

## Evidence for Negative Binding Cooperativity within CCR5-CCR2b Heterodimers

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### ABSTRACT

It is well established that most G protein-coupled receptors are able to form homo- and heterodimers, although the functional consequences of this process often remain unclear. CCR5 is a chemokine receptor that plays an important role in inflammatory diseases and acts as a major coreceptor for human immunodeficiency viruses. CCR5 was previously shown to homodimerize and heterodimerize with CCR2b, a closely related receptor. In the present study, we have analyzed the functional consequences of this dimerization process, in terms of ligand binding, stimulation of intracellular cascades, and internalization. Bioluminescence resonance energy transfer and coimmunoprecipitation assays demonstrated that CCR5 and CCR2b heterodimerize with the same efficiency as they homodimerize. In contrast to what has been reported previously, no cooperative signaling was observed after costimulation of the two receptors by their re-

spective ligands. However, we observed that CCR5-specific ligands that are unable to compete for monocyte chemoattractant protein (MCP-1) binding on cells expressing CCR2b alone efficiently prevented MCP-1 binding when CCR5 and CCR2b were coexpressed. The extent of this cross-competition was correlated with the amount of CCR5 expressed in cells, as determined by fluorescence-activated cell sorting analysis. Similar observations were made for the CCR2b-selective ligand MCP-1 that competed efficiently for macrophage inflammatory protein-1 $\beta$  binding on cells expressing both receptors. Internalization assays did not allow us to demonstrate cointernalization of the receptors in response to agonist stimulation. Together, our observations suggest that CCR5 and CCR2b form homo- and heterodimers with similar efficiencies and that a receptor dimer can only bind a single chemokine.

G protein-coupled receptors (GPCRs) were in the past considered as acting as monomers. However, dimerization has recently been described for a number of GPCRs, and it is presently well accepted that most, if not all GPCRs, are able

to form homodimers. Heterodimers have been described as well for a growing number of receptors. In a few situations, dimerization was shown to be essential for receptor trafficking and functional response (Kaupmann et al., 1998; White et al., 1998) or to generate a binding site with a distinct pharmacology, compared with the monomeric (or homodimeric) receptors (Kaupmann et al., 1998). In most other instances, however, the significance of the heterodimerization process observed in artificial conditions of heterologous overexpression has not been established, and the functional consequences of this process remain obscure.

CCR5 is a member of the chemokine receptor family that binds MIP-1 $\alpha$  (CCL3), MIP-1 $\beta$  (CCL4), RANTES (CCL5), MCP-2 (CCL8), and a truncated form of hemofiltrate CC chemokine-1 (CCL14) with high affinity (Blanpain et al., 1999b). CCR5 shares 75% identity with another chemokine

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**ABBREVIATIONS:** GPCR, G protein-coupled receptor; CCR, chemokine receptor; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; RANTES, related on activation normal T cell expressed and secreted; HIV, human immunodeficiency virus; BRET, bioluminescence resonance energy transfer; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; EYFP, enhanced yellow fluorescent protein; HEK, human embryonic kidney; FACS, fluorescence-activated cell sorting; mAb, monoclonal antibody; PE, phycoerythrin; Rluc, *Renilla reniformis* luciferase.

receptor, CCR2b. Most of the amino acid differences are located within the extracellular domains and in the C-terminal intracellular tail. Within their predicted transmembrane segments, CCR5 and CCR2b share 91% identity. The CCR5 and CCR2b genes are located as a head-to-tail array in the CC-chemokine receptor cluster, in the p21 region of the human genome, and result presumably from a relatively recent duplication (Samson et al., 1996a). Yet the pharmacology of the two receptors is clearly different, because CCR2b does not bind MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES, but binds MCP-1 (CCL2) with high affinity. MCP-2 is shared by the two receptors. The ligand binding specificity of these two receptors has been mapped to the extracellular domains, particularly the second extracellular loop, in agreement with the sequence variability found in these regions. In addition to its role as a chemokine receptor involved in the recruitment of leukocytes in a number of pathological situations (e.g., rheumatoid arthritis, graft rejection, and neurodegenerative diseases), CCR5 also constitutes the major coreceptor for macrophage-tropic strains of human immunodeficiency virus (HIV), which allows, together with CD4, binding of the viral particles to the cell surface through its envelope protein gp120, triggering the membrane fusion process (Berger et al., 1999; Gerard and Rollins, 2001). The presence in human populations of nonfunctional CCR5 genes, particularly the  $\Delta 32$  allele, which encodes a truncated variant retained in the secretory pathway, and providing to its carriers an almost complete resistance to HIV, have highlighted the essential and nonredundant role of this receptor in HIV entry (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996b; Michael et al., 1997; Smith et al., 1997). CCR2b and MCP-1 were shown to play a significant role in the development of atherosclerotic lesions (Boring et al., 1998). As for other chemokine receptors, CCR5 and CCR2b are coupled to the G $_i$  class of heterotrimeric G proteins, and they inhibit adenylyl cyclase, promote intracellular calcium mobilization, stimulate the mitogen-activated protein kinase pathways, and promote actin cytoskeleton remodeling and chemotaxis.

As for other receptors belonging to the same family, CCR5 and CCR2b were shown to homo-oligomerize (Rodriguez-Frade et al., 1999; Vila-Coro et al., 2000; Issafras et al., 2002), using a variety of techniques, including immunoprecipitation and fluorescence or bioluminescence resonance energy transfer (BRET) techniques. CCR5 and CCR2b were also reported to form heterodimers (Mellado et al., 2001; Hernanz-Falcon et al., 2004). However, there are conflicting reports regarding the influence of receptor activation on the dimerization process and the functional consequences of receptor dimerization. Some studies have reported agonist-induced dimerization (Rodriguez-Frade et al., 1999; Mellado et al., 2001), whereas no effects of ligands were observed in others (Benkirane et al., 1997; Issafras et al., 2002). A study suggested that CCR5-CCR2b heterodimerization increased the sensitivity and dynamic range of the chemokine response (Mellado et al., 2001). No modification of the binding properties of CCR5 or CCR2b, as a consequence of the dimerization process, has however been described so far, nor for other chemokine receptors.

Both CCR2b and CCR5 are expressed on memory T lymphocytes and the monocyte-macrophage lineage (Frade et al., 1997; Rabin et al., 1999; Murphy et al., 2000). If heterodimerization between these receptors has functional consequences,

either on the pharmacological binding profile, the signaling efficacy, or other aspects of receptor function, such as internalization or recycling, it might have important implications in the physiology of leukocyte recruitment, the development of a variety of human diseases, and the therapeutic approaches targeting these receptors. We have therefore investigated the homo- and heterodimerization properties of CCR5 and CCR2b, as well as its functional consequences, to clarify the conflicting reports in the recent literature.

## Materials and Methods

**Antibodies.** The anti-CCR5 monoclonal antibodies 2D7 and 2D7-PE were obtained from Invitrogen (Carlsbad, CA), and MC-5 was kindly provided by Mathias Mack (Medizinische Poliklinik, University of Munich, Munich, Germany). The CCR2 antibodies Doc-2, Doc-3, and Doc-4 were provided by Mathias Mack; 1D9 was a gift from Millennium Pharmaceuticals (Cambridge, MA); SC-20 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and N20-PE was from R&D Systems (Minneapolis, MN).

**Cell Lines and Leukocyte Populations.** CHO-K1 cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum (Invitrogen), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin (Invitrogen), and 250  $\mu$ g/ml Zeocin (Invitrogen). The CCR2b coding sequence was cloned between the BamHI and XbaI sites of the bicistronic expression vector pEFIB3, as described previously (Samson et al., 1996a, 1997). The pEFIB3-CCR2b construct was transfected by lipofection into a CHO-K1 cell line expressing apoaequorin and G $_{\alpha 16}$ , or the same cell line expressing the wild-type CCR5 receptor (Blanpain et al., 1999a). Cells expressing CCR2 were selected by 10  $\mu$ g/ml blasticidin (Invitrogen) for 14 days and further maintained in the presence of 5  $\mu$ g/ml blasticidin. Cells expressing CCR5 were maintained in the presence of 400  $\mu$ g/ml G418 (Invitrogen). The population of CHO-K1 cells coexpressing CCR5 and CCR2b were cloned by limiting dilution, and the resulting clones were characterized by flow cytometry analysis and  $^{125}$ I-MIP-1 $\beta$  and  $^{125}$ I-MCP-1 saturation binding assays. Clones expressing different levels of receptors were selected on this basis. Human peripheral blood lymphocytes were isolated from buffy coats of healthy blood donors by centrifugation on Ficoll. CD4 $^{+}$  T lymphocytes were then isolated by negative selection by using a magnetic bead cell sorting kit (130-091-155; Milleniy Biotec, Sunnyvale, CA). After this procedure, CD4 $^{+}$  blasts were generated by incubating the lymphocytes with a plate-coated anti-CD3 antibody (1:1000) and a soluble anti-CD25 (1:1000) antibody for 3 days. Cells were maintained in a medium supplemented with recombinant human IL-2 (2 ng/ml; R&D Systems) for an additional 7 days. Throughout the culture period, the cell density was kept around  $2 \times 10^6$  cells/ml by appropriate dilution.

**Immunoprecipitation Assays.** Cells expressing the receptor(s) were washed with ice-cold PBS, harvested and lysed in a solubilization buffer containing 1% Cymal-5 as described previously (Mirzabekov et al., 1999). G-Sepharose beads were preincubated for 4 h at 4°C with the anti-CCR5 or anti-CCR2b antibodies, and the cell lysates were incubated overnight at 4°C with the beads. The beads were then washed three times in the solubilization medium, pelleted, resuspended in an equal volume of SDS-polyacrylamide gel electrophoresis sample buffer, and incubated for 1 h at 55°C under slow agitation, before being loaded on 10% SDS-polyacrylamide gels. Proteins were electrotransferred to nitrocellulose membranes, and free binding sites were blocked by incubation in Tris-HCl-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk. CCR5 and CCR2b were detected by using MC5 (1/1000), SC20 (1/200), or 1D9 (1/1000) monoclonals, followed by horseradish peroxidase-conjugated goat anti-mouse IgG (MC5 and 1D9) or donkey anti-goat IgG (SC-20) antibodies, and visualized by chemiluminescence (Lumilight+ Western blotting kit; Roche Diagnostics, Mannheim, Germany).

**Binding Assays.** Cells expressing receptors were grown near to confluence, collected from plates in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS, centrifuged for 5 min at 1500g, and washed with PBS. Cells resuspended in buffer A (15 mM Tris-HCl, pH 7.5, 2 mM  $\text{MgCl}_2$ , 0.3 mM EDTA, and 1 mM EGTA) and homogenized in a glass homogenizer. The homogenates were first centrifuged for 5 min at 500g, and the resulting supernatants at 40,000g for 30 min at 4°C. The cell membrane pellet was washed in buffer A and resuspended in buffer B (75 mM Tris-HCl, pH 7.5, 12.5 mM  $\text{MgCl}_2$ , 0.3 mM EDTA, 1 mM EGTA, and 250 mM sucrose) at a protein concentration of approximately 1 mg/ml. Protein content was assayed using the bicinchoninic acid reagent (Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard. Crude membrane fractions were stored at  $-80^\circ\text{C}$  before use. Saturation binding experiments were carried out in Minisorb tubes (Nunc GmbH & Co. KG, Wiesbaden, Germany) in 0.1-ml final volume of assay buffer (50 mM HEPES, pH 7.4, 1 mM  $\text{CaCl}_2$ , 5 mM  $\text{MgCl}_2$ , and 0.5% bovine serum albumin), containing 2  $\mu\text{g}$  of membrane proteins and increasing concentrations of  $^{125}\text{I}$ -MIP-1 $\beta$  or  $^{125}\text{I}$ -MCP-1 (specific activity, 2200 Ci/mmol; Amersham Biosciences Inc., Piscataway, NJ). Total binding was measured in the absence of competitor, and nonspecific binding was measured with a 100-fold excess of unlabeled ligand. For competition binding experiments, the assay buffer was supplemented with 0.2 nM  $^{125}\text{I}$ -MIP-1 $\beta$  or 0.1 nM  $^{125}\text{I}$ -MCP-1 as tracers and with variable concentrations of MIP-1 $\beta$ , MIP-1 $\alpha$ , RANTES, MCP-1, MCP-2, or the 2D7 or Doc-4 monoclonal antibodies as unlabeled competitors. Samples were incubated for 90 min at  $27^\circ\text{C}$ , and then bound tracer was separated by filtration through GF/B filters presoaked 0.5% polyethylenimine. Filters were counted in a  $\beta$ -scintillation counter. Binding parameters were determined with the Prism software (GraphPad Software, Inc., San Diego, CA) using nonlinear regression applied to a single site, or two sites, binding model. The software compared the sum of squares and the degrees of freedom of each regression by using the F-test and selected the most appropriate equation.

**Intracellular Calcium Mobilization Assay.** The functional response to chemokines was analyzed with an aequorin-based assay as described previously (Blanpain et al., 1999a). In brief, cells were harvested from plates with  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free DMEM supplemented with 5 mM EDTA and centrifuged for 2 min at 1000g. The pellet was resuspended in DMEM at a density of  $5 \times 10^6$  cells/ml and incubated for 4 h in the dark in the presence of 5  $\mu\text{M}$  coelenterazine H (Promega, Madison, WI). Cells were then diluted 5-fold before use. Variable concentrations of chemokines in a volume of 50  $\mu\text{l}$  of DMEM were added to 50  $\mu\text{l}$  of cell suspension (25,000 cells) per well. Luminescence was measured for 30 s in an EG&G Berthold luminometer (PerkinElmer Life and Analytical Sciences, Boston, MA). Half-maximal effective concentrations ( $\text{EC}_{50}$ ) were determined with the GraphPad Prism software using nonlinear regression applied to a sigmoidal dose-response model. The reported values are the mean  $\pm$  S.E.M. of at least three independent experiments.

**BRET Assays.** The cDNAs encoding EYFP and a humanized form of *Renilla reniformis* luciferase were fused in frame to the 3' end of CCR5 and CCR2b cDNAs in the pcDNA3.1 vector, as described previously (Issafras et al., 2002). A BRET protocol adapted to cell monolayers was developed (U. Andrieu, G. Vassart, S. Costagliola, manuscript in preparation). In brief, human embryonic kidney (HEK-293) cells, maintained in DMEM supplemented with 10% fetal bovine serum, were seeded at a density of  $3 \times 10^6$  cells per 100-mm plates. The next day, the cells were transfected by the calcium phosphate precipitation method, using a constant amount of plasmid DNA but various ratios of plasmids encoding the fusion protein partners (Jordan et al., 1996). The different combinations tested were CCR5-hRluc with CCR5-EYFP and CCR2b-hRluc with CCR2b-EYFP for homodimerization studies, and CCR5-hRluc with CCR2b-EYFP and CCR2b-hRluc with CCR5-EYFP for heterodimerization studies. The empty vector pcDNA3.1 was used as carrier when necessary. A control corresponding to mock-transfected cells was included to subtract the raw basal luminescence from the data.

Twenty-four hours after transfection, the cells were collected, resuspended in the same medium without phenol red at a density of 300,000 cells/ml, and distributed in 96-well optical bottom plates (30,000 cells/well; Nunc GmbH & Co. KG).

Forty-eight hours after transfection, the medium was replaced by BRET buffer (PBS containing 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , and 1% glucose). The expression of the EYFP fusion proteins was estimated by measuring the fluorescence of the cells at 535 nm after excitation at 485 nm, using a Mithras LB 940 multilabel reader (Berthold Technologies, Bad Wildbad, Germany). Fluorescence was recorded as fold over background (mock-transfected cells). The cells were incubated for 15 min with 2.5  $\mu\text{M}$  coelenterazine H (Promega), and luminescence of luciferase was recorded at 1, 5, 10, and 15 min. In parallel, BRET was measured as the fluorescence of the cells at 535 nm at the same time points. The BRET ratio is defined as [(emission at 510–590)/(emission at 440–500)] – Cf, where Cf corresponds to (emission at 510–590)/(emission at 440–500) for the *-hRluc* construct expressed alone in the same experiment.

## Results

**Characterization of Cell Lines Expressing CCR5 and CCR2b.** To characterize CCR5/CCR2b dimerization, CHO-K1 cell lines stably coexpressing CCR5 and/or CCR2b were isolated and analyzed for the level of expression of the two receptors at the cell surface by FACS, using receptor-specific monoclonal antibodies, and by saturation binding assays using, respectively,  $^{125}\text{I}$ -MIP-1 $\beta$  and  $^{125}\text{I}$ -MCP-1 as tracers (data not shown). Four cell lines were selected for the different studies performed in the present work. A parental CCR5-expressing CHO-K1 cell line (C5) was previously characterized as expressing about 2.7 pmol/mg membrane proteins in binding assays (Blanpain et al., 1999a). This cell line was used as the recipient for CCR2b coexpression, and two clones (C25-12 and C25-15) expressing both receptors were selected on the basis of FACS and binding assays. FACS analysis did not discriminate between the parental line and the two daughter lines in terms of CCR5 expression. In a  $^{125}\text{I}$ -MIP-1 $\beta$  saturation binding assay, the  $B_{\text{max}}$  value was estimated to 2.9 pmol/mg protein for clone C25-12 and 3.4 pmol/mg protein for clone C25-15. CCR2b expression was determined by  $^{125}\text{I}$ -MCP-1 saturation binding assay to 0.62 pmol/mg proteins for clone C25-12 and 2.4 pmol/mg proteins for clone C25-15. As a control, a CCR2b-expressing CHO-K1 cell line (C2) established independently was characterized by a  $B_{\text{max}}$  value of 0.51 pmol/mg protein, similar to that of clone C25-12. FACS analysis, using anti-CCR2b mAbs, confirmed the relative expression level of this receptor in the three lines. The estimated  $K_D$  value of CCR5 and CCR2b for their respective ligands in binding assays (data not shown) was consistent with values described previously. The FACS analysis demonstrated that the clones were homogeneous in terms of receptor expression, and regular testing confirmed stable expression over time.

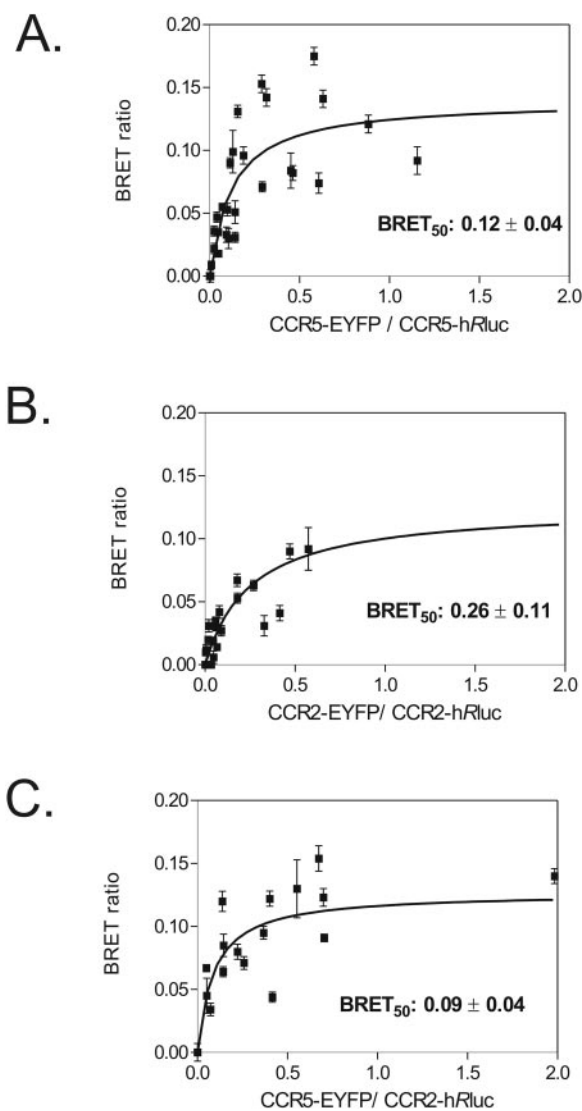
**Coimmunoprecipitation of CCR5 and CCR2b and BRET Assays.** We next investigated whether CCR5 and CCR2b associate with each other by conducting coimmunoprecipitation experiments with specific mAbs on whole-cell lysates. CCR2b immunoprecipitation by the Doc-2 mAb, followed by CCR2b immunodetection by the SC-20 mAb, confirmed binding and FACS data, by showing that clone C25-15 expresses more CCR2b than clone C25-12 (not shown). Immunodetection of CCR5 after CCR2b immunoprecipitation indicated that CCR5 was successfully coprecipitated with



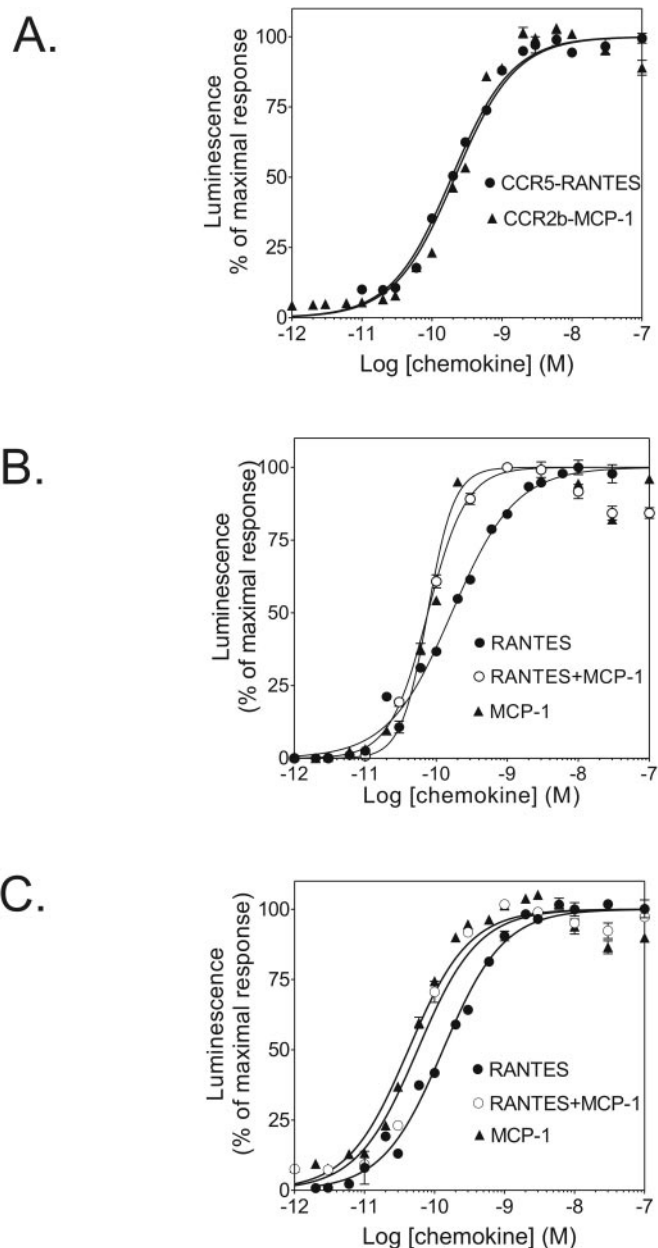
CCR2b, with a single immunoreactive band detected at 45 kDa. Coimmunoprecipitation of CCR5 was also observed when the Doc-2 monoclonal was added before lysis of the clone C25-12 cells (data not shown), demonstrating the association of CCR5 and CCR2b at the cell surface.

To study the homo and heterodimerization of CCR5 and CCR2b in living cells, we applied the BRET technique. CCR5 and CCR2b were fused at their C terminus with either luciferase (hRluc) or EYFP, and the constructs were cotransfected to assess homo- or heterodimerization. As described previously (Issafras et al., 2002), energy transfer was observed between CCR5-hRluc and CCR5-EYFP, in a ligand-independent manner. A similar energy transfer was observed be-

tween CCR2b-hRluc and CCR2b-EYFP, as well as between CCR2b-hRluc and CCR5-EYFP (Fig. 1, B and C). In these cases as well, addition of ligands did not affect the energy transfer either positively or negatively (data not shown). The BRET<sub>50</sub> value, which is considered to constitute a measure of the "affinity" between the two studied proteins, was found to



**Fig. 1.** BRET assays. HEK-293 cells were transfected with a constant amount of the hRluc fusion and increasing amounts of the EYFP fusion, and homo- and heterodimerization of CCR5 and CCR2b were investigated by measuring the energy transfer between the two partners. A, CCR5-hRluc and CCR5-EYFP constructs. B, CCR2b-hRluc and CCR2b-EYFP constructs. C, CCR2b-hRluc and CCR5-EYFP constructs. The abscissa represents the measured expression of CCR5-EYFP or CCR2b-EYFP divided by the measured expression of CCR5-hRluc or CCR2b hRluc. The ordinate is a measure of the fraction of the hRluc fusion partner involved in an interaction with the EYFP fusion partner, defined as the BRET ratio. The BRET<sub>50</sub> values were calculated by nonlinear regression using a single-site saturation binding model.



**Fig. 2.** Aequorin-based functional assays. Functional responses of receptors in cells coexpressing CCR5/CCR2b (clones C25-12 and C25-15) or each receptor alone were measured using the aequorin-based functional assay. Clones C25-12 (B) and C25-15 (C) were incubated with a range of concentrations of RANTES, MCP-1, or a mixture of both chemokines, and luminescence was recorded for 30 s. CHO-K1 cells expressing CCR5 or CCR2b alone were incubated with their respective ligands and used as positive controls (A). The results were normalized for the basal luminescence of the cells in absence of agonist (0%) and the maximal response obtained for each receptor with the reference chemokine (100%). The functional parameters ( $EC_{50}$  and  $E_{max}$ ) were determined by nonlinear regression using the GraphPad Prism software and a sigmoidal dose-response model. The displayed data are representative of three independent experiments. All data points were performed in duplicates (error bars indicate S.E.M.).

be similar for CCR5-CCR5 ( $0.12 \pm 0.04$ ), CCR2b-CCR2b ( $0.26 \pm 0.11$ ), and CCR5-CCR2b ( $0.09 \pm 0.04$ ) interactions. As controls, the thyrotropin receptor (TSHR) and GABA<sub>B2</sub> receptor fused to EYFP were used in combination with CCR5-hRluc and CCR2b-hRluc. A much lower energy transfer was observed in these situations, with a slower saturation for which no reliable BRET<sub>50</sub> could be determined (data not shown).

**Aequorin-Based Functional Assay.** CCR5 and CCR2b were described previously to act synergistically when coexpressed in the same cells. Indeed, it was reported that costimulation by ligands of both receptors (RANTES and MCP-1) resulted in the activation of the cells (calcium mobilization) for chemokine concentrations 10- to 100-fold lower than those necessary to activate cells expressing a single receptor (Mellado et al., 2001). We therefore repeated these experiments in our cell lines expressing one or the two receptors, by establishing detailed concentration-action curves using the aequorin-based calcium mobilization assay. MCP-1 and RANTES alone induced calcium mobilization with EC<sub>50</sub> consistent with earlier reports (Blanpain et al., 1999b). The values were not affected by the coexpression of the two receptors, because similar values were obtained for the lines expressing a single receptor or both (Fig. 2, A and B; Table 1). Furthermore, the simultaneous stimulation by MCP-1 and RANTES did not modify significantly these values (Fig. 2, A–C). Finally, we also showed that the sensitivity of Ca<sup>2+</sup> signaling to pertussis toxin was similar whether the receptors were expressed individually, coexpressed but stimulated individually, or costimulated (data not shown). Therefore, our data do not support the cooperativity hypothesis among CCR5/CCR2b heterodimers, but rather an independent behavior of both receptors in terms of functional stimulation of intracellular cascades.

**Binding Assays.** We next examined the ability of the ligands of each receptor to compete for <sup>125</sup>I-MIP-1β and <sup>125</sup>I-MCP-1 binding to membranes of cells expressing CCR5 and/or CCR2b. As expected, MIP-1α (data not shown), MIP-1β, RANTES (data not shown), and MCP-2 inhibited the binding of <sup>125</sup>I-MIP-1β on membranes containing CCR5 alone (Fig. 3A), whereas MCP-1 competed with a low affinity (IC<sub>50</sub> = 212 nM), as described previously (Blanpain et al., 1999b). Likewise, MCP-1 and MCP-2 competed for the binding of <sup>125</sup>I-MCP-1 on membranes containing CCR2b alone (Fig. 4A), whereas MIP-1α (data not shown), MIP-1β (Fig. 4A), and RANTES (data not shown) were unable to compete, in agreement with the previously described pharmacology of the receptor (Charo et al., 1994). Unexpectedly, however, modifications in the competition patterns were observed on membranes prepared from cell lines coexpressing CCR5 and CCR2b. In the <sup>125</sup>I-MIP-1β binding assay, MIP-1α, MIP-1β, RANTES, and MCP-2 behaved similarly, but the apparent affinity of MCP-1 was considerably increased (Fig. 3, B and

C), and the profile of the curve for the C25-12 clone (Fig. 3B) suggested two binding sites with high and low affinities (IC<sub>50</sub> value of 0.5 and 150 nM, respectively). The parameters of these high- and low-affinity sites correspond to the affinities of MCP-1 for, respectively, CCR2b and CCR5. However, the existence of two sites was barely detectable for the C25-15 clone that expresses a higher level of CCR2b. On the other hand, in the <sup>125</sup>I-MCP-1 binding assay, competition by MCP-1 and MCP-2 was unaffected by CCR5 coexpression (Fig. 4, B and C), whereas a significant competition by MIP-1α (not shown), MIP-1β (Fig. 4, B and C), and RANTES (not shown) was now observed. The calculated IC<sub>50</sub> value obtained for clones c12 and c15 were similar to those obtained for these chemokines in the <sup>125</sup>I-MIP-1β binding assay. However, the competition was incomplete, because it represented only 55% of the specific binding for clone c15 that expresses high levels of CCR2b, and 70% for clone c12 that expresses lower levels of this receptor. These results suggest therefore that ligands from one receptor are able to compete for the binding of the tracer on the other, in a manner that seems to correlate with the expected proportion of heterodimers. We showed that a truncated variant of RANTES, [10-68]-RANTES, that acts as a partial agonist on CCR5, and the N-terminally extended variant Met-RANTES, a CCR5 antagonist, were able to compete for MCP-1 binding as efficiently as RANTES itself on cells coexpressing CCR5 and CCR2b (Fig. 5; data not shown).

To investigate whether steric hindrance at the surface of the receptor might explain this observation, we tested in both binding assays monoclonal antibodies that bind to CCR5 or CCR2b and that were previously shown to prevent chemokine binding without activating the receptors (Lee et al., 1999; J. Y. Springael, K. Wagner, L. El-Asmar, M. Godard, M. Parmentier, and M. Mack, manuscript in preparation). The anti-CCR5 mAb 2D7, which is directed against the second extracellular loop of CCR5, prevented <sup>125</sup>I-MIP-1β binding on membranes containing CCR5 or the two receptors, but it did not affect binding of <sup>125</sup>I-MCP-1 (Fig. 6). Likewise, the anti-CCR2b mAb Doc-4, which recognizes a multidomain epitope of CCR2b (J. Y. Springael, K. Wagner, L. El-Asmar, M. Godard, M. Parmentier, and M. Mack, manuscript in preparation), prevented <sup>125</sup>I-MCP-1 binding, but it did not prevent <sup>125</sup>I-MIP-1β binding (Fig. 6).

Finally, we tested whether such cross-competition could be demonstrated for cells that endogenously express CCR2b and CCR5 at physiological levels. We isolated human CD4<sup>+</sup> T lymphocytes and activated them with an anti-CD3 antibody and IL-2, a procedure known to increase their CCR5 and CCR2b content. The relative expression of both receptors was monitored by FACS on each blast preparation. Using <sup>125</sup>I-MCP-1 as a tracer, specific binding on CCR2b was obtained, and the anti-CCR2 mAb DOC3 inhibited this <sup>125</sup>I-MCP-1 binding, demonstrating further its specificity (Fig. 7). In the

TABLE 1

Functional parameters of cell lines

The EC<sub>50</sub> values (nanomolar) of the functional response of the cell lines was determined by nonlinear regression using the GraphPad Prism software and a sigmoidal dose-response model. The displayed data are representative of at least three independent experiments (mean  $\pm$  S.E.M.).

EC <sub>50</sub>	C5	C2	C25-12	C25-15
RANTES	0.19 $\pm$ 0.04		0.17 $\pm$ 0.02	0.13 $\pm$ 0.03
MCP-1		0.21 $\pm$ 0.06	0.07 $\pm$ 0.01	0.040 $\pm$ 0.006
RANTES + MCP-1			0.055 $\pm$ 0.005	0.065 $\pm$ 0.007

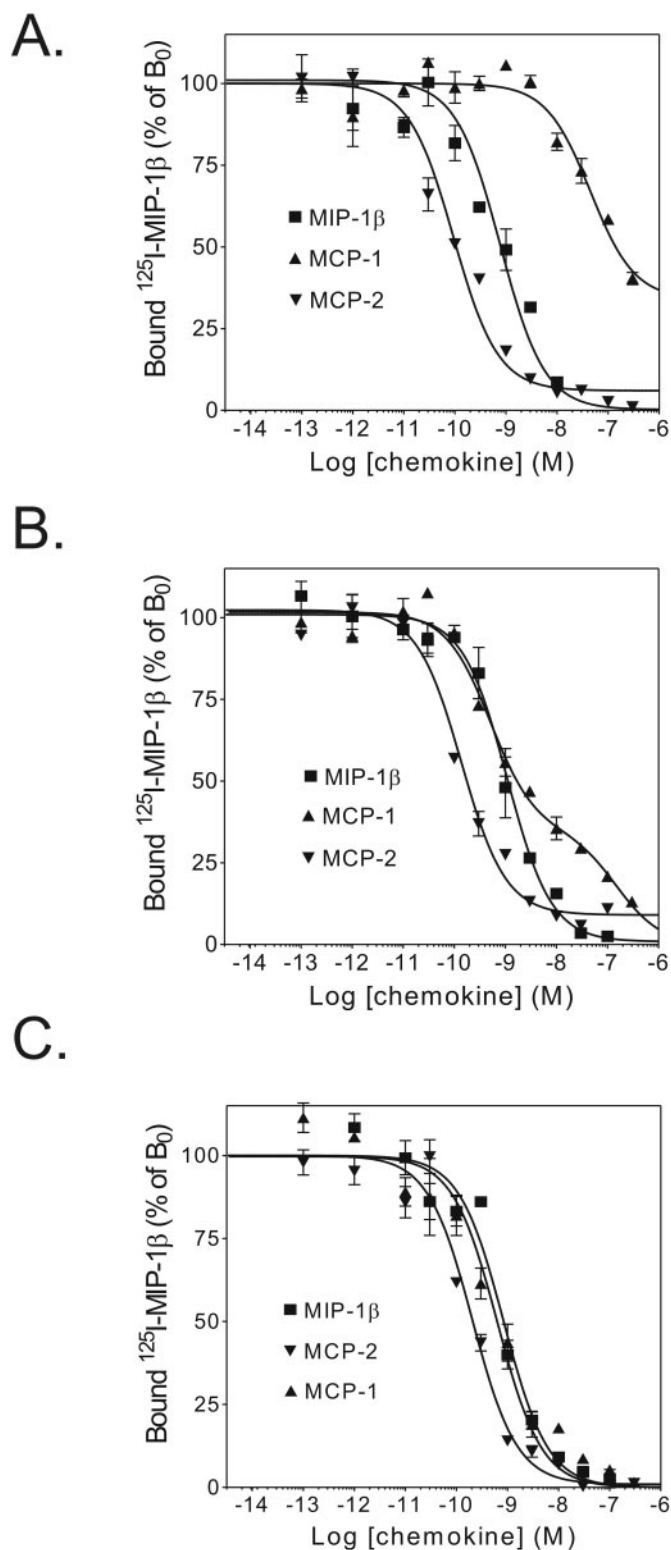
same assay, the addition of 300 nM MIP-1 $\beta$  inhibited about 25% of the specific  $^{125}$ I-MCP-1 binding (Fig. 7). These data suggest that CCR2b/CCR5 heterodimers do indeed exist in native cells and are therefore physiologically relevant.

## Discussion

We have analyzed in the present work the dimerization properties of CCR5 and CCR2b and their functional consequences, after coexpression of the two receptors in a heterologous system. As previously reported by different groups, CCR5 is able to form homodimers, as determined by immunoprecipitation and BRET assays. CCR5 is also able to heterodimerize with CCR2b. The BRET<sub>50</sub> values calculated for CCR5 homodimers, CCR2b homodimers, or CCR5-CCR2b heterodimers are in the same range, suggesting that the propensity of a CCR5 receptor to associate with another CCR5 molecule, or with a CCR2 receptor, is similar. As a consequence, the formation of homo- or heterodimers is expected to be strictly dependent on the level of expression of the two receptors in the cells. The derived BRET<sub>50</sub> values suggest that homo- and heterodimerization would occur for levels of expression found in leukocytes. Heterodimerization between CCR5 and CCR2b was also confirmed by coimmunoprecipitation, although the low recovery of immunoprecipitated heterodimers, contrasting with the high BRET signal observed between the two receptors suggests that most dimers dissociate during the immunoprecipitation procedure.

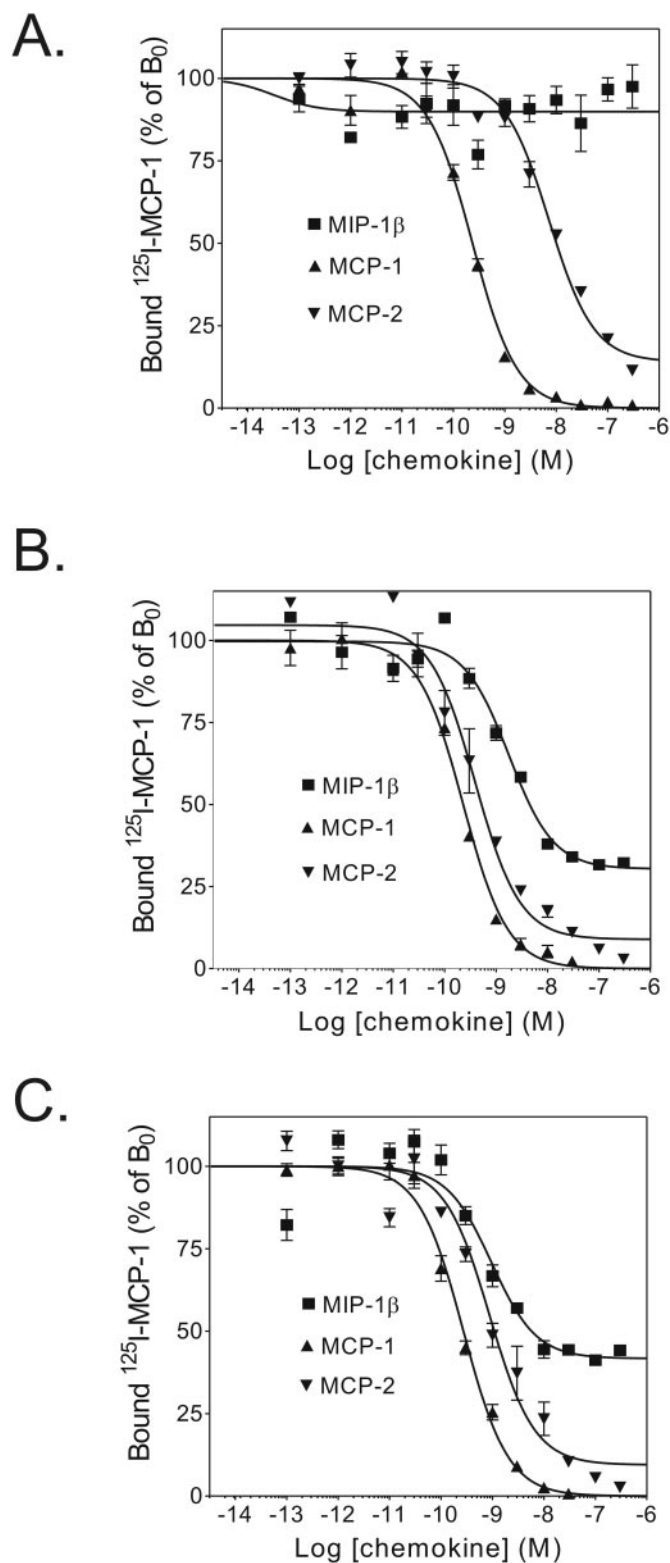
In contrast to what was reported previously (Mellado et al., 2001; Hernanz-Falcon et al., 2004), the homo- and heterodimerization process was found to be independent from agonist-induced stimulation of the receptors. Although agonist stimulation-independent CCR5 homo-oligomerization has been demonstrated previously (Benkirane et al., 1997; Issafras et al., 2002), our experiments have shown here that CCR2b homodimers can also form in the absence of stimulation by agonists. The discrepancies between these various studies might partially be explained by the different experimental approaches used to demonstrate dimerization. The immunoprecipitation experiments performed by Mellado et al. (2001) reporting the strict requirement of a ligand for dimer detection were performed after treatment with a cross-linking agent. Fluorescence resonance energy transfer experiments performed by the same group failed to confirm the effect of agonists on CCR5 dimerization (Hernanz-Falcon et al., 2004). In our hands, the proportion of dimers after immunoprecipitation of CCR5 in the absence of a cross-linking agent was not influenced by ligand treatment (unpublished observations). However, the low recovery of dimers suggests a dissociation of the complexes during the immunoprecipitation procedure. It is therefore possible that the presence of a chemokine ligand bound to a receptor dimer increases the efficiency of cross-linking (possibly by bridging the chemokine to both receptors), compared with an unoccupied dimer. The consequence would be a higher recovery of dimers on gels, leading to the false interpretation that the agonist promotes dimerization.

Synergistic effects have previously been suggested after costimulation of CCR5 and CCR2b expressed in the same cells (Mellado et al., 2001). We did not observe such a synergistic effect, because the functional responses obtained for low concentrations of CCR5 and CCR2b ligands were strictly additive when applied together. This is actually not surpris-



**Fig. 3.** MIP-1 $\beta$  competition binding assay. Competition binding assays were performed on clone C5 expressing CCR5 alone (A) or clones C25-12 (B) and C25-15 (C) coexpressing CCR5 and CCR2b, using  $^{125}$ I-MIP-1 $\beta$  as tracer, and MIP-1 $\beta$ , MCP-1, and MCP-2 as competitors. The data were normalized for nonspecific binding determined in the presence of 300 nM MIP-1 $\beta$  (0%) and specific binding in the absence of competitor (100%). The displayed data are representative of at least three independent experiments. All data points were performed in triplicate (error bars indicate S.E.M.).

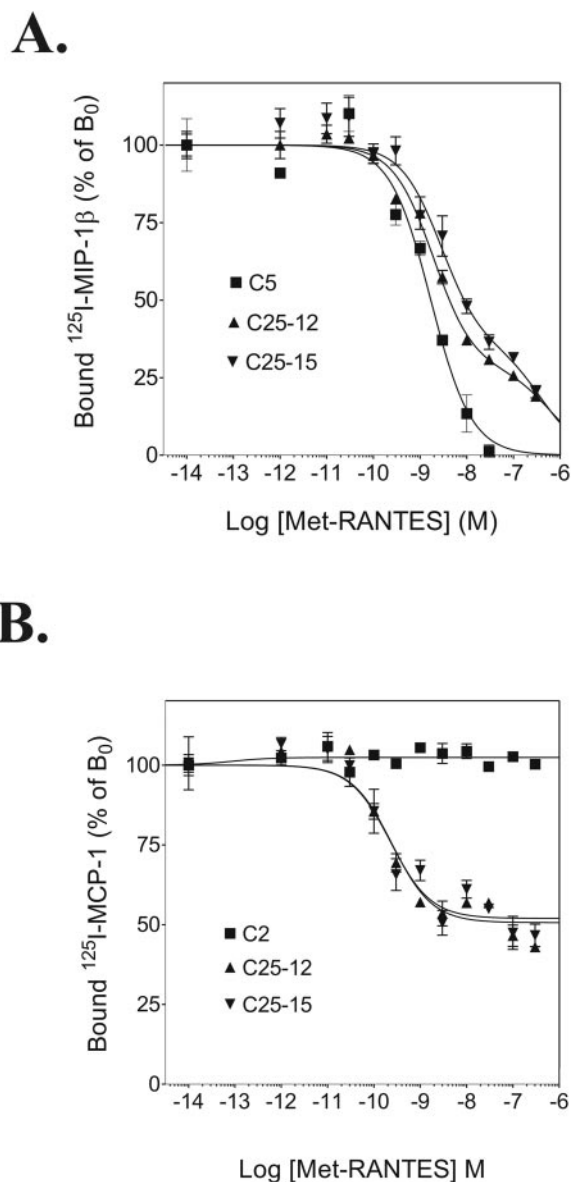




**Fig. 4.** MCP-1 competition binding assay. Competition binding assays were performed, on clone C2 expressing CCR2b alone (A) or clones C25-12 (B) and C25-15 (C) coexpressing CCR5 and CCR2b, using  $^{125}\text{I}$ -MCP-1 as tracer, and MIP-1 $\beta$ , MCP-1, and MCP-2 as competitors. The data were normalized for nonspecific binding determined in the presence of 300 nM MCP-1 (0%) and specific binding in the absence of competitor (100%). The displayed data are representative of at least three independent experiments. All data points were performed in triplicate (error bars indicate S.E.M.).

ing to us. If positive cooperativity was occurring within CCR5/CCR2b heterodimers, similar cooperativity would be expected within CCR5 and CCR2b homodimers. The coactivation of both partners of a heterodimer by different chemokines, or the coactivation of the two partners of a homodimer by a single chemokine, would indeed result in a similar functional response.

Binding assays led, however, to a previously unreported observation either in homo- or heterodimers. Cross-inhibition of ligand binding was observed between CCR5 and CCR2b coexpressed in the same cell line. Because this observation was made on membrane preparations, the involvement of intracellular cascades can be excluded. We therefore



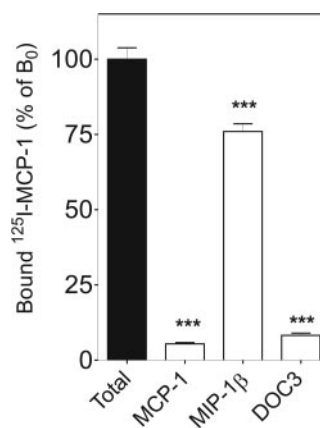
**Fig. 5.** Competition binding assays using CCR5 antagonist. Competition binding assays were performed on clone C2 or C5 expressing CCR2b or CCR5 alone and clones C25-12 or C25-15 coexpressing CCR5 and CCR2b, using  $^{125}\text{I}$ -MIP-1 $\beta$  (A) or  $^{125}\text{I}$ -MCP-1 (B) as tracer and Met-RANTES as competitors. The data were normalized for nonspecific binding determined in the presence of 300 mM Met-RANTES (0%) and specific binding in the absence of competitor (100%). The displayed data are representative of at least three independent experiments. All data points were performed in triplicate (error bars indicate S.E.M.).

attribute these observations to the formation of heterodimers between CCR5 and CCR2b, as seen in BRET experiments. The partial competition observed in the  $^{125}\text{I}$ -MCP-1 binding assay for MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES was found to be correlated to the expected proportion of CCR5/CCR2b heterodimers. Indeed, on membranes from clone C25-15 that expresses equal amounts of CCR5 and CCR2b, the expected proportion of CCR2b molecules engaged in the formation of heterodimers is 50%, considering that no preference exists for the formation of homodimers. On this clone, CCR5-specific ligands competed for 55% of specific MCP-1 binding. Clone C25-12 expresses CCR5 at a level about 5-fold higher than CCR2b, and the expected proportion of CCR2b molecules involved in heterodimers is in the range of 80%, in agreement with the higher level of competition observed in this situation (about 70% of specific binding). The situation is similar in the  $^{125}\text{I}$ -MIP-1 $\beta$  binding assay, considering that MCP-1 is able to bind CCR5 with low affinity. Therefore, in coexpression conditions, full competition is observed for MCP-1, with a high-affinity site corresponding to the indirect competition through the dimer and a low-affinity site corresponding to direct competition.

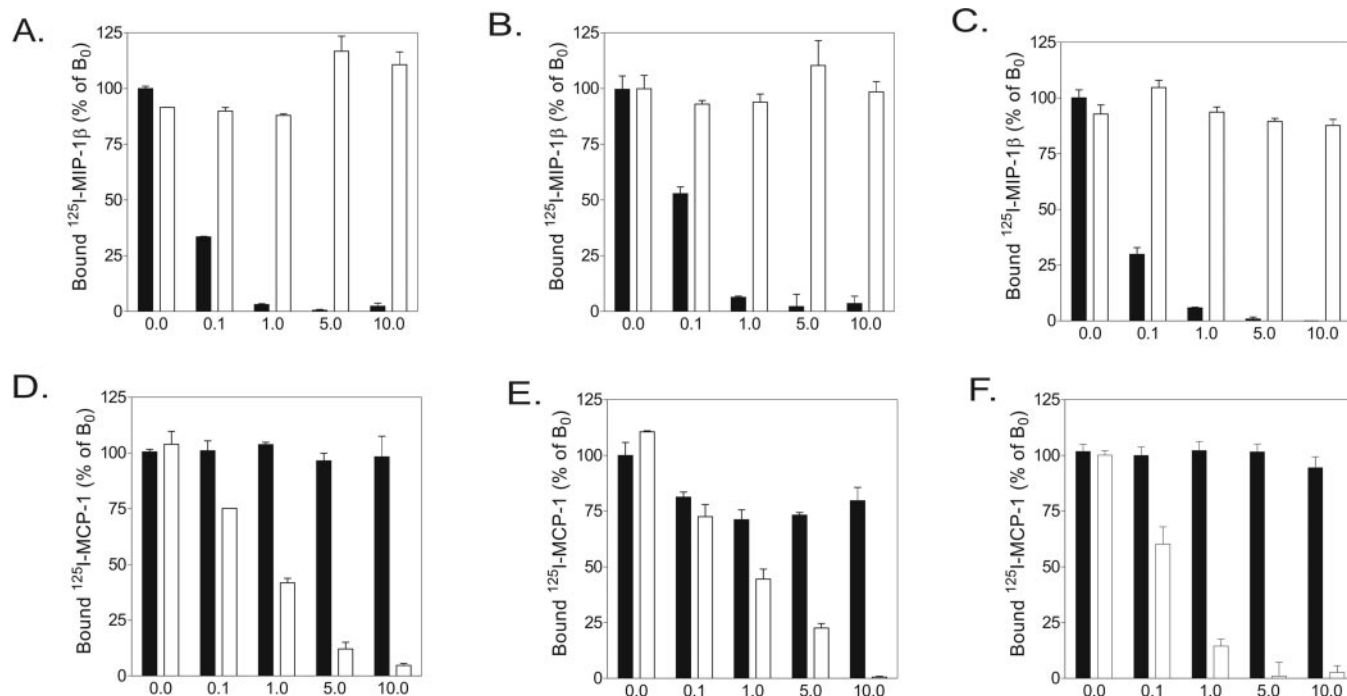
Similar cross-competition takes place in native cells coexpressing both receptors at physiological levels. Indeed, MIP-1 $\beta$  was able to compete for 25% of specific  $^{125}\text{I}$ -MCP-1 binding on human lymphoblasts. To our knowledge, this is the first evidence that CCR2b/CCR5 heterodimers do exist in native cells. The use of recombinant cells coexpressing CCR2b and CCR5 for further characterization of the pharmacological properties of dimers therefore constitutes a valid model of the *in vivo* situation.

Previous studies have reported modifications of the pharmacological profile of receptors after their coexpression with

other receptors and the formation of heterodimers. Changes in the ligand-binding properties of the adenosine A<sub>1</sub> and the purinergic P2Y<sub>1</sub> receptors have been described previously (Yoshioka et al., 2001). Ligand-binding studies have revealed a significant reduction of affinity for A<sub>1</sub> agonists and antagonists on membranes of cotransfected cells, and a 400-fold increase in the affinity of ADP $\beta$ S, a P2Y<sub>1</sub> agonist. Modifications of the functional responses to agonists were also re-



**Fig. 7.** MCP-1 competition binding assay on CD4<sup>+</sup> lymphoblasts. Competition binding assays were performed on CD4<sup>+</sup> by using  $^{125}\text{I}$ -MCP-1 as tracer and MCP-1 (300 nM), MIP-1 $\beta$  (300 nM), or DOC-3 (5  $\mu\text{g}/\text{ml}$ ) as competitors. The data were normalized for specific binding in the absence of competitor (100%). The displayed data are representative of three independent experiments performed with lymphoblasts from different donors. Values of bound  $^{125}\text{I}$ -MCP-1 in the presence of competitors compared with the condition in the absence competitors were statistically different according to a two-way analysis of variance test followed by Tukey's test (\*\*\*,  $P < 0.001$ ). All data points were performed in triplicate (error bars indicate S.E.M.).



**Fig. 6.** Competition binding assays using CCR5- and CCR2b-specific mAbs. Different concentrations of 2D7 (■) and Doc-4 (□, 0, 0.1, 1, 5, and 10  $\mu\text{g}/\text{ml}$ ) were tested for their ability to compete with the binding of  $^{125}\text{I}$ -MIP-1 $\beta$  (A–C) or  $^{125}\text{I}$ -MCP-1 (D–F) to clones C12-25 (B, E) and C15-25 (C, F) and CHO-K1 cells expressing CCR5 (A) or CCR2b (D) alone. The data were normalized for nonspecific binding determined in the presence of 300 nM MIP-1 $\beta$  or MCP-1 (0%) and specific binding in the absence of competitor (100%). The displayed data are representative of at least three independent experiments. All data points were performed in triplicate (error bars indicate S.E.M.).



ported, as measured in an adenylyl cyclase assay. Likewise, the  $\kappa$  and  $\delta$  opioid receptors were shown to heterodimerize, thereby creating a binding site with an original pharmacological profile, distinct from individual receptors expressed independently, both in terms of binding and of functional responses. This new binding site could be activated synergistically by selective ligands of both receptors (Jordan and Devi, 1999). These situations are however different from the observations reported here. First, we did not detect alterations of the functional responses of the coexpressed receptors; and second, no evidence for binding competition on one receptor by the specific ligands of the other has been reported in these other studies. Although dimerization is probably driving the modifications of the pharmacological properties in these three situations, the mechanisms involved in each case is probably different.

The mechanisms by which *trans*-binding competition occurs within CCR5/CCR2b heterodimers is not determined precisely so far. In theory, it might result from a variety of interactions across the members of the dimer. First, the formation of heterodimers might modify the structure of the individual binding sites, so that the CCR2b site that would bind MCP-1 in the monomeric and homodimeric states now accommodates the CCR5-specific ligands as well. This hypothesis, however, seems unlikely, because the binding properties of the bona fide ligands of each receptor seem unchanged after coexpression. In addition, no competition for  $^{125}\text{I}$ -MIP-1 $\beta$  was observed using an anti-CCR2b mAb or for  $^{125}\text{I}$ -MCP-1 using an anti-CCR5 mAb, demonstrating that each iodinated tracer continues to bind to its specific receptor within the dimer. A second hypothesis would be that a chemokine bound to one of the receptors overlaps onto the binding site of the other receptor of the dimer, thereby preventing the simultaneous binding of a second chemokine onto the dimer. The fact that mAbs bound to one receptor do not prevent binding of a chemokine onto the other might be taken as an argument against this hypothesis. A third hypothesis would be that the binding of an agonist to one of the receptors induces a conformational change in both members of the dimer, thereby modifying the binding site of the other receptor. The fact that [10-68]-RANTES, a CCR5 ligand with weak agonist properties, is as effective as RANTES in competing for MCP-1 binding suggests, however, that full activation of the receptor is not required. As mentioned above, a last hypothesis that would involve postreceptor signaling pathways, may be discarded, because our observations were made on membranes in which limited signaling events may occur. Our remaining working hypotheses are therefore steric hindrance and induced conformational change within receptor dimers.

The functional significance of the homo- and heterodimerization of CCR5 and CCR2b remains to be determined. Our observations regarding the binding properties of CCR5/CCR2b heterodimers suggest that heterodimers and presumable homodimers as well are able to bind a single chemokine. It has recently been described that dimers of the leukotriene B<sub>4</sub> receptor form in vitro a complex with a single heterotrimeric G protein (Baneres and Parelo, 2003).

Heterodimerization does not seem to modify the efficiency of signaling of chemokine receptors in CHO-K1 cells through the calcium release pathway. However, our present experiments do not allow to exclude that the dimerization state

(homo- or hetero-) might be required for efficient signaling. Receptors impaired in their dimerization potential should be designed to test this possibility. It is also not excluded that heterodimerization of CCR5 and CCR2b might modify the range and/or efficacy of signaling cascades, other than the intracellular calcium release mediated by G<sub>i</sub> proteins.

Finally, the functional consequences of the fact that a chemokine receptor dimer is able to bind a single chemokine will have to be studied further. Indeed, most of our knowledge related to chemokine receptor pharmacology and function has been derived from the study of receptors expressed in heterologous systems, in which they probably form homodimers. The design of a set of obligate receptor monomers and the use of receptor mutants deficient in one of its natural properties (e.g., binding, activation, and desensitization) will be necessary for evaluating further the functional relevance of the dimerization process. Heterodimers between CCR5 and CCR2b, characterized by their different pharmacology, will be an ideal model to understand the mechanisms underlying the negative binding cooperativity. Because such a mechanism might involve the induction of conformational changes between the dimer partners, these studies might also help to understand the conformational changes associated with receptor activation, with broad implications in the GPCR field in general.

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