

# Human Interferon-Inducible 10-kDa Protein and Human Interferon-Inducible T Cell $\alpha$ Chemoattractant Are Allotopic Ligands for Human CXCR3: Differential Binding to Receptor States

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## ABSTRACT

The human CXC chemokines IP-10 (10-kDa interferon-inducible protein), MIG (monokine induced by human interferon- $\gamma$ ), and I-TAC (interferon-inducible T cell  $\alpha$  chemoattractant) attract lymphocytes through activation of CXCR3. In the studies presented here, we examined interaction of these chemokines with human CXCR3 expressed in recombinant cells and human peripheral blood lymphocytes (PBL). IP-10, MIG, and I-TAC were agonists in stimulating [ $^{35}$ S]GTP $\gamma$ S binding in recombinant cell and PBL membranes but had no effect in the absence of hCXCR3 expression.  $^{125}$ I-IP-10 and  $^{125}$ I-I-TAC bound hCXCR3 with high affinity, although the  $^{125}$ I-I-TAC  $B_{\max}$  value in saturation bindings was 7- to 13-fold higher than that measured with  $^{125}$ I-IP-10. Coincubation with unlabeled chemokines decreased  $^{125}$ I-IP-10 binding with a single discernible affinity. However, with  $^{125}$ I-I-TAC, competition with IP-10 or MIG was incomplete, and multiple binding affinities were evident. Moreover, in contrast to I-TAC, IP-10 and MIG binding  $IC_{50}$  values

did not increase predictably with increased  $^{125}$ I-I-TAC concentration in competition bindings, suggesting that these chemokines are noncompetitive (i.e., allotopic) ligands. Uncoupling of hCXCR3 eliminated  $^{125}$ I-IP-10 binding but only decreased  $^{125}$ I-I-TAC binding 30 to 80%, indicating that unlike IP-10, I-TAC binds with high affinity to uncoupled (**R**) and coupled (**R** $^*$ ) hCXCR3. To examine chemokine binding to **R** $^*$ , we tested the effect of anti-hCXCR3 antibody on I-TAC- and IP-10-stimulated [ $^{35}$ S]GTP $\gamma$ S binding. The antibody attenuated [ $^{35}$ S]GTP $\gamma$ S binding in response to IP-10 but not to I-TAC, suggesting that the two chemokines bind differently to **R** $^*$ . Moreover, increased occupancy of **R** $^*$  with a >75-fold increase in  $^{125}$ I-IP-10 concentration did not increase the I-TAC binding  $IC_{50}$  value, and I-TAC increased the dissociation rate of  $^{125}$ I-IP-10. From these data, we conclude that the binding of IP-10 and I-TAC to the **R** $^*$  state of hCXCR3 is allotopic.

Chemoattractant cytokines (chemokines) stimulate leukocyte chemotaxis by activation of various G protein-coupled receptors. As such, chemokines are important mediators of inflammatory responses. Interferon-inducible 10-kDa protein (IP-10) and monokine induced by human interferon- $\gamma$  (MIG) are CXC chemokines originally characterized as potent chemoattractants for activated T and natural killer cells (Luster and Leder, 1993; Farber, 1997). IP-10 and MIG attract leukocytes through activation of CXCR3 expressed on these cells. Recently, a novel non-ELR (glutamate-leucine-arginine) CXC chemokine, interferon-inducible T cell  $\alpha$  chemoattractant (I-TAC), was also identified as a potent hCXCR3 agonist in human and mouse (Cole et al., 1998; Widney et al., 2000). Interestingly, there is evidence that I-TAC expression and its regulation differ from that of MIG

and IP-10 (Mach et al., 1999), suggesting that these chemokines are more than just redundant ligands for hCXCR3.

Cole et al. (1998) used both signaling assays and binding studies with  $^{125}$ I-I-TAC to examine I-TAC pharmacology at hCXCR3. They found that I-TAC was more potent and efficacious than IP-10 or MIG as a chemoattractant and in stimulating calcium flux and receptor desensitization. Indeed, these findings led them to propose that I-TAC is the dominant ligand for this receptor. Competition bindings with  $^{125}$ I-I-TAC, human IP-10, and human MIG generated binding profiles that seem non-Michaelian. The authors suggested that these nonclassical binding curves reflected the poor affinity for IP-10 and MIG relative to I-TAC for hCXCR3. The studies presented here elucidate I-TAC and IP-10 binding at hCXCR3 expressed in recombinant or hu-

**ABBREVIATIONS:** IP-10, 10-kDa interferon-inducible protein; GTP $\gamma$ S, guanosine-5'-O-(3-thio)triphosphate; I-TAC, interferon-inducible T cell  $\alpha$  chemoattractant; MIG, monokine induced by human interferon- $\gamma$ ; PBL, peripheral blood lymphocytes; WGA-SPA, wheat germ agglutinin bead-scintillation proximity assay.

man peripheral blood lymphocytes. Using a variety of pharmacological approaches, we demonstrate that IP-10 and I-TAC have vastly different affinities for uncoupled hCXCR3 and are allotropic ligands for coupled hCXCR3.

## Experimental Procedures

**Cells and Cell Culture.** The cDNA encoding human CXCR3 was generated as described previously (Jen et al., 1999) and cloned into the mammalian expression vector pME18Sneo, a derivative of the SR $\alpha$  expression vector (Takebe et al., 1988). Interleukin-3-dependent mouse pro-B cells (Ba/F3) were transfected to express hCXCR3 (Ba/F3-hCXCR3) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100  $\mu$ g/ml streptomycin, 1 g/ml G418 (Life Technologies, Gaithersburg, MD), and 100 U/ml penicillin, 50  $\mu$ M  $\beta$ -mercaptoethanol, and 2 ng/ml of recombinant mouse Interleukin-3 (BioSource International, Camarillo, CA). 293EBNA (Epstein Barr Nuclear Ag) cells (Invitrogen, Carlsbad, CA) transfected to express hCXCR3 (293-hCXCR3; Jen et al., 1999) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100  $\mu$ g/ml streptomycin and 100 U/ml penicillin, 100  $\mu$ g/ml G418, and 300  $\mu$ g/ml hygromycin (Roche Molecular Biochemicals, Indianapolis, IN). Human peripheral blood lymphocytes (PBL) were prepared by Ficoll-Hypaque centrifugation, depleted of monocytes, (Wahl and Smith, 1991) and stimulated for 2 days with 1  $\mu$ g/ml phytohemagglutinin (Murex Diagnostics, Dartford, UK) and 100 U/ml interleukin-2 (Sigma, St. Louis, MO) in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100  $\mu$ g/ml streptomycin, 100 U/ml penicillin, 1% nonessential amino acids, and 2 mM HEPES. After stimulation, PBL were cultured in above media containing 5% conditioned media (Sigma) for up to 15 days.

**Cell Membrane Preparation.** Ba/F3-hCXCR3 were pelleted and resuspended in lysis buffer containing 10 mM HEPES, pH 7.5, and Complete protease inhibitors (1 tablet/100 ml) (Roche Molecular Biochemicals) at a cell density of  $20 \times 10^6$  cells/ml. After a 5-min incubation on ice, the cells were transferred to a 4639 cell disruption bomb (Parr Instrument, Moline, IL) and applied with 1500 psi nitrogen for 30 min on ice. Large cellular debris was removed by centrifugation at 1000g. Cell membrane in the supernatant was pelleted at 100,000g. The membrane was resuspended in lysis buffer supplemented with 10% sucrose and stored at  $-80^\circ\text{C}$ . PBL and 293-hCXCR3 membranes were prepared as described previously (Hipkin et al., 1997). Cells were pelleted by centrifugation and incubated in homogenization buffer (10 mM Tris-HCl, 5 mM EDTA, 3 mM EGTA, pH 7.6) and 1 mM phenylmethylsulfonyl fluoride on ice for 30 min. The cells were then lysed with a Dounce homogenizer using a stirrer type RZR3 polytron homogenizer (CafraMo, Warton, ON) with 10 to 20 strokes at 900 rpm. The intact cells and nuclei were removed by centrifugation at 500g for 5 min. The cell membranes in the supernatant were then pelleted by centrifugation at 10,000g for 30 min. The membranes were then resuspended in glycol buffer (20 mM glycylglycine, 1 mM MgCl<sub>2</sub>, 250 mM sucrose, pH 7.2), aliquoted, quick frozen, and stored at  $-80^\circ\text{C}$ . Protein concentration in membrane preparations was determined using the method of Bradford (1976).

**Radioligand Bindings.** <sup>125</sup>I-I-TAC and carrier-free <sup>125</sup>I-IP-10 (specific activity, 986-2200 and 2200 Ci/mmol, respectively) were obtained from PerkinElmer Life Sciences (Boston, MA). A scintillation proximity assay was used for radioligand competition and saturation binding assays. For each assay point, 0.5 to 2  $\mu$ g of membrane was preincubated for 1 h at room temperature with 300  $\mu$ g of wheat germ agglutinin-coated scintillation proximity assay beads (WGA-SPA; Amersham Pharmacia Biotech, Piscataway, NJ) in SPA binding buffer (50 mM HEPES, 1 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 125 mM NaCl, 0.002% NaN<sub>3</sub>, 1.0% bovine serum albumin). The beads and membranes were transferred to a 96-well Isoplate (Wallac, Gaith-

ersburg, MD) and incubated at room temperature with <sup>125</sup>I-IP-10 or <sup>125</sup>I-I-TAC and the indicated concentrations of chemokines for 3 h. Where indicated, membranes were incubated in the absence or presence of purified mouse anti-hCXCR3 monoclonal antibody (clone 1/C6/CXCR3; PharMingen International, San Diego, CA) or isotype control antibody (mouse IgG<sub>1</sub>,  $\kappa$ ) before addition of ligands and radioligand. Ligand affinities from competition bindings were calculated from binding IC<sub>50</sub> values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

**[<sup>35</sup>S]GTP $\gamma$ S Binding.** Cell membranes were resuspended in GTP $\gamma$ S binding buffer [20 mM HEPES, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, and 0.2% (w/v) bovine serum albumin (factor V, lipid free), pH 7.4] and kept on ice. Membranes in 96-well plates (1–2  $\mu$ g/point, in triplicate) were incubated with 1  $\mu$ M GDP, 0.3 nM guanosine 5'- $\gamma$ -<sup>35</sup>S-triphosphate ([<sup>35</sup>S]GTP $\gamma$ S, triethylammonium salt; specific activity, 1250 Ci/mmol; PerkinElmer Life Sciences) in the presence or absence of various ligands for 30 to 60 min at 30°C. The reaction was terminated by placing the plates on ice and filtering the membranes through a UniFilter GF/B filter plate (Packard Instrument Co., Meriden, CT) using a Tomtec 96-well cell harvester (Hamden, CT). The filters and membranes were washed 10 times at room temperature with 20 mM HEPES and 10 mM sodium pyrophosphate. Membrane-bound [<sup>35</sup>S]GTP $\gamma$ S was measured by liquid scintillation using a TopCount NXT Microplate scintillation and luminescence counter (Packard Instrument Co.). In some experiments, [<sup>35</sup>S]GTP $\gamma$ S bindings were performed, and membranes were preincubated for 60 min at room temperature in the absence or presence of 1C6/CXCR3 or isotype control antibody (see above) before addition of ligands and [<sup>35</sup>S]GTP $\gamma$ S. In these experiments, the bindings were done in SPA binding buffer (as described above) containing 1  $\mu$ M GDP and 0.3 nM [<sup>35</sup>S]GTP $\gamma$ S. Membrane-bound [<sup>35</sup>S]GTP $\gamma$ S was measured by scintillation proximity assay.

**Materials.** Chemokines were purchased from R & D Systems Inc. (Minneapolis, MN). Nonlinear regression analysis of the data was performed using Prism 2.0b (GraphPad Software, San Diego, CA). All other reagents were of the best grade available and purchased from common suppliers.

## Results

**Effect of Chemokines on [<sup>35</sup>S]GTP $\gamma$ S Exchange in Ba/F3-hCXCR3, 293-hCXCR3, and Peripheral Blood Lymphocyte Membranes.** Activation of G protein-coupled receptors with agonists stimulates the molecular exchange of GTP for GDP on the active site of the  $\alpha$  protein (Gilman, 1987). By substituting the nonhydrolyzable GTP analog [<sup>35</sup>S]GTP $\gamma$ S for GTP, agonist activation and subsequent guanylyl nucleotide exchange in cell membranes results in an increase in [<sup>35</sup>S]GTP $\gamma$ S binding (Hilf et al., 1989; Lorenzen et al., 1993; Gonsiorek et al., 2000). To this end, a [<sup>35</sup>S]GTP $\gamma$ S exchange assay was instituted to measure chemokine agonism in membranes from two cell lines transfected to over-express hCXCR3 or from activated PBL. Ba/F3-hCXCR3 (Fig. 1, left) or PBL (Fig. 1, right) membranes were incubated with 0.3 nM [<sup>35</sup>S]GTP $\gamma$ S, 1  $\mu$ M GDP, and the indicated concentrations of IP-10, I-TAC, or MIG for 60 min at 30°C, upon which the reaction was terminated by filtration (as described under *Experimental Procedures*). Under these assay conditions, IP-10, I-TAC, and MIG were all full agonists in stimulating [<sup>35</sup>S]GTP $\gamma$ S exchange (Fig. 1) but varied considerably in their potency (Ba/F3-hCXCR3, EC<sub>50</sub> =  $0.3 \pm 0.08$ ,  $0.07 \pm 0.05$ , and  $11 \pm 1$  nM, respectively;  $n = 3$ ; PBL, EC<sub>50</sub> =  $5.0 \pm 3.7$ ,  $0.24 \pm 0.08$ , and  $70.0 \pm 1.1$  nM, respectively;  $n = 2$ ). [<sup>35</sup>S]GTP $\gamma$ S exchange assays within 293-hCXCR3 membranes produced similar results (data not shown; IP-10

$EC_{50} = 0.19 \pm 0.15$ , I-TAC =  $0.08 \pm 0.05$ , and MIG =  $13.5 \pm 6.3$  nM;  $n = 2$ ). There was no stimulation of [ $^{35}$ S]GTP $\gamma$ S binding in membranes from untransfected Ba/F3 and 293 cells, which do not bind  $^{125}$ I-IP-10 or  $^{125}$ I-I-TAC (data not shown). Therefore, we conclude that the stimulation of [ $^{35}$ S]GTP $\gamma$ S binding upon incubation with the chemokines is mediated through binding to hCXCR3.

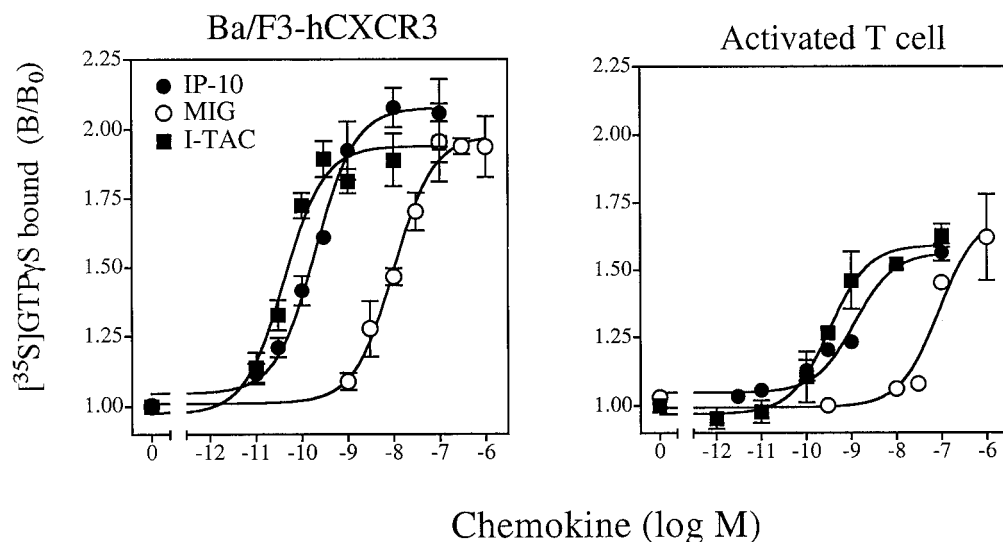
**Saturation and Competition Binding Analysis with  $^{125}$ I-IP-10 and  $^{125}$ I-I-TAC.**  $^{125}$ I-IP-10 and  $^{125}$ I-I-TAC affinities for hCXCR3 were measured in Ba/F3-hCXCR3, 293-hCXCR3, and human PBL membranes by saturation binding analyses (as described under *Experimental Procedures*). Membranes were incubated at room temperature with the indicated concentrations of radioligand in the presence or absence of 30 nM I-TAC.  $^{125}$ I-IP-10 bound with one discernable affinity in membranes from both recombinant cells (Ba/F3-hCXCR3,  $65 \pm 28$  pM; 293-hCXCR3,  $180 \pm 42$  pM; Fig. 2) and PBL ( $235 \pm 190$  pM). Parallel saturation analysis with  $^{125}$ I-I-TAC showed that it bound hCXCR3 with slightly higher affinity than did  $^{125}$ I-IP-10 ( $30 \pm 4$ ,  $99 \pm 7$ , and  $26 \pm 11$  pM, respectively). More strikingly, the calculated  $B_{max}$  value with  $^{125}$ I-I-TAC was 7- to 13-fold higher in both recombinant cell and PBL membranes (Fig. 2) and in intact PBL (data not shown). This incongruity in specific binding suggests that I-TAC and IP-10 are not binding to the identical receptor population.

In competition binding analysis with Ba/F3-hCXCR3 membranes and  $^{125}$ I-IP-10 (Fig. 3, left), human I-TAC, human IP-10, and human MIG all competed for binding with the expected high affinities ( $K_i \pm S.D. = 79 \pm 27$  pM,  $33 \pm 6$  pM, and  $1.2 \pm 0.4$  nM, respectively;  $n = 2-3$ ). As can be seen in the representative experiment shown in Fig. 3 (center), human IP-10 and MIG also inhibited  $^{125}$ I-I-TAC binding, although multiple affinities were apparent, especially in competition with human IP-10 (25%,  $K_i = 1.0$  nM; 75%,  $K_i = 86$  nM;  $n = 3$ ). In addition, competition with human IP-10 and MIG was incomplete relative to that seen with I-TAC. Moreover, the I-TAC  $K_i$  value calculated from competition with  $^{125}$ I-I-TAC ( $K_i = 480 \pm 35$  pM) was considerably lower than that calculated with  $^{125}$ I-IP-10 competition (see above). Competition binding in human PBL membranes using  $^{125}$ I-I-TAC, IP-10, and I-TAC (Fig. 3, right) generated a binding

profile similar to that seen in the recombinant cell membranes. I-TAC competed with a similar affinity ( $0.52 \pm 0.2$  nM), whereas IP-10 was ineffective in competing for binding ( $n = 3$ ). We next measured the potency of IP-10 and I-TAC to inhibit binding in Ba/F3-hCXCR3 membranes in the face of increasing concentrations of  $^{125}$ I-I-TAC (33, 333, or 1300 pM). As shown in Fig. 4, the binding  $IC_{50}$  value for I-TAC competition increased when the  $^{125}$ I-I-TAC concentration was elevated such that the calculated  $K_i$  value remained constant ( $K_i = 50-60$  pM), consistent with the binding of competitive ligands. In contrast, IP-10 did not fully compete for binding with  $^{125}$ I-I-TAC, and the potency of binding inhibition varied with the concentration of  $^{125}$ I-I-TAC. The inconsistent  $K_i$  values ( $0.3-52$  nM) generated from the binding  $IC_{50}$  values using the Cheng-Prusoff calculation again suggests that IP-10 and I-TAC bind in an allotopic (noncompetitive) manner to hCXCR3.

**Effect of Functional Uncoupling of hCXCR3 on I-TAC and IP-10 Binding.** We assessed the effect of hCXCR3 uncoupling on IP-10 and I-TAC binding. Ba/F3-hCXCR3 membranes were incubated with  $^{125}$ I-I-TAC or  $^{125}$ I-IP-10 and the indicated concentrations of GTP $\gamma$ S, 10 nM IP-10, or 30 nM I-TAC. As shown in Fig. 5A, GTP $\gamma$ S decreased the binding of both  $^{125}$ I-I-TAC and  $^{125}$ I-IP-10 with  $IC_{50}$  values of  $97 \pm 33$  and  $18 \pm 6$  nM, respectively ( $n = 2$ ). GTP $\gamma$ S inhibited  $^{125}$ I-IP-10 binding to the same extent as did excess unlabeled IP-10;  $^{125}$ I-I-TAC binding was decreased by only  $35 \pm 2\%$  versus  $93 \pm 1\%$  binding inhibition with 30 nM I-TAC. These data indicate that I-TAC has a higher affinity for uncoupled hCXCR3 than does IP-10. To more fully investigate this observation, we performed competitions in Ba/F3-hCXCR3 membranes using  $^{125}$ I-I-TAC in the presence or absence of GTP $\gamma$ S (Fig. 5B). Again, GTP $\gamma$ S decreased I-TAC affinity by 30 to 40%, consistent with the decrease in total  $^{125}$ I-I-TAC binding. In the absence of GTP $\gamma$ S, IP-10 competes for  $^{125}$ I-I-TAC binding with two apparent affinities ( $27 \pm 2\%$ ,  $IC_{50} = 0.7 \pm 0.4$  nM;  $73 \pm 2\%$ ,  $IC_{50} = 80 \pm 6$  nM). However, in the presence of GTP $\gamma$ S, IP-10 competes for  $^{125}$ I-I-TAC binding at a single low-affinity binding site ( $83 \pm 16$  nM;  $n = 2$ ).

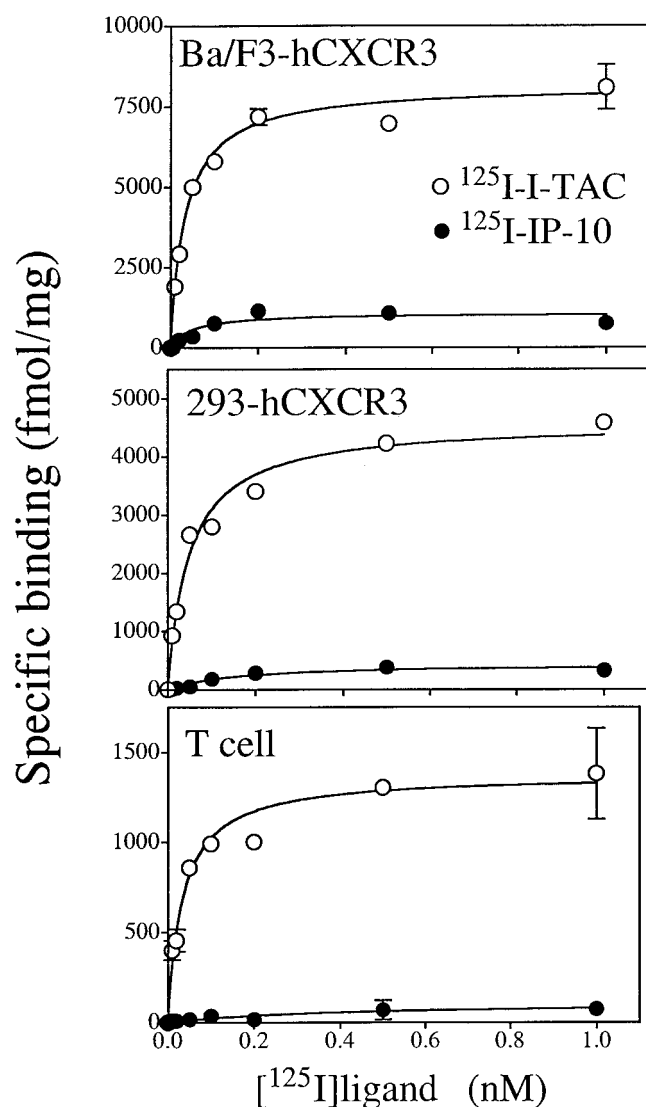
As an alternative approach to uncouple hCXCR3, 293-hCXCR3 cells and activated PBL were incubated for 18 to 20 h in the absence or presence of 100 ng/ml of pertussis



**Fig. 1.** The effect of chemokines on [ $^{35}$ S]GTP $\gamma$ S exchange in Ba/F3-hCXCR3 and PBL membranes. Ba/F3-hCXCR3 (left) or PBL membranes (right) were incubated for 60 min at 30°C in GTP $\gamma$ S binding buffer (as described under *Experimental Procedures*) containing 0.3 nM [ $^{35}$ S]GTP $\gamma$ S, 1  $\mu$ M GDP, the indicated concentrations of I-TAC (■), IP-10 (●), or MIG (○). After filtration, membrane-associated radioactivity was measured by liquid scintillation. Data, expressed relative to basal binding, represent the mean total binding  $\pm$  range of triplicate determinations from two to four independent experiments.



toxin. Pertussis toxin ADP-ribosylates the C-terminal region of  $G_{i/o}$ , preventing the interaction of these G proteins with their receptors (Passador and Iglewski, 1994). Membranes from these cells were then incubated with 20 pM  $^{125}$ I-I-TAC and the indicated concentrations of IP-10 and I-TAC or 10  $\mu$ M GTP $\gamma$ S. As shown in Fig. 5C, in pertussis toxin-pretreated 293-hCXCR3 membranes, total  $^{125}$ I-I-TAC binding decreased approximately 80% ( $n = 2$ ), consistent with a 5-fold decrease in I-TAC affinity ( $K_i = 0.62 \pm 0.13$  nM) relative to that seen in control membranes ( $K_i = 0.14 \pm 0.02$  nM). IP-10 binding affinity was also lower in membranes from pertussis toxin-pretreated cells ( $K_i = 194 \pm 24$  nM) relative to that measured in control membranes ( $K_i = 32 \pm 4$  nM;  $n = 2$ ). Not surprisingly, there was no measurable specific binding of  $^{125}$ I-IP-10 in membranes from pertussis toxin-pretreated cells (data not shown). Uncoupling of hCXCR3 in



**Fig. 2.** Saturation binding in Ba/F3-hCXCR3, 293-hCXCR3, and PBL membranes. For saturation binding analysis, membranes (2–4  $\mu$ g/well) from Ba/F3-hCXCR3 (top), 293-hCXCR3 (center), and peripheral blood cells (bottom) were incubated in binding buffer at room temperature with the indicated concentrations of  $^{125}$ I-IP-10 (●) or  $^{125}$ I-I-TAC (○)  $\pm$  30 nM I-TAC. Radioligand binding to the membranes was measured by WGA-SPA scintillation. The mean specific binding  $\pm$  S.E.M. of triplicate determinations from a representative experiment is shown ( $n = 2$ –3).

pertussis toxin-pretreated cell membranes was complete as 10  $\mu$ M GTP $\gamma$ S did not further decrease total  $^{125}$ I-I-TAC binding, indicating that hCXCR3 interacts only with  $G_{i/o}$ . In PBL membranes (Fig. 5D), I-TAC competed with  $^{125}$ I-I-TAC binding ( $K_i = 0.19 \pm 0.13$  nM), whereas IP-10 binding was incomplete and low affinity ( $IC_{50} = 35 \pm 10$  nM; see Fig. 3). Pertussis toxin pretreatment of PBL again decreased total  $^{125}$ I-I-TAC binding approximately 80% ( $n = 2$ ).

From these data, we conclude that  $^{125}$ I-I-TAC binds with high affinity to both the uncoupled (**R**) and coupled (**R**<sup>\*</sup>) hCXCR3 conformations, whereas  $^{125}$ I-IP-10, for all practical purposes, binds only to **R**<sup>\*</sup>.

**Effect of Anti-hCXCR3 Antibody on I-TAC and IP-10 Binding.** We assessed the effect of a monoclonal antibody raised against a peptide corresponding to the first 37 amino acids of the N-terminal region of hCXCR3 on IP-10 and I-TAC binding. This antibody (clone 1/C6;  $\alpha$ -CXCR3) has been reported to block the binding of human IP-10 to hCXCR3 (Qin et al., 1998). Ba/F3-hCXCR3 membranes were incubated with the indicated concentrations of  $\alpha$ -CXCR3 or its isotype control for 60 min before a 3-h incubation with either  $^{125}$ I-I-TAC or  $^{125}$ I-IP-10. The isotype control had no effect on the binding of either radioligand, whereas  $\alpha$ -CXCR3 blocked  $^{125}$ I-IP-10 binding to **R**<sup>\*</sup> with an  $IC_{50}$  value of 0.55 nM (Fig. 6A).  $^{125}$ I-I-TAC binding was also potently inhibited by the antibody ( $IC_{50} = 1.1$  nM), although binding was inhibited only 30 to 40%, whereas  $^{125}$ I-IP-10 binding decreased 70 to 80%. As  $^{125}$ I-I-TAC binds to both **R**<sup>\*</sup> and **R**, we conducted experiments to assess the ability of the antibody to inhibit I-TAC binding to the different receptor conformations. Membranes from 293-hCXCR3 cells pretreated in the absence or presence of pertussis toxin (see above) were incubated with  $^{125}$ I-I-TAC and the indicated concentrations of  $\alpha$ -CXCR3 antibody (Fig. 6B). Again, the antibody potently ( $IC_{50} = 1.2$  nM) but incompletely inhibited  $^{125}$ I-I-TAC binding in control membranes. Interestingly, in membranes from pertussis toxin-pretreated cells,  $^{125}$ I-I-TAC binding was completely inhibited by the  $\alpha$ -CXCR3 antibody ( $IC_{50} = 0.6$  nM). These data suggest that the antibody is more efficient at inhibiting I-TAC binding to uncoupled hCXCR3 and that the I-TAC binding site(s) on coupled and uncoupled receptor differ. Next, 293-hCXCR3 membranes were incubated with  $^{125}$ I-I-TAC and the indicated concentrations of GTP $\gamma$ S in the absence or presence of 80 nM  $\alpha$ -CXCR3 or its isotype control. As shown in Fig. 7, with the isotype control, GTP $\gamma$ S displaced  $52 \pm 3\%$  of the specific binding. Incubation with  $\alpha$ -CXCR3 antibody alone decreased specific binding ( $51 \pm 0.5\%$ ;  $n = 2$ ). Coincubation with GTP $\gamma$ S further decreased specific  $^{125}$ I-I-TAC binding  $78 \pm 2\%$ . Therefore, in the presence of the  $\alpha$ -CXCR3, a higher percentage of the remaining  $^{125}$ I-I-TAC binding represents interaction with coupled receptor. This observation is consistent with the hypothesis that this antibody preferentially inhibits the binding of I-TAC to uncoupled receptor.

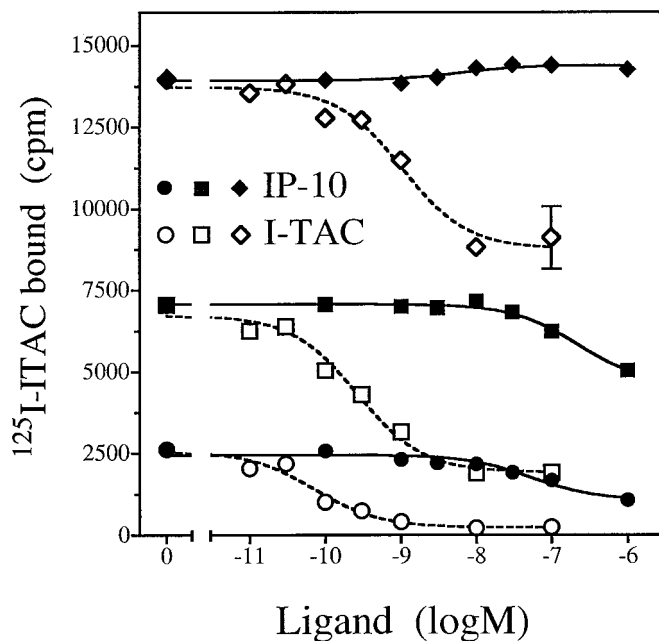
**Effect of Anti-CXCR3 Antibody on hCXCR3 Activation.** We assessed the effect of  $\alpha$ -CXCR3 on hCXCR activation by both IP-10 and I-TAC. Ba/F3-hCXCR3 membranes prebound to WGA-SPA beads were incubated with 12.5  $\mu$ g/ml of  $\alpha$ -CXCR3 or its isotype control for 60 min before incubation with the indicated concentrations of IP-10 or I-TAC and 0.3 nM [ $^{35}$ S]GTP $\gamma$ S for 45 min at 30°C (as described under *Experimental Procedures*). As shown in Fig. 8,  $\alpha$ -CXCR3 inhib-

ited activation of the receptor by IP-10 in a competitive manner (isotype control,  $EC_{50} = 0.45 \pm 0.06$  nM;  $\alpha$ -CXCR3,  $EC_{50} = 11.3 \pm 3.1$  nM;  $n = 3$ ). In contrast, the antibody had only a small effect on the potency of receptor activation by I-TAC (isotype control,  $EC_{50} = 34 \pm 14$  pM;  $\alpha$ -CXCR3,  $EC_{50} = 74 \pm 31$  pM;  $n = 2-3$ ). These data are consistent with the binding data, which showed that  $\alpha$ -CXCR3 blocked the binding of IP-10 to  $R^*$  but was less effective in interfering with I-TAC binding to the active receptor conformation.

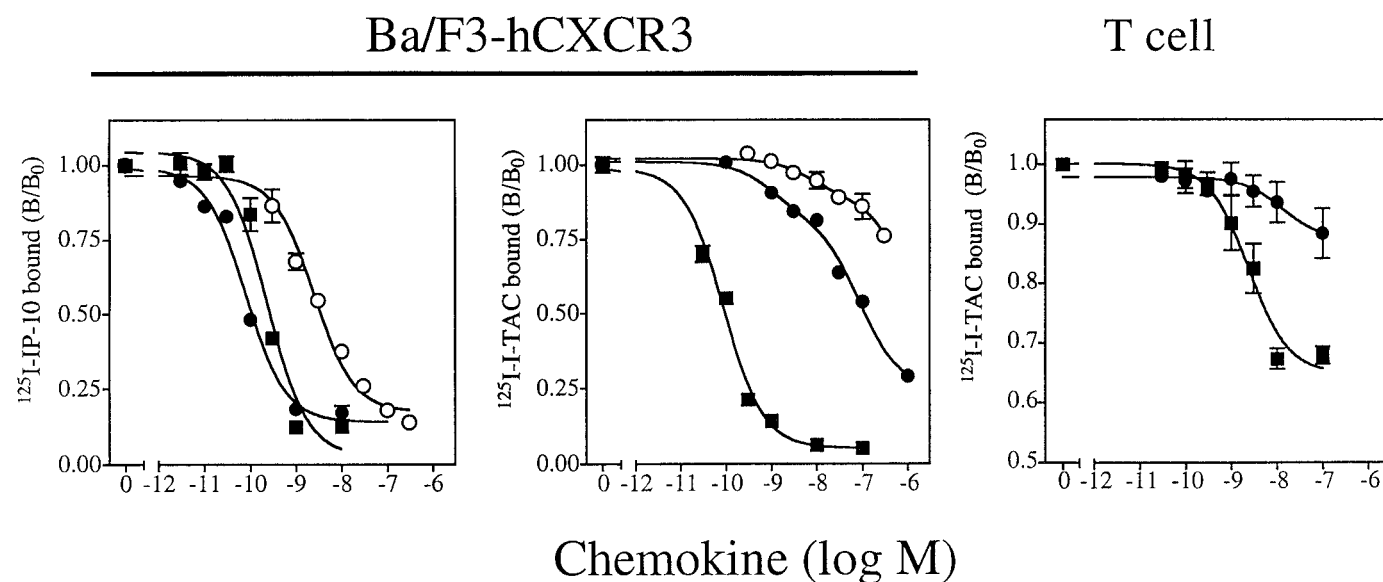
**Effect of  $^{125}$ I-IP-10 Concentration on the Apparent Binding  $K_i$  Value for IP-10 and I-TAC on hCXCR3  $R^*$ .** The antibody experiments (Figs. 6–8) suggest that IP-10 and I-TAC are allotopic ligands on the  $R^*$  conformation of hCXCR3. To test this hypothesis, competition bindings were set up with Ba/F3-hCXCR3 membranes using increasing concentrations of  $^{125}$ I-IP-10 (22, 173, or 1730 pM) and the indicated concentrations of I-TAC. As would be expected, total radioligand binding increased as  $^{125}$ I-IP-10 concentration was raised (Fig. 9, left). However, the I-TAC binding  $IC_{50}$  value remained unchanged despite the >75-fold increase in  $^{125}$ I-IP-10 concentration (Fig. 9, right). Cheng-Prusoff analysis generated  $K_i$  values that decreased as the  $^{125}$ I-IP-10 concentration increased. This is inconsistent with the binding of competitive ligands. The binding  $IC_{50}$  values for both IP-10 and MIG increased predictably with the concentration of  $^{125}$ I-IP-10 (data not shown) such that binding  $K_i$  values remained constant (IP-10,  $0.021 \pm 0.01$  nM; MIG,  $4.2 \pm 0.9$  nM;  $n = 2$ ), confirming that these ligands bind competitively with  $^{125}$ I-IP-10. The large population of uncoupled hCXCR3 in the membrane preparations and the relatively small difference in affinities of I-TAC for coupled and uncoupled receptor precluded attempting analogous studies with  $^{125}$ I-I-TAC.

**Effect of I-TAC on  $^{125}$ I-IP-10 Dissociation Rate from hCXCR3  $R^*$ .** We assessed whether I-TAC decreased  $^{125}$ I-IP-10 binding from the  $R^*$  conformation by increasing its dissociation rate from hCXCR3. Ba/F3-hCXCR3 membranes

were incubated with  $^{125}$ I-IP-10, pelleted, washed, and resuspended in cold binding buffer. The membranes and bound radioligand were then incubated at 30°C with various concentrations of I-TAC for the indicated times (as described under *Experimental Procedures*). As shown in Fig. 10, receptor-bound  $^{125}$ I-IP-10 dissociated at two discrete rates ( $t_{1/2} =$



**Fig. 4.** The effect of  $^{125}$ I-I-TAC concentration on competition with I-TAC or IP-10 in Ba/F3-hCXCR3 membranes. Membranes (1  $\mu$ g/well) from Ba/F3-hCXCR3 cells were incubated in binding buffer containing 33 pM (circles), 333 pM (squares), or 1.3 nM  $^{125}$ I-I-TAC (diamonds) and the indicated concentrations of I-TAC (open symbols) or IP-10 (closed symbols). Radioligand binding was measured by WGA-SPA scintillation. Data represent the mean  $\pm$  S.E.M. of triplicate determinations from an experiment representative of two independent experiments. Ligand affinities from competition bindings were calculated from binding  $IC_{50}$  value using the Cheng-Prusoff equation.

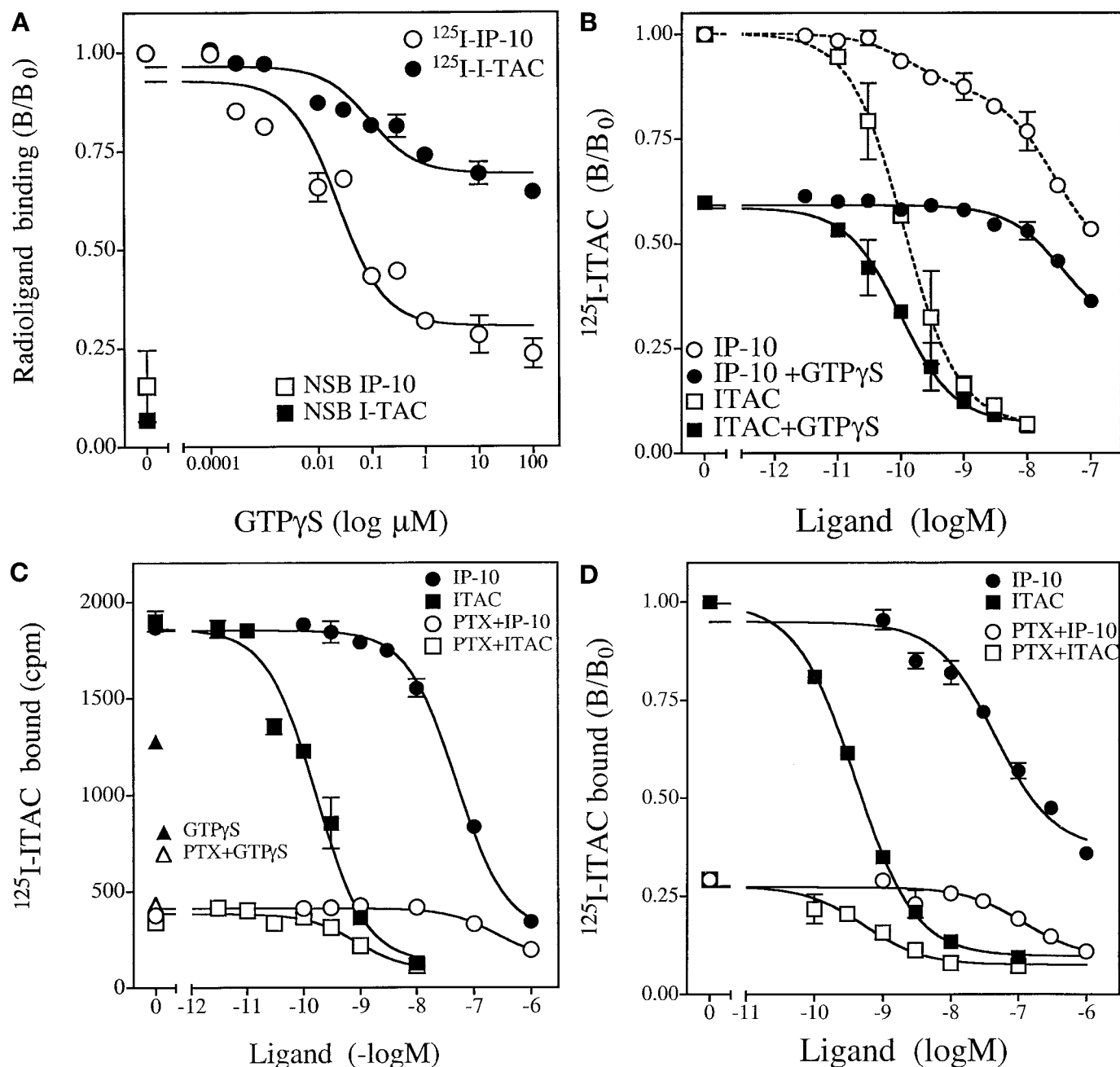


**Fig. 3.** Competition bindings in Ba/F3-hCXCR3 or PBL membranes using  $^{125}$ I-IP-10 or  $^{125}$ I-I-TAC. Membranes (2–4  $\mu$ g/well) from Ba/F3-hCXCR3 (left and center) and peripheral blood cells (right) were incubated in binding buffer containing 50 to 100 pM  $^{125}$ I-IP-10 and  $^{125}$ I-I-TAC and the indicated concentrations of I-TAC (■), IP-10 (●), or MIG (○). Radioligand binding to the membranes was measured by WGA-SPA scintillation. Data represent the mean  $\pm$  S.E.M. of triplicate determinations from a representative experiment (Ba/F3-hCXCR3) or three to four independent experiments. Ligand affinities from competition bindings were calculated from binding  $IC_{50}$  value using the Cheng-Prusoff equation.

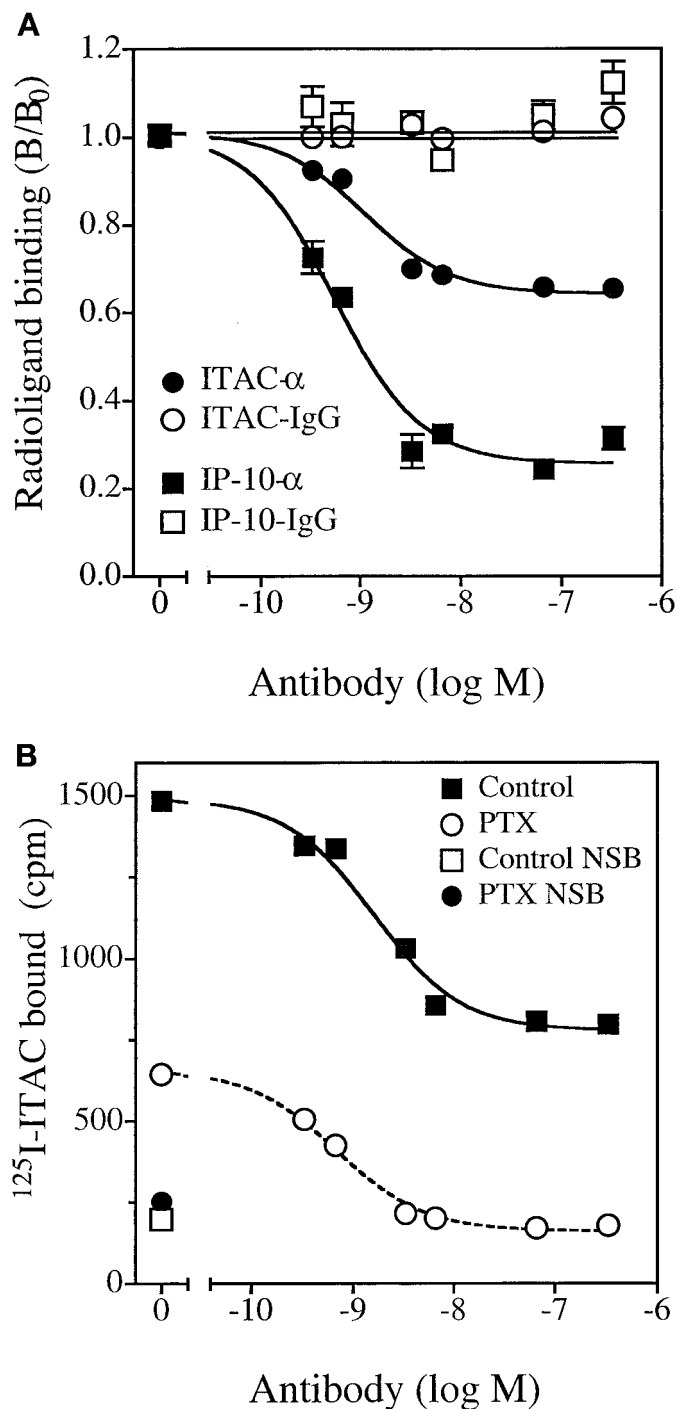
$6 \pm 3$  min and  $9.4 \pm 1.3$  h). Incubation with I-TAC stimulated dissociation of  $^{125}\text{I}$ -IP-10 binding from the high-affinity site in a concentration-dependent manner. The half-time of dissociation from the high-affinity site decreased to  $3.4 \pm 1.0$  h with 0.01 nM I-TAC and to  $22 \pm 6$  min with 10 nM ( $n = 2$ ). The dissociation of  $^{125}\text{I}$ -IP-10 by 10 nM I-TAC was complete in that binding was reduced to nonspecific levels (data not shown). Taken together, we conclude that IP-10 and I-TAC bind to the  $\text{R}^*$  conformation of hCXCR3 in an allotropic manner.

## Discussion

The studies presented herein used functional, pharmacological, and immunological approaches to characterize hCXCR3 interaction with its chemokine ligands. As has been reported previously (Cole et al., 1998), we found that I-TAC, IP-10, and MIG are potent agonists at hCXCR3. Surprisingly, we found that only IP-10 and MIG bound to hCXCR3 in a competitive manner. Both binding and functional studies using an anti-hCXCR3 antibody showed that I-TAC binds



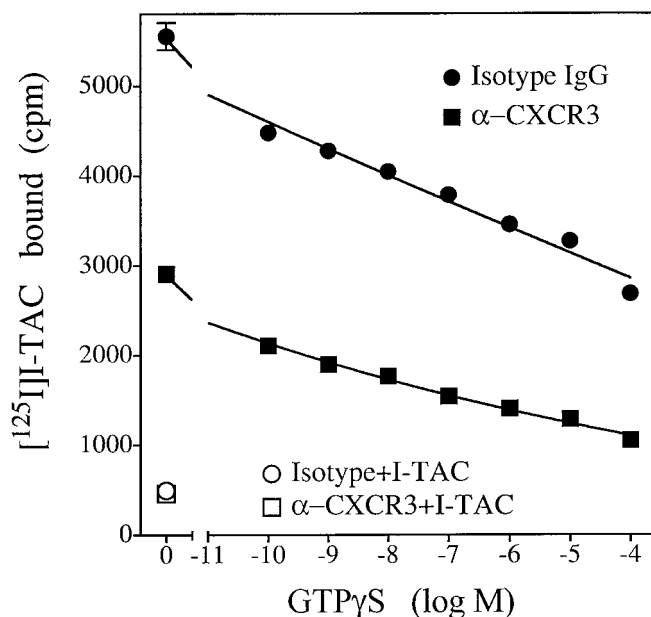
**Fig. 5.** The effect of hCXCR3 uncoupling on  $^{125}\text{I}$ -IP-10 and  $^{125}\text{I}$ -I-TAC binding. **A**, Ba/F3-hCXCR3 membranes (1–2  $\mu\text{g}/\text{well}$ ) in binding buffer were incubated with 100 pM  $^{125}\text{I}$ -IP-10 ( $\circ$ ) or  $^{125}\text{I}$ -I-TAC ( $\bullet$ ) and the indicated concentrations of  $\text{GTP}\gamma\text{S}$ , 10 nM IP-10 ( $\square$ ), or 30 nM I-TAC ( $\blacksquare$ ). **B**, Ba/F3-hCXCR3 membranes (1  $\mu\text{g}/\text{well}$ ) were incubated in binding buffer in the absence (open symbols) or presence (closed symbols) of 100  $\mu\text{M}$   $\text{GTP}\gamma\text{S}$  and the indicated concentrations of IP-10 ( $\circ$  and  $\bullet$ ) or I-TAC ( $\square$  and  $\blacksquare$ ). Membranes from 293-hCXCR3 cells (**C**) or PBL (**D**) pretreated overnight in the presence (open symbols) or absence (closed symbols) of 100 ng/ml of pertussis toxin were incubated with 100 pM  $^{125}\text{I}$ -I-TAC and the indicated concentrations of IP-10 ( $\circ$  and  $\bullet$ ), I-TAC ( $\square$  and  $\blacksquare$ ), or 100  $\mu\text{M}$   $\text{GTP}\gamma\text{S}$  (**C**,  $\triangle$  and  $\blacktriangle$ ). Radioligand binding to the membranes was measured by WGA-SPA scintillation. Data represent the mean total binding  $\pm$  S.E.M. of triplicate determinations from an experiment representative of two to four independent experiments. Ligand affinities from competition bindings were calculated from binding  $\text{IC}_{50}$  value using the Cheng-Prusoff equation.



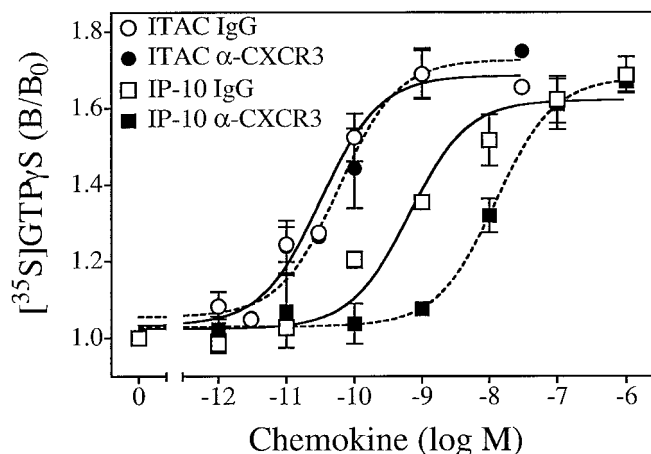
**Fig. 6.** The effect of  $\alpha$ -hCXCR3 antibody on chemokine binding to hCXCR3. **A**, Ba/F3-hCXCR3 membranes ( $2 \mu\text{g}/\text{well}$ ) were incubated with  $50 \text{ pM}$   $^{125}\text{I}$ -IP-10 (□ and ■) or  $^{125}\text{I}$ -I-TAC (○ and ●) and the indicated concentrations of  $\alpha$ -hCXCR3 (clone 1/C6, closed symbols) or isotype control antibody (open symbols). Radioligand binding to the membranes was measured by WGA-SPA scintillation. Data, expressed as a fraction of the specifically bound radioligand, represent the mean  $\pm$  S.E.M. of triplicate determinations from an experiment representative of two independent experiments. **B**, 293-hCXCR3 membranes ( $2 \mu\text{g}/\text{well}$ ) from cells pretreated in the presence (○ and □) or absence of pertussis toxin (● and ■) were incubated with  $200 \text{ pM}$   $^{125}\text{I}$ -I-TAC and the indicated concentrations of  $\alpha$ -hCXCR3 (clone 1/C6) or  $30 \text{ nM}$  I-TAC (● and □). Radioligand binding to the membranes was measured by WGA-SPA scintillation. Data represent the mean total binding  $\pm$  S.E.M. of triplicate determinations from an experiment representative of three independent experiments. Affinities for the  $\alpha$ -hCXCR3 antibody were derived from binding  $\text{IC}_{50}$  value using the Cheng-Prusoff equation.

allotopically with IP-10 and MIG to the active conformation of hCXCR3 ( $\text{R}^*$ ). Moreover, I-TAC was the only ligand of the three that also bound with high affinity to uncoupled receptor ( $\text{R}$ ).

In competition binding with  $^{125}\text{I}$ -I-TAC, the  $K_i$  value for I-TAC remained constant in the face of increased radioligand concentration, which would be expected if I-TAC bound competitively with  $^{125}\text{I}$ -I-TAC. However, IP-10 and MIG were ineffective and impotent in displacing  $^{125}\text{I}$ -I-TAC and became



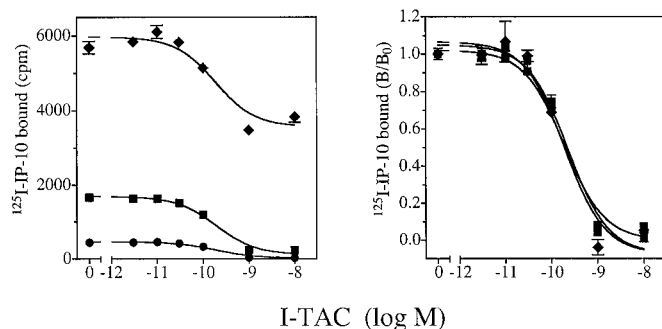
**Fig. 7.** The effect of receptor uncoupling on the inhibition of  $^{125}\text{I}$ -I-TAC binding with  $\alpha$ -hCXCR3 antibody. 293-hCXCR3 membranes ( $3 \mu\text{g}/\text{well}$ ) were incubated with  $50 \text{ pM}$   $^{125}\text{I}$ -I-TAC, the indicated concentrations of GTP $\gamma$ S, and  $80 \text{ nM}$   $\alpha$ -hCXCR3 (■) or isotype control antibody (●). Radioligand binding to the membranes was measured by WGA-SPA scintillation. Data represent the mean total binding  $\pm$  S.E.M. of triplicate determinations from an experiment representative of three independent experiments.



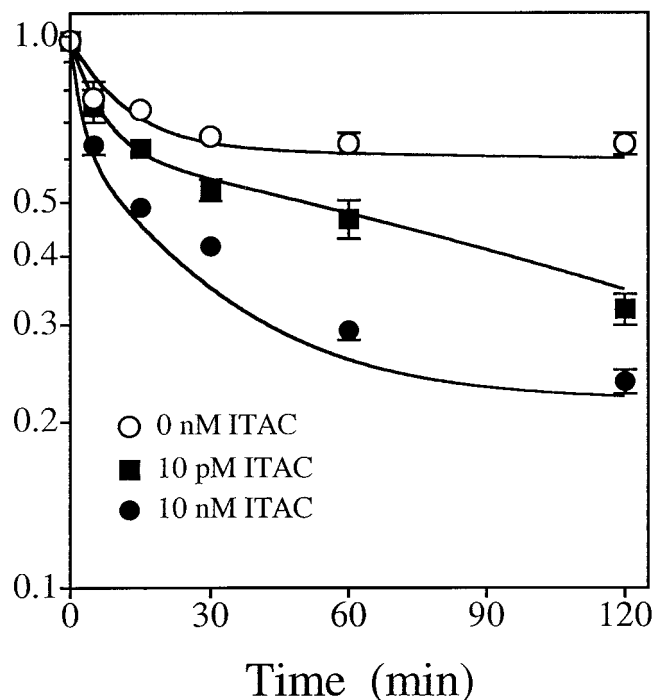
**Fig. 8.** The effect of  $\alpha$ -hCXCR3 antibody on hCXCR3 activation by IP-10 and I-TAC. Ba/F3-hCXCR3 membranes ( $2 \mu\text{g}/\text{well}$ ) were incubated for  $60 \text{ min}$  at  $30^\circ\text{C}$  with the indicated concentrations of IP-10 (squares) or I-TAC (circles) in the presence of  $80 \text{ nM}$   $\alpha$ -hCXCR3 (● and ■) or isotype control antibody (○ and □) in binding buffer containing  $0.3 \text{ nM}$   $^{35}\text{S}$ GTP $\gamma$ S and  $1 \mu\text{M}$  GDP. Radioligand binding to the membranes was measured by WGA-SPA scintillation. Data, expressed relative to basal binding, represent the mean total binding  $\pm$  S.E.M. of triplicate determinations from two independent experiments.



increasingly so as the concentration of  $^{125}\text{I}$ -I-TAC was increased. This was our first indication that I-TAC and IP-10/MIG bound at discrete sites on the receptor. Cole et al. (1998) also noted an unusual binding profile but suggested that it resulted from differences in the affinity with which I-TAC binds hCXCR3 relative to IP-10 and MIG. Our data suggest that the relative ineffectiveness of IP-10/MIG to displace I-TAC reflects the high affinity with which the latter binds to functionally uncoupled receptor. Using GTP $\gamma$ S or pertussis



**Fig. 9.** The effect of  $^{125}\text{I}$ -IP-10 concentration on competition with I-TAC in Ba/F3-hCXCR3 membranes. Membranes (2  $\mu\text{g}/\text{well}$ ) were incubated in binding buffer containing 22 pM ( $\bullet$ ), 173 pM ( $\blacksquare$ ) or 1.73 nM ( $\blacklozenge$ ) of  $^{125}\text{I}$ -IP-10 and the indicated concentrations of I-TAC. Radioligand binding to the membranes was measured by WGA-SPA scintillation. Data represent the mean total bound radioligand  $\pm$  S.E.M. or (right) as a fraction of the specifically bound radioligand  $\pm$  S.E.M. of triplicate determinations from a study representative of three independent experiments. Ligand affinities from competition bindings were calculated from binding  $\text{IC}_{50}$  value using the Cheng-Prusoff equation.



**Fig. 10.** The effect of I-TAC on the dissociation rate constant for  $^{125}\text{I}$ -IP-10 in Ba/F3-hCXCR3 membranes. Membranes (4  $\mu\text{g}/\text{point}$ ) were incubated in binding buffer containing 200 pM  $^{125}\text{I}$ -IP-10 in the absence or presence of 30 nM I-TAC, pelleted, washed, and further incubated at  $30^\circ\text{C}$  for various times with 0 ( $\circ$ ), 0.01 ( $\blacksquare$ ), or 10 nM I-TAC ( $\bullet$ ). Membranes were filtered and bound radioligand measured by liquid scintillation. Data represent the mean total binding  $\pm$  range of duplicate determinations from two independent experiments. Nonlinear regression analysis of the data was performed using Prism 2.0b (GraphPad).

toxin, we showed that only I-TAC bound with high affinity to both  $\text{R}^*$  and  $\text{R}$ . Surprisingly, a large population of uncoupled CXCR3 was present in our membrane preparations such that the  $^{125}\text{I}$ -IP-10  $B_{\text{max}}$  value (i.e., coupled receptor) in membranes (and Ba/F3-hCXCR3 cells; data not shown) represented approximately 10 to 20% of  $^{125}\text{I}$ -I-TAC  $B_{\text{max}}$  value (i.e., coupled + uncoupled receptor). Why a large percentage of receptor is uncoupled in both recombinant and normal cells is not known but may reflect a relative deficiency in the expression of the appropriate G protein. If so, G protein expression may be a limiting factor in cellular responsiveness to agonists, and changes in transducer expression may be functionally significant. At the  $^{125}\text{I}$ -I-TAC concentration used in our studies, approximately 60 to 70% of the total binding represents binding to uncoupled receptor. Therefore, the incomplete competition of I-TAC binding by IP-10 and MIG probably reflects their inability to displace radioligand binding from the uncoupled receptor population.

By using  $^{125}\text{I}$ -IP-10 in competitions, however, it is possible to characterize the binding of these chemokines to the  $\text{R}^*$  conformation of hCXCR3. Using this approach we found that the  $\text{IC}_{50}$  value for I-TAC displacement remained constant as the concentration of the  $^{125}\text{I}$ -IP-10 increased 75-fold such that the calculated I-TAC  $K_i$  values decreased progressively. In contrast, the IP-10 and MIG binding  $\text{IC}_{50}$  value predictably increased with increased radioligand such that the calculated  $K_i$  value remained unchanged. These data suggest that although IP-10 and MIG are competitive, I-TAC binds hCXCR3 at a nonoverlapping region(s) on the receptor protein. This hypothesis was further tested by assessing the effect of unlabeled I-TAC on the dissociation rate of  $^{125}\text{I}$ -IP-10 from  $\text{R}^*$ . Indeed, we found that incubation with I-TAC caused a 24-fold increase in the dissociation rate of  $^{125}\text{I}$ -IP-10. As competitive ligands will not affect ligand  $K_{\text{off}}$ , these data support the hypothesis that IP-10 and I-TAC bind alloptically to the active conformation of CXCR3. Presumably, binding of the ligands occurs within distinct regions of the N-terminal and/or extracellular loops of the receptor. At this point, however, we cannot exclude the possibility that the binding of these ligands occurs on separate receptor proteins within a receptor multimer. A number of G protein-coupled receptors are thought to form homo- or heterodimers (Jordan and Devi, 1999; Lee et al., 2000), including the metabolic glutamate receptor (Romano et al., 1996) and the chemokine receptor, CCR5 (Vila-Coro et al., 2000). It is tempting to speculate that hCXCR3 may also form multimers. By whichever mechanism, the evidence supports the hypothesis that IP-10 (and possibly MIG) and I-TAC binding occur within discrete regions in the active receptor conformation.

Although the binding data make a compelling argument for differential binding to CXCR3, we also undertook biochemical studies using an antibody reported to block IP-10 binding and activation of CXCR3 (Qin et al., 1998). Indeed,  $\alpha$ -hCXCR3 antibody completely blocked  $^{125}\text{I}$ -IP-10 binding and competitively inhibited IP-10 stimulation of  $[\text{S}] \text{GTP}\gamma\text{S}$  exchange. Interestingly, the same antibody also inhibited specific  $^{125}\text{I}$ -I-TAC binding but only by 50%, the majority of which ( $\approx 80\%$ ) could be displaced with GTP $\gamma$ S. However, when coincubated with the isotype control antibody (or in the absence of antibody; Fig. 5, top right), binding of  $^{125}\text{I}$ -I-TAC was inhibited by approximately 50% with GTP $\gamma$ S. These data suggest that, unlike IP-10,  $\alpha$ -CXCR3 preferentially inhibited



I-TAC binding to uncoupled CXCR3 and that I-TAC binding to **R\*** was largely undisturbed. This observation was consistent with functional studies in which the  $\alpha$ -CXCR3 antibody competitively inhibited IP-10 stimulation of [ $^{35}$ S]GTP $\gamma$ S exchange (see above), whereas I-TAC stimulation was not significantly affected. Taken together, these data also suggest that the point(s) of I-TAC interaction with uncoupled and coupled receptor differ. Furthermore, the point(s) of interaction between I-TAC and **R** may overlap with the region of IP-10-**R\*** binding. The biological consequence(s) of the allotopic interaction of chemokines with CXCR3 is not clear. What is apparent, however, is that I-TAC is a more potent agonist than either IP-10 or MIG and effectively displaces IP-10 from the active conformation of hCXCR3. Therefore, in the face of equal concentrations of the three chemokines, I-TAC would be the dominant ligand for hCXCR3 in vivo.

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