

Allosteric Modulation of Binding Properties between Units of Chemokine Receptor Homo- and Hetero-Oligomers

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ABSTRACT

We have demonstrated previously 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 bioluminescence resonance energy transfer 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 unlabeled 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 phenomenon does not require the full conformational change associated with receptor activa-

tion. Finally, we show that G protein coupling is required for high-affinity binding of macrophage inflammatory protein-1 β (CCL4) to CCR5 and that the dissociation from G proteins, after incubation with Gpp(NH)p, promotes the release of rebound radiolabeled chemokines with kinetics similar to those measured after the addition of an excess of unlabeled chemokines. These observations suggest that the association with G proteins probably participates in the negative cooperativity observed between receptor monomers. We propose that negative cooperativity within homo- and heterodimers of chemokine receptors and probably other G protein-coupled receptors will probably have major implications in their pharmacology in vivo and in the physiopathology of the diseases with which they are associated.

The chemokine receptors CCR5 and CCR2 are closely related members of class 1 G protein-coupled receptors (GPCRs). 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-1 α (CCL3), MIP-1 β (CCL4), RANTES (CCL5), MCP-2 (CCL8), and a truncated form of hemofiltrate CC chemo-

kine-1 (CCL14) with high affinity (Blanpain et al., 1999), whereas 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 coreceptor for macrophage-tropic strains of human immunodeficiency virus, 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 lin-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; RANTES, regulated on activation normal T cell expressed and secreted; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; CHO, Chinese hamster ovary; EYFP, enhanced yellow fluorescent protein; BRET, bioluminescence resonance energy transfer; DMEM, Dulbecco's modified Eagle's medium; RT, room temperature; mAb, monoclonal antibody; PTX, pertussis toxin; hRLuc, humanized form of *Renilla reniformis* luciferase; TAK-779, *N,N*-dimethyl-*N*-(4-(((2-(4-methylphenyl))-6,7-dihydro-5H-benzocyclohepten-8-yl)carbonyl)amino)benzyl)tetrahydro-2H-pyran-4-aminium chloride.

eage (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; El Asmar et al., 2005; Percherancier et al., 2005). Subcellular fractionation followed by bioluminescence resonance energy transfer (BRET) measurements have suggested that homodimerization of CCR5 occurs shortly after synthesis in the endoplasmic reticulum (Issafras et al., 2002). In a previous study, we investigated the functional consequences of dimerization in recombinant cells expressing CCR2, CCR5, or both receptors. We showed that CCR5-specific ligands (MIP-1 α , MIP-1 β , 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 coexpressed. Similar observations were made for the CCR2-selective ligand MCP-1 that competed efficiently for MIP-1 β 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 seemed important for understanding how a receptor dimer works, its molecular bases were poorly characterized. In the present study, we used ligand dissociation assays after "infinite" dilution of the tracer to demonstrate that the negative cooperativity between receptor monomers is of an 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.

Materials and Methods

Cell Lines, Leukocyte 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 are described elsewhere (El Asmar et al., 2005). Human T CD₄⁺ 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 reniformis* 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 were performed as described previously (Urizar et al., 2005). Human embryonic kidney 293 cells were transfected by the calcium phosphate precipitation method with different receptor combinations. A control corresponding to mock-transfected cells was included to subtract the raw basal luminescence. Forty-eight hours after transfection, the BRET measurement was performed using a Mithras LB 940 Multilabel Reader (Berthold Technologies, Bad Wildbad, Germany) as described previously (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.

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 [¹²⁵I]-MCP-1 or [¹²⁵I]-MIP-1 β in a final volume of 500 μ l of assay buffer (50 mM HEPES, pH 7.4, 1 mM

CaCl₂, 5 mM MgCl₂, and 0.5% bovine serum albumin). The estimated K_d value for CCR5 and CCR2 for their respective ligands (MIP-1 β and MCP-1) in binding assays were consistent with values described previously (respectively, 180 \pm 10 and 52 \pm 8 pM; Samson et al., 1997). After 1 h of incubation with the tracer, the membranes were centrifuged for 5 min at 15,000g 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 concentration of unlabeled MCP-1 or MIP-1 β . At different time points after resuspension, aliquots were collected, the bound tracer was separated by filtration through GF/B filters presoaked for 1 h in 0.5% polyethylenimine, and the filters were counted for 1 min in a γ -scintillation counter. Total and bound radioligands were also measured immediately after membrane resuspension 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 5-fold dilution of the incubation medium after membrane resuspension, the tracer dilution factors were on average 60 and 72 for, respectively, the MIP-1 β 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 reassociation (data not shown). The data are presented as the ratio between bound counts per minute at the various dissociation time points and total bound counts per minute at time 0 of dissociation. The curves were fitted with the use of Prism software (version 4.0; GraphPad Software Inc., San Diego, CA) using nonlinear regression and a single-phase decay model. The statistical analysis of $t_{1/2}$ values was performed by the same software using a two-way analysis of variance test followed by Tukey's test.

Intracellular Calcium Mobilization Assays. The functional response to chemokines was analyzed with an aequorin-based assay as described previously (El Asmar et al., 2005). In brief, cells were harvested from plates with Ca²⁺- and Mg²⁺-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 10⁶ cells/ml and incubated for 4 h in the dark in the presence of 5 μ M coelenterazine H (Promega, Madison, WI). Cells were then diluted 5-fold before use. Variable concentrations of chemokines in a volume of 50 μ l of DMEM were added to 50 μ 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). EC₅₀ values were determined with the GraphPad Prism software using nonlinear regression coupled to a sigmoidal dose-response model. The reported values are the mean \pm S.E.M. of at least three independent experiments.

Results

Influence of the Stimulation by Chemokines on the CCR2/CCR5 Heterodimerization. We have reported previously 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 con-

ditions has not yet been investigated. As described previously (El Asmar et al., 2005), energy transfer was observed between CCR5-hRLuc and CCR2-EYFP (Fig. 1) and CCR2-hRLuc and CCR5-EYFP (data 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-hRLuc and CCR2-hRLuc. 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, whereas the unrelated receptors ($GABA_{B2}$) did not (data not shown). As shown previously for the CCR5 (Issafras et al., 2002) and CCR2 homodimers (J.-Y. Springael and M. Parmentier unpublished observations), we observed that the addition of ligands specific for CCR5 (MIP-1 β) 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 binding has no influence on the homo- and heterodimerization states of chemokine receptors (Benkirane et al., 1997; Issafras et al., 2002; El Asmar et al., 2005; Springael et al., 2005).

Negative Binding Cooperativity within CCR2/CCR5 Heterodimers. We next tested further the pharmacological

properties of CCR5/CCR2 heterodimers using binding assays. We demonstrated previously 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). To determine more precisely the mechanism underlying these effects, we built on the model of chemokine receptor dimers and performed dissociation kinetics experiments after extensive ligand dilution, a procedure that constitutes the classic way of investigating allosteric interactions between distinct binding sites (de Meyts et al., 1973; Christopoulos et al., 1997). 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 radiolabeled chemokine tracer was allowed to bind to the receptors at equilibrium, after which the free tracer was removed by aspiration, and the incubation medium was further diluted 5-fold. Radiolabeled ligand dissociation was assayed over time in the assay buffer, in the absence or presence of unlabeled chemokines at various concentrations. This protocol allows the 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 prebound ^{125}I -MCP-1 was slow in the absence but strongly accelerated in the presence of 100 nM concentration of unlabeled MCP-1 ($t_{1/2}$, 19 ± 3 min; Fig. 2). Kinetics performed over longer periods showed slow but substantial dissociation of MCP-1 over time ($t_{1/2}$, approximately 350 min; Fig. 3A). Complete dissociation was obtained by 24 h (data not shown). MIP-1 β , a specific CCR5 ligand, had no effect in these conditions, whereas it completely dissociated bound ^{125}I -MCP-1 from cells coexpressing CCR2 and CCR5 ($t_{1/2}$, 20 ± 8 min, $P < 0.001$). Likewise, MCP-1 at 25 nM, a concentration well lower than its K_d on CCR5, promoted a rapid dissociation of ^{125}I -MIP-1 β from CCR5 in cells in which CCR2 was coexpressed compared with cells expressing CCR5 alone ($t_{1/2}$, 27.7 ± 7.1 and 122 ± 13 min, respectively, $P < 0.01$). The slow dissociation rate of ^{125}I -MIP-1 β 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 unlabeled chemokines (Fig. 3B). The concentration required for half-dissociation seemed 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-1 β (see below). We could show in BRET experiments that the R126N mutant is able to het-

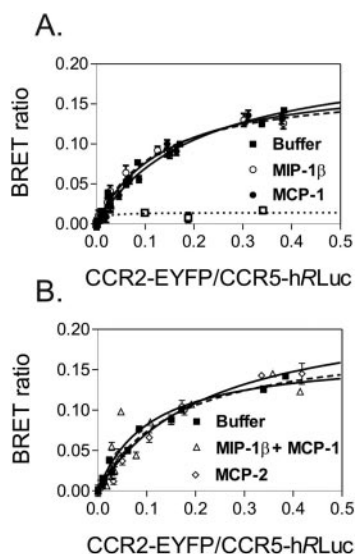


Fig. 1. Influence of stimulation by chemokines of CCR5/CCR2 heterodimers as measured in a BRET assay. Human embryonic kidney 293T cells were transfected with a constant amount of the CCR5-hRLuc plasmid and increasing amounts of the CCR2-EYFP or $GABA_{B2}$ -EYFP as negative control (\square , broken line). Heterodimerization was investigated by measuring the energy transfer between the two partners in the absence of agonist (\blacksquare , dotted curve) or 5 min after the addition of 100 nM MIP-1 β (\circ), MCP-1 (\bullet), MCP-2 (\diamond), or 50 nM concentration of each MIP-1 β and MCP-1 (\triangle) at room temperature. The graphs represent the BRET ratio (see *Materials and Methods*) over the relative level of expression of CCR2-EYFP or $GABA_{B2}$ -EYFP and CCR5-hRLuc. The analysis was performed using the GraphPad Prism software (version 4.0) using non-linear regression and assuming a single-site saturation binding model. This figure is representative of three independent experiments. Error bars represent the S.E.M. of triplicate data points.

erodimerize with CCR2 as efficiently as wild-type CCR5 (data not shown). In cells coexpressing both receptors, however, MIP-1 β was unable to increase the dissociation rate of 125 I-MCP-1 (Fig. 3C). Finally, to explore whether this phenomenon could take place in primary cells in which CCR2 and CCR5 are naturally coexpressed, we performed dissociation kinetics of 125 I-MIP-1 β on membranes prepared from human CD4 $^{+}$ T lymphoblasts. Dissociation of prebound 125 I-MIP-1 β was accelerated in the presence of unlabeled MCP-1 or MIP-1 β (Fig. 3D), suggesting that CCR2/CCR5 heterodimers do indeed exist in native cells and that negative binding cooperativity among homo- and heterodimers is physiologically relevant.

Effects of an Inverse Agonist and Monoclonal Antibodies on Chemokine Binding. 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-1 β , RANTES and MCP-2 also increased 125 I-MIP-1 β 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 nonpeptidic inverse agonist, also increased the dissociation rate of 125 I-MIP-1 β (Fig. 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 α -helices and to inhibit chemokine binding in a noncompetitive, allosteric manner (Christopoulos and Kenakin, 2002). 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 (Cloue et al., 1990). However, it is largely accepted that only the monomeric forms of chemo-

kines 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-1 β were described previously (Laurence et al., 2001). To ascertain whether our observations were independent of ligand dimerization, we tested the effects of two such monovalent chemokines, P8A-MIP-1 β and F13A-MIP-1 β , on 125 I-MIP-1 β dissociation and found that both mutant chemokines increased the tracer dissociation rate (Fig. 4B). We next tested the influence of monoclonal antibodies recognizing specifically CCR5 or CCR2. The functional properties of some of these antibodies have been described elsewhere (Lee et al., 1999; Blanpain et al., 2002). The anti-CCR5 2D7 and MC-1, which are directed against the second extracellular loop of CCR5, increased the dissociation rate of 125 I-MIP-1 β , 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 (Fig. 5). In C25-12 cells coexpressing CCR2 and CCR5 (El Asmar et al., 2005), the anti-CCR5 mAb 2D7 increased the dissociation rate of 125 I-MIP-1 β 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-1 β dissociation (Fig. 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

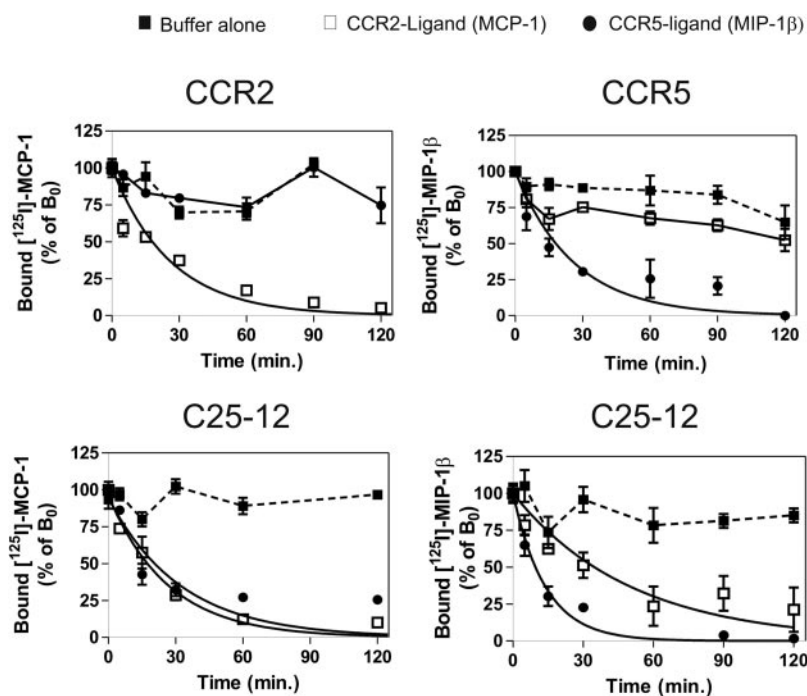


Fig. 2. Dissociation of radiolabeled chemokine tracers from chemokine receptor homo- or heterodimers. CHO-K1 cell lines expressing CCR2, CCR5, or both receptors (C25-12) were described previously (El Asmar et al., 2005). The B_{max} values measured in saturation binding assays were 0.51 and 0.62 pmol/mg of protein for CCR2 in CCR2 and C25-12 cell lines, respectively, and 2.7 and 2.9 pmol/mg of protein for CCR5 in CCR5 and C25-12 cell lines, respectively. After 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-1 β (●) for 2 h, and the bound tracer was measured at different time points. The analysis was performed using the GraphPad Prism software (version 4.0) using nonlinear regression and assuming a single-phase decay model. This figure is the compilation of three 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-way analysis of variance test followed by Tukey's test.

activated/coupled form of the receptor that displays the highest affinity for its agonists (De Lean et al., 1980; Kenakin, 1996). 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

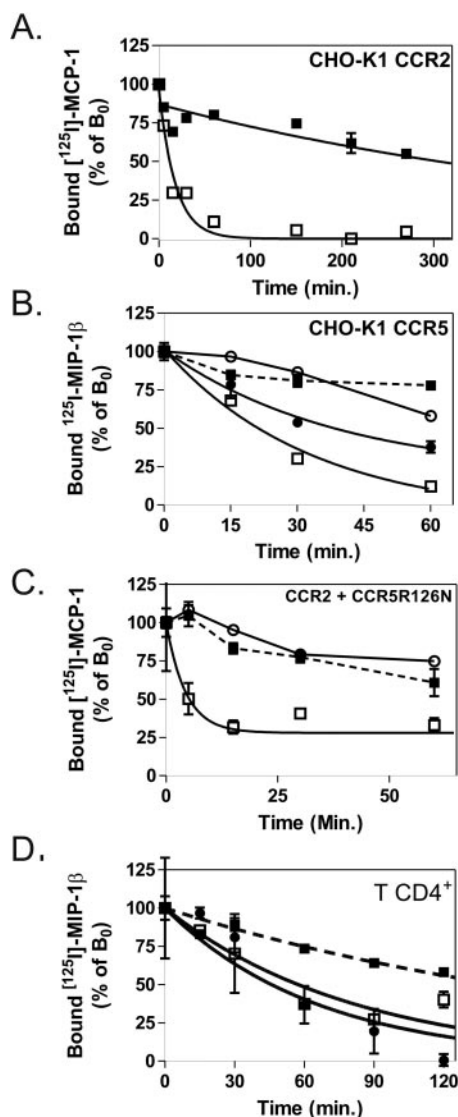


Fig. 3. A, dissociation of radiolabeled MCP-1 over a 5-h period. After binding of ¹²⁵I-MCP-1 (0.1 nM) up to equilibrium and removal of the free tracer, the membranes expressing CCR2 were incubated with buffer (■) or MCP-1 at the final concentration of 100 nM (□), and the bound tracer was measured at different time points. B, dissociation of radiolabeled MIP-1β in the presence of various concentrations of unlabeled chemokine. After binding of ¹²⁵I-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 1 h, and the bound tracer was measured at different time points. C, dissociation of radiolabeled MCP-1 from cells coexpressing CCR2 and CCR5R126N. After binding of ¹²⁵I-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 radiolabeled MIP-1β on T CD4⁺ lymphoblasts. After binding of ¹²⁵I-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 2 h, and the bound tracer was measured at different time points. The analysis was performed as for Fig. 2. The displayed data are representative of three independent experiments carried out with triplicate data points (error bars indicate S.E.M.).

investigated the effects of G protein coupling on the binding of radiolabeled MIP-1β to CCR5. For this purpose, we measured the binding of ¹²⁵I-MIP-1β on CCR5 from cells pretreated or not with pertussis toxin (PTX) and showed that PTX decreased strongly ¹²⁵I-MIP-1β binding down to undetectable levels (Fig. 7A). We next explored the binding of ¹²⁵I-MIP-1β to membrane preparations in the presence or absence of Gpp(NH)p, a nonhydrolyzable analog of GTP. As shown in Fig. 7B, the addition of Gpp(NH)p in the binding assay reduced in a concentration-dependent way the binding of ¹²⁵I-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 (Staudinger et al., 2001). One could therefore predict that mutations preventing

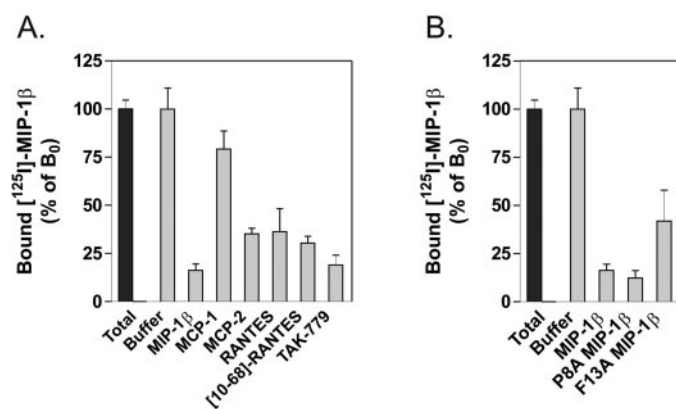


Fig. 4. A, dissociation of radiolabeled MIP-1β in the presence of various CCR5 and CCR2 ligands. After binding of ¹²⁵I-MIP-1β (0.1 nM) up to equilibrium, and removal of the free tracer, the membranes expressing CCR5 were incubated with buffer alone, various chemokines at 100 nM or TAK-779 at the final concentration of 1 μM, and the bound tracer was measured 90 min later. B, dissociation of radiolabeled MIP-1β in the presence of monomeric chemokine variants. After binding of ¹²⁵I-MIP-1β (0.1 nM) up to equilibrium 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 min later. The displayed data are representative of three independent experiments carried out with triplicate data points (error bars indicate S.E.M.).

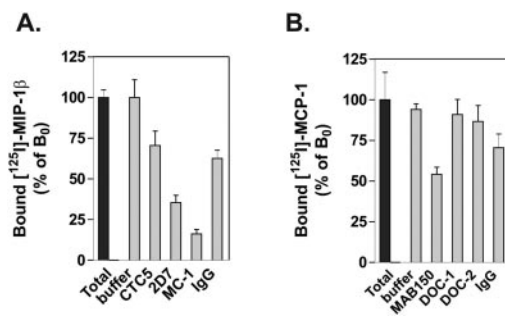


Fig. 5. A, dissociation of radiolabeled MIP-1β in the presence of anti-CCR5 monoclonal antibodies. After binding of ¹²⁵I-MIP-1β (0.1 nM) up to equilibrium and removal of the free tracer, the membranes expressing CCR5 were incubated with buffer, anti-CCR5 antibodies, or a control IgG at the final concentration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

G protein-coupling would affect chemokine binding. To test this hypothesis, ^{125}I -MIP-1 β 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-1 β to this mutant was found to be strongly decreased compared with wild-type CCR5 (Fig. 7C). We have

shown above that MIP-1 β could not increase ^{125}I -MCP-1 dissociation from cells coexpressing CCR2 and the R126N CCR5 mutant. This observation, however, might be due either to the lack of coupling of the mutant or to its low affinity for chemokines. To investigate further whether G protein coupling might influence the allosteric regulation within receptor dimers, we carried out ^{125}I -MIP-1 β dissociation experiments in the presence or absence of Gpp(NH)p. As shown in Fig. 8, the addition of Gpp(NH)p in the assay buffer increased the dissociation rate of ^{125}I -MIP-1 β from CCR5, with a kinetics similar to that resulting from the addition of unlabeled MIP-1 β . The addition of Gpp(NH)p in combination with MIP-1 β increased slightly the dissociation rate. Taken together, these data suggest that G protein uncoupling is sufficient to promote MIP-1 β dissociation, probably 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 apoequoprin-expressing CCR5R^{126N} mutant CHO-K1 cells with CCR2 or CCR5 receptors and analyzed the functional response of CCR2 and CCR5. The coexpression of the R126N mutant did not impair significantly CCR2 or CCR5 signaling, indicating the absence of dominant-negative effect of the nonfunctional mutant. These data support the view that a single G protein is sufficient for promoting signaling through a dimer.

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 in other receptor families. In the present study, we analyzed in detail the heterodimerization of CCR5 and CCR2 as a model of

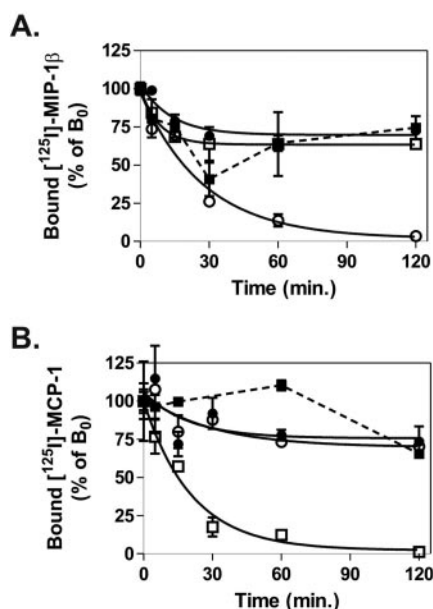


Fig. 6. Dissociation of radiolabeled chemokines in the presence of anti-CCR5 and anti-CCR2 monoclonal antibodies from cells coexpressing CCR5 and CCR2. **A**, After binding of ^{125}I -MIP-1 β (0.1 nM) up to equilibrium and removal of the free tracer, the membranes were incubated with buffer (■), the anti-CCR5 2D7 (○), the anti-CCR2 MAB150 (□), or a control IgG (●) at the final concentration of 10 $\mu\text{g}/\text{ml}$, and the bound tracer was measured at different time points. **B**, After 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 $\mu\text{g}/\text{ml}$, and the bound tracer was measured at different time points. The analysis was performed as for Fig. 2. The displayed data are representative of three independent experiments carried out with triplicate data points (error bars indicate S.E.M.).

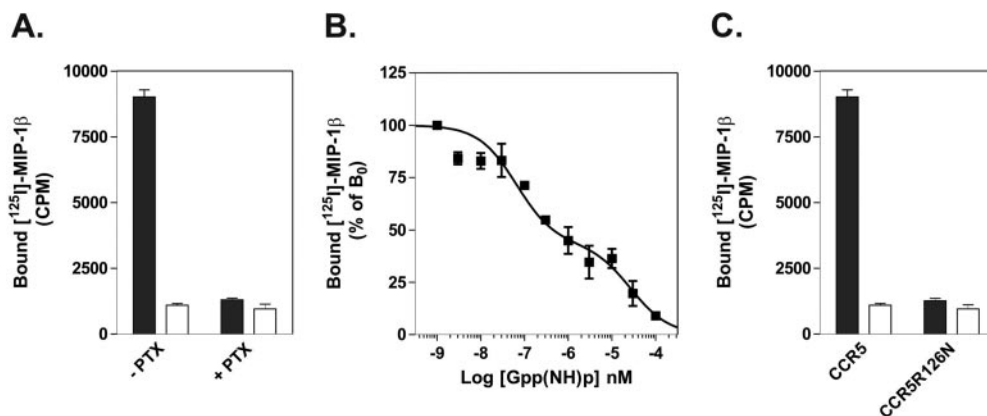


Fig. 7. MIP-1 β competition binding assay. **A**, competition binding assays performed on cells expressing CCR5 alone and pretreated with PTX at 0.2 $\mu\text{g}/\text{ml}$ using ^{125}I -MIP-1 β as tracer and unlabeled MIP-1 β as competitor. Binding is determined in the absence of competitor (■) or in the presence of 300 nM MIP-1 β (□). **B**, effect of Gpp(NH)p on MIP-1 β binding. Equilibrium binding assay performed on cells expressing CCR5 alone, using ^{125}I -MIP-1 β 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 radiolabeled MIP-1 β to CCR5 and the mutant CCR5R126N. Competition binding assays performed on cells expressing CCR5 or the nonsignaling mutant CCR5R126N using ^{125}I -MIP-1 β as tracer and unlabeled MIP-1 β as competitor. Binding was determined in the absence of competitor (■) or in the presence of 300 nM MIP-1 β (□).

chemokine receptor dimerization, and the mechanisms associated with the negative binding cooperativity. CCR5 and CCR2 are particularly well adapted for this purpose, because they share an almost identical transmembrane helix bundle but display 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 (Benkirane et al., 1997; Issafras et al., 2002; El Asmar et al., 2005; Springael et al., 2005) 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 after the addition of agonists has been attributed to conformational changes within CXCR4 homodimers and CXCR4/CCR2 heterodimers (Percherancier et al., 2005). Because 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 dimers are stable and nonexchangeable is consistent with the current view prevailing for other classes of GPCRs (Bulenger et al., 2005).

We have shown previously 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 coexpressed. Similar observations were made for the CCR2-selective ligand MCP-1, which competed efficiently for MIP-1 β binding on cells expressing both receptors but was 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 classic approach to demonstrate negative cooperativity across two distinct binding sites has been to perform dissociation kinetics experiments after extensive dilution of the tracer, as first proposed for the insulin receptor (de Meyts et al., 1973; Kostenis and Mohr, 1996; Christopoulos et al., 1997; Pizard

et al., 1998; Urizar et al., 2005). 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 unlabeled 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 *Materials and Methods*). MIP-1 β 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 cells coexpressing CCR5 and CCR2, the tracer dissociation rate from one receptor was increased by unlabeled 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-1 β from CCR5 was faster and stronger, suggesting the involvement of the high-affinity binding site of CCR2. The inability of MIP-1 β to 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 strict requirements. 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 (Fig. 9A). When considering CCR5 or CCR2 homodimers, the amount of radiolabeled chemokine remaining bound after the fast dissociation promoted by the unlabeled agonist is very close to the nonspecific 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 (Mercier et al., 2002; Fotiadis et al., 2003; Guo et al., 2003). The ability of unlabeled ligands to promote complete tracer dissociation from cells coexpressing 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 (Fig. 9B) (Levitzki, 1974; Fotiadis et al., 2003). Alternatively, an exchange of subunits between homo- and heterodimeric complexes, in the course of the experiment, could also account for the observation (Fig. 9B) (Gouldson et al., 1998), although the stability of the BRET signal after 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 coexpress 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. These results might be due to the presence of other proteins in lymphoblast membranes that are not

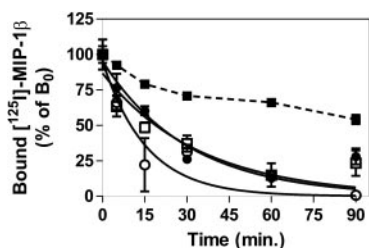


Fig. 8. Dissociation of radiolabeled MIP-1 β in the presence of Gpp(NH)p. After binding of ^{125}I -MIP-1 β (0.1 nM) up to equilibrium and removal of the free tracer, the membranes expressing CCR5 were incubated with buffer supplemented (\square) or not (\blacksquare) with Gpp(NH)p at 100 μM or after the addition of unlabeled MIP-1 β at the final concentration of 100 nM supplemented (\circ) or not (\bullet) 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.).

present at the same relative level in CHO cells but potentially affect the function of receptor oligomers and modify 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 occurring in native cells.

We showed that negative binding cooperativity does not require chemokine dimerization. Indeed, monovalent forms of MIP-1 β , which were shown previously 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, which is 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). Some monoclonal antibodies, particularly those shown previously 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 coexpressed 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 that involve interactions within higher order receptor oligomers cannot be excluded. It was shown previously that the bridging capabilities of anti-CCR5 antibodies were important for some of their biological properties, because their monovalent forms could bind the receptor but were devoid of functional activity (Blanpain et al., 2002; Issafras et al., 2002).

Our results suggest that under most physiological conditions (i.e., at low agonist concentration), a single mole-

cule 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. On the other hand, 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 Parelo, 2003; Filipek et al., 2004; Goudet et al., 2005; Hlavackova 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 asymmetrical, 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, whereas the other monomer (R2) remains in an "uncoupled", low-affinity conformation. During dissociation kinetics, the binding of unlabeled 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 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 prebound tracer from CCR5, with a

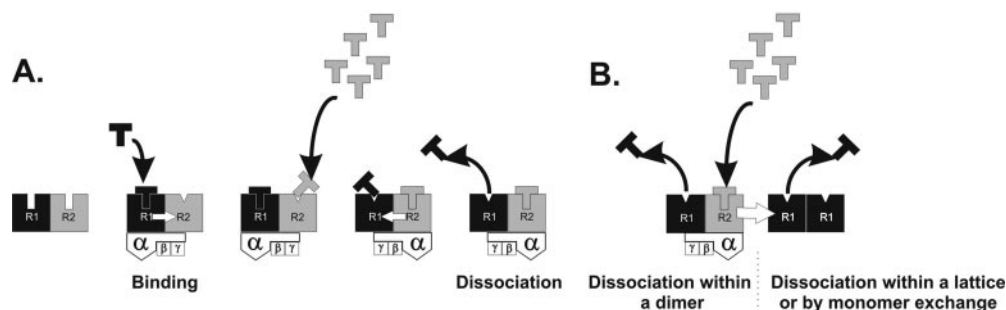


Fig. 9. A, model of allosteric interactions between GPCR monomers. High-affinity binding of ligand 1 (black) to monomer R1 is linked to G protein interaction with R1 and conformational changes in monomer R2. Subsequent binding of ligand 2 (gray) to monomer R2 triggers conformational changes resulting 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 coexpressing CCR2 (R1) and CCR5 (R2). Ligand 1 binds monomer R1 within both homo- and heterodimers. After 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.

kinetics similar to that observed after the addition of unlabeled 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 1970s (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 coexpressed in a particular cell type. This notion could directly affect drug discovery programs which until now were based essentially on the 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 is 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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