson, D. A., Cormier, E. G., Kajumo, F. A., Maxwell, E., Lin, S. W., Ying, W., Smith, S. O., Sakmar, T. P., and Moore, J. P. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5639–5644
46. Paterlini, M. G. (2002) Biophys. J. 83, 3012–3031
47. Watson, C., Jenkinson, S., Kazmierski, W., and Kenakin, T. (2005) Mol. Pharmacol. 67, 1268–1282
48. Lee, S., Lapham, C. K., Chen, H., King, L., Manischewitz, J., Romantseva, T., Mostowski, H., Stantchev, T. S., Broder, C. C., and Golding, H. (2000) J. Virol. 74, 5016–5023
49. Del Real, G., Jimenez-Baranda, S., Lacalle, R. A., Mira, E., Lucas, P., Gomez-Mouton, C., Carrera, A. C., Martinez, A. C., and Manes, S. (2002) J. Exp. Med. 196, 293–301
50. Fragoso, R., Ren, D., Zhang, X., Su, M. W., Burakoff, S. J., and Jin, Y. J. (2003) J. Immunol. 170, 913–921
51. Zaitseva, M., Romantseva, T., Manischewitz, J., Wang, J., Goucher, D., and Golding, H. (2005) J. Leukocyte Biol. 78, 1306–1317
52. Regoes, R. R., and Bonhoeffer, S. (2005) Trends Microbiol. 13, 269–277
53. Matthias, L. J., Yam, P. T., Jiang, X. M., Vandegraaff, N., Li, P., Pembourgos, P., Donoghue, N., and Hogg, P. J. (2002) Nat. Immunol. 3, 727–732
54. Freed, E. O. (2004) Trends Microbiol. 12, 170–177
55. Shnaper, S., Sackett, K., Gallo, S. A., Blumenthal, R., and Shai, Y. (2004) J. Biol. Chem. 279, 18326–18343
56. Nguyen, D. H., and Taub, D. (2002) Blood 99, 4298–4306
57. Nguyen, D. H., and Taub, D. (2002) J. Immunol. 168, 4121–4126
58. Madore, N., Smith, K. L., Graham, C. H., Jen, A., Brady, K., Hall, S., and Morris, R. (1999) EMBO J. 18, 6917–6926
59. Schuck, S., Honsho, M., Ekroos, K., Shevchenko, A., and Simons, K. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5795–5800
60. Ceazanne, L., Lecat, S., Lagane, B., Millot, C., Vollmer, J. Y., Matthes, H., Galzi, J. L., and Lopez, A. (2004) J. Biol. Chem. 279, 45057–45067
61. Daumas, F., Destainville, N., Millot, C., Lopez, A., Dean, D., and Salome, L. (2003) Biophys. J. 84, 356–366