IP10 and MIG are two members of the CXC branch of the chemokine superfamily whose expression is dramatically up-regulated by interferon (IFN)-γ. The proteins act largely on natural killer (NK)-cells and activated T-cells and have been implicated in mediating some of the effects of IFN-γ and lipopolysaccharides (LPSs), as well as T-cell-dependent anti-tumor responses. Recently both chemokines have been shown to be functional agonists of the same G-protein-coupled receptor, CXCR3. We now report the pharmacological characterization of CXCR3 and find that, when heterologously expressed, CXCR3 binds IP10 and MIG with Kᵢ values of 0.14 and 4.9 nM, respectively. The receptor has very modest affinity for SDF-1α and little or no affinity for other CXC-chemokines. The properties of the endogenous receptor expressed on activated T-cells are similar. Surprisingly, several CC-chemokines, particularly eotaxin and MCP-4, also compete with moderate affinity for the binding of IP10 to CXCR3. Eotaxin does not activate CXCR3 but, in CXCR3-transfected cells, can block IP10-mediated receptor activation. Eotaxin, therefore, may be a natural CXCR3 antagonist.

Chemokines are a superfamily of small secreted proteins that play an important role in the selective trafficking of leukocytes (for review, see Ref. 1). Most members of the superfamily can be divided into two groups depending on the organization of the first cysteine pair: the CC branch in which the cysteines are adjacent, and the CXC-branch in which they are separated by a single amino acid. Two members of the CXC branch of the superfamily, IP10 and MIG, were initially identified because of their dramatically enhanced expression in monocytes activated by IFN-γ or LPS (2–4). The biological actions of IP10 and MIG are largely restricted to activated T- and natural killer (NK)-cells for which both are potent chemoattractants (5, 6). These properties suggest that the two chemokines may mediate some of the lymphocyte-directed effects of IFN-γ and LPS, a hypothesis that is supported by the observation that IP10 elicits a potent T-cell-dependent antitumor response (5). IP10 and MIG are also strongly angiostatic (7), a property which may be related to their antitumor activity.

Chemokines elicit their biological functions by binding to specific G protein-coupled receptors expressed on the appropriate cells types. Like the chemokines, the receptors can be largely divided into two sub-families: the CXC receptors (CXCRs), which bind CXC chemokines, and the CC receptors (CCRs), which bind CC-chemokines (1). Recently, Loetscher et al. (8) identified a new member of the CXCR subfamily, CXCR3, which when recombinantly expressed mediates chemotaxis and Ca²⁺ mobilization in response to both IP10 and MIG. However, these authors were unable to show binding of either ligand to CXCR3. We now demonstrate that CXCR3 does bind both IP10 and MIG with affinities consistent with the concentrations of the chemokines required to elicit cellular responses. In addition, CXCR3 has some avidity for the CCRX4 ligand, SDF-1, and rather surprisingly has considerable affinity for several CC-chemokines, particularly the CCR3 ligands eotaxin and MCP-4. The pharmacological properties of the recombinantly expressed receptor mirror those of the native receptor expressed on activated human T-cells.

**EXPERIMENTAL PROCEDURES**

Materials—All human chemokines were from Peprotech (Rocky Hill, NJ) except for SDF-1α, which was from Gryphon Sciences (South San Francisco, CA). Radioactive chemokines were from NEN Life Science Products. Venous whole blood or plasmapheresed leukocytes from normal human donors was obtained from the New York Blood Center or the University of Pennsylvania Medical Center.

Cloning of CXCR3—The cDNA encoding CXCR3 was cloned by PCR using lymph node cDNA (CLONTECH, Palo Alto, CA) as a template. The PCR primers were designed based on the published sequence (8). The PCR product was digested with EcoRI and NotI and ligated to similarly digested and linearized pBluescript KS II (Promega). The sequence of the coding region of the receptor was verified by sequencing, then excised from pBS-CXCR3 by digestion with HindIII and NotI, and then ligated into mammalian expression vector pBlu-neo (9).

Expression of CXCR3 in CHO and RBL-2H3 Cells—10⁶ CHO cells (ATCC: CCL-61) were transfected with 20 μg of DNA using a standard calcium phosphate procedure (Specialty Media, Lavallette, NJ). The DNA was incubated with cells at 37 °C, 6% CO₂ for 6 h, whereupon the cells were glycerol-shocked (15% glycerol shock solution, Specialty Media) and re-fed with selection media containing 0.4 mg/ml Geneticin (Life Technologies, Inc.). Concurrently, RBL-2H3 cells were electroporated with the CXCR3 expression plasmid as described previously (13) and selected in 1 mg/ml Geneticin. After 10 days, the surviving CHO or RBL foci were pooled. Stable expression of CXCR3 was verified by determining that an aliquot of cells bound radiolabeled IP10 (see binding parameters below). The remaining cells were cloned by limiting dilution in 96-well microtiter plates, and the cells were expanded. Stable cell lines were derived from individual clones selected on the basis of binding and functional assays.

**Binding Assays**—Binding of ³¹⁻IP10 (2200 Ci/mmol, typically 20 pM) in the presence of unlabeled ligands was initiated by adding intact cells (75,000 cells/pot) as described previously (10). After incubation at room temperature for 30 min, the cells were filtered through GF/F filters treated with 0.33% polyethylenimine and washed with buffer (10).

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‡ The abbreviations used are: IFN, interferon; LPS, lipopolysaccharide; CXCR, CXC receptor; CCR, CC receptor; PCR, polymerase chain reaction; CHO, Chinese hamster ovary.
contain 25 mM Hepes, 0.02% NaN₃, and 0.5 mM NaCl, pH 7.2. Microphysiometry—Functional assays were performed using microphysiometry (11, 12). Briefly, untransfected CHO-KI cells or CHO-KI cells stably transfected with human chemokine receptor CXCR3 were seeded onto the Transwell cell capsule cups (Molecular Devices, Sunnyvale, CA) at a density of 0.33 × 10⁶/ml in fresh media containing IL-2 at 200 units/ml for the specified times. Following overnight culture, the capsules were transferred to microphysiometer sensor chambers (Cytosensor, Molecular Devices) and allowed to equilibrate for 2 h, during which time they were perfused with running medium (1 mM phosphate-buffered RPMI 1640 medium, pH 7.4 (Molecular Devices) plus 0.1% bovine serum albumin (Life Technologies, Inc.)). Once stable acidification rates were established, cells were exposed for 6 min to various concentrations of chemokine diluted in running medium. A flow rate of 100 μl/min was used, and acidification rates were measured at 2 min intervals.

Calcium Flux—Measurements were carried out with transfected cells or purified T-cells labeled with Indo-1 (Molecular Probes, Eugene, OR) as described previously (13).

T-cell Activation—T-cells were purified from a mononuclear cell preparation by E-rosetting with neuraminidase-treated sheep red blood cells, followed by overnight incubation at 37 °C. The T-cells were washed and incubated in plastic flasks at 2 × 10⁶/ml in media containing 400 units/ml of human recombinant IL-2 (Biosource International, Camarillo, CA) for 1–2 days, and further maintained at a density of 2–4 × 10⁶/ml in fresh media containing IL-2 at 200 units/ml for the specified times.

RESULTS AND DISCUSSION

Characterization of the Binding Properties of CXCR3 on CHO Cells Stably Expressing the Receptor—Because untransfected CHO cells do not bind ¹²⁵I-IP10, lines stably expressing CXCR3 were established by monitoring the gain of this activity (see "Experimental Procedures"). The binding properties of a representative CHO clone (C1.17) were assessed by competition of various chemokines against ¹²⁵I-IP10 (Fig. 1 and Table I). The clone exhibits a single high affinity binding site (Kᵣ = 0.14 nM) for IP10 and expresses approximately 50,000 sites/cell. As expected, the receptor also binds MIG, although with an affinity (Kᵣ = 4.9 nM) that is substantially lower than that found for IP10. Other CXC-chemokines, notably IL-8, which binds to both CXCR1 and CXCR2 (14, 15), and GROα and NAP2, which bind to CXCR2, have little or no affinity for CXCR3 (Table I). SDF-1, the ligand for CXCR4 (16, 17), does show very slight affinity for CXCR3 with a Kᵣ = 400 nM.

Table I

| Ligand | CHO line | Activated T-cells | RBL line |
|--------|----------|------------------|----------|
| IP10   | 0.14 ± 0.04 | 0.47 ± 0.16     | ND       |
| MIG    | 4.9 ± 0.8 | 0.8 ± 0.2       | 3.3 ± 1.3 |
| SDF-1α | 1000     | 170 ± 20       | ND       |
| GROα   | >1500    | ND               | ND       |
| MCP-3  | 250 ± 100 | 100 ± 20       | ND       |
| RANTES | 420 ± 20  | 130 ± 30       | ND       |
| MIP-3α | 140 ± 20  | ND               | ND       |
| MIP-3β | 1300 ± 400| ND              | ND       |

Kᵣ values were determined from competition binding experiments carried out against ¹²⁵I-IP10 as described in the legends of Figures 1 and 2 and in the "Experimental Procedures" section. All values are given in nM and are the averages of two to three experiments. NB is no binding; ND not determined.

Binding of IP10 and Other CXCR3 Ligands to Purified IL-2 Activated T-cells—Since a number of factors, including differences in the endogenous complement of G-proteins or various other host cell-specific restrictions, may influence the way in which ligands bind to their receptors, we compared the pharmacology of the receptor recombinantly expressed in CHO cells to endogenously expressed CXCR3 and to receptor expressed in RBL-2H3 cells.

It has been reported that transcripts for CXCR3 are virtually absent in resting T-cells but are present in IL-2-activated T-cells (8). In our hands, the ability of human T-cells to bind IP10 is consistent with these observations. Freshly isolated T-cells show variable but low binding activity, an activity that is substantially up-regulated by a number of activation procedures including treatment with anti-CD3/anti-CD28, phorbol 12-myristate 13-acetate/ionomycin, or IL-2 (data not shown). To characterize the pharmacology of CXCR3 on primary cells, we chose to use T-cells treated with IL-2 for 6–8 days, a protocol that generates maximal binding of IP10 (data not shown). As shown in Fig. 2 and Table I, the properties of the receptor on the primary cells closely mirrors that of the recombinant molecule expressed in CHO cells. The Kᵣ values are 2–3-fold lower on average, but the receptors in both cell types bind the same ligands with the same rank order of potencies. Similar affinities were also obtained for receptor recombinantly expressed in RBL-2H3 cells (Table I).
tem was used to monitor ligand-induced increases in cellular acidification rate as a measure of functional activation (11).

As illustrated in Fig. 3 both IP10 and MIG induce dose-dependent increases in the acidification rate. As expected from the binding data, IP10 is more potent than MIG with EC_{50} values of ~10 and 100–200 nM, respectively. In fact, the ratio of EC_{50} values for the two ligands (10–20) is consistent with the relative binding affinities (~30, Table I). In comparison, eotaxin failed to generate any response, even at a concentration of 1 μM (Fig. 3B), a value 15-fold greater than its K_{i} as determined in binding studies (Table I).

We also examined the ability of IP10, MIG, and eotaxin to stimulate functional responses in activated human T-cells and transfected RBL cells. For these studies, increases in intracellular Ca^{2+} levels were used to monitor responses. The data are consistent with the results from the recombinant CHO lines. As shown in Fig. 4, in activated primary T-cells, both IP10 and MIG induced a flux at a concentration of 100 nM, whereas eotaxin failed to generate a response, even at a concentration of 1 μM. Interestingly, since a subset of Th2 T-cells has been reported to express CCR3 (19), the primary receptor for eotaxin, the lack of response to eotaxin also suggests that our T-cell preparations contain a very low level of this subset. High concentrations of eotaxin (up to 1 μM) also failed to induce a Ca^{2+}-flux in our stable RBL CXCR3 transfected line (Fig. 5), whereas the EC_{50} of IP10 for this response was 10 nM (data not shown).

The binding and functional data suggest that eotaxin, under appropriate circumstances, should act as a receptor antagonist. To test this hypothesis, we examined the ability of eotaxin to inhibit an IP10-induced Ca^{2+}-flux in the transfected RBL cells. As shown in Fig. 5, eotaxin does inhibit the response to 10 nM IP10 with and IC_{50} of about 1 μM, a potency consistent with the 100-fold difference in binding affinities (Table I). As a control, IL-8 had no effect at concentrations as high as 10 μM.

The chemokine system consists of more than 50 ligands and 13 receptors. The specificities of the ligand/receptor interactions are complex as each receptor binds multiple chemokines and most chemokines bind to more than one receptor. However, a general rule has been that a chemokine receptor binds either CC- or CXC-chemokines but not both. The one exception has been DARC, a highly promiscuous, ubiquitously expressed, nonsignaling receptor whose function is unclear (20, 21). Thus it is surprising to find that CXCR3 has moderate affinity for several CC-chemokines, particularly those which bind to CCR3. In fact, those affinities are higher than for any of the CXC-chemokines examined here, except IP10 and MIG. It would appear, therefore, that overall protein sequence homologies are not a sufficient means from which to predict the class of a chemokine.
of ligand (CC or CXC) a given receptor may bind. Moreover, since the primary CXCR3 ligands, IP10 and MIG, also show moderate affinities for CCR3 (Kᵢ values of 100 and 30 nM, respectively), it is also likely that CXCR3 and CCR3 possess structural homology that enables them to present key interactions to shared ligands.

An important but as yet unanswered question, given the modest affinities that the CCR3 ligands show for CXCR3 and vice versa, is whether these overlapping ligand specificities are physiologically relevant. It is tempting to believe they are. In vivo, there is a correlation between strong TH2 responses and the diminished accumulation of TH1 cells (22, 23). Since CCR3 is expressed on cells characteristic of TH2 responses, including a subset of TH2 T-cells (19, 24) and whereas CXCR3 is found predominately on TH1 T-cells (24), the putative antagonistic effects of the CCR3 ligands on CXCR3 could impair the accumulation of TH1 cells and in part account for some of the inhibitory activity of TH2 responses. Considerable additional evidence is needed to support this speculation, including the direct demonstration that CCR3 ligands do indeed antagonize the effects of IP10 and MIG on TH1 cells. Moreover, sufficient local concentrations of the CCR3 chemokines have to be achieved in order for such antagonism to occur. In this regard, it is currently thought that much of the chemokine generated in vivo is bound to surface proteoglycan, a modality that has been argued to greatly increase the local concentration, particularly because the interaction with the receptor occurs on a solid phase (25).

Regardless of whether the above argument is true, our data suggest that chemokines may play a dual regulatory role, as agonists for some responses and antagonists for others.

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[3] Y. Weng, S. J. Siciliano, K. E. Waldburger, A. Sirotina-Meisher, M. J. Staruch, B. L. Daugherty, S. L. Gould, Martin S. Springer, and Julie A. DeMartino, unpublished observations.

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