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HIV-1 Coreceptor Activity of CCR5 and Its Inhibition by Chemokines: Independence from

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HIV-1 infection requires the presence of specific chemokine receptors on CD4+ target cells to enable the fusion reactions involved in virus entry. CCR5 is a major fusion coreceptor for macrophage-tropic HIV-1 isolates. HIV-1 entry and fusion are mediated by the viral envelope glycoprotein (Env) and are inhibited by CCR5 ligands, but the mechanisms are unknown. Here, we test the role of G protein signaling and CCR5 surface downmodulation by two separate approaches: direct inactivation of CCR5 signaling by mutagenesis and inactivation of G_i-type G proteins with pertussis toxin. A CCR5 mutant lacking the last 45 amino acids of the cytoplasmic C-terminus ($CCR5_{306}$) was created that was expressed on transfected cells at levels comparable to cells expressing CCR5 and displayed normal chemokine binding affinity. CCR5 ligands induced calcium flux and receptor downmodulation in cells expressing CCR5, but not in cells expressing $CCR5_{306}$. Nevertheless, CCR5 or $CCR5_{306}$, when coexpressed with CD4, supported comparable HIV-1 Env-mediated cell fusion. Consistent with this, treatment of CCR5-expressing cells with pertussis toxin completely blocked ligand-induced transient calcium flux, but did not affect Env-mediated cell fusion or HIV-1 infection. Also, pertussis toxin did not block chemokine inhibition of Env-mediated cell fusion or HIV-1 infection. However, chemokines inhibited Env-mediated cell fusion less efficiently for $CCR5_{306}$ than for CCR5. We conclude that the C-terminal domain of CCR5 is critical for G protein signaling and receptor downmodulation from the surface, but that neither function is required for CCR5 fusion coreceptor activity. The contrasting phenotypes of CCR5 and $CCR5_{306}$ suggest that coreceptor downmodulation and direct blockage of Env interaction sites both contribute to chemokine inhibition of HIV-1 infection.

INTRODUCTION

HIV initiates infection of CD4+ cells by a process of direct fusion between the virion membrane and the target cell membrane. In addition to CD4, the target cell must express additional human-specific cofactors to allow fusion mediated by HIV-1 Env (Alkhatib *et al.,* 1996a; Ashorn *et al.,* 1990; Broder *et al.,* 1993; Clapham *et al.,* 1991; Dragic *et al.,* 1992; Harrington *et al.,* 1993; Maddon *et al.,* 1986; Nussbaum *et al.,* 1994; Ramarli *et al.,* 1993; Weiner *et al.,* 1991). Recent discoveries have revealed that the fusion cofactors for HIV-1 are specific chemokine receptors (reviewed in Berger, 1997). The first cofactor discovered was a putative G protein-coupled receptor (designated "fusin") that was identified by a functional cDNA cloning strategy focused on the cofactor for T-cell line-tropic (TCL-tropic) HIV-1 isolates (Feng *et al.,* 1996). The protein was subsequently renamed CXCR4 upon the discovery that it is a receptor for the CXC chemokine stromal cell-derived factor-1 (SDF-1), which blocks TCL-tropic HIV-1 strains (Bleul *et al.,* 1996; Oberlin *et al.,* 1996). The identification of a CXC chemokine receptor as a fusion cofactor for TCL-tropic strains, coupled with an earlier demonstration that certain CC chemokines suppress infection by macrophage-tropic HIV-1 strains (Cocchi *et al.,* 1995), quickly led to the identification of the CC chemokine receptor CCR5 (Combadiere *et al.,* 1996; Samson *et al.,* 1996) as a fusion cofactor for macrophage-tropic HIV-1 strains (Alkhatib *et al.,* 1996b; Choe *et al.,* 1996; Deng *et al.,* 1996; Doranz *et al.,* 1996; Dragic *et al.,* 1996). Coreceptor activity has also been reported for the CC chemokine receptors CCR3 and CCR2b (Choe *et al.,* 1996; Doranz *et al.,* 1996) as well as for an "orphan" receptor with unknown ligand specificity (Liao *et al.,* 1997). With the recent findings that direct molecular interactions occur between the fusion cofactors, Env and CD4, (Lapham *et al.,* 1996; Trkola *et al.,* 1996; Wu *et al.,* 1996), it is appropriate to view these molecules as coreceptors.

The mechanism of action of the coreceptors in the fusion process, and the mode of inhibition by the chemokine ligands, are poorly understood. Of particular interest are the possible roles of G protein signal transduction and surface receptor downmodulation in these pro-

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cesses. The present study addresses these questions by analysis of fusion under conditions where signaling is blocked by mutation of the coreceptor or by treatment with an inhibitor of signal transduction. Our results demonstrate that coreceptor function is independent of G protein signaling; furthermore they suggest two mechanisms by which chemokines block the fusion process: surface receptor downmodulation and direct blocking.

MATERIALS AND METHODS

Cell lines, viruses, and materials

Human HeLa, human embryonic kidney HEK 293, and murine fibroblast NIH 3T3 cell lines (American Type Culture Collection, Rockville, MD) were cultured in DMEM-10 [Dulbecco's modified Eagle's medium (Quality Biologicals, Gaithersburg, MD) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT), 2 m*M* L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin]. The human PM1 T-cell line (Lusso *et al.,* 1995) was obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD) and was grown in RPMI-10 [RPMI 1640 medium (Quality Biologicals) containing 10% FBS, 10 m*M* Hepes, 2 m*M* glutamine, and antibiotics]. Recombinant vaccinia virus stocks were prepared by standard procedures (Earl *et al.,* 1991). Pertussis toxin was obtained from List (Campbell, CA). Recombinant chemokines were purchased from Peprotech (Rocky Hill, NJ). Fura 2-AM and propidium iodide were obtained from Molecular Probes (Eugene, OR). Sodium azide and ATP were from Sigma (St. Louis, MO).

CCR5 constructs

We created epitope-tagged variants of CCR5 to enable detection by the M5 monoclonal antibody (Kodak, Rochester, NY). We amplified the CCR5 open reading frame from the clone 8.5 cDNA (Combadiere *et al.,* 1996) by PCR using the following primers: (1) for full-length CCR5 (designated CCR5): a 3′-oligonucleotide containing (from 3′ to 5′) 27 bases complementary to the last 9 codons of CCR5, 3 bases for the stop codon, 6 bases for an *Xho*I restriction site, and 8 miscellaneous bases; (2) for CCR5 lacking most of the cytoplasmic C-terminus (designated $CCR5_{306}$): a 3′-oligonucleotide containing (from 3′ to 5′) 27 bases complementary to codons 298–306 of CCR5, 3 bases for a stop codon, 6 bases for an *Xho*I restriction site, and 8 miscellaneous bases; and (3) for both constructs: a 5′-oligonucleotide containing (from 5′ to 3′) 8 miscellaneous bases, 6 bases for a *Hin*dIII site, 3 bases for the start codon, 24 bases encoding the flag epitope DYKDDDDK, and 27 bases complementary to CCR5 codons 2 to 10. The resulting two PCR products were digested and subcloned between the *Hin*dIII and *Xho*I sites of the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA); this vector contains both human cytomegalovirus and bacteriophage T7 promoters. The nucleotide sequence was confirmed by double-stranded DNA sequencing using the Sequenase DNA sequencing kit version 2.0 (USB, Cleveland, OH).

Expression of recombinant CCR5 and $CCR5_{306}$

Stable cell lines were prepared expressing the recombinant chemokine receptors. HEK 293 cells were grown to log phase in DMEM-10, and 10^7 cells were electroporated using a GenePulser (Bio-Rad Laboratories, Hercules, CA) with 20 μg of plasmid DNA containing the appropriate cDNA insert. Colonies resistant to 2 g/L G-418 (Gibco BRL) were isolated and expanded in the same media supplemented with 2 g/L G-418.

As an alternative method, recombinant chemokine receptors were produced along with CD4 using the vaccinia virus expression system. NIH 3T3 cells were transfected using DOTAP lipofection (Boehringer Mannheim, Indianapolis, IN) with pcDNA3-based plasmids which contained cDNAs encoding CCR5 or $CCR5_{306}$ linked to the bacteriophage T7 promoter; control cells were transfected with the pcDNA3 vector alone. After 4 hr at 37°, the cells were coinfected with vTF7-3 encoding T7 RNA polymerase (Fuerst *et al.,* 1993) and vCB-3 encoding CD4 (Broder *et al.,* 1993). In both viruses the foreign genes were linked to vaccinia early/late promoters; the multiplicity of infection was 10 PFU/cell for each virus. Cell cultures were incubated at 31° overnight to allow expression of the vaccinia-encoded proteins and then washed and resuspended.

Immunocytochemistry

Rabbit polyclonal antisera were raised against a synthetic peptide corresponding to amino acids 1–28 of the wild-type CCR5 sequence conjugated to KLH. HEK 293 cells were detached by incubation in PBS at 37° for 15 min, washed twice in flow cytometry buffer (HBSS supplemented with 0.5% FBS and 0.02% sodium azide), and resuspended in 100 μl flow cytometry buffer at 10^7/ml. Cells were then incubated with a 1:50 dilution of rabbit antisera at 4° for 30 min. They were then washed twice, resuspended in 100 μl ice-cold flow cytometry buffer in the presence of FITC-conjugated anti-rabbit IgG (Sigma) and incubated at 4° for 30 min. Finally, cells were washed twice, resuspended in 500 μl ice-cold flow cytometry buffer containing propidium iodide and analyzed in a FACScan cytometer (Beckton–Dickinson). Alternatively, cells were suspended in a 1:1 (vol:vol) mixture of 2.5% E-MEM and PBS containing 0.1% BSA. Each sample was incubated with rabbit antiserum at a 1:50 dilution for 45 min at room temperature. Subsequently, cells were washed with PBS and incubated with a 1:10 dilution of FITC-conjugated F(ab′)2 goat anti-rabbit IgG (Boehringer Mannheim) for 30 min at room temperature.

Ligand binding assay

NIH 3T3 cells (2×10^6) expressing vaccinia-encoded CCR5 or $CCR5_{306}$ (and CD4) were incubated in triplicate with 0.25 n*M* ^{125}I-labeled MIP-1α (sp act 2200 Ci/mmol, DuPont NEN) plus varying concentrations of unlabeled recombinant human MIP-1α (Peprotech, Rocky Hill, NJ) in 20 μl of binding buffer (HBSS with 1 mg/ml bovine serum albumin plus sodium azide, pH 7.4). After 1 hr incubation at room temperature cells were washed with binding buffer containing 0.5 *M* sodium chloride, and cell pellet-associated counts were measured in a gamma counter.

Calcium flux assay

Receptor activation was assessed by real time measurement of $[Ca^{2+}]_i$ changes using a MSIII fluorimeter (Photon Technology International, S. Brunswick, NJ) in HEK 293 cell lines expressing receptor constructs as previously described (Combadiere *et al.,* 1996). Briefly, cells were loaded with 2 μ*M* FURA-2AM at 37° for 45 min, washed twice, and resuspended at 10^6 cells/ml in HBSS, pH 7.4. Two milliliters of the cell suspension were placed in a stirred, water-jacketed cuvette at 37°, and excited sequentially at 340 and 380 nm. Fluorescence emission was monitored at 510 nm before and after addition of agonists. For some experiments, cells were incubated with 250 ng/ml pertussis toxin for 3 hr prior to functional assay.

Cell fusion assay

Fusion between effector cells expressing HIV-1 Env and target cells expressing CD4 was quantitated by a vaccinia-based reporter gene assay in which β-galactosidase is produced selectively in fused cells (Nussbaum *et al.,* 1994). As effector cells, HeLa cells were coinfected with vCB-21R, which encodes the *Escherichia coli LacZ* gene under control of the bacteriophage T7 promoter (Alkhatib *et al.,* 1996a), and a recombinant vaccinia virus encoding one of the following HIV-1 Envs (Broder and Berger, 1995): M-tropic Envs Ba-L (vCB-43; note this is a correction of the nomenclature used for this virus in Broder and Berger, 1995), ADA (vCB-39), SF-162 (vCB-32), and JR-FL (vCB-28); and Unc, an uncleavable mutant of IIIB (vCB-16). In one protocol, the target cells were the HEK 293 cell transfectants stably expressing the indicated CCR5 contructs. These cells were coinfected with vCB-3 encoding human CD4 and vTF7-3 encoding T7 RNA polymerase. In another protocol, the targets were NIH 3T3 cells coexpressing vaccinia-encoded CD4, the indicated CCR5 constructs, and T7 RNA polymerase. Duplicate samples of 10^5 Env-expressing effector cells and 10^5 $CD4^+$/$coreceptor^+$ target cells were mixed in 96-well microtiter plates in the presence of cytosine arabinoside (40 μg/ml) and incubated at 37° for 2 hr. β-galactosidase activity produced in response to cell fusion was quantitated by colorimetric assay of detergent cell lysates. To analyze the effects of chemokines on cell fusion activity, chemokines were added individually or in combination to the target cells expressing CCR5 or $CCR5_{306}$, and incubated for 1 hr at 37° before mixing with Env-expressing HeLa cells. In some experiments, the stably transfected HEK 293 cell lines, or the transiently transfected NIH 3T3 cells, were incubated for 3 hr at 37° with pertussis toxin prior to infection with vaccinia viruses. The cells were then incubated overnight at 31° in the presence of pertussis toxin.

HIV-1 infection

PM1 cells were preincubated for 1.5 hr at 37° in the presence or absence of RANTES and pertussis toxin. Cell-free HIV-1 Ba-L was added and the mixtures were distributed to multiple wells of 96-well microtiter plates (1×10^5 cells/well) and incubated at 37°. The following day, 50-μl aliquots of cell suspension were collected; every 3–4 days thereafter, 100-μl aliquots of cell suspension were removed and replaced with fresh medium containing the same agents. Samples were assayed for p24 content by enzyme-linked immunosorbent assay (Dupont).

RESULTS

Signaling by G protein-coupled receptors is thought to involve conformational changes of the intracellular domains induced by ligand binding (Lefkowitz, 1993; Strader *et al.,* 1994). As one approach to assess the importance of G protein signaling in CCR5's function as an HIV-1 coreceptor, we examined a mutant form of CCR5 truncated at amino acid 306, designated $CCR5_{306}$. This receptor variant lacks almost the entire predicted cytoplasmic C-terminal segment. The corresponding domain has been shown previously to be a determinant of signaling for several related 7 transmembrane domain chemoattractant receptors, including the chemokine receptors CXCR2 and CCR2b (Ben-Baruch *et al.,* 1995; Franci *et al.,* 1996). Previously, we have shown that stimulation of cells expressing CCR5 with its ligands MIP-1α, MIP-1β, or RANTES induces a calcium flux response that can be completely blocked by pretreatment of the cells with pertussis toxin (Combadiere *et al.,* 1996), suggesting coupling to G_i-type G proteins (Simon *et al.,* 1991). Therefore, to further assess the role of signaling we also examined the effects of pertussis toxin on the HIV-1 coreceptor activity of CCR5.

The C-terminal domain of CCR5 is not required for cell surface expression or chemokine binding

Cell surface expression of CCR5 and $CCR5_{306}$ was analyzed by flow cytometry using as the probe either a

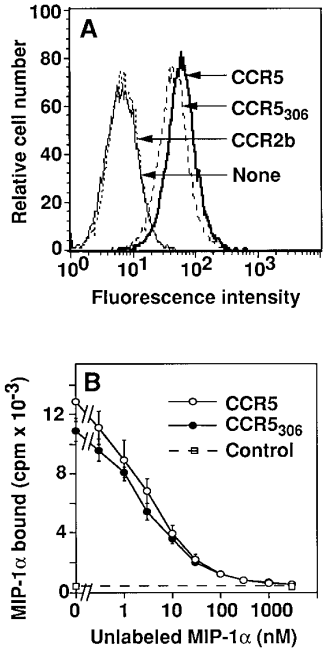

FIG. 1. Cytoplasmic C-terminal domain of CCR5 is not required for cell surface expression or ligand binding. (A) Surface expression. HEK 293 transfectant cell lines stably expressing CCR5, $CCR5_{306}$, or CCR2b were stained with a polyclonal rabbit antiserum raised against amino acids 1-28 of CCR5, and analyzed by flow cytometry. "None" refers to parental HEK 293 cells. Expression of CCR2b was verified by strong calcium flux responses induced by MCP-1 and MCP-3 (not shown), consistent with our previous report (Combadiere *et al.,* 1995). (B) Ligand binding. NIH 3T3 cells expressing vaccinia-encoded CD4 and CCR5 (open circles) or $CCR5_{306}$ (closed circles) were incubated with 0.25 n*M* ^{125}I-labeled MIP-1α alone, or in the presence of increasing concentrations of unlabeled MIP-1α. Receptor-positive cells represented 12% of the population for each construct, as assessed by flow cytometry using the rabbit polyclonal antiserum directed against CCR5. Cells transfected with the control vector gave a low background of binding that was not competed by cold MIP-1α (open squares). All values are the average of triplicate determinations $\pm$SD.

rabbit polyclonal antiserum generated against a synthetic peptide representing the predicted extracellular amino terminal domain of CCR5 (amino acids 1–28) or a mAb recognizing the Flag epitope. Figure 1A shows analysis of HEK 293 cells stably transfected with various chemokine receptor constructs. Specific cell surface staining at comparable intensity was obtained when cells stably transfected with either CCR5 or $CCR5_{306}$ were incubated with the anti-CCR5 antiserum. In contrast, cells stably transfected with the closely related receptor CCR2b (75% amino acid identity) gave only background fluorescence equivalent to that observed with the parental HEK 293 cells. With preimmune serum, only low background staining was observed with all cell lines (not shown). Staining with the anti-Flag mAb gave concordant results (not shown). We conclude that the rabbit antiserum is specific for the CCR5 amino terminus, that this domain is exposed extracellularly, and that the intracellular C-terminal domain is not required for efficient cell surface expression. Similar results were obtained in analyses of CCR5 and $CCR5_{306}$ produced transiently using the vaccinia expression system (see below). These results with CCR5 are consistent with those reported for other chemoattractant receptors (Ben-Baruch *et al.,* 1995; Murphy, 1996).

We also examined the effects of removal of the C-terminal domain of CCR5 on binding of chemokine ligands. As shown in Fig. 1B, both CCR5 and $CCR5_{306}$ bound radioinodinated MIP-1α; moreover, the IC_{50} for inhibition by unlabeled MIP-1α was equivalent for both molecules ($\sim$3 n*M*). We conclude that the cytoplasmic C-terminal domain of CCR5 is not required for high affinity ligand binding.

Loss of G protein signaling by truncation of the CCR5 C-terminus

We next tested whether $CCR5_{306}$ could support agonist-dependent calcium flux responses. This G protein-dependent response is characteristic of chemokine receptor activation, is highly associated with other chemokine-induced cell responses, and is a convenient way of monitoring receptor activation in real time using calcium-sensitive dyes such as Fura-2, which can be loaded into living cells (Grynkiewicz *et al.,* 1985). The HEK 293 transfectant expressing CCR5 exhibited a robust response to the CCR5 ligand RANTES, whereas a RANTES-induced response was not detectable in the transfectant expressing $CCR5_{306}$ (Fig. 2). The same dichotomy was observed when the other CCR5 ligands, MIP-1α and MIP-1β, were tested (not shown). As controls, the cells expressing CCR5 and $CCR5_{306}$ exhibited similar responses to ATP (Fig. 2), which activates an endogenous signaling pathway in HEK 293 cells (probably mediated by P2 purinergic receptors). We have also confirmed our previous reported result that the calcium flux response mediated by CCR5 can be completely blocked by pertussis toxin (Combadiere *et al.,* 1996 and data not shown). Taken together, the results presented so far indicate that the C-terminal segment of CCR5 beyond amino acid 306 contains determinants critical for signal transduction, probably by mediating interaction with G_i-type G proteins.

CCR5 signaling through G proteins is not required for HIV-1 coreceptor activity

The results presented above provided an opportunity to test whether G protein signaling is required for the HIV-1 coreceptor activity of CCR5. We used a quantitative vaccinia-based reporter gene assay of HIV-1 Env-mediated cell fusion (Nussbaum *et al.,* 1994). HEK 293 cell transfectants expressing CCR5 or $CCR5_{306}$ (along with vaccinia-encoded CD4) were tested for their ability to fuse with HeLa cells expressing vaccinia-encoded Envs from several M-tropic strains. As shown in Fig. 3, comparable levels of fusion occurred with CCR5 and $CCR5_{306}$

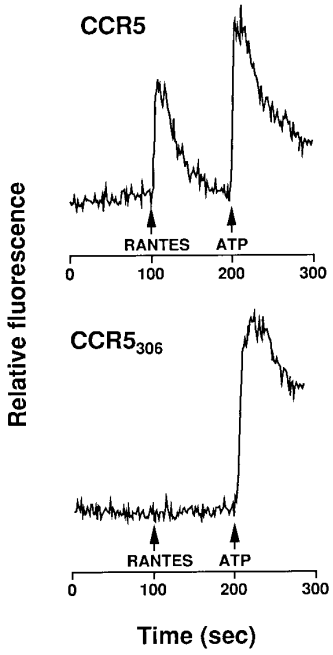

FIG. 2. The C-terminal domain of CCR5 is a major determinant of G protein signaling. Relative fluorescence was monitored in CCR5- and $CCR5_{306}$-expressing HEK 293 transfectant cell lines loaded with Fura 2 (top and bottom panels, respectively), before and during sequential stimulation with 50 n*M* RANTES and 50 n*M* ATP at the times indicated by the arrows. The tracings shown are from the same experiment, which is representative of greater than ten separate experiments using at least five separate clones for each receptor. In each case, similar expression levels of CCR5 and $CCR5_{306}$ were verified by staining with the anti-CCR5 rabbit antiserum.

for each Env tested. Similar results were obtained in an alternative protocol whereby CCR5 and $CCR5_{306}$ were coexpressed with CD4 on NIH 3T3 cells using the transient vaccinia expression system (not shown). Thus, the C-terminal truncation that abolished the G protein signal transduction activity of CCR5 had no effect on fusion coreceptor activity.

Pertussis toxin provided an alternative means to test the requirement for G protein signal transduction in the fusion coreceptor activity of CCR5. The experiment shown in Fig. 4 was performed with the Jurkat-derived T-cell line PM1 as the target. PM1 cells express CD4 and are highly susceptible to M-tropic HIV-1 strains (Lusso *et al.,* 1995). Moreover, CCR5 mRNA is expressed in these cells (Alkhatib *et al.,* unpublished data), and infection by M-tropic isolates is potently inhibited by CCR5 ligands, suggesting that the major native coreceptor for M-tropic HIV-1 on PM1 cells is CCR5 (Cocchi *et al.,* 1996; Oravecz *et al.,* 1996). When PM1 cells were used as target cells in the cell fusion assay with effector HeLa cells expressing the Ba-L Env, the efficient fusion observed was completely resistant to 500 ng/ml pertussis toxin (Fig. 4A). We also tested the effects of pertussis toxin on productive HIV-1 infection of PM1 cells (Fig. 4B). In the continuous presence of pertussis toxin (500 ng/ml), we observed robust infection by the M-tropic Ba-L isolate. We extended these analyses to cells expressing recombinant CCR5. As shown in Fig. 5, fusion of Env-expressing cells with HEK 293 CCR5 transfectant cells coexpressing vaccinia-encoded CD4 was unaffected by 500 ng/ml pertussis toxin. Similar results documented no effect of pertussis toxin on cell fusion when CCR5 was expressed (along with CD4) using the vaccinia expression system (data not shown). Thus, pertussis toxin at concentrations that potently block G protein-mediated signal transduction had minimal effect on either Env-mediated cell fusion or productive infection. These results parallel earlier reports that pertussis toxin did not block entry/infection by TCL-tropic (Orloff *et al.,* 1991) or M-tropic (Cocchi *et al.,* 1996) strains.

Chemokine inhibition of fusion coreceptor activity does not require G protein signaling or the C-terminal domain of CCR5

RANTES, MIP-1α, and MIP-1β suppress productive HIV-1 infection (Cocchi *et al.,* 1995) by inhibiting CCR5-

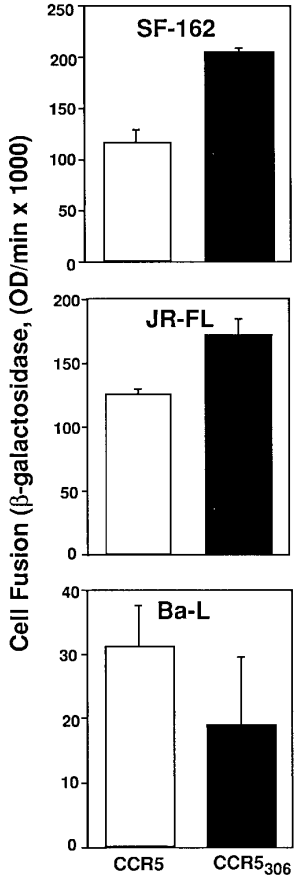

FIG. 3. The cytoplasmic C-terminal domain of CCR5 is dispensable for HIV-1 coreceptor activity. CCR5- and $CCR5_{306}$-expressing HEK 293 transfectant cell lines also expressing vaccinia-encoded CD4 were tested for their ability to fuse with HeLa cells expressing the indicated HIV-1 Envs. Cell fusion was measured by quantitation of β-galactosidase activity in cell lysates. The results are the mean $\pm$ SEM of a single experiment representative of at least five independent experiments. Background fusion activity, defined using HeLa cells expressing the Unc Env as effectors, was consistently below 5 in all experiments.

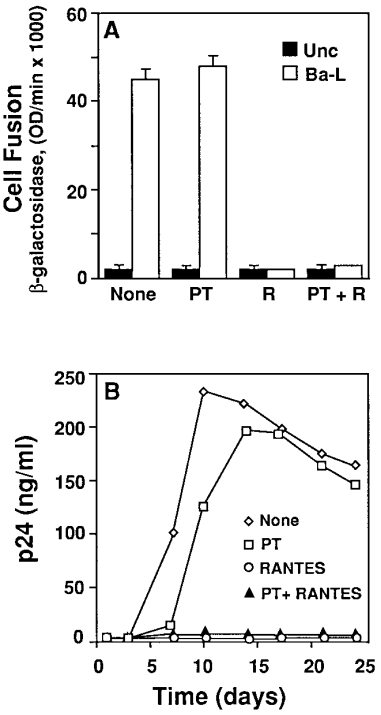

FIG. 4. Blockade of G protein signaling by pertussis toxin does not affect CCR5 coreceptor activity or chemokine inhibition of coreceptor activity in the PM1 T-cell line. (A) Cell fusion. Target PM1 cells were mixed with effector HeLa cells expressing the HIV-1 Envs Unc or Ba-L, in the presence of the indicated combinations of 500 ng/ml pertussis toxin (PT) and 500 n*M* RANTES (R). Cell fusion was measured by quantitation of β-galactosidase activity in cell lysates. Results are the mean $\pm$ SEM from a single experiment representative of three independent experiments. (B) Productive HIV infection. PM1 cells were infected with HIV-1 Ba-L in the presence of the indicated combinations of 500 ng/ml pertussis toxin and 500 n*M* RANTES. Cell-free supernatants were analyzed for p24 content.

dependent Env-mediated membrane fusion and virus entry (Alkhatib *et al.,* 1996b; Choe *et al.,* 1996; Deng *et al.,* 1996; Doranz *et al.,* 1996; Dragic *et al.,* 1996). We wished to test the role of G protein signal transduction in this process and again used pertussis toxin and $CCR5_{306}$ as probes. With PM1 cells expressing endogenous CCR5, pertussis toxin did not impair the blocking effects of RANTES on cell fusion with the Ba-L Env (Fig. 4A) or productive infection with the Ba-L strain of HIV-1 (Fig. 5B). These results parallel and extend previous reports by other laboratories studying native coreceptors on PM1 cells (Cocchi *et al.,* 1996; also stated in Oravecz *et al.,* 1996 with no data shown). Similarly with cells expressing recombinant CCR5, pertussis toxin had little effect on the ability of RANTES to inhibit cell fusion with the HEK 293 CCR5 transformant (Fig. 5) or with NIH 3T3 cells expressing vaccinia-encoded CCR5 (data not shown).

We also examined the effects of chemokines on Env-mediated fusion in cells expressing the C-terminal-truncated variant of CCR5. RANTES inhibited Env-mediated fusion in a dose-dependent fashion in HEK 293 cell transfectants expressing either CCR5 or the signaling-incompetent truncation mutant $CCR5_{306}$ (along with CD4, Fig. 6A). Similar effects were seen when the coreceptors and CD4 were expressed on NIH 3T3 cells using the vaccinia system: RANTES, MIP-1α, and MIP-1β each inhibited Env-mediated fusion with both CCR5 and $CCR5_{306}$ (Fig. 6B). We consistently observed that fusion inhibition was less efficient with the $CCR5_{306}$ variant compared to CCR5, as shown by the higher concentrations required for an equivalent percentage of inhibition (Fig. 6A) and the less extensive inhibition observed at high concentrations of each chemokine (Fig. 6B). These findings, coupled with the results presented in the following section, have important implications for the mechanisms of chemokine inhibition of HIV-1 entry (see Discussion).

The C-terminus of CCR5 is essential for surface downmodulation by chemokines

Binding of ligands to G protein-coupled receptors in general, and chemokine receptors in particular, often results in reduced cell surface expression of the receptor (Sabroe *et al.,* 1997), a process known as receptor sequestration or downmodulation. This process is thought to explain in part the phenomenon of receptor desensitization (Lefkowitz, 1993) and could be important either for HIV-1 Env-dependent membrane fusion and/or chemokine inhibition of fusion. Figure 7 shows that CCR5 was strongly downmodulated by chemokine ligands, whereas the truncated $CCR5_{306}$ receptor was unaffected. Thus, in addition to containing critical determinants of signaling, the C-terminal domain of CCR5 also contains essential determinants for chemokine-mediated downmodulation. Further study will be required to learn whether CCR5 downmodulation is obligatorily linked to G protein signaling and whether the C-terminal domain determinants that support each function are the same or different.

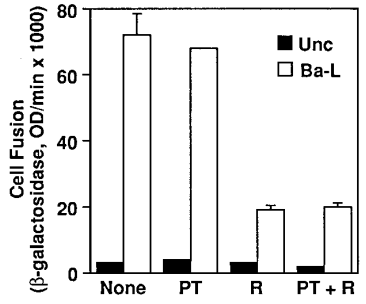

FIG. 5. Blockade of G protein signaling by pertussis toxin does not affect chemokine inhibition of CCR5's HIV-1 coreceptor activity. Target CCR5-expressing HEK 293 transfectant cells coexpressing vaccinia-encoded CD4 were mixed with effector HeLa cells expressing the HIV-1 Envs Unc or Ba-L in the presence of the indicated combinations of 500 ng/ml pertussis toxin (PT) and 500 n*M* RANTES (R). Cell fusion was measured by quantitation of β-galactosidase activity in cell lysates. Results are the mean $\pm$ SEM from a single experiment representative of three independent experiments.

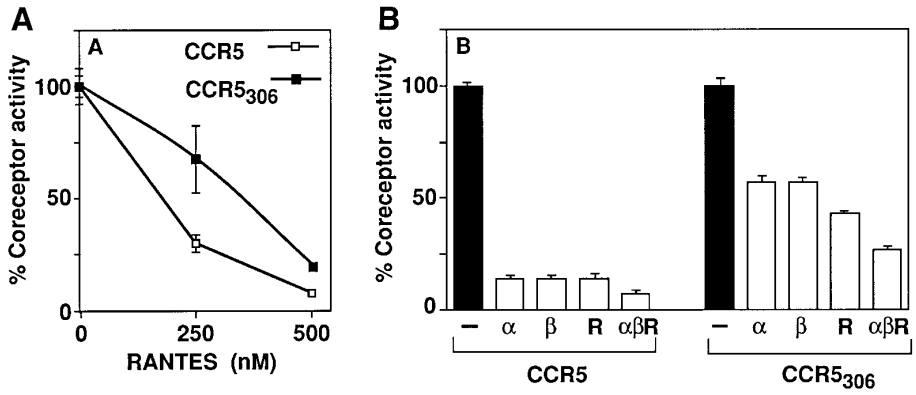

FIG. 6. Less efficient chemokine inhibition of HIV-1 coreceptor activity with CCR5 lacking the cytoplasmic C-terminal domain. Target cells expressing CD4 plus the indicated chemokine receptors were preincubated with the designated chemokines and then mixed with effector HeLa cells expressing the Ba-L Env. Cell fusion was measured by quantitation of β-galactosidase activity in cell lysates. Results are the mean $\pm$ SEM from a single experiment. (A) The HEK 293 transformant cell lines were preincubated with various concentrations of RANTES. (B) NIH 3T3 cells expressing vaccinia-encoded CD4 and CCR5 or $CCR5_{306}$ were preincubated with MIP-1α (α), MIP-1β (β), RANTES (R), all three chemokines ($\alpha\beta$R), or no chemokines (−). Each chemokine was tested individually at 500 n*M* or together at 170 n*M* each. Similar expression levels of CCR5 and $CCR5_{306}$ was verified by flow cytometry with the anti-CCR5 rabbit antiserum.

DISCUSSION

The results presented in this paper demonstrate that G protein signaling is not absolutely required for chemokine inhibition of Env-mediated cell fusion or HIV-1 infection. Moreover, they demonstrate that the absence of signaling by the truncated receptor $CCR5_{306}$ is not associated with altered binding of chemokine ligands, consistent with a previous report that removal of the cytoplasmic C-terminus of the CXC chemokine receptor CXCR2 has no effect on ligand binding affinity (Ben-Baruch *et al.,* 1995). In this respect, our results go beyond a recent study of CCR5/CCR2b chimeric receptors, one of which displayed HIV-1 coreceptor activity but did not signal in response to chemokines (Atchison *et al.,* 1996); the significance of this finding was unclear in view of the fact that extracellular regions of the receptors were exchanged, and no data on chemokine interaction were presented. Very recently, Farzan *et al.* described three CCR5 point mutants (D76N, D125N/R126N, and R126N) that had a similar phenotype as our truncated receptor (lack of MIP-1beta-induced calcium flux activity; intact HIV-1 coreceptor activity) (Farzan *et al.,* 1997).

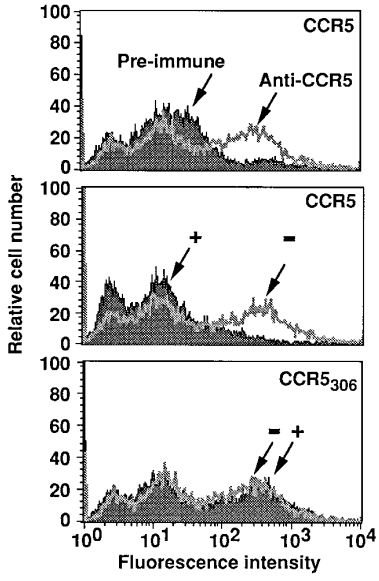

FIG. 7. Role of the cytoplasmic C-terminal domain of CCR5 in ligand-induced surface downmodulation. Flow cytometry analysis of surface expression of vaccinia-encoded CCR5 (top and middle panels) and $CCR5_{306}$ (bottom panel) on NIH 3T3 cells, preincubated at 37° for 30 min in the presence (+) or absence (−) of a mixture of MIP-1α, MIP-1β and RANTES (170 n*M* each). The top panel indicates cells stained with either the polyclonal antiserum (Anti-CCR5) or the preimmune serum from the same rabbit; the middle and bottom panels indicate cells stained with the anti-CCR5 polyclonal rabbit antiserum. The analysis shown was performed in parallel with the fusion experiment shown in Fig. 6B.

Our data do not exclude the possibility that alternative signaling pathways may be involved in the fusion process or its inhibition by chemokines. In particular, the pertussis toxin data must be viewed in the context of recent findings that some chemokine receptors are capable of interacting with pertussis toxin-insensitive G proteins, at least when overexpressed together in heterologous cell types (Kuang *et al.,* 1996). However, the ability of pertussis toxin to completely block calcium flux in response to chemokine activation of CCR5 (Combadiere *et al.,* 1996) suggests that pertussis toxin-insensitive G proteins are either not available or not used by CCR5 in HEK 293 cells; alternatively they may be used but not coupled to a calcium mobilizing pathway.

Ligand-induced downmodulation of surface CCR5 represents an obvious mechanism by which chemokines inhibit fusion/infection. However, the ability of chemokines to inhibit fusion even when downmodulation is prevented (i.e., with $CCR5_{306}$) suggests a second mechanism: a direct blocking effect. In view of our finding that truncation of the C-terminus of CCR5 prevents chemo-

kine-induced downmodulation and reduces the efficiency of chemokine inhibition of coreceptor activity, it is likely that both surface downmodulation and direct blocking contribute to the inhibitory effects. Both mechanisms therefore must be taken into account in mechanistic interpretations of chemokine inhibition experiments. These considerations also have implications for designing novel chemokine receptor-targeted therapies for HIV-1, since they suggest that an agent capable of both downmodulating CCR5 and physically blocking its interaction with Env may be most efficacious.

Our data, suggesting a direct blocking mechanism, are consistent with recent studies demonstrating physical interactions between Env, coreceptors, and CD4 (Lapham *et al.,* 1996; Trkola *et al.,* 1996; Wu *et al.,* 1996). A favored model envisions multiple, probably sequential, interactions whereby gp120 binds first to CD4, then to the fusion coreceptors; ultimately it is presumed that the cryptic hydrophobic "fusion peptide" at the N-terminus of gp41 is exposed so that it can interact with the target cell membrane to promote the fusion event. Despite the noninvolvement of G protein signaling in fusion, it is interesting to speculate that Env interactions with coreceptors (CCR5, CXCR4, etc.) might trigger signaling processes unrelated to entry that contribute to the pathogenic sequelae associated with HIV infection.

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