Murine CXCR1 Is a Functional Receptor for GCP-2/CXCL6 and Interleukin-8/CXCL8*

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Functional interleukin-8 (IL-8) receptors (IL-8RA and IL-8RB: CXCR1 and CXCR2, respectively) have been described in human, monkey, dog, rabbit, and guinea pig. Although three IL-8 homologues have been found in rat, only one of these, rat CXCR2, appears to be functional based on responsiveness to ligands. Similarly, CXC chemokines induce biological responses through the murine homolog of CXCR2, but the identification of functional rodent CXCR1 homologues has remained elusive. We have identified and characterized the mouse CXCR1 homologue (mCXCR1). Murine CXCR1 shares 68 and 88% amino acid identity with its human and rat counterparts, respectively. Similar to the tissue distribution pattern of rat CXCR1, we found murine CXCR1 mRNA expression predominantly in lung, stomach, bone marrow, and leukocyte-rich tissues. In contrast to previous reports, we determined that mCXCR1 is a functional receptor. We show predominant engagement of this receptor by mouse GCP-2/CXCL6, human GCP-2, and IL-8/CXCL8 by binding, stimulation of GTPγS exchange, and chemotaxis of mCXCR1-transfected cells. Furthermore, murine CXCR1 is not responsive to the human CXCR2 ligands ENA-78/CXCL5, NAP-2/CXCL7, GRO-α, -β, -γ/CXCL1–3, or rat CINC-1–3. In addition, we show concomitant elevation of mCXCR1 and its proposed major ligand, GCP-2, positively correlated with paw swelling in murine collagen-induced arthritis. This report represents the first description of a functional CXCR1-like receptor in rodents.

Chemokines and chemokine receptors are well preserved through various mammalian species (1, 2). However, a few animal chemokines (e.g. regakine-1 and lungkine) (3) exist for which a homologue has not yet been identified and might indeed be absent in humans. Reciprocally, homologues of some human chemokines, e.g. IL-8³/CXCL8 and pulmonary and activation-regulated chemokine/CCL18 are probably missing in specific rodents, e.g. mice. Determination of the contribution of chemokines to pathology using animal models of inflammation is hampered by this fact. Hence, murine models of inflammatory disease have not been considered fully representative or equivalent to human disease with respect to evaluation of neutrophil-mediated inflammation induced by IL-8/CXCL8. However, other potent neutrophil chemotaxants exist in the mouse, such as GCP-2, KC/GROα, MIP-2α/GROβ, and MIP-2β/GROγ. Thus, it is assumed that neutrophil migration in the mouse is mediated by these chemokines in the place of IL-8/CXCL8.

Disparity between human and rodent systems is further implied by the presence of two functional human IL-8 receptors, CXCR1 and CXCR2 (4, 5). In the human system, IL-8/CXCL8 and GCP-2/CXCL6 signal through both CXCR1 and CXCR2, whereas the other ELR⁺ CXC chemokines are agonists only for CXCR2 (6). Absence of IL-8 in the mouse system may suggest a concordant absence of one of the IL-8-specific receptors, e.g. CXCR1 as rodent GCP-2 and GRO-α, -β, and -γ counterparts may still mediate neutrophil chemotactic effects through CXCR2. Rat homologues of both CXCR1- and CXCR2-like receptors have previously been identified by molecular cloning and sequence identity (7). Activation of the proposed rat CXCR2 was confirmed by MIP-2-induced Ca²⁺ flux. However, the lack of detectable response to either MIP-2 or KC/CINC ligand by the second rat IL-8 receptor homologue suggested that it was probably a nonfunctional receptor.

To date, only one IL-8 receptor has been identified and functionally characterized in the mouse (8, 9). This receptor was found to bind KC and MIP-2 with high affinity. Despite its high potency on murine neutrophils, hIL-8/CXCL8 was a poor agonist for the mouse IL-8R. This suggests that this mouse IL-8 type B receptor (8) was likely the mouse homologue of CXCR2, and another functional murine CXCR1 (mCXCR1) remained to be found. Conflicting evidence surrounded the potential

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∥ The abbreviations used are: IL-8, interleukin-8; IL-8R, IL-8 receptor; CIA, collagen-induced arthritis; GTPγS, guanosine 5′-3-O-(thio)triphosphate; m-, murine; h, human; SPA, scintillation proximity assay; WGA, wheat germ agglutinin; MPO, myeloperoxidase; MIP-2, macrophage inflammatory protein-2; GCP-2, granulocyte chemotactic protein-2.
existence of a second, as yet undefined mIL-8-R that would represent mCXCR1. Speculation around such a receptor comes from detection of a second, albeit weaker band by Southern blot analysis when probing with the murine IL-8R sequence (8), although a second report suggested that only one mIL-8R exists, as based on Southern analysis with mouse genomic DNA (10). In addition, targeted gene disruption of mouse IL-8RB resulted in loss of neutrophil chemotactic response and intracellular calcium flux to mMIP-2 and hIL-8/CXCL8 (11, 12), suggesting that mCXCR2 is the primary (or only) chemokine receptor for these ligands on mouse neutrophils. Recently, two different groups described the cloning and characterization of mCXCR1 (13, 14). The studies established that mCXCR1 mRNA was expressed in bone marrow, peripheral mononuclear cells, CD4+ and CD8+ T cells, and certain lymphoid cell lines. However, neither group successfully identified cognate ligand(s) for mCXCR1, although many of the known CXCR1/CXCR2 ligands were tested. As a result, it was proposed that mCXCR1 was activated by a hitherto unknown CXC chemokine (14).

Therefore, it was of interest to determine definitively whether the CXCR1 homologue, encoded in the murine genome, is a functional receptor, as determined by responsiveness to autologous and heterologous IL-8R ligands. The studies presented herein identify and characterize the murine homologue of CXCR1 using ligand binding assays, GTPγS exchange, and chemotaxis assays with Ba/F3 cells transfected to express mCXCR1. In contrast to what has been described with murine CXCR1, mCXCR1 is a functional receptor, specifically activated by mGCP-2, hGCP-2/CXCL6, and hIL-8/CXCL8. Furthermore, we show elevated expression of mCXCR1 and corresponding murine ligands GCP-2, KC, and MIP-2 during chronic inflammation in murine collagen induced arthritis (mCIA). We propose that this receptor is the functional murine homologue of hCXCR1.

**EXPERIMENTAL PROCEDURES**

**Chemical Reagents**—Chemokines were purchased from R&D Systems Inc. (Minneapolis, MN). All other reagents were of the best grade available and purchased from common suppliers. Recombinant murine (15) and human (16) GCP-2/CXCL6 were cloned from fibroblasts and expressed in Escherichia coli as previously described.

**Cells and Cell Culture**—Murine IL-3-dependent pro-B cells (Ba/F3) were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μg/ml streptomycin, 50 μg/ml penicillin, 0.1% bovine serum albumin, 0.002% NaN3, and 1 ng/ml of recombinant mouse IL-3 (Sham Biosciences) in SPA binding buffer (1 mM CaCl2, 5 mM MgCl2, 50 mM NaCl, 0.1% bovine serum albumin, 0.002% NaN3, 50 mM Hepes, pH 7.6). The cells were then cultured and centrifuged, incubated in homogenization buffer (10 mM Tris-HCl, 5 mM EDTA, 3 mM EGTA, 0.1% SDS, pH 8.0), and nuclei were removed by centrifugation at 4000 g for 10 min at 4 °C. The cell membranes in the supernatant were then pelleted by centrifugation (20 000 g for 30 min) at 4°C. The cell membranes in the supernatant were then pelleted by centrifugation at 100,000 g for 30 min. The membranes were then resuspended in glycyglycine buffer (20 mM glycyglycine, 1 mM MgCl2, 250 mM sucrose, pH 7.2), aliquoted, quick frozen, and stored at −80 °C. Protein concentration in membrane preparations was determined using the method of Bradford (19).

**Cloning of Mouse CXCR1 Gene**—A BLASTN search of murine expressed sequence tags was conducted using the cDNA sequence of human CXCR1. A murine cDNA sequence corresponding to human CXCR1 was identified. The predicted murine mRNA sequence was derived from the genomic DNA sequence. Using this sequence, we designed PCR primers spanning the whole length of the open reading frame, forward 5′-ATG GCC GAG GCT GAA TAT TTC-3′, and reverse 5′-TTA ATA AAT AGC GGT GAG AGA-3′, that were used to amplify the full-length cDNA from a mouse BALB/c lung cDNA library. This 600-bp open reading frame fragment was subcloned into pCR2.1 cloning vector (Invitrogen) and sequenced using the PE Automated Sequencer ABI 377 (Applied Biosystems, Foster City, CA).

**Cloning and Stable Expression of mCXCR1 and mCXCR2**—The murine CXCR1 open reading frame with Kozak sequence in front of the first ATG was subcloned into pME18Sneo vector, a derivative of the Srα expression vector (17), containing the CD8 signal peptide, Sr-alpha promoter, and SV40 poly(A) signal. The unique ScaI site in the plasmid backbone was used to linearize the vector. Mouse CXCR2 was PCR-amplified from NIH 3T3 cell genomic DNA using primers GCA TGG TAC CTC AAA GAT GGG AGA ATT C and CGA TCT CGA GGG GCC ACC GTT TAG G. The product was cut with Kpn1 (5′) and Xhol (3′) and ligated into pBS SK+. The cDNA were cloned into the mammalian expression vector pME18Sneo, a derivative of the Srα expression vector (17). Ba/F3 cells were transfected by electroporation and a stable population selected by resistance to G418 (1 mg/ml, Invitrogen). Clonal cell lines expressing mCXCR1 were then established by limiting dilution of stable transfecants.

**Flow Cytometric Analysis of Surface mCXCR1 and mCXCR2 Expression**—Expression of surface receptor expression was measured by flow cytometry. Cells were collected and centrifuged at 400 × g 5 min at 4 °C. Following blocking with normal mouse serum (Sigma), cells were stained with 10 ng of biotinylated M2 anti-FLAG antibody (Sigma) followed by streptavidin-PE (BD Pharmingen). Cells were then analyzed on the BD-FACScan (BD Biosciences Immunocytometry Systems, Mountain View, CA) using CellQuest software.

**Cell Membrane Preparation**—Ba/F3-mCXCR1 and Ba/F3-mCXCR2 membranes were prepared as previously described (18). Cells were pelleted by centrifugation, incubated in homogenization buffer (10 mM Tris-HCl, 5 mM EDTA, 3 mM EGTA, pH 7.6) and 1 mM phenylmethylsulfonyl fluoride for 30 min on ice. The cells were then lysed with a Dounce homogenizer using stirrer type RZR3 Polytron homogenizer (Cafaro, Wiarton, Ontario, Canada) with 12 strokes at 900 rpm. The intact cells and nuclei were removed by centrifugation at 500 × g for 5 min. The cell membranes in the supernatant were then pelleted by centrifugation at 100,000 × g for 30 min. The membranes were then resuspended in glyglucose buffer (20 mM glycyglycine, 1 mM MgCl2, 250 mM sucrose, pH 7.2), aliquoted, quick frozen, and stored at −80 °C. Protein concentration in membrane preparations was determined using the method of Bradford (19).

**[35S]GTPγS Exchange Assay**—Guanosine 5′-[γ-35S]triphosphate ([35S]GTPγS, triethylammonium salt, specific activity = 1250 Ci/mmol, PerkinElmer Life Sciences) exchange was measured using a scintillation proximity assay (SPA) as previously described (20). For each assay point, 2–4 μg of membrane was preincubated for 30 min at room temperature with 300 μg of wheat germ agglutinin-coated SPA beads (WGA-SPA, Amersham Biosciences) in SPA binding buffer (1 mM CaCl2, 5 mM MgCl2, 50 mM NaCl, 0.1% bovine serum albumin, 0.002% NaN3, 50 mM Hepes, pH 7.6). The beads and membranes were transferred to a 96-well Isoplate (Wallac, Gaithersburg, MD) and
incubated with 1 μM guanosine 5’-diphosphate in the presence or absence of various chemokines for 60 min at room temperature. The incubation was continued for another 60 min following the addition of 0.1 nM [35S]GTP S. Membrane-bound [35S]GTPyS was measured using a 1450 Microbeta Trilux counter (Wallac).

Radioiodinl Binding Assay—Carrier-free 125I-IL-8/hCXCL8 (specific activity = 2200 Ci/mmol) was purchased from PerkinElmer Life Sciences. Radioligand competition and saturation binding assays were done using SPA technology (as described above). Membranes (10 μg per assay point) in SPA binding buffer were preincubated for 30 min at room temperature with 800 μg of WGA-SPA, transferred to a 96-well Isoplate, and further incubated at room temperature with the indicated concentrations of [125I]hIL8/CXCL8 and chemokines for 1–6 h. Ligand affinities from competition bindings were calculated from binding IC₅₀ using the Cheng-Prusoff equation (21).

Chemotaxis—The assays were performed as previously described (22) using 96-well ChemoTx microplates (NeuroProbe®, Inc., Gaithersburg, MD) with a 5 μm filter as per manufacturers’ instructions. Cells were allowed to migrate toward the indicated concentrations of chemokines for 1.5 h at 37 °C in a humidified CO₂ (5%) chamber. Cell numbers were quantitated using a CellTiter-Glo Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI) according to the manufacturer’s instructions. Chemotaxis is expressed as a percentage of total cells.

Quantitative PCR Analysis—RNA from various tissues and immune cells were extracted using the TRI reagent RNA isolation kit from MRC, Inc. (Cincinnati, OH) following specifications from the manufacturer. cDNA was generated by reverse transcription using random hexamers (Promega) and oligo(dT) primers (Invitrogen). Quantitative PCR analysis (TaqMan) was performed on an ABI 7700 (Applied Biosystems) sequence detection instrument following the manufacturer’s instructions. For TaqMan analysis, 25 ng of cDNA was used together with primers at 0.9 μM final concentration, and a 6-carboxyfluorescein-labeled diagnostic probe at a final concentration of 0.25 μM. Primers/probes sequences were designed using the Primers Express software version 1.5 (Applied Biosystems) as shown in Table 1. Ribosomal RNA primers/probe (PE Applied Biosystems) were used as an internal control. Quantitative PCR conditions were as follows: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 1 min. A plasmid containing the mouse CXCR1 gene was used as a standard, ranging from 100 pg to 0.1 fg. Data were analyzed using Sequence Detection Systems software version 1.7 (Applied Biosystems).

Relative quantitation of CXCR1 and CXCR2 mRNA in mouse hemopoietic cell lines and paws from mice with collagen-induced arthritis (CIA) was performed using SYBR green master mix PCR kit as recommended by the manufacturer (Applied Biosystems). Each 20-μl reaction contained 2× SYBR Green mastermix (Applied Biosystems), 50 ng of cDNA, and 500 nM of each primer. Ubiquitin was used as an internal control. Two independent primer sets were used to confirm specificity (Table 1).

CIA—Murine CIA was established as previously described (23). Briefly, 12-week-old male B10.RII mice (Jackson Laboratories) were immunized intradermally at five sites with bovine type II collagen (Elastin Products, Owensville, MO) emulsified with an equal volume of complete Freund’s adjuvant. Complete Freund’s adjuvant was comprised of a mixture of incomplete Freund’s adjuvant (Difco) and heat-killed, freeze-dried Mycobacterium tuberculosis (Ministry of Agriculture, Fisheries & Food, Surrey, England). Each mouse received 300 μg/ml bovine type II collagen and 0.5 mg/ml complete Freund’s adjuvant. Mice were boosted intraperitoneally with 100 μg of bovine type II collagen on day 21. Disease incidence and disease score were monitored for 43 days. Mice were euthanized at time points as indicated. RNA was isolated from whole individual paws using Tri reagent according to the manufacturer’s instructions. Quantitation of CXCR1 and CXCR2 mRNA was determined as described above.

Data Analysis—Nonlinear regression analysis of the data and calculation of EC₅₀ and Kᵣ was performed using Prism 2.0c (GraphPad Software, San Diego, CA.). Analysis of CXCR1 and CXCR2 mRNA expression was conducted using Student’s t test. Results were considered significant where p < 0.05.

RESULTS

Identification of the Mouse Homologue of the Human CXCR1 Gene—The mouse CXCR1 gene was cloned from a BALB/c lung cDNA library. The gene has an open reading frame of 1056 nucleotides, encoding a protein of 352 amino acids (Fig. 1). Sequencing analysis showed that the cloned murine CXCR1 gene matched the predicted sequence. As summarized in Table 2, the murine CXCR1 gene is 88 and 68% homologous to the rat (accession P70612) and human (accession P25024) CXCR1 genes, respectively. The sequence isolated herein matched 100% mouse genomic sequence on chromosome 1 position from 75759265 to 75760320. Fig. 1 shows the comparison between mouse, rat, and human CXCR1 proteins. Boxed regions indicate areas of amino acid identity. The seven putative transmembrane domains common to this class of receptors are conserved in the murine sequence, as indicated by hatched lines. Similar to human and rat CXCR1, murine CXCR1 protein shows conservation of nine cysteine residues, and an abundance of acidic residues at the N-terminal, features common to many chemokine receptors.

Ligand Identification—Chemokine activation of mCXCR1 was initially assessed using GTPγS exchange in membranes from a
stable clonal Ba/F3-mCXCR1 cell line. Membranes were incubated with 1 μM GDP, 0.1 nM [35S]GTPγS, and up to 1 μM of various human and rodent chemokines as described under “Experimental Procedures.” Only mGCP-2, hGCP-2/CXCL6, and hIL-8/CXCL8 stimulated [35S]GTPγS exchange (Fig. 2). The CXC chemokines lungkine, mBRAK, hGRO-α/CXCL1 (data not shown), hGRO-β/CXCL2, GRO-β (39–107), hGRO-γ/CXCL3, hENA-78/CXCL5, hNAP-2/CXCL7, rCINC-3, and rCINC-1 had no measurable affect. Interestingly, the mCXCR2 ligands, mKC and mMIP-2, were also inactive in stimulating [35S]GTPγS exchange in mCXCR1 membranes (data not shown), although both of these chemokines were potent agonists in parallel [35S]GTPγS assays with Ba/F3 cells transfected to express mCXCR2 (Ba/F3-mCXCR2, Fig. 3). Based on these data, we assessed the potency of hIL-8/CXCL8 (77 and 72 amino acids), mGCP-2, hGCP-2/CXCL6, and their truncated isoforms, mGCP-2-(9–78) and hGCP-2-(9–77). As shown in Fig. 4, mGCP-2 was less potent than was mGCP-2-(9–78), whereas hGCP-2-(9–77) was more potent than both forms of hIL-8/CXCL8 or mGCP-2.

We next assessed the potency and efficacy of chemokines in stimulating chemotaxis of a stable, non-clonal Ba/F3-mCXCR1 cell line. As can be seen in Fig. 5, the cells migrated toward mGCP-2-(9–78) with a typical bell-shaped chemotaxis dose curve. Parental Ba/F3 cells were unresponsive while both parental and transfected cells migrated toward hSDF-1α/CXCL12 through mCXCR4, which is endogenously expressed in Ba/F3 cells (inset, Fig. 5A). Pretreatment of cells with pertussis toxin abolished the chemotaxis of transfectants to mGCP-2-(9–78) (Fig. 5B, inset). Pretreatment of cells with pertussis toxin abolished the chemotaxis of parental cells to hSDF-1α/CXCL12 (Fig. 5B, inset). Taken together, these data show that mCXCR1 is a functional receptor for mGCP-2 via pertussis toxin-sensitive G proteins.

To facilitate more extensive study of mCXCR1 functionality, we selected higher expressing clonal cell lines from the non-clonal Ba/F-mCXCR1 cells based on surface expression of the FLAG-tagged receptor. Of the higher expressing lines (data not shown), clone #9 was selected to assess the chemotactic activity of the chemokines identified as mCXCR1 agonists in GTPγS exchange assays (Fig. 4). As can be seen in the representative experiments shown in Fig. 6, Ba/F-mCXCR1 (#9) cells migrated most efficiently to the naturally occurring truncated variants hGCP-2-(9–77) and hGCP-2-(9–78) (Fig. 5B) or parental Ba/F3 cells to hSDF-1α/CXCL12 (Fig. 5B, inset). Taken together, these data show that mCXCR1 is a functional receptor for mGCP-2 via pertussis toxin-sensitive G proteins.
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**FIGURE 2.** Effect of chemokines on [35S]GTP\(_S\) exchange in Ba/F-mCXCR1 membranes. Membranes (2 \mu g/well) from Ba/F-mCXCR1 cells were incubated in binding buffer containing 1 \mu M GDP with 0.1 nM [35S]GTP\(_S\) for 60 min at room temperature. The incubations were continued for another 60 min following the addition of 0.1 nM [35S]GTP\(_S\). Binding of [35S]GTP\(_S\) to the membranes was measured by WGA-SPA scintillation. Data represent mean specific binding \pm S.E. of triplicate determinations from a representative experiment (n = 2). The box in dotted line represents 95% confidence interval values.

**FIGURE 3.** Effect of mMIP-2, mKC, and mGCP-2 on [35S]GTP\(_S\) exchange in Ba/F-mCXCR2 membranes. Membranes (2 \mu g/well) from Ba/F-mCXCR2 cells were incubated in binding buffer containing 1 \mu M GDP with 0.1 nM [35S]GTP\(_S\) with the indicated concentrations of mMIP-2, mKC, or mGCP-2. After 60 min at room temperature, the incubations were continued for another 60 min following the addition of 0.1 nM [35S]GTP\(_S\). Binding of [35S]GTP\(_S\) to the membranes was measured by WGA-SPA scintillation. Data represent mean specific binding \pm S.E. of triplicate determinations from a representative experiment (n = 2).

potent than full-length mouse GCP-2. However, consistent with the hGCP-2 data, mGCP-2-(9–78) was more potent than full-length mGCP-2. In agreement with the [35S]GTP\(_S\) data (Fig. 4), hGCP-2-(3–77) and hGCP-2-(9–77) were both more potent than mGCP-2-(9–78) (Fig. 6B).

**Chemokine Binding Affinity for mCXCR1**—Saturation binding analysis showed that mCXCR1 bound \(^{125}\text{I}\)-hIL-8/CXCL8 with relatively low affinity (K\(_d\) ~ 10 nM, data not shown). We measured the chemokine affinity by competition binding analyses in Ba/F-mCXCR1 membranes using 200 pM \(^{125}\text{I}\)-hIL-8/CXCL8 and SPA-WGA beads (Fig. 7 and Table 3). The affinity of the chemokines for mCXCR1 reflected their potency in chemotaxis and GTP\(_S\) exchange such that hGCP-2-(9–77) bound with the highest affinity while mMIP-2 and mKC bound with the lowest affinity.

**Mouse CXCR1 Expression Profile**—Murine CXCR1 mRNA expression was determined by quantitative real-time PCR analysis (TaqMan) on cDNA derived from various tissues and immune cells. Similar to the expression patterns reported for rat CXCR1-like receptor (7) and a previous report on mCXCR1 (13), we found mCXCR1 was highly expressed (>300 RNA molecules/25 ng of cDNA) in lung (Fig. 8). Mouse CXCR1 mRNA was also relatively high in placenta, bone marrow, and testes, whereas weaker expression (>50 RNA molecules/25 ng of cDNA) was observed in mesenteric and peripheral lymph nodes (Fig. 8, MLN and PLN), spleen, and stomach. No detectable expression was found in total brain, small intestine, liver, and pituitary gland.

We next quantified mCXCR1 and mCXCR2 mRNA expression in various mouse hemopoietic cell lines, including T cell lymphomas (BW5147, 32D, and EL4), B cell lymphomas (L1.2 and A20), mouse monocytes, RAW cells, and bone marrow-derived immature dendritic JAWSII cells (Fig. 8B). Spleen (mostly lymphocytes and some neutrophils) and bone marrow (undifferentiated myeloid cells and neutrophils) were used as an internal positive control for receptor message. Of the T cell lines tested, only EL4 cells expressed appreciable amounts of both mCXCR1 and mCXCR2 mRNA (30–35 RNA molecules/25 ng of cDNA), although mCXCR2 mRNA was readily detectable in BW5147 cells (~900 RNA molecules/25 ng of cDNA). The 32D and RAW cells expressed little if any of either receptor mRNA. The JAWSII dendritic cells expressed both mCXCR1 and mCXCR2 message at reasonable levels (~100 RNA molecules/25 ng of cDNA), which is consistent with a previous study that detected receptor message by Northern blot analysis (16). Of the two B cell lymphomas tested, only A20 cells expressed receptor mRNA at appreciable levels (1000–1300 RNA molecules/25 ng of cDNA). These data differ from previously published Northern blot analyses, which failed to detect mRNA for either receptor in these cells (16). The one or more reasons for this difference are unclear, although Northern blot analysis is not as sensitive and quantitative as is real-time PCR analysis.

**Expression of mCXCR1 and Chemokines during Mouse CIA**—Following induction of CIA, inflamed paws were used to determine the temporal expression levels of mCXCR1 and mCXCR2.
mRNA over the disease course. The paw swelling measured following the collagen boost (day 21) in these experiments is shown (Fig. 9A). Both mCXCR1 and mCXCR2 mRNA (relative to ubiquitin) increased gradually over time (Fig. 9, B and C) and substantially increased coincident with the onset of paw swelling between days 16–21, and immediately prior to boost on day 21 (p < 0.05). mCXCR1 and mCXCR2 mRNA peaked on days 24–27 and 27–34, respectively (i.e. coincident with maximal swelling), subsided with time, but remained elevated through day 41. The increase in mCXCR1 expression above day 0 was 15- to 20-fold that of mCXCR2, and the expression of both receptors paralleled paw swelling (Fig. 9, D and E, p < 0.001).

We also measured the expression of various chemokines in inflamed paws. As shown in Fig. 10, temporal expression of mKC (top left), mMIP2 (top right), and mGCP-2 mRNA (bottom left) mirrored that of the receptors with maximal elevation in mRNA during peak swelling (p < 0.05). mGCP-2 and mCXCR1 were elevated ~300-fold above baseline during disease, whereas mKC, mMIP2, and mCXCR2 mRNA increased by 40- to 50-fold over baseline. Myeloperoxidase (MPO) can be used as an indirect measure of neutrophil infiltration. Interestingly, MPO was elevated prior to the onset of paw swelling (Fig. 10, bottom right) and reverted to baseline following the day 21 boost when mCXCR1, mCXCR2, mKC, mMIP2, and mGCP-2 mRNA were elevated. Consistent with other reports, these data would suggest that neutrophil activity is involved in early initiation of CIA, which is then amplified by local T and B cell responses (23, 24). This finding may also suggest that mCXCR1 (and mCXCR2) may be expressed on infiltrating lymphocytes in the later stages of mouse CIA when maximal swelling is observed following boost with antigen.

**DISCUSSION**

The studies presented herein demonstrate that mCXCR1 is a functional chemokine receptor activated by mGCP-2 and suggest that this receptor is indeed the functional homologue of hCXCR1 in the mouse. Cells transfected to express mCXCR1 bound hIL-8/CXCL8 with reasonable affinity and functionally responded to hIL8/CXCL8, hGCP-2/CXCL6, and mGCP-2. Ba/F3-mCXCR1 chemotaxis was more pronounced in response to truncated hGCP-2/CXCL6-(9–78), compared with intact chemokine. Selective CXCR2 ligands such as hGRO-α, -β, and -γ (CXCL1, -2, and -3), hENA-78/ CXCL5 isofoms, hNAP-2/CXCL7, mKC, mMIP-2, rCINC-3, and rCINC-1 were ineffective ligands for mCXCR1 as measured by [35S]GTPγS exchange and chemotaxis assays. Although CXCR2 has been well characterized in mice (8, 12) and rats (7), this is the first example of a functional homologue of human CXCR1 in rodents. Three IL-8R-like receptors have previously been identified in the rat, one of which was found to be the functional rat CXCR2
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receptor, whereas the other two were reported to be either pseudoreceptors or non-functional, based on lack of response to mouse KC and MIP-2 and rat CINC (7). In the human system, IL-8/CXCL8 and GCP-2/CXCL6 can bind and activate both CXCR1 and CXCR2 (6). However, hENA-78/CXCL5, hNAP-2/CXCL7, and hGRO-α, -β, and -γ/CXCL1, -2, and -3 are potent agonists only for CXCR2 (1). There have been conflicting reports in the literature regarding the existence of a second mouse IL-8R homologue. By genomic hybridization, Bozic et al. (8) found evidence of two bands on blots, but these authors showed no functional or sequence data on these two species. In a subsequent report, Lee et al. (12) also described a weak second band and while targeted deletion of mCXCR2 ablated the chemotactic response to mMIP-2, mKC, and hIL-8/CXCL8, the authors speculated that a second receptor may exist on neutrophils that mediate functions via the other CXC ligands not tested. Recently, two groups did identify the mCXCR1 homologue (13, 14). However, these studies did not attempt to characterize the pharmacology of this receptor (13) or were unsuccessful in demonstrating chemokine binding or receptor

**TABLE 3**

| Chemokine       | $K_i$  | $n$  |
|-----------------|--------|------|
| hGCP-2-(9–77)   | 0.88 ± 0.14 | 2 |
| hGCP-2-(1–77)   | 5.2 ± 1.4   | 4 |
| mGCP-2          | 16.7 ± 7.9  | 2 |
| mGCP-2-(9–78)   | 59 ± 11     | 3 |
| mKC             | 337 ± 73    | 2 |
| mMIP-2          | 643 ± 158   | 2 |

$K_i$ ± S.E. For duplicate experiments. $K_i$ ± range.

$^a$ Experiments/data set.
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FIGURE 9. Time course of paw swelling and mCXCR1 and mCXCR2 expression in mCIA. Representative data from three independent experiments shows the extent and time course of paw swelling post-antigen challenge. A, paw size in function of time of induction of CIA. B and C, kinetics of CXCR1 and CXCR2 expression. D and E, -fold expression of CXCR1 and CXCR2 in relation to paw size. Disease is initiated on day 0, followed by a boost on day 21. Onset of swelling follows boost and is sustained until termination of experiment on day 41. Bars represent a mean of 3–6 paws/group ± S.D. A–C, -fold expression relative to paw thickness (millimeters) is shown in graphs (D and E) in which each point represents an individual paw.

FIGURE 10. Expression of inflammatory mediators in mCIA paws. As per Fig. 9, RNA was extracted from individual paws obtained from mice during the course of mCIA and expression of mKC, mMIP-2, mGCP-2, and MPO were determined by quantitative reverse transcription-PCR. The -fold expression versus time (days) is shown. Each point represents an individual paw.

activation (14). No obvious explanation exists for the failure of Moepps et al. (14) to demonstrate mCXCR1 activation by a host of chemokines. In that study, mCXCR1 or mCXCR2 and G protein subunits (human β1, bovine γ2, and rat α2) were expressed recombinantly via baculovirus infection of Sf9 cells, and receptor activation was assayed by GTPγS exchange. Although activation of mCXCR2 was demonstrated using this system, it is possible that the expressed mCXCR1 protein was misfolded or had a relatively poor affinity for rat α2. Also, GTPγS exchange assays tend to be a less sensitive functional readout relative to whole cell assays such as chemotaxis (25). If the expression of the receptor protein is not sufficiently high, little if any GTPγS exchange will occur in response to an agonist.

Although chemokines and their receptors are often functionally conserved across species, exceptions have been reported in species traditionally used for animal models of human inflammatory diseases. Functional IL-8/CXCL8 and GCP-2/CXCL6 and CXCR1 and CXCR2 have been identified in monkey, chicken, rabbit, and guinea pig (26–28). Human, monkey, and rabbit IL-8 and GCP-2 are able to mediate function through both CXCR1 and CXCR2 (6, 29). An IL-8 equivalent has not been identified in mice or rats, although both species possess chemokines (KC and CINC-1) with shared properties of both human IL-8/CXCL8 and GRO-α/CXCL1 such as neutrophil activation (degranulation and respiratory burst) and migration (30). In addition, it has been suggested that the role of IL-8 in neutrophil recruitment has been replaced by GCP-2 in mice (31). Like its counterparts in human, rabbit, and guinea pig, mGCP-2 has been shown to activate CXCR2 in mouse and to cross-desensitize or activate CXCR1 from other species (32). Similar to published data on GCP-2 from other species, this study demonstrates that N-terminally processed mGCP-2 (9–78), which is a potent neutrophil agonist (32), can activate both mCXCR1 and mCXCR2.

Generally, tissue expression of mCXCR1 message was found to correlate well with the expression of hCXCR1, being localized in lung, bone marrow, spleen, and placenta (Fig. 8). Mouse CXCR1 was detected by reverse transcription-PCR analysis of polymorphonuclear neutrophils from mouse bone marrow (Fig. 8) (14) and from isolated neutrophils (13). Nevertheless, neutrophils from mCXCR2 knock-out mice had little (if any) chemotactic response to hIL-8/CXCL8 at concentrations that would be predicted to activate mCXCR1 (12). Moreover, mouse CXCR2 knock-out mice have defective transepithelial neutrophil migration in response to experimental urinary tract infection (33), a function predominantly mediated by CXCR1 in human urinary tract infections (34). Expression analysis of inflamed paws from mCIA (Figs. 9 and 10) showed that up-regulation of mCXCR1 and mCXCR2 mRNA occurred after neutrophil MPO mRNA levels reverted to baseline. This finding suggested that mCXCR1 and mCXCR2 were expressed on cells other than neutrophils (such as macrophages, T and B cells) in the latter stages of mCIA when maximal swelling is observed following boost. This hypothesis is consistent with mCXCR1
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and mCXCR2 mRNA expression in spleen (mostly lymphocytes), CD4+ /CD8+ T cells, CD11b+ macrophages, and certain B and T lymphoma cell lines (Fig. 8) (13, 14). The observation that MPO mRNA levels occurred prior to paw swelling (p > 0.05) is consistent with reports that neutrophils likely play a crucial role in initiating the inflammatory cascade and drawing in macrophages and lymphocytes (24, 35), but it conflicts with reports indicating significant elevation of mMIP-2 and MPO protein levels on day 35 of mCIA correlated with development of arthritis and neutrophil infiltration by histological examination (24, 36). Ultimately, defining mCXCR1 protein expression on leukocyte populations and tissues will require the generation of anti-mCXCR1 antibodies.

In conclusion, we have demonstrated for the first time that a functional homologue for hCXCR1 exists in the mouse. Further, our data support the idea that hIL-8/CXCL8 has been functionally replaced by GCP-2/CXCL6 in the mouse (31) and that MGC-2 and mCXCR1 play a key role in the mouse autoimmune model of CIA.

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