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Allosteric Regulation of CCR5 by Guanine Nucleotides and HIV-1 Envelope[1]

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The chemokine receptor CCR5 is the principal coreceptor for R5 (macrophage-tropic) strains of HIV-1. CCR5 uses G-proteins as transducing elements. Here we report the biochemical consequences of the interaction between CCR5 and G-proteins. Macrophage inflammatory protein-1β (MIP-1β) binding to CCR5 was potently and specifically inhibited by guanine nucleotides. The molecular mechanism of this inhibitory effect was shown to be a dose-dependent reduction in MIP-1β receptors. We also show that the MIP-1β binding site is allosterically regulated by monovalent cations and that binding of this endogenous agonist is highly temperature sensitive and dependent on divalent cations, characteristic of a G-protein-coupled receptor (GPCR). HIV-1 envelope glycoprotein decreased the affinity of CCR5 for MIP-1β but also altered the kinetics of MIP-1β binding to CCR5, proving that it interacts with a distinct, but allosterically coupled binding site. The findings described herein contribute to our understanding of how CCR5 interacts with chemokines and HIV-1 envelope.

Key Words: **chemokine receptor; CCR5; G-protein-coupled receptor; HIV-1 envelope; macrophage inflammatory protein-1β; guanine nucleotides.**

Chemokines provide directional cues for the movement of leukocytes in development, homeostasis, and inflammation (1–5). The dramatic increase in the secretion of chemokines during inflammation results in the selective recruitment of leukocytes, including T-lymphocytes, into inflamed tissues. Generally, chemokines act on more than one type of leukocyte, and *in vitro* responses include chemotaxis, enzyme release from intracellular stores, oxygen-radical formation, cytoskeletal rearrangements, generation of lipid mediators and induction of adhesion to endothelium or extracellular matrix proteins (3–5). More recently, chemokines and their receptors have been also shown to play a key role in the regulation of angiogenesis and neuronal cell regulation (6, 7). In 1996, the chemokine receptor CCR5 was identified as the principal HIV-1 coreceptor for R5 tropic isolates (8–12). This discovery was based on observations that a number of CC chemokines can suppress infection of T-cells with R5 tropic, but not X4 tropic HIV-1 strains (13). The importance of chemokine receptors in the events leading to HIV-1 infection was reinforced by the discovery that homozygosity for a 32-base-pair deletion in the human CCR5 gene, which produces a truncated protein that is not expressed on the cell surface, was found to confer resistance to infection by HIV-1 (14–16). HIV-1 viral envelope glycoprotein has also been shown to inhibit chemokine binding to CCR5, however the mechanism of this inhibitory effect has not been investigated.

Chemokine receptors belong to the superfamily of GTP-binding protein coupled receptors (GPCR) (3). Activation of a GPCR by an agonist engenders a conformational change in the receptor that promotes interaction with G-protein, which results in substitution of GDP with GTP and subsequent hydrolysis of GTP. In the presence of GTP, the complex is not stable and the G-protein dissociates into subunits, and the receptor is uncoupled from the G-protein (17, 18). Hereby, guanine nucleotides regulate the interaction of ligand with receptor by promoting the conversion of the receptor from a high-affinity to a low-affinity state. This has been documented for many receptors, the most thoroughly studied of which has been the β-adrenergic receptor (19). Here we describe that guanine nucleotides modulate the number of binding sites for MIP-1β without the appearance of a detectable low-affinity

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binding state. We also show that the gp120–sCD4 complex inhibits chemokine binding to CCR5 by interaction with a distinct, allosterically coupled binding site.

MATERIALS AND METHODS

Materials

Recombinant soluble CD4 (sCD4) was produced in CHO cells (Intracel, Issaquah, WA), ^{125}I-MIP-1β (sp act 2200 Ci/mmol) was purchased from New England Nuclear, R5 (macrophage-tropic) HIV gp120 (strain W61D) originates from Dutch Patient 320 from the Amsterdam cohort and was a generous gift from Dr. Gerald Voss (Smith-Kline Beecham, Brussels, Belgium).

Cell Culture

HOS–CCR5 cells were obtained from the AIDS Reagent Reference Program. Cells were grown in DMEM containing 10% fetal bovine serum, 4 mM glutamine, 50 μg/ml of penicillin and streptomycin, and 1 μg/ml of puromycin.

Binding Studies with Membrane-Bound CCR5

Membrane preparation. Crude membranes from HOS–CCR5 cells were prepared as described (20). Briefly, cells were rinsed with phosphate-buffered saline (PBS), resuspended in lysis buffer (50 mM Hepes, pH 7.4, 1 mM EGTA containing protease inhibitor cocktail) and then homogenized with 40 strokes with a tight pestle in a Dounce homogenizer. Nuclei and unbroken cells were then pelleted by a low speed centrifugation (800*g* for 10 min at 4°C). The supernatant was centrifuged at 45,000*g* for 30 min at 4°C. The crude membrane pellet was washed once and then resuspended in above buffer with the aid of a Dounce homogenizer.

General Assay Conditions

All binding studies were performed in 20 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 1% BSA, in a final assay volume of 0.1–0.25 ml. The incubation temperature was 20°C, unless otherwise specified. ^{125}I-MIP-1β (72–272 pM) was incubated with 0.048–0.17 mg/ml HOS–CCR5 membrane protein for 60 min. The definition of nonspecific binding was 250 nM MIP-1β. Gp120 and sCD4 were preincubated for 30 min at 20°C before addition to the binding assay. Receptor-bound radioligand was separated from unbound ligand by filtration through Whatman GF/C filters. Filters were washed twice with 4 ml of ice-cold incubation buffer containing 500 mM NaCl.

Kinetic Studies

For association kinetics of CCR5, $gp120_{W61D}$ and sCD4 (100 nM each) were preincubated with membranes for 30 min at 20°C and the association was started with ^{125}I-MIP-1β. The reaction was terminated at the indicated times by filtration. The data were normalized using the linearized, integrated second-order rate equation

$$[1/(L_0 - R_0) * \ln[(L_0 - x)/(R_0 - x)] = k_{+1} * t + A,$$

where R_0 and L_0 are the total concentrations of receptor and radioligand, x is the receptor–ligand complex at the indicated times, t, and A is $[1/(L_o - R_o)] * \ln(L_0/R_0)$.

In dissociation experiments ^{125}I-MIP-1β was incubated with HOS–CCR5 membranes in the absence and presence of 100 nM $gp120_{W61D}$–sCD4 at 20°C for 60 min. Subsequently, dissociation was initiated by the addition of 250 nM unlabeled MIP-1β. The reaction was terminated at the indicated times by filtration. Data were transformed according to the following equation: $\ln[B_t/B_e] = -k_{-1} * t$, where B_t is the specifically bound radioligand at the indicated time after initiation of the dissociation by unlabeled ligand, and B_e is the concentration of radioligand specifically bound at equilibrium.

Data Analysis

Binding inhibition curves were parameter optimized using the general dose–response equation according to DeLean *et al.* (21). The dissociation constant (K_D) and the maximal density of binding sites (B_{max}) were obtained by linear regression analysis after Scatchard transformation of the equilibrium saturation binding data.

RESULTS

Interactions between CCR5 and G-Proteins in Membranes

One of the defining characteristics of a G-protein mediated signal transduction pathway is the negative heterotropic interaction between the binding of a guanine nucleotide to the G-protein and the binding of the agonist to its receptor (17, 18). In the absence of guanine nucleotides, MIP-1β bound with high affinity to a single class of binding sites in HOS–CCR5 membranes. A typical homologous competition curve is shown in Fig. 1A. Half maximal inhibition occurred at 492 ± 108 pM (mean IC_{50} from six independent experiments), which is in perfect agreement with the previously reported IC_{50} value of MIP-1β binding to cellular CCR5 (22). Scatchard analysis (Fig. 1B) of the equilibrium saturation data revealed a homogeneous CCR5 receptor populations with a computed K_D of 225 ± 65 pM and a B_{max} of 1.23 ± 0.23 pmol/mg of membrane protein. The effects of several nucleotides on binding of MIP-1β to HOS–CCR5 membranes are shown in Fig. 2A. GTP was a potent inhibitor of MIP-1β binding with an IC_{50} of 115 nM (Fig. 2B). The inhibitory effect of nucleotides was specific to GTP [or its nonhydrolyzable analogs GIDP, 5′-guanylylimidodiphosphate and GTPγS, guanosine 5′-*O*-(3-thiotriphosphate)] and GDP, which are known to bind to G-protein (17, 18), while GMP and ATP were without effect. More comprehensive studies of the interaction between CCR5 and G-proteins were carried out with GTP. The effects of GTP on the equilibrium binding properties of MIP-1β were determined from homologous competition binding studies (Fig. 2B). In these experiments, membranes were pre-incubated with the indicated concentrations of GTP for 30 min. As can be seen in Fig. 2C, GTP caused a concentration-dependent decrease in the computed number of binding sites (B_{max}), but had no effect on the affinity of the residual receptor sites. We did not observe the appearance of a low-affinity state to accompany the loss of high-affinity sites.

Temperature Dependence of MIP-1β Binding to CCR5

An agonist, in order to cause a biological effect, induces an energy dependent conformational change in

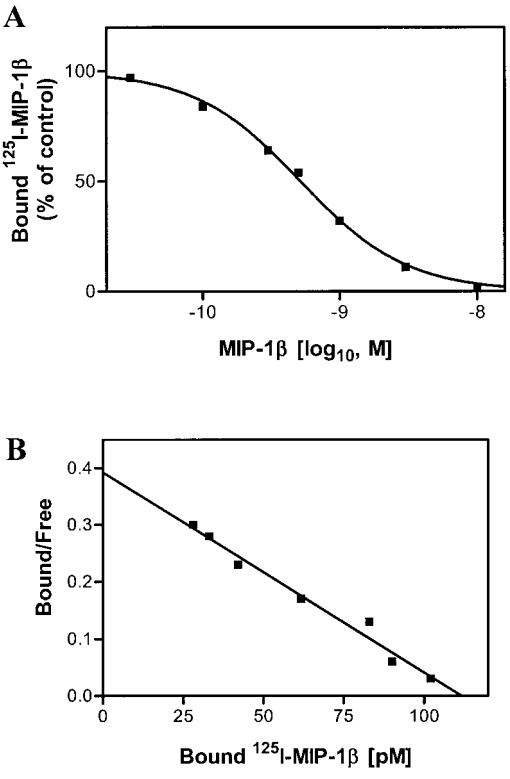

FIG. 1. Equilibrium binding parameters of ^{125}I-MIP-1β to HOS–CCR5 membranes. (A) 84 pM ^{125}I-MIP-1β were incubated with 0.08 mg/ml of membrane protein in the presence of the indicated concentrations of unlabeled MIP-1β at 20°C for 60 min. IC_{50} value and slope (n_H values) were calculated as described under Materials and Methods and the best fit curves drawn with the following parameters: IC_{50} = 550 pM, n_H = 1.12. (B) Scatchard transformation of equilibrium binding parameters. K_D and B_{max} values were calculated by linear regression analysis: K_D = 290 pM, B_{max} = 1.40 pmol/mg, r = 0.98.

the receptor (17). We therefore examined the temperature dependence of MIP-1β binding to CCR5. The interaction of CCR5 with its chemokine was extremely sensitive to changes in incubation temperature. Incubation at 4°C decreased binding by 95% (data not shown).

Effect of Cations on MIP-1β Binding to CCR5

Since divalent cations promote agonist binding to many GPCR in membranes by favoring the formation of a high-affinity agonist/receptor/G-protein complex (17), we examined the effect of EDTA on MIP-1β binding to CCR5. Inclusion of 10 mM EDTA inhibited binding by 97% (Fig. 3A). Ca^{2+} and Mg^{2+} were essential for MIP-1β binding (data not shown), whereas Cd^{2+} and Mn^{2+} blocked MIP-1β binding (Fig. 3A). It is well recognized that the interaction of receptor and G-protein is allosterically regulated by Na^+ and other monovalent cations (23). The site of Na^+ action has been localized to an aspartate residue in transmembrane helix #2, which is highly conserved among GPCRs and reduces precoupling of the receptor (24). We therefore examined the effect of Na^+, Li^+, and K^+ on MIP-1β binding to CCR5 (Fig. 3A). The dose–response curve for Na^+ is shown in Fig. 3B. The steep slope (pseudo-Hill coefficient, n_H = 1.80) is indicative of an allosteric modulation of the MIP-1β binding site.

Gp120–CD4 Complex Decreases the Affinity for MIP-1β

It has been shown that gp120, in the presence of sCD4, inhibits binding of chemokines to CCR5 (25, 26), but the mechanism of this inhibitory effect has not been clarified. In the absence of sCD4 the R5 tropic $gp120_{W61D}$ had no effect on MIP-1β binding (data not shown), which confirms previous reports that binding of HIV-1 viral envelope to CCR5 is absolutely dependent on the interaction with CD4 (27). In the presence of sCD4, 100 nM $gp120_{W61D}$ inhibited ^{125}I-MIP-1β binding to CCR5 by 56 ± 9%. We investigated the molecular mechanism of this inhibitory effect of the $gp120_{W61D}$–CD4 complex. Homologous competition experiments were carried out in the absence and presence of 100 nM $gp120_{W61D}$–sCD4. Scatchard analysis of the equilibrium binding data revealed that gp120–sCD4 decreases the affinity of CCR5 for MIP-1β (K_D = 425 ± 72 pM) with only a slight decrease in receptor density (B_{max} 1.08 ± 0.21 pmol/mg). A typical experiment is shown in Fig. 4.

$Gp120_{W61D}$–sCD4 Affects Kinetics of MIP-1β Binding to CCR5

Since gp120–sCD4 had mostly a K_D effect on MIP-1β binding to CCR5 in equilibrium binding experiments, we examined this effect using kinetic studies. Dissociation kinetics revealed that $gp120_{W61D}$–sCD4 accelerated the decay of the MIP-1β–CCR5 complex (Fig. 5A), proving that $gp120_{W61D}$-CD4 is not a simple competitive ligand for the MIP-1β binding site (28). $Gp120_{W61D}$–sCD4 also decelerated the association reaction of MIP-1β to CCR5 by 2.2-fold (Fig. 5B). The dissociation constants derived from kinetic constants were as follows: 112 pM in the absence and 340 pM in the presence of 100 nM $gp120_{W61D}$–sCD4.

DISCUSSION

GPCRs serve as therapeutic targets for approximately 60% of the pharmaceutical agents currently used in clinical practice (29). Chemokines not only play major roles in inflammatory pathology and may be good targets for anti-inflammatory drugs, but their receptors are also necessary cofactors for HIV-1 entry. HIV-1 entry blockers have received increasing attention, because they appear ideal therapeutic agents,

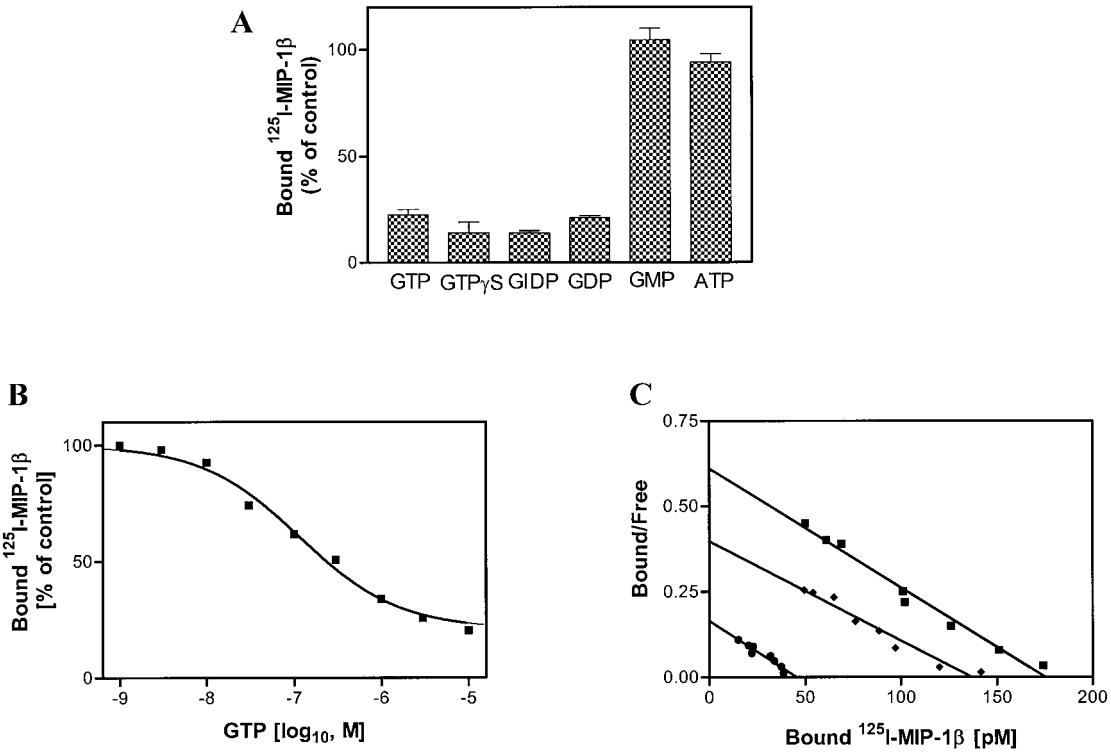

FIG. 2. Effects of nucleotides on MIP-1β binding to CCR5. In these experiments HOS–CCR5 membranes were preincubated with the indicated concentrations of nucleotides for 30 min at 20°C and the reaction was started with ^{125}I-MIP-1β. (A) 0.15 mg/ml HOS–CCR5 membrane protein were incubated with 265 pM ^{125}I-MIP-1β in the absence and presence of 100 μM nucleotide. (B) GTP inhibition of MIP-1β binding to CCR5. Half maximal inhibition (IC_{50}) occurred at 115 nM. Inhibition was incomplete (80%), $n_H = 0.76$. (C) Scatchard analysis of the saturation isotherms of MIP-1β binding to HOS–CCR5 membranes in the absence (■), $K_D = 274$ pM, $B_{max} = 1.12$ pmol/mg, $r = 0.97$ and presence of 0.3 μM GTP (◆), $K_D = 299$ pM, $B_{max} = 0.68$ pmol/mg, $r = 0.98$ and 3 μM GTP (●), $K_D = 272$ pM, $B_{max} = 0.26$ pmol/mg, $r = 0.96$.

since they prevent infection a priori. For an effective therapy to be developed, the molecular pathways need to be characterized. It has become clear that G-proteins play an obligatory role in the activation of immune cells by chemokines. Pertussis toxin (PTX), which ADP-ribosylates G, has been shown to inhibit a variety of MIP-1β-stimulated responses in cells (30–33). On the other hand, PTX was shown not to interfere with the capacity of HIV-1 to infect T cell lines *in vitro* (30, 32, 34, 35). Also, truncation of the C-terminal tail of CCR5, which blocked chemokine-dependent activation of classic second messengers, intracellular calcium fluxes, and the cellular response of chemotaxis, did not alter the ability of CCR5 to act as an HIV-1 coreceptor (36). These results lead to the conclusion that the initiation of signal transduction, the prototypic function of GPCR, is not involved in the process of HIV-1 fusion. Surprisingly, a recent report described the important finding that PTX specifically inhibited entry of HIV-1 through CCR5 in primary T cells and inhibited replication of R5 as well as X4 HIV-1 isolates (37). These results underscore the importance of the studies of the interaction of CCR5 and G proteins, not only for its role as a chemokine receptor, but also as an HIV-1 coreceptor.

In this paper we have characterized the effects of the CCR5 interaction with G-protein on its binding properties for MIP-1β. We have shown that CCR5 in its role as a chemokine receptor is profoundly affected by the association with G-protein. In the absence of guanine nucleotide we observed a single class of high-affinity binding sites for MIP-1β. Addition of GTP or the nonhydrolyzable analog GTPγS potently and specifically inhibited binding of MIP-1β to CCR5 in membrane preparations. In the presence of GTP the G-protein is unstable and dissociates into its subunits (17, 18). Uncoupled from its G-protein, CCR5 is unable to bind MIP-1β. We suggest that the incomplete loss of binding (85% inhibition observed) is, in large part, due to the inaccessibility of some of the relevant G-proteins to GTP rather than receptor heterogeneity, as was also suggested for the C5a receptor (38). Because the binding sites for MIP-1β and GTP lie on opposite sides of the plasma membrane, receptor–G-protein complexes will not be accessible to GTP on sealed vesicles. Addition of GTP resulted in a dose dependent reduction in the number of MIP-1β binding sites. It is well established that guanine nucleotides regulate the interaction of ligand with receptor by promoting the conversion of the receptor from a high-affinity to a low-affinity

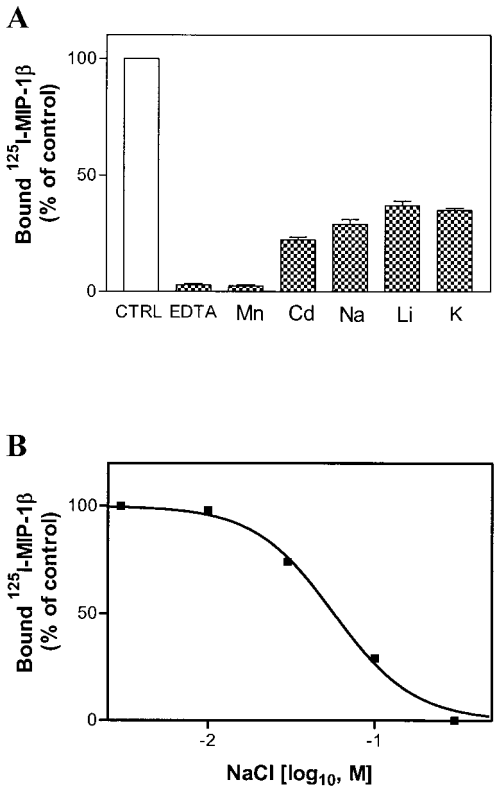

FIG. 3. Modulation of MIP-1β binding to HOS–CCR5 membranes by cations. (A) 180 pM ^{125}I-MIP-1β were incubated with 0.15 mg/ml membrane protein in the buffer described under Materials and Methods, 10 mM EDTA or 100 mM of indicated cations (chloride salts) were included in the buffer. (B) Inhibitory effect of NaCl; IC_{50} = 57 mM, n_H = 1.80.

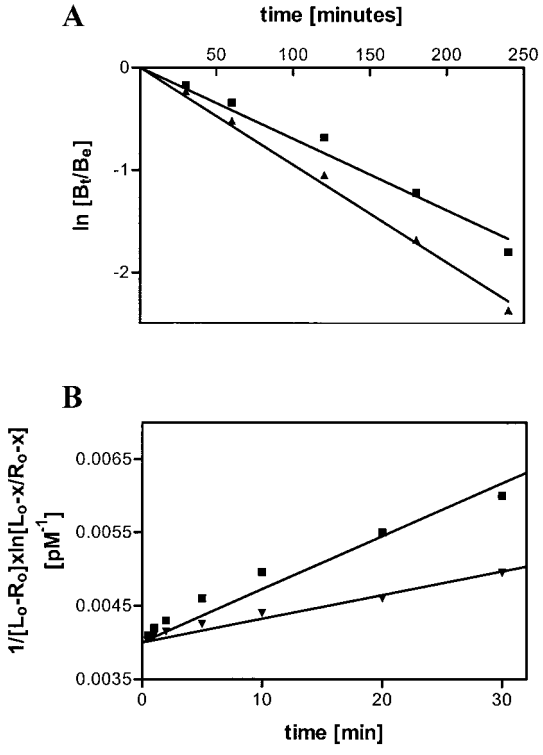

FIG. 5. Effect of gp120–sCD4 on the kinetics of MIP-1β binding to CCR5. (A) Dissociation kinetics of ^{125}I-MIP-1β for CCR5. 0.15 mg/ml HOS–CCR5 membrane protein was labeled in the absence (control) and presence of 100 nM $gp120_{W61D}$–sCD4 with 173 pM ^{125}I-MIP-1β at 20°C for 60 min. Dissociation was initiated by the addition of 250 nM unlabeled MIP-1β. Dissociation rate constants were determined as described under Materials and Methods: control (■), k_{-1} = 0.0067 min^{-1}, r = 0.98; presence of 100 nM $gp120_{W61D}$–sCD4 (▲), k_{-1} = 0.0092 min^{-1}, r = 0.99. (B) Linear transformation of the association data according to the second order rate equation (see Materials and Methods) to determine the association rate constant k_{+1}. Association was carried out in the absence (■) and presence of 100 nM $gp120_{W61D}$–sCD4 (▲). The slopes of the lines, as estimates of the association rate constants were: control (■), 0.000060 pM^{-1} × min^{-1}; r = 0.97; presence of 100 nM $gp120_{W61D}$–sCD4 (▲), 0.000027 pM^{-1} × min^{-1}, r = 0.99.

state (17–19). As we did not observe the appearance of low-affinity MIP-1β receptors to accompany the loss of high-affinity binding sites, the K_D of the low-affinity state must be beyond the limits of detection in the binding assay, again resembling the C5a receptor (38). In other aspects, CCR5 behaved like a typical GPCR. Binding of the agonist MIP-1β was allosterically regulated by monovalent cations, dependent on Ca^{2+}/Mg^{2+} and extremely temperature sensitive. Given the important recent findings that PTX interferes with entry of R5 strains of HIV-1 in primary T cells (37), it will be of great importance to determine the requirement of G-protein coupling for CCR5 to act as an HIV-1 coreceptor. Studies of guanine nucleotide regulation of the gp120 binding site of CCR5 are currently underway in this laboratory.

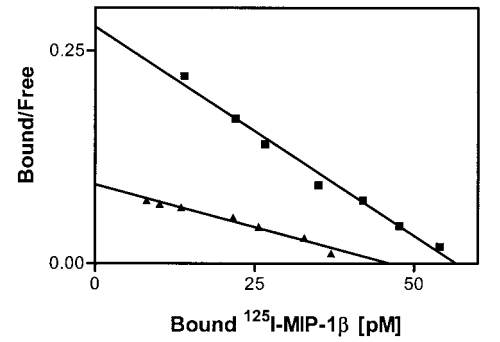

FIG. 4. Effect of gp120–sCD4 on equilibrium binding of MIP-1β binding to CCR5. Scatchard analysis of homologous competition binding: HOS–CCR5 membranes (0.048 mg/ml) were incubated with 117 pM ^{125}I-MIP-1β in the presence of increasing concentrations of unlabeled MIP-1β at 20°C for 60 min. Control (■), K_D = 204 pM, B_{max} = 1.18 pmol/mg, r = 0.99; presence of 100 nM $gp120_{W61D}$–sCD4 (▲). K_D = 497 pM, B_{max} = 0.95 pmol/mg, r = 0.99.

Finally, we examined the interaction of the chemokine- and HIV-1 viral envelope binding sites of CCR5. It has been known that gp120 inhibits MIP-1 binding to CCR5 (25, 26). The molecular mechanism of this inhibitory effect has not been elucidated. We show

that the gp120/sCD4 complex decreases the affinity of CCR5 for MIP-1β with only a minor effect on receptor density (B_{max}) in equilibrium binding studies. However, the gp120/sCD4 complex altered the kinetics of MIP-1β to CCR5, proving that it is an allotropic antagonist to MIP1-β (28). Therefore, the binding sites for gp120/sCD4 and MIP-1β are distinct, but allosterically coupled. Using a panel of receptor chimeras, it has been determined that regions of CCR5 involved in chemokine ligand specificity and in the cofactor usage for various HIV-1 strains are not identical (39, 40). More recently, however, alanine-scanning mutagenesis and N-terminal truncation experiments suggested an overlapping binding site of chemokines and gp120 on the CCR5 amino terminus (22). Others found only partially overlapping binding sites (41). However, it is not known whether chemokines or gp120 interact directly with these regions or whether these mutations affect overall CCR5 structure. Therefore, the results of our binding studies add important information, since they demonstrate that gp120–sCD4 binds to a site distinct from the MIP-1β binding site and that the inhibitory effect of gp120–CD4 must therefore be achieved by a conformational change in CCR5. Because CCR5 chemokines and analogs are potent inhibitors of HIV-1 infection, understanding how these two binding sites interact could help in the design of more potent inhibitors of virus entry.

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