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Allosteric modulation of binding properties between units of chemokine receptor homo- and hetero-oligomers

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Running title: Allosteric properties of chemokine receptor oligomers

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Abreviations : GPCR, G protein coupled receptor; RANTES, Related on activation normal T cell expressed and secreted; MCP, Monocyte chemoattractant protein; MIP, Macrophage inflammatory protein; HCC, Hemofiltrate CC chemokine; CHO, Chinese hamster ovary; HEK, Human embryonic kidney; BSA, Bovine serum albumin; PBS, Phosphate buffer saline; EYFP, Enhanced yellow fluorescent protein; BRET, Bioluminescence resonance energy transfer - 2 -

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ABSTRACT

We have previously demonstrated that the chemokine receptors CCR2 and CCR5 form homo- and heterodimers, and that dimers can only bind a single chemokine molecule with high affinity. We provide here evidence from BRET experiments that stimulation by chemokines does not influence the CCR2/CCR5 heterodimerization status. In addition, we show that the rate of radioligand dissociation from one unit of the heterodimer in "infinite" tracer dilution conditions is strongly increased in the presence of an unlabelled chemokine ligand of the other unit. These results demonstrate unambiguously that the interaction between heterodimer units is of allosteric nature. Agonists, but also some monoclonal antibodies, could promote such negative binding cooperativity, indicating that this phenomenom does not require the full conformational change associated with receptor activation. Finally, we show that G protein coupling is required for high affinity binding of MIP-1β (CCL4) to CCR5, and that the dissociation from G proteins, following incubation with Gpp(NH)p, promotes the release of pre-bound radiolabelled chemokines with kinetics similar to that measured after the addition of an excess of unlabelled chemokines. These observations suggest that the association with G proteins likely participates in the negative cooperativity observed between receptor monomers. We propose that negative cooperativity within homo- and hetero-dimers of chemokine receptors and probably other GPCRs will likely have major implications in their pharmacology in vivo and the physiopathology of diseases with which they are associated.

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INTRODUCTION

The chemokine receptors CCR5 and CCR2 are closely related members of class 1 G protein-coupled receptors (GPCR). They share 75% overall identity, but most differences are located within the extracellular domains and the C-terminal tail, and this percentage goes up to 91% when considering transmembrane segments only. Yet, the pharmacology of the two receptors is clearly different. CCR5 binds MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), MCP-2 (CCL8) and a truncated form of HCC-1 (CCL14) with high affinity (Blanpain *et al.*, 1999) while CCR2 binds MCP-1 (CCL2) and MCP-2 (which is thus 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 (Samson *et al.*, 1997). In addition to its role as a chemokine receptor involved in the recruitment of leukocytes in a number of pathological situations (rheumatoid arthritis, graft rejection, neurodegenerative diseases, etc), CCR5 also constitutes the major co-receptor 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). Both CCR2 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).

CCR2 and CCR5 were shown to form both homo- and heterodimers using a variety of approaches (Benkirane *et al.*, 1997;Mellado *et al.*, 2001;Issafras *et al.*, 2002;Hernanz-Falcon *et al.*, 2004;Percherancier *et al.*, 2005;El Asmar *et al.*, 2005). Subcellular fractionation followed by bioluminescence resonance energy transfer (BRET) measurements has suggested that homodimerization of CCR5 occurs shortly after synthesis in the endoplasmic reticulum (Issafras *et al.*, 2002). In a previous study, we have investigated the functional consequences

of dimerization in recombinant cells expressing CCR2, CCR5 or both receptors. We showed that CCR5-specific ligands (MIP-1α, MIP-1β, RANTES) were unable to compete for MCP-1 binding on cells expressing CCR2 alone but efficiently inhibited MCP-1 binding when CCR5 and CCR2 were co-expressed. Similar observations were made for the CCR2-selective ligand MCP-1 that competed efficiently for MIP-1β binding on cells expressing both receptors. These results demonstrated negative binding cooperativity between the binding pockets of the two receptors and showed that a receptor heterodimer, and most probably a homodimer as well, could only bind a single chemokine with high affinity. Although this phenomenon appeared important for understanding how a receptor dimer works, its molecular bases were poorly characterized. In the present study, we used ligand dissociation assays following "infinite" dilution of the tracer, in order to demonstrate that the negative cooperativity between receptor monomers is of allosteric nature. Furthermore, we investigated the contribution of G protein coupling to high affinity ligand binding and negative binding cooperativity, and we suggest that the negative allosteric regulation across the dimer might involve the positioning of the G protein heterotrimer relative to the liganded monomer.

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MATERIAL AND METHODS

Cell lines, leucocyte populations and antibodies

CHO-K1 cells expressing CCR2, CCR5 or both receptors (line C25-12), and membrane preparations made from these cells for radioligand binding assays were described elsewhere (El Asmar *et al.*, 2005). Human T CD_4^+ lymphoblasts were isolated, prepared and used as described previously (El Asmar *et al.*, 2005).

BRET assays

The cDNAs encoding enhanced yellow fluorescent protein (EYFP) and a humanized form of *Renilla* luciferase (hRluc) were fused in frame to the 3' end of CCR5 and CCR2 cDNAs in the pcDNA3.1 vector, as described previously (Issafras *et al.*, 2002). A BRET protocol adapted to cell monolayers was developed, and the BRET experiments performed as described (Urizar *et al.*, 2005). Human embryonic kidney (HEK-293) cells were transfected by the calcium phosphate precipitation method with different receptor combinations. A control corresponding to mock-transfected cells was included in order to subtract the raw basal luminescence. Forty-eight hours after transfection, the BRET measurement was performed using a Mithras LB 940 Multilabel Reader (Berthold) as described (El Asmar *et al.*, 2005;Urizar *et al.*, 2005). 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.

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Dissociation kinetics experiments

For ligand dissociation experiments, membrane preparations of cells expressing the chemokine receptors CCR5 and/or CCR2 were first incubated at room temperature (RT) with 0.1 nM ^{125}I-MCP-1 or ^{125}I-MIP-1β in a final volume of 500 µl of assay buffer (50 mM Hepes pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% BSA). The estimated equilibrium dissociation constant (K_d) of CCR5 and CCR2 for their respective ligands (MIP-1β and MCP-1) in binding assays were consistent with previously described values (respectively 180 ±10 pM and 52 ±8 pM, Samson et al. 1997). After one hour of incubation with the tracer, the membranes were centrifuged for 5 min at 15,000 x g at RT, and the unbound radioligand was removed by aspiration. The membrane pellet was washed once with assay buffer at RT, and then resuspended in 2.5 ml of assay buffer at RT, with or without 100 nM unlabelled MCP-1 or MIP-1β. At different time points after resuspension, aliquots were collected, the bound tracer was separated by filtration through GF/B filters pre-soaked for 1 hour in 0.5% polyethylenimine and the filters were counted for 1 minute in a γ-scintillation counter. Total and bound radioligands were also measured immediately after membrane resuspension in order to determine the actual concentration of tracer during the dissociation phase of the assay and the initial bound fraction. In all experiments, total binding and total tracer remaining at the initiation of the dissociation phase represented 10% or less of the amount of tracer engaged initially. Considering the percentage of remaining tracer and the five-fold dilution of the incubation medium following membrane resuspension, the tracer dilution factors were on average 60 and 72 for respectively the MIP-1β and MCP-1 dissociation binding assays. Increasing the overall dilution factor up to 200-fold resulted in similar results, indicating that initial dilution was sufficient to fulfill the "infinite" dilution conditions and to prevent radioligand re-association (data not shown). The data are presented as the ratio between bound cpm at the various dissociation time points and total bound cpm at time zero of dissociation. The curves were fitted with the GraphPad Prism software (v4.0) using non-linear regression and a single phase decay model. The statistical analysis

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of $t_{1/2}$ values was performed by the same software, using a two ways ANOVA test followed by Tukey's test.

Intracellular calcium mobilization assays

The functional response to chemokines was analyzed with an aequorin-based assay as previously described (El Asmar *et al.*, 2005). Briefly, cells were harvested from plates with Ca^{2+}- and Mg^{2+}-free DMEM supplemented with 5 mM EDTA and centrifuged for 2 min at 1000 g. The pellet was resuspended in DMEM at a density of 5 x 10^6 cells/ml, and incubated for 4 h in the dark in the presence of 5 µM coelenterazine H (Promega Corporation). Cells were then diluted 5-fold before use. Variable concentrations of chemokines in a volume of 50 µl of DMEM were added to 50 µl of cell suspension (25,000 cells) per well. Luminescence was measured for 30 sec in an EG&G Berthold luminometer (PerkinElmer Life Sciences). Half-maximal effective concentrations (EC_{50}) were determined with the GraphPad Prism software using nonlinear regression coupled to a sigmoidal dose-response model. The reported values are the mean ± S.E.M. of at least three independent experiments.

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RESULTS

Influence of the stimulation by chemokines on the CCR2/CCR5 heterodimerization

We have previously reported BRET and binding data demonstrating that the structurally related chemokine receptors CCR5 and CCR2 can homo- and heterodimerize (Issafras *et al.*, 2002;El Asmar *et al.*, 2005). Before studying further the functional consequences of this dimerization process in terms of receptor cooperativity, we tested the influence of various ligands on the heterodimerization of CCR5 and CCR2 in living cells using the BRET technique. Indeed, it has been shown previously that the homodimerization status of CCR5 and CCR2 was not influenced by their stimulation by agonists (Issafras *et al.*, 2002), but the stability of heterodimers in these conditions was not investigated yet. As described previously (El Asmar *et al.*, 2005), energy transfer was observed between CCR5-h*R*Luc and CCR2-EYFP (Figure 1) and CCR2-h*R*Luc and CCR5-EYFP (not shown) in the absence of ligands. We confirmed that the parameters of energy transfer ($BRET_{50}$, $BRET_{MAX}$) were in the same range for homo and heterodimers (El Asmar *et al.*, 2005), with the consequence that the formation of homo- and heterodimers is dictated by the relative expression levels of the two receptors. As controls for the specificity of the energy transfer, the $GABA_{B2}$ receptor fused to EYFP was used in combination with CCR5-h*R*Luc and CCR2-h*R*Luc. A much lower level of energy transfer was observed in these situations (Fig. 1 and data not shown). Furthermore, in BRET competition experiments, only increasing amounts of CCR5 or CCR2 were able to decrease the BRET signal resulting from heterodimerization, while the unrelated receptors ($GABA_{B2}$) did not (data not shown). As previously shown for the CCR5 (Issafras *et al.*, 2002) and CCR2 homodimers (our unpublished observations), we observed that the addition of ligands specific for CCR5 (MIP-1β) or CCR2 (MCP-1), alone or in combination, did not affect the energy transfer among heterodimers. Similar results were obtained with MCP-2, which binds to both receptors with high affinity (Fig. 1). These results support the view that ligand

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binding has no influence on the homo- and heterodimerization states of chemokine receptors (Springael *et al.*, 2005;Issafras *et al.*, 2002;El Asmar *et al.*, 2005;Benkirane *et al.*, 1997).

Negative binding cooperativity within CCR2/CCR5 heterodimers

We next tested further the pharmacological properties of CCR5/CCR2 heterodimers using binding assays. We have previously demonstrated that CCR2/CCR5 heterodimers can only bind a single chemokine with high affinity (El Asmar *et al.*, 2005). These observations suggested either an overlap between the two chemokine binding sites of the monomers or some kind of negative allosteric interaction across the dimer interface (Springael *et al.*, 2005). It was also shown for another class I receptor (TSHr) that a single ligand molecule binds to a receptor dimer (Urizar *et al.*, 2005), and a similar observation has been made recently for a receptor belonging to class 3 (Kniazeff *et al.*, 2004;Urizar *et al.*, 2005). In order to determine more precisely the mechanism underlying these effects, we built on the model of chemokine receptor dimers and performed dissociation kinetics experiments following extensive ligand dilution, a procedure that constitutes the classical way of investigating allosteric interactions between distinct binding sites (Christopoulos *et al.*, 1997;de Meyts *et al.*, 1973). CHO-K1 cell lines expressing CCR2, CCR5, or both receptors (C25-12) have been generated and characterized previously (El Asmar *et al.*, 2005). Using membranes prepared from these cell lines, a radiolabelled chemokine tracer was allowed to bind to the receptors at equilibrium, following which the free tracer was removed by aspiration and the incubation medium further diluted five fold. Radiolabelled ligand dissociation was assayed over time in the assay buffer, in the absence or presence of unlabelled chemokines at various concentrations. This protocol allows measurement of the effect of excess ligand on the kinetics of tracer dissociation. Results showed that, on membranes prepared from cells expressing CCR2 alone, dissociation of pre-bound ^{125}I-MCP-1 was slow in the absence but strongly accelerated in the presence of 100 nM unlabelled MCP-1 ($t_{1/2}$: 19 ± 3 min, Figure 2). Kinetics performed over longer periods showed slow but substantial dissociation of MCP-1 over time ($t_{1/2}$: around 350 min,

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Figure 3A). Complete dissociation was obtained by 24h (data not shown). MIP-1β, a specific CCR5 ligand, had no effect in these conditions, whereas it completely dissociated bound ^{125}I-MCP-1 from cells co-expressing CCR2 and CCR5 ($t_{1/2}$: 20 ± 8 min, P<0.001). Similarly, MCP-1 at 25 nM, a concentration well below its K_d on CCR5, promoted a rapid dissociation of ^{125}I-MIP-1β from CCR5 in cells where CCR2 was coexpressed compared to cells expressing CCR5 alone ($t_{1/2}$: 27.7 ± 7.1 min and 122 ± 13 min respectively, p<0.01). The slow dissociation rate of ^{125}I-MIP-1β from CCR5-expressing cells incubated in the presence of MCP-1 can be attributed to the ability of MCP-1 to bind CCR5 with low affinity (Blanpain et al. 1999). In addition, we showed that the dissociation rate correlated with the concentration of unlabelled chemokines (Figure 3B). The concentration required for half dissociation appeared to be higher than the K_d value, supporting the involvement of a low affinity binding site in the observed phenomenon. This is consistent with recent data reporting both high and low affinity sites on glycoprotein receptor dimers, for which negative binding cooperativity was demonstrated as well (Urizar *et al.*, 2005). These data suggest that ligand binding in one monomer induces conformational changes in the partner, resulting in faster dissociation of the bound ligand. As a control, we combined the expression of CCR2 and the R126N mutant of CCR5 that is unable to signal and displays a reduced affinity for MIP-1β (see below). We could show in BRET experiments that the R126N mutant is able to heterodimerize with CCR2 as efficiently as wild-type CCR5 (not shown). In cells coexpressing both receptors, MIP-1β was however unable to increase the dissociation rate of ^{125}I-MCP-1 (Figure 3C). Finally, in order to explore whether this phenomenon could take place in primary cells in which CCR2 and CCR5 are naturally co-expressed, we performed dissociation kinetics of ^{125}I-MIP-1β on membranes prepared form human $CD4^+$ T lymphoblasts. Dissociation of pre-bound ^{125}I-MCP-1 was accelerated in the presence of unlabelled MCP-1 or MIP-1β (Figure 3D), suggesting that CCR2/CCR5 heterodimers do indeed exist in native cells, and that negative binding cooperativity among homo- and heterodimers is physiologically relevant.

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Effects of an inverse agonist and monoclonal antibodies on chemokine binding

In order to better characterize the mechanisms involved in negative binding cooperativity, we tested the influence of additional ligands on tracer dissociation. We showed that, in addition to MIP-1β, RANTES and MCP-2 also increased ^{125}I-MIP-1β dissociation from cells expressing CCR5. A truncated variant of RANTES, [10-68]-RANTES, that acts as a weak partial agonist on CCR5, and TAK-779, a non peptidic inverse agonist, increased also the dissociation rate of ^{125}I-MIP-1β (Figure 4A). Even very partial agonists are therefore able to increase the dissociation rate of the tracer. Chemical antagonists of CCR5 or other chemokine receptors have been shown to bind to the bundle of transmembrane alpha-helices and to inhibit chemokine binding in a non-competitive, allosteric, manner (Christopoulos and Kenakin, 2002a). Allosteric interaction within a monomer can therefore explain the observations, although interaction with the unbound monomer and allosteric interaction across the dimer could contribute also to the dissociation induced by TAK-779.

It is well established that CC-chemokines are able to form homo- and heterodimers (Clore *et al.*, 1990). However, it is largely accepted that only the monomeric forms of chemokines are able to bind and activate their receptors (Gong and Clark-Lewis, 1995;Gong *et al.*, 1996;Paavola *et al.*, 1998), and functionally active monomeric mutants of MIP-1β were described previously (Laurence *et al.*, 2001). In order to ascertain whether our observations were independent of ligand dimerization, we tested the effects of two such monovalent chemokines, P8A-MIP-1β and F13A-MIP-1β, on ^{125}I-MIP-1β dissociation and found that both mutant chemokines increased the tracer dissociation rate (Figure 4B). We next tested the influence of monoclonal antibodies recognizing specifically CCR5 or CCR2. The functional properties of some of these antibobies have been described elsewhere (Lee *et al.*, 1999;Blanpain *et al.*, 2002). The anti-CCR5 2D7 and MC-1, which are directed against the

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second extracellular loop of CCR5, increased the dissociation rate of ^{125}I-MIP-1β whereas the anti-CCR5 CTC5, recognizing the very N-terminus of the receptor (Lee *et al.*, 1999), and a control IgG had no significant effects. Conversely, the ^{125}I-MCP-1 dissociation rate from cells expressing CCR2 alone was increased by one of the anti-CCR2 monoclonals, MAB150 (uncharacterized epitope), but not by DOC-1 and DOC-2, two mAbs recognizing extracellular loops (data not shown) or a control IgG (Figure 5). Interestingly, in C25-12 cells co-expressing CCR2 and CCR5 (El Asmar *et al.*, 2005), the anti-CCR5 mAb 2D7 increased the dissociation rate of ^{125}I-MIP-1β but not that of ^{125}I-MCP-1. Conversely, the anti-CCR2 MAB150 increased dissociation of ^{125}I-MCP-1 but had no effect on ^{125}I-MIP-1β dissociation (Figure 6). Although these observations are not fully understood, they indicate that specific mAbs, some of which were described to promote receptor internalization ((Blanpain *et al.*, 2002), can promote negative allosteric regulations within homodimers, without affecting heterodimers.

Influence of G protein coupling on negative binding cooperativity

It is generally accepted that the active state of some GPCRs is stabilized by its coupling to a guanine-nucleotide free G protein α subunit, and that it is this activated/coupled form of the receptor that displays the highest affinity for its agonists (Kenakin, 1996;De Lean *et al.*, 1980). In keeping with this concept, one could assume that negative binding cooperativity might require some conformational change within the receptor/G protein complex. We first investigated the effects of G protein coupling on the binding of radiolabeled MIP-1β to CCR5. For this purpose, we measured the binding of ^{125}I-MIP-1β on CCR5 from cells pretreated or not with *pertusssis* toxin (PTX), and showed that PTX decreased strongly ^{125}I-MIP-1β binding down to undetectable levels (Figure 7A). We next explored the binding of ^{125}I-MIP-1β to membrane preparations in the presence or absence of Gpp(NH)p, a non-

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hydrolysable analogue of GTP. As shown in Figure 7B, the addition of Gpp(NH)p in the binding assay reduced in a concentration-dependent way the binding of ^{125}I-MIP-1β to membranes of CCR5-expressing cells. These data demonstrate that, as for other GPCRs, G protein coupling of CCR5 is required for high affinity chemokine binding, in line with a previous study (Standinger *et al.*, 2001). One could therefore predict that mutations preventing G protein-coupling would affect chemokine binding. To test this hypothesis, ^{125}I-MIP-1β binding was assessed on cells expressing the R126N mutant of CCR5, which displays no detectable constitutive activity and a strong decrease of functional response to agonists (Lagane *et al.*, 2005). The binding of ^{125}I-MIP-1β to this mutant was found to be strongly decreased as compared to wild-type CCR5 (Figure 7C). We have shown above that MIP-1β could not increase ^{125}I-MCP-1 dissociation from cells co-expressing CCR2 and the R126N CCR5 mutant. This observation might however be due either to the lack of coupling of the mutant, or to its low affinity for chemokines. In order to investigate further whether G protein coupling might influence the allosteric regulation within receptor dimers, we carried out ^{125}I-MIP-1β dissociation experiments in the presence or absence of Gpp(NH)p. As shown in Figure 8A, the addition of Gpp(NH)p in the assay buffer increased the dissociation rate of ^{125}I-MIP-1β from CCR5, with a kinetics similar to that resulting from the addition of unlabelled MIP-1β. The addition of Gpp(NH)p in combination with MIP-1β increased slightly the dissociation rate. Taken together, these data suggest that G protein uncoupling is sufficient to promote MIP-1β dissociation, likely by shifting the receptor from a high to a low affinity state. This led us to investigate the functional response of CCR2 when this receptor is coexpressed with a partner unable to interact with G proteins. We transfected apoaequoprin-expressing CCR5R^{126}N mutant CHO-K1 cells with CCR2 or CCR5 receptors and analysed the functional response of CCR2 and CCR5. The co-expression of the R126N mutant did not impair significantly CCR2 or CCR5 signalling, indicating the absence of dominant negative

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effect of the non-functional mutant. These data support the view that a single G protein is sufficient for promoting signalling through a dimer.

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DISCUSSION

Previous experiments performed with heterodimers of the chemokine receptors CCR2 and CCR5 showed that they can only bind a single chemokine with high affinity (El Asmar *et al.*, 2005). These experiments suggested either an overlap between binding sites of the two chemokines, or some kind of negative allosteric regulation across the dimer interface. Although not demonstrated formally, the negative binding cooperativity in a heterodimer of closely related receptors suggested that similar interactions would take place in homodimers of chemokine receptors, and possibly other receptor families. In the present study, we have analyzed in details the heterodimerization of CCR5 and CCR2 as a model of chemokine receptor dimerization, and the mechanisms associated with the negative binding cooperativity. CCR5 and CCR2 are particularly well adapted for this purpose, as they share an almost identical transmembrane helix bundle, while displaying a very different pharmacology. They therefore form heterodimers as efficiently as homodimers, and can be probed independently by specific tracers, agonists and monoclonal antibodies. By using BRET, we showed that the stimulation of the receptors by agonists specific for CCR5 or CCR2, alone or in combination, did not affect the energy transfer among heterodimers. These results extend similar observations made previously for homodimers (Springael *et al.*, 2005;Issafras *et al.*, 2002;El Asmar *et al.*, 2005;Benkirane *et al.*, 1997) and support the view that ligand binding has no influence on the homo- and heterodimerization states of chemokine receptors. In some cases however, modification of BRET signals following the addition of agonists has been attributed to conformational changes within CXCR4 homodimers and CXCR4/CCR2 heterodimers (Percherancier *et al.*, 2005). As stimulation of chemokine receptors results in their internalization, the absence of modification of BRET signal suggests also the internalization of heterodimers as such, and the absence of significant monomer exchange between dimers. The hypothesis that

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dimers are stable and non-exchangeable is consistent with the current view prevailing for other classes of GPCRs (Bulenger *et al.*, 2005).

We have previously shown that CCR5-specific ligands which were unable to compete for MCP-1 binding on cells expressing CCR2 alone, efficiently inhibited MCP-1 binding when CCR5 and CCR2 were co-expressed. Similar observations were made for the CCR2-selective ligand MCP-1, that competed efficiently for MIP-1β binding on cells expressing both receptors, while being a poor competitor on cells expressing CCR5 alone (El Asmar *et al.*, 2005). These results are highly suggestive of negative binding cooperativity between the two binding pockets of the dimers. However, negative cooperativity cannot be distinguished from binding site heterogeneity by equilibrium binding experiments alone. A classical approach to demonstrate negative cooperativity across two distinct binding sites has been to perform dissociation kinetics experiments following extensive dilution of the tracer, as first proposed for the insulin receptor (Christopoulos *et al.*, 1997;Urizar *et al.*, 2005;Kostenis and Mohr, 1996;Pizard *et al.*, 1998;de Meyts *et al.*, 1973). Using this approach, we have built on our previous observations and showed that the rate of radioligand dissociation from dimers was strongly increased in the presence of unlabelled ligands. In our experimental conditions, the total concentration of tracer remaining during the dissociation kinetics represented between 1 and 2% of the concentration used during the equilibrium phase, preventing therefore significant tracer reassociation (see methods). MIP-1β dissociation from CCR5 was promoted by high concentrations of MCP-1, in agreement with the previously reported ability of MCP-1 to bind CCR5 with low affinity (Blanpain et al. 1999). Moreover, when dissociation was performed on cell co-expressing CCR5 and CCR2, the tracer dissociation rate from one receptor was increased by unlabelled ligands binding to the other, demonstrating unambiguously that the observation was the consequence of the formation of heterodimers between CCR2 and CCR5. In these conditions, MCP-1-promoted dissociation of MIP-1β from CCR5 was faster and stronger, suggesting the involvement of the high affinity binding site of CCR2. The inability of MIP-1β to

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increase MCP-1 dissociation in cells coexpressing CCR2 and the CCR5R126N mutant, despite the formation of heterodimers, confirms that binding of the chemokine and/or G protein coupling to the dimer partner are a strict requirement. Taken together, our data confirm negative binding cooperativity in both homo- and heterodimers, and demonstrate that this interaction between binding sites is allosteric in nature. They suggest that ligand binding in one monomer induces conformational changes in the partner, resulting in faster dissociation of the bound ligand (Figure 9A). When considering CCR5 or CCR2 homodimers, the amount of radiolabelled chemokine remaining bound after the fast dissociation promoted by the unlabelled agonist is very close to the non-specific binding. This is in complete agreement with the view that almost all chemokine receptors are involved in homodimers, and more generally with the rare reports that have been able to quantify the proportion of receptors present as dimers at the cell surface (Guo *et al.*, 2003;Mercier *et al.*, 2002;Fotiadis *et al.*, 2003). The ability of unlabelled ligands to promote complete tracer dissociation from cells co-expressing CCR5 and CCR2 suggests on the other hand that homodimers and heterodimers do interact. This observation supports the idea that the conformational changes associated with negative cooperativity are propagated in hetero-oligomeric complexes possibly reminiscent of rhodopsin lattices (Figure 9B) (Fotiadis *et al.*, 2003;Levitzki, 1974). Alternatively, exchange of subunits between homo and heterodimeric complexes, in the course of the experiment, could also account for the observation (Figure 9B) (Gouldson *et al.*, 1998), although the stability of the BRET signal following receptor activation supports rather the idea that heterodimers are stable. Additional experiments will certainly be required to understand better the molecular mechanisms underlying this phenomenon. An allosteric interaction between binding sites was also shown to take place in membrane preparations from lymphoblasts that co-express both receptors at physiological levels, supporting the existence of CCR2/CCR5 heterodimers in native cells. The rate of tracer dissociation in the absence of chemokines was somehow faster in these conditions than with membrane preparations of CHO-K1 cells. This

results might be due to the presence in lymphoblast membranes of other proteins, not present at the same relative level in CHO cells but potentially affecting the function of receptor oligomers and modifying as a consequence the spontaneous dissociation rate of chemokines. Nevertheless, these results clearly indicate that recombinant cells coexpressing CCR2 and CCR5 constitute a valid model for studying the interactions occuring in native cells.

We showed that negative binding cooperativity does not require chemokine dimerization. Indeed, monovalent forms of MIP-1β, which were previously shown to bind and activate CCR5, increase efficiently the rate of tracer dissociation. This is not unexpected, given the prior demonstration that chemokines activate their receptors as monomers, but confirms the absence of relation between the ability of chemokines to dimerize, and the allosteric regulation mechanism within dimers of receptors. We also showed that the truncated mutant [10-68]-RANTES, which acts as a weak partial agonist of CCR5, increases the rate of tracer dissociation with the same efficiency as wild-type RANTES. This finding suggests that full activation of one monomer is not required for the allosteric regulation of the other, in agreement with recent results showing that allosteric regulation of glycoprotein hormone receptor dimers is not directly related to receptor activation (Urizar *et al.*, 2005). Interestingly, some monoclonal antibodies, particularly those previously shown to antagonize chemokine binding (MC-1, 2D7, MAB150) and to promote receptor internalization (MC-1) were also shown to promote full tracer dissociation from their target receptor but not from the co-expressed receptor. The phenomenon observed here might therefore be different from what is observed with chemokines. Indeed, an antibody-driven allosteric regulation across the heterodimer interface cannot be demonstrated. The complete tracer dissociation from the antibody-targeted protomer might be explained by an allosteric effect within the protomer and would suggest that the antagonist activity of these monoclonal antibodies is not competitive. Other explanations which involve interactions within higher order receptor oligomers cannot be excluded. It was previously shown that the bridging capabilities of anti-CCR5 antibodies were

important for some of their biological properties, as their monovalent forms could bind the receptor but were devoid of functional activity (Blanpain *et al.*, 2002;Issafras *et al.*, 2002).

Our results suggest therefore that under most physiological conditions (i.e. at low agonist concentration), a single molecule of agonist binds to a dimer. Negative cooperativity has been described as a way for a system to respond with maximal sensitivity in the lower concentration range, while still responding to very high concentrations (Koshland, 1996).

The exact molecular mechanism of negative binding cooperativity is not known for sure. This allosteric interaction might be mediated directly through the dimer interface. Indeed, GPCR activation has been shown to involve the relative movement of transmembrane helical segments, particularly the cytosolic part of helix 6 (Farrens *et al.*, 1996;Gether, 2000), and the modification of the dimer interface might induce a concerted structural change in the other monomer. Alternatively, the allosteric modulation might involve the interaction of the receptor dimer with the G protein. It has been suggested that a GPCR dimer interacts, in an asymmetrical manner, with a single heterotrimeric G protein only (Baneres and Parello, 2003;Hlavackova *et al.*, 2005;Filipek *et al.*, 2004;Goudet *et al.*, 2005) and that G proteins are precoupled to the receptors in the absence of agonist (Gales *et al.*, 2005). According to such a model, the interaction of a G protein heterotrimer with a receptor dimer is necessarily asymetrical, and there are two opposite ways for the G protein to contact the dimer. In addition, we have shown here that G protein coupling is required for high affinity binding of chemokines, and affects the allosteric regulation observed in receptor dimers.

We therefore propose the following mechanism to explain the negative cooperativity between binding sites (Fig. 9). Activation of one of the monomers (R1) is expected to favor the interaction of the receptor dimer with the G protein in such a way that the high affinity ligand binding state on the occupied monomer is stabilized, while the other monomer (R2) remains in an "uncoupled", low-affinity, conformation. During dissociation kinetics, the binding of unlabelled agonist to the monomer R2 (in a low affinity conformation), might promote a shift of the G protein from one

monomer to the other, in such a way that it induces now the release of agonist bound to the first monomer (R1). A major component of the change of receptor-G protein interaction might be the position of the α subunit relative to the agonist-occupied monomer. In keeping with this, we have shown that incubation with Gpp(NH)p is sufficient to promote the release of pre-bound tracer from CCR5, with a kinetics similar to that observed after addition of unlabelled chemokines.

In conclusion, we have demonstrated in the present study the allosteric behavior of chemokine receptor dimers. Allosterism between GPCR monomers could constitute a general molecular mechanism underlying many previous observations of negative binding cooperativity reported since the 70's (Limbird *et al.*, 1975;de Meyts, 1976;Carayon *et al.*, 1979) to the present (Christopoulos and Kenakin, 2002;Park *et al.*, 2004). This property might have important implications in physiology and pharmacology. For receptors expressed in the same cell that are capable of heterodimerization, it would allow cross-inhibition of distinct regulatory signals: one agonist being able to decrease the effect of the other, while exerting its own effects. Allosteric regulation within oligomers suggests that the pharmacological properties of a given receptor could be influenced by the nature of its partners co-expressed in a particular cell type. This notion could directly affect drug discovery programs which until now are based essentially on expression of the receptor of interest in a single standardized cell type thus ignoring putative effects of partners. As a corollary, drugs designed to target a specific receptor could exhibit side effects via allosteric phenomena on the function of the untargeted interacting receptor. Future work will be required to identify, at the molecular level, the conformational changes implicated in GPCR allosterism. In the meantime, it is expected that this functional consequence of GPCR oligomerization will greatly modify current pharmacological practice.

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Footnotes

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LEGENDS FOR FIGURES

Figure 1. Influence of stimulation by chemokines of CCR5/CCR2 heterodimers, as measured in a BRET assay. HEK-293T cells were transfected with a constant amount of the CCR5-h*R*Luc plasmid and increasing amounts of the CCR2-EYFP or $GABA_{B2}$-EYFP as negative control (□, dotted line). Heterodimerization was investigated by measuring the energy transfer between the two partners in the absence of agonist (■, dotted curve) or 5 min after addition of 100 nM MIP-1β (○), MCP-1 (●), MCP-2 (◊) or 50 nM of each MIP-1β and MCP-1 (Δ) at room temperature. The graphs represent the BRET ratio (see materials and methods) over the relative level of expression of CCR2-EYFP or GABAbR2-EYFP and CCR5-h*R*Luc. The analysis was performed using the GraphPad Prism software (v4.0) using non-linear regression and assuming a single-site saturation binding model. This figure is representative of 3 independent experiments. Error bars represent the S.E.M. of triplicate data points.

Figure 2. Dissociation of radiolabelled chemokine tracers from chemokine receptor homo- or heterodimers. CHO-K1 cell lines expressing CCR2, CCR5 or both receptors (C25-12) were described previously (El Asmar *et al.*, 2005). The B_{max} measured in saturation binding assays were 0.51 and 0.62 pmoles/mg protein for CCR2 in CCR2 and C25-12 cell lines respectively, and 2.7 and 2.9 pmoles/mg protein for CCR5 in CCR5 and C25-12 cell lines respectively. Following binding of the tracer (0.1 nM) up to equilibrium, and removal of the free tracer, the membranes were incubated with buffer (■), MCP-1 (□) or MIP-1β (●) for two hours, and the bound tracer was measured at different time points. The analysis was performed using the GraphPad Prism software (v 4.0) using non-linear regression and assuming a single-phase decay model. This figure is the compilation of 3 independent experiments carried out with triplicate data points (error bars indicate S.E.M.). The calculated $t_{1/2}$ values were statistically different according to a two ways ANOVA test followed by Tukey's test.

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Figure 3. A. Dissociation of radiolabelled MCP-1 over a 5 hours period. Following binding of ^{125}I-MCP-1 (0.1 nM) up to equilibrium, and removal of the free tracer, the membranes expressing CCR2 were incubated with buffer (■) or MCP-1 at the final concentration of 100 nM (□) and the bound tracer was measured at different time points. **B. Dissociation of radiolabelled MIP-1β in the presence of various concentrations of unlabelled chemokine.** Following binding of ^{125}I-MIP-1β (0.1 nM) up to equilibrium, and removal of the free tracer, the membranes expressing CCR5 were incubated with buffer (■) or MIP-1β at the final concentration of 100 nM (□), 1 nM (●) or 0.01 nM (○) for one hour, and the bound tracer was measured at different time points. **C. Dissociation of radiolabelled MCP-1 from cells coexpressing CCR2 and CCR5R126N.** Following binding of ^{125}I-MCP-1 (0.1 nM) up to equilibrium, and removal of the free tracer, the cells were incubated with buffer (■), MCP-1 (□) or MIP-1β (○) at 50 nM, and the bound tracer was measured at different time points. **D. Dissociation of radiolabelled MIP-1β on T CD_4^+ lymphoblasts**. Following binding of ^{125}I-MCP-1 (0.1 nM) up to equilibrium, and removal of the free tracer, membranes were incubated with buffer (■), 100 nM MCP-1 (□) or MIP-1β (●) for two hours, and the bound tracer was measured at different time points. The analysis was performed as for Figure 2. The displayed data are representative of 3 independent experiments carried out with triplicate data points (error bars indicate S.E.M.).

Figure 4. A. Dissociation of radiolabelled MIP-1β in the presence of various CCR5 and CCR2 ligands. Following binding of ^{125}I-MIP-1β (0.1 nM) up to equilibrium, and removal of the free tracer, the membranes expressing CCR5 were incubated with buffer alone, various chemokines at 100 nM or TAK-779 at the final concentration of 1 μM, and the bound tracer was measured 90 minutes later. **B. Dissociation of radiolabelled MIP-1β in the presence of monomeric chemokine variants**. Following binding of ^{125}I-MIP-1β (0.1 nM) up to equilibrium,

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and removal of the free tracer, the membranes expressing CCR5 were incubated with buffer alone or chemokines at the final concentration of 100 nM, and the bound tracer was measured 90 minutes later. The displayed data are representative of 3 independent experiments carried out with triplicate data points (error bars indicate S.E.M.).

Figure 5. A. Dissociation of radiolabelled MIP-1β in the presence of anti-CCR5 monoclonal antibodies. Following binding of ^{125}I-MIP-1β (0.1 nM) up to equilibrium, and removal of the free tracer, the membranes expressing CCR5 were incubated with buffer, anti-CCR5 antibodies or a control IgG at the final concentration of 10 μg/ml, and the bound tracer was measured 90 minutes later. **B. Dissociation of radiolabelled MCP-1 in the presence of anti-CCR2 monoclonal antibodies**. Following binding of ^{125}I-MCP-1 (0.1 nM) up to equilibrium, and removal of the free tracer, the membranes expressing CCR2 were incubated with buffer, anti-CCR2 antibodies or a control IgG at the final concentration of 10 μg/ml, and the bound tracer was measured 90 minutes later. The displayed data are representative of 3 independent experiments carried out with triplicate data points (error bars indicate S.E.M.).

Figure 6. Dissociation of radiolabelled chemokines in the presence of anti-CCR5 and anti-CCR2 monoclonal antibodies from cells coexpressing CCR5 and CCR2. A. Following binding of ^{125}I-MIP-1β (0.1 nM) up to equilibrium, and removal of the free tracer, the membranes were incubated with buffer (■), the anti-CCR5 2D7 (●), the anti-CCR2 MAB150 (□) or a control IgG (○) at the final concentration of 10 μg/ml, and the bound tracer was measured at different time points. **B.** Following binding of ^{125}I-MCP-1 (0.1 nM) up to equilibrium, and removal of the free tracer, the membranes were incubated with buffer (■), the anti-CCR5 2D7 (●), the anti-CCR2 MAB150 (□) or a control IgG (○) at the final concentration of 10 μg/ml, and the bound tracer was measured at different time points. The analysis was performed as for Figure 2. The displayed data

are representative of 3 independent experiments carried out with triplicate data points (error bars indicate S.E.M.).

Figure 7. MIP-1β competition binding assay. A. Competition binding assays performed on cells expressing CCR5 alone and preteated with *pertussis* toxin (PTX) at 0.2 µg/ml using ^{125}I-MIP-1β as tracer and unlabelled MIP-1β as competitor. Binding is determined in the absence of competitor (filled bars) or in the presence of 300 nM MIP-1β (open bars). **B**. **Effect of Gpp(NH)p on MIP-1β binding.** Equilibrium binding assay performed on cells expressing CCR5 alone, using ^{125}I-MIP-1β as tracer in the presence of various concentrations of Gpp(NH)p. The data were normalized for binding in the presence of 100 µM Gpp(NH)p (0%), and specific binding in the absence of Gpp(NH)p (100%). **C. Binding of radiolabelled MIP-1β to CCR5 and the mutant CCR5R126N.** Competition binding assays performed on cells expressing CCR5 or the non-signaling mutant CCR5R126N, using ^{125}I-MIP-1β as tracer and unlabelled MIP-1β as competitor. Binding was determined in the absence of competitor (filled bars) or in the presence of 300 nM MIP-1β (open bars).

Figure 8. A. Dissociation of radiolabelled MIP-1β in the presence of Gpp(NH)p. Following binding of ^{125}I-MIP-1β (0.1 nM) up to equilibrium, and removal of the free tracer, the membranes expressing CCR5 were incubated with buffer supplemented (□) or not (■) with Gpp(NH)p at 100 µM, or after addition of unlabelled MIP-1β at the final concentration of 100 nM supplemented (○) or not (●) with Gpp(NH)p at 100 µM, and the bound tracer was measured at different time points. The displayed data are representative of two independent experiments carried out with duplicate data points (error bars indicate S.E.M.).

Figure 9. A. Model of allosteric interactions between GPCR monomers. High affinity binding of ligand 1 (black) to monomer R1 is linked to G-protein interaction with R1 and conformational changes in monomer R2. Subsequent binding of ligand 2 (gray) to monomer R2 triggers conformational changes resulting in a switch of G protein interaction from R1 to R2, followed by ligand 1 dissociation. Ligands 1 and 2 are specific for receptor R1 and R2, respectively. White arrows indicate allosteric interactions between monomers. A similar model can be applied to receptor homodimers. **B. Working hypothesis to explain complete ligand dissociation in cells co-expressing CCR2 (R1) and CCR5 (R2).** Ligand 1 binds monomer R1 within both homo- and heterodimers. Following the binding of ligand 2 to monomer R2, our results suggest that ligand 1 dissociates from monomer R1 in both homo- and heterodimers. Binding of ligand 2 to R2 homodimers might also contribute to this phenomenon (not illustrated). In our model, R2 could induce ligand 1 dissociation either by direct contact with R1 homodimer or less probably by monomer exchange.

Figure 1

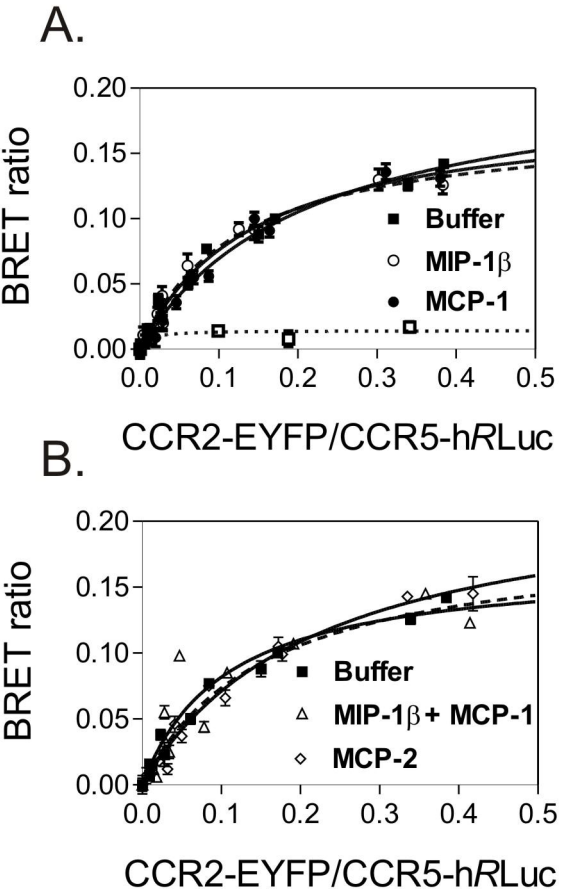

A.
BRET ratio
0.20
0.15
0.10
0.05
0.00
0.0
0.1
0.2
0.3
0.4
0.5
Buffer
MIP-1β
MCP-1
CCR2-EYFP/CCR5-hRLuc
B.
BRET ratio
0.20
0.15
0.10
0.05
0.00
0.0
0.1
0.2
0.3
0.4
0.5
Buffer
MIP-1β+ MCP-1
MCP-2
CCR2-EYFP/CCR5-hRLuc

Figure 2.

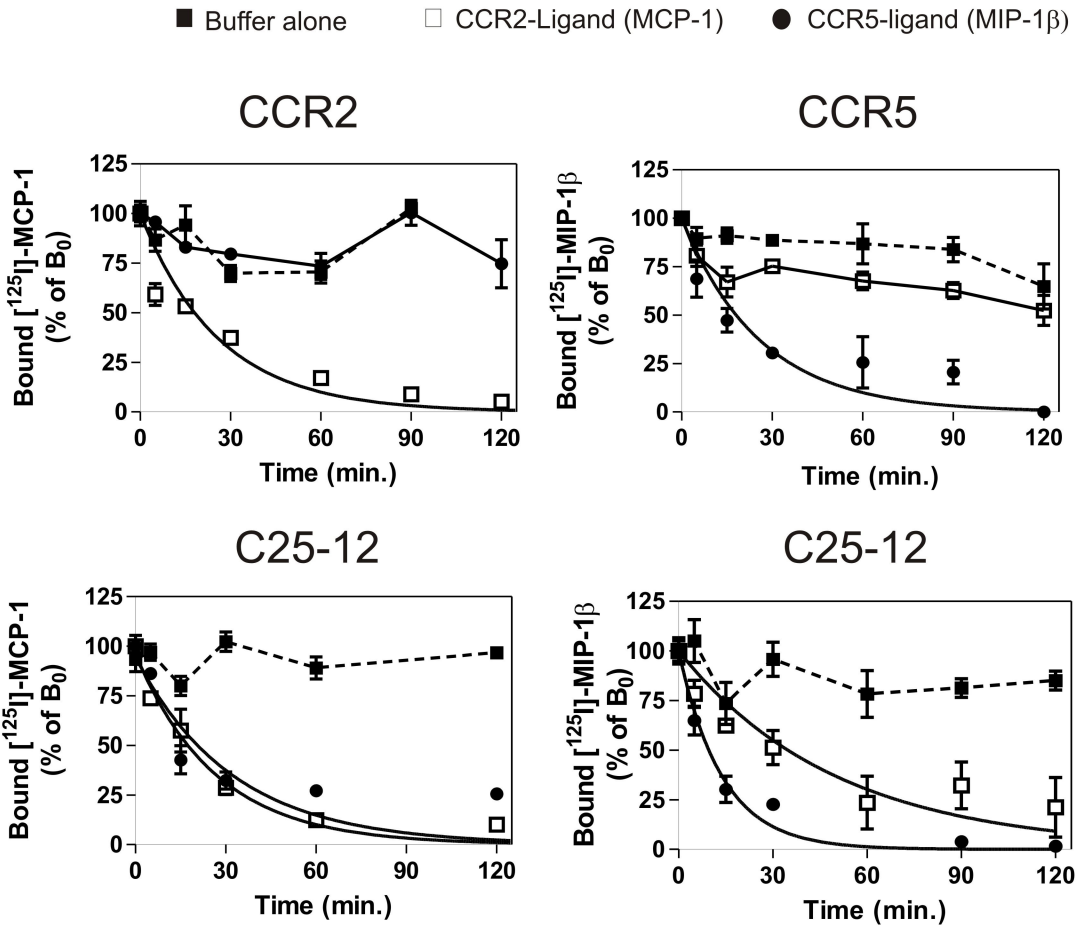

Buffer alone
CCR2-Ligand (MCP-1)
CCR5-ligand (MIP-1β)
CCR2
CCR5
C25-12
C25-12
Bound [125I]-MCP-1 (% of B0)
Bound [125I]-MIP-1β (% of B0)
Time (min.)
0
25
50
75
100
125
0
30
60
90
120

Figure 3

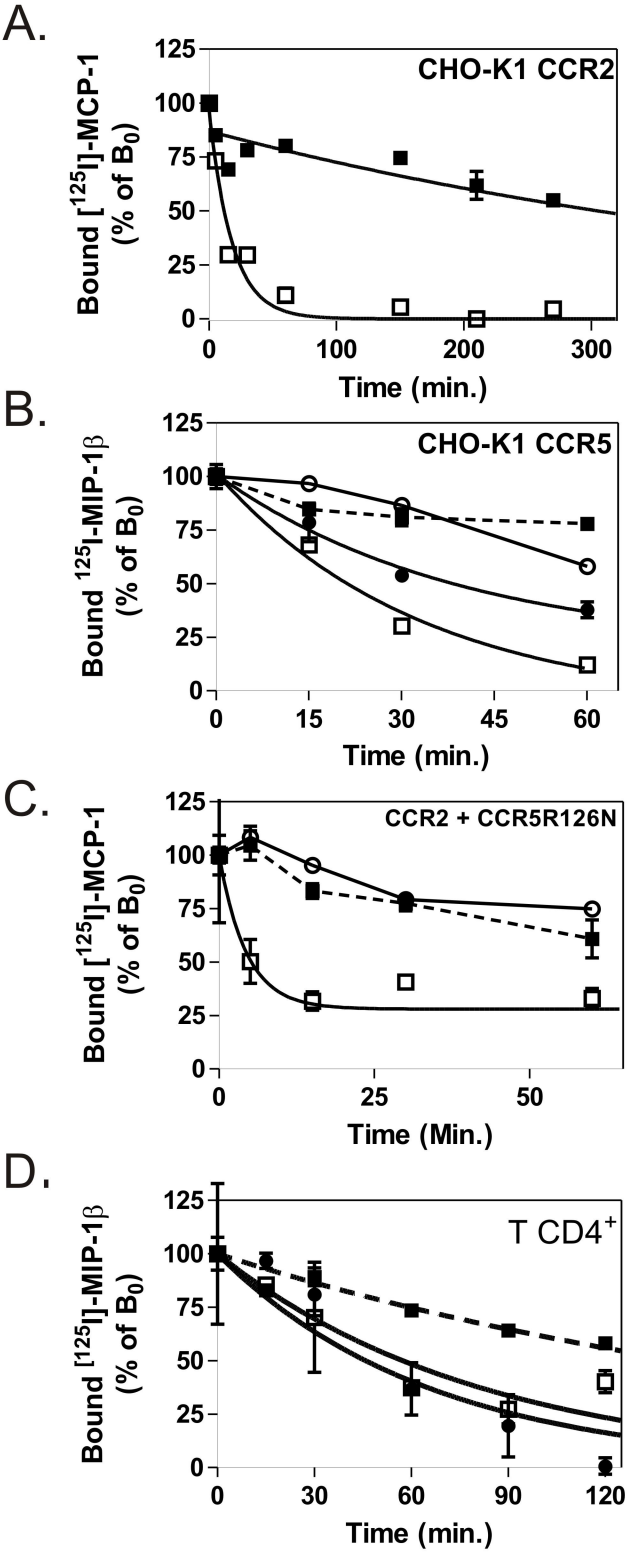

A.
CHO-K1 CCR2
Bound [125I]-MCP-1 (% of B0)
Time (min.)
B.
CHO-K1 CCR5
Bound 125I-MIP-1β (% of B0)
Time (min.)
C.
CCR2 + CCR5R126N
Bound [125I]-MCP-1 (% of B0)
Time (Min.)
D.
T CD4+
Bound [125I]-MIP-1β (% of B0)
Time (min.)

Figure 4

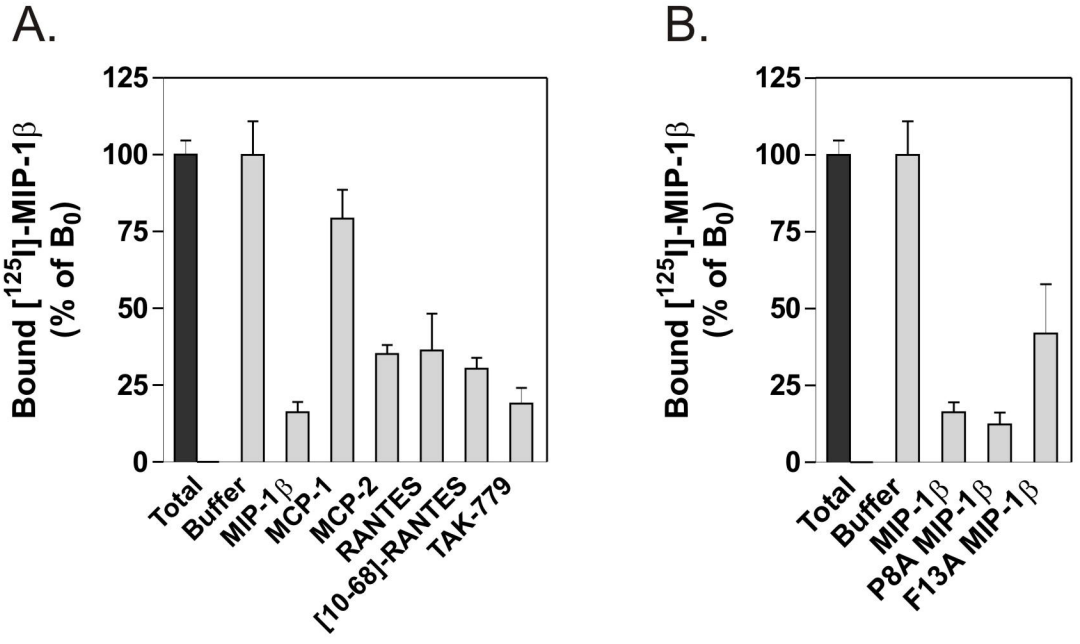

A.
B.
Bound [125I]-MIP-1β (% of B0)
125
100
75
50
25
0
Total
Buffer
MIP-1β
MCP-1
MCP-2
RANTES
[10-68]-RANTES
TAK-779
P8A MIP-1β
F13A MIP-1β

Figure 5

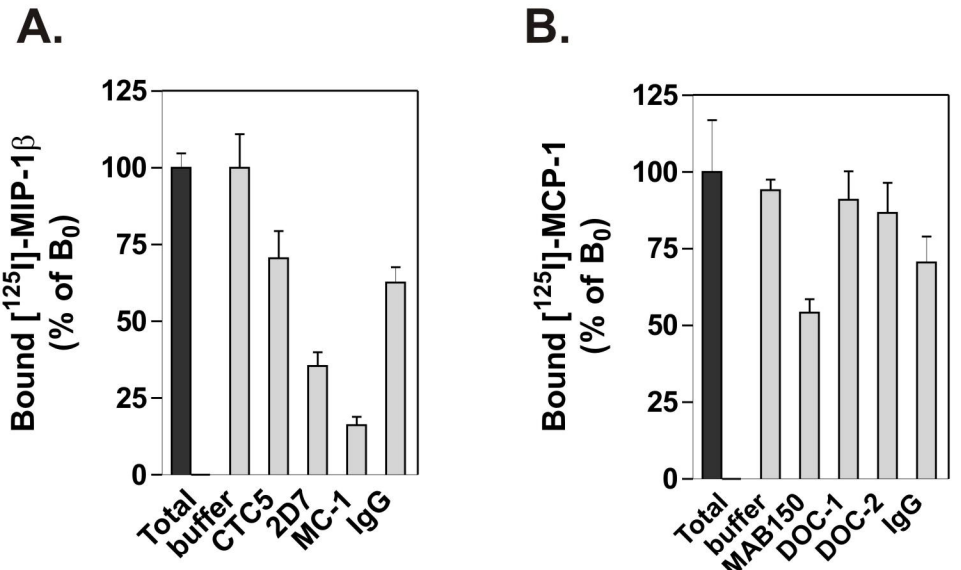

A.
Bound [125I]-MIP-1β (% of B0)
125
100
75
50
25
0
Total
buffer
CTC5
2D7
MC-1
IgG
B.
Bound [125I]-MCP-1 (% of B0)
125
100
75
50
25
0
Total
buffer
MAB150
DOC-1
DOC-2
IgG

Figure 6

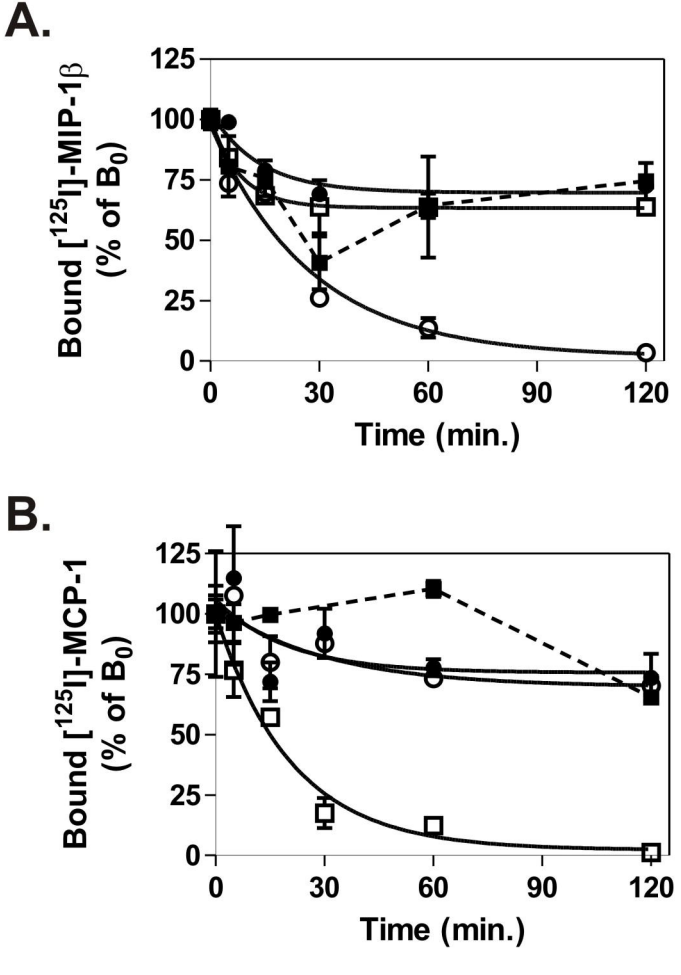

A.
Bound [125I]-MIP-1β
(% of B0)
125
100
75
50
25
0
0
30
60
90
120
Time (min.)
B.
Bound [125I]-MCP-1
(% of B0)
125
100
75
50
25
0
0
30
60
90
120
Time (min.)

Figure 7

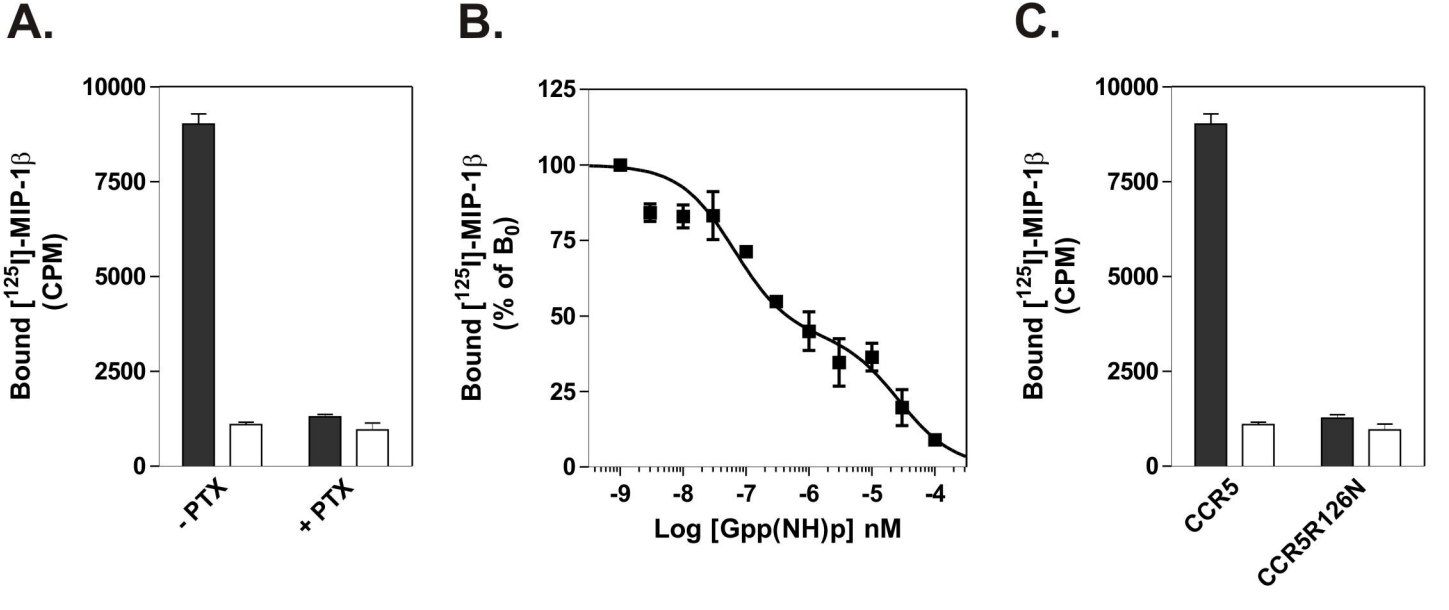

A.
B.
C.
10000
7500
5000
2500
0
Bound [125I]-MIP-1β (CPM)
- PTX
+ PTX
125
100
75
50
25
0
Bound [125I]-MIP-1β (% of B0)
-9
-8
-7
-6
-5
-4
Log [Gpp(NH)p] nM
CCR5
CCR5R126N

Figure 8

A.

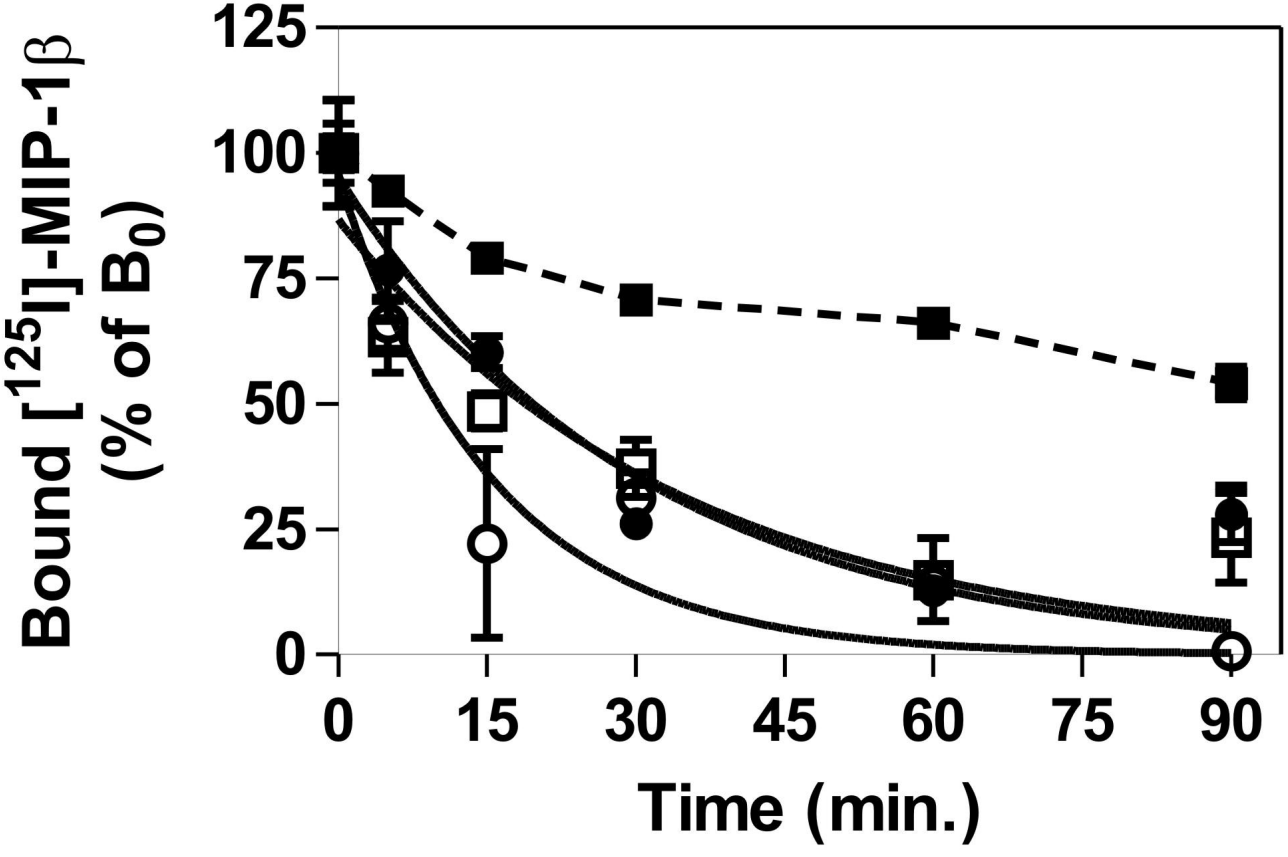
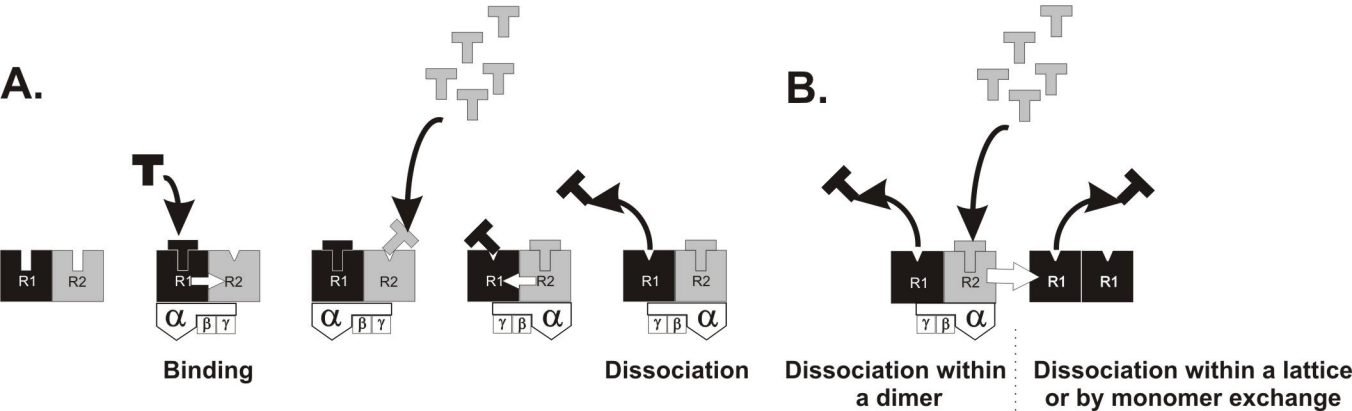

Bound [125I]-MIP-1β
(% of B0)
125
100
75
50
25
0
0
15
30
45
60
75
90
Time (min.)

Figure 9


A.
B.
T
R1
R2
α
β
γ
Binding
Dissociation
Dissociation within a dimer
Dissociation within a lattice or by monomer exchange