Molecular Cloning and Functional Expression of Murine JE (Monocyte Chemoattractant Protein 1) and Murine Macrophage Inflammatory Protein 1α Receptors

EVIDENCE FOR TWO CLOSELY LINKED C-C CHEMOKINE RECEPTORS ON CHROMOSOME 9

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We have isolated cDNA clones that encode two closely related, murine C-C chemokine receptors. Both receptors are members of the G-protein-coupled, seven-transmembrane domain family of receptors and are most closely related to the human monocyte chemoattractant protein 1 receptor. Expression of each of the receptors was detected in murine macrophage cell lines, but not in nonhematopoietic lines. Expression of these receptors in Xenopus oocytes revealed that one receptor signaled in response to low nanomolar concentrations of murine JE, whereas the second receptor was activated by murine macrophage inflammatory protein (MIP) 1α and the human chemokines MIP-1β and RANTES. Binding studies revealed high affinity binding of radiolabeled mJE to the mJE receptor and murine MIP-1α to the second receptor. Chromosomal localization indicated that the two receptor genes were clustered within 80 kilobases of each other on mouse chromosome 9. Creation of receptor chimeras suggested that the amino terminus was critically involved in mediating signal transduction and ligand specificity of the mJE receptor, but not the mMIP-1α receptor. The identification and cloning of two functional murine chemokine receptors provides important new tools for investigating the roles of these potent cytokines in vivo.

Leukocyte trafficking plays an important role in immune system surveillance and chronic inflammation. Locally produced chemoattractant cytokines, known as chemokines, are thought to play a critical role in this directed migration (see Refs. 1–4 for recent reviews). Human monocyte chemoattractant protein 1 (hMCP-1)† and its murine homolog, JE (mJE), are members of the C-C family of chemokines, in which the first two of four conserved cysteines are adjacent to each other. Other C-C chemokines include macrophage inflammatory protein 1α and 1β (MIP-1α, MIP-1β) and RANTES (regulated on activation, normal T cell-expressed). In general, C-C chemokines are potent monocyte and lymphocyte chemoattractants. A C-C chemokine that is a chemoattractant for eosinophils, eotaxin (5), has recently been described, as well as a novel lymphocyte chemokine containing two, rather than four cysteines, known as lymphotaxin (6).

The murine JE gene was originally identified by virtue of its dramatic induction in murine fibroblasts by platelet-derived growth factor and other growth factors (7). Characterization of the gene by Rollins et al. (8) revealed important similarities to known cytokines such as macrophage colony-stimulating factor, interferon α, and interleukin (IL) 6. Murine JE and hMCP-1 are 62% identical over their amino-terminal domains, but mJE extends an additional 49 amino acids beyond the carboxyl end of hMCP-1. This carboxyl-terminal extension, which is extensively glycosylated, is not required for the chemoattractant activity of mJE (9). Further, mJE and hMCP-1 have similar chemoattractant activity for human monocytes (9). Murine JE is thus a structural and functional analog of hMCP-1.

MCP-1 has been implicated in the pathogenesis of diseases characterized by mononuclear infiltrates, including psoriasis (10), pulmonary fibrosis (11), rheumatoid arthritis (12), and atherosclerosis (13, 14). In mice, mJE has been shown to be up-regulated by infusion of minimally oxidized low density lipoproteins (15) and thus may play a role in the accumulation of monocyte/macrophages in early atherosclerotic lesions. A possible role for mJE in tumor suppression in vivo was suggested by Rollins et al. (16) who found that expression of hMCP-1 or mJE in Chinese hamster ovary cells suppressed the ability of the cells to form tumors in nude mice.

Human receptors for IL-8 (Type A and Type B (17, 18)) and a single receptor that binds both RANTES and MIP-1α (19, 20) have been cloned and shown to be members of the seven-transmembrane domain superfamily of receptors. We have recently reported the cloning and expression of two alternatively spliced forms of the human MCP-1 receptor, which differ only in their terminal carboxyl tails (21) and which couple to Gα in TM, transmembrane; HEK, human embryonic kidney; RFLV, restriction fragment length variant; MIP-1α-R, macrophage inflammatory protein 1α receptor; kb, kilobase(s); PUC, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid.

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a pertussis toxin-sensitive manner (22). To investigate further the roles of chemokines in vivo, considerable effort has been focused recently on the cloning of their murine receptors. In contrast to the situation in the human, a single receptor appears to exist for murine IL-8 (23). Extramedullary myelopoiesis, as well as a decreased neutrophil response after injection of thioglycolate, was noted in mice in which the IL-8 receptor was deleted by homologous recombination (23). These studies suggest that IL-8, and perhaps other chemokines, are involved in the regulation of myelopoiesis. Gao and Murphy (24) have very recently reported the cloning of a murine MIP-1α receptor, as well as two orphan receptors. In this paper, we report the cloning and functional expression of a murine JE/MCP-1 receptor, as well as a second, closely related receptor that signals in response to mMIP-1α, hMIP-1β, and hRANTES.

MATERIALS AND METHODS

Reagents—Recombinant chemokines were obtained from R & D Systems, Inc. (Minneapolis, MN). Initial experiments used full-length mouse (9) and rat JE purified from supernatants of stably transfected Chinese hamster ovary cells (kindly provided by T. Yoshimura, National Cancer Institute, Frederick, MD, and B. Rollins, Dana Farber Cancer Institute, Boston, MA). Murine JE expressed in Escherichia coli (R & D Systems, Inc.) was used in subsequent experiments. No differences in activity were observed between the JE expressed in Escherichia coli versus mammalian cells. LipofectAMINE and G418 sulfate were from Life Technologies, Inc. Restriction enzymes were from Boehringer Mannheim. CaCl2 was obtained from Amersham. All other reagents were obtained from Sigma.

Tissue Culture, Calcium Fluorimetry, and Stable Transfections—WEHI 3, WEHI 274.1, and WEHI 265.1 cells were obtained from the American Type Culture Collection (Bethesda, MD) and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT) and 1% penicillin/streptomycin. WEHI cells were stably transfected with the murine JE that was radiolabeled using the Bolton-Hunter procedure (DuPont NEN) to a specific activity of 2200 Ci/mmol. After incubation for 90 min at 27°C with gentle shaking, the membranes were collected onto a glass fiber filter (presoaked in 0.3% polyethyleneimine, 0.2% bovine serum albumin for 1 h), using a SKA-TRON filtration harvester (Sterling, VA), set at a vacuum pressure of 500 mm Hg. Unbound ligand was removed by the addition of 4 ml of wash buffer (10 mM Hepes, 0.5 mM NaCl, 0.5% bovine serum albumin) over an interval of 10 s. Bound 32P-labeled JE was quantitated by counting γ emissions. Essentially identical results were obtained using intact transfected HEK-293 cells, but with a higher level of nonspecific binding. In the case of the mMIP-1α receptor, binding was performed using (125I)-Bolton-Hunter-labeled mMIPα and stably transfected HEK-293 cells that were homogenized and subjected to calcium efflux assay using the program LIGAND (27). Binding data were plotted, and I50 values were determined using the program Prism (by Graph Pad, San Diego, CA).

Southern and Northern Blot Analysis—For Southern blots, 10 μg of mouse genomic DNA was digested with restriction enzymes, fractionated by size on a 0.6% agarose gel, and transferred to Zetaprobe GT (Bio-Rad) nylon membranes according to the manufacturer’s instructions. The membranes were sequentially hybridized with probes specific for the murine MIP-1α and JE receptors. These probes were derived from the 3’- untranslated regions of the corresponding cDNAs, and their specificities were confirmed by hybridization to increasing concentrations of both cDNAs spotted on a nylon membrane. Prior to reprobing, the membranes were washed in 0.1 × SSC at 65°C for 30 min, followed by washing in 0.1 × SSC/0.1% SDS at 65°C for 2 h. The membrane was also hybridized with a probe that detects both receptors (corresponding to nucleotides 335–620 of the mJE receptor cDNA) which encode the highly conserved region from transmembrane 1 through transmembrane 3 (TM3). Probe (32P)labeled to a specific activity of ~8.0 × 109 cpm/μg and used at a concentration of 4.0 × 106 cpm/ml. Hybridizations were performed in 50% deionized formamide, 5 × SSC, 5 × Denhardt’s, 1.0% SDS, and 100 μg/ml denatured salmon sperm DNA at 42°C for 16 h. The membrane was washed in 0.1 × SSC/0.1% SDS at 65°C for 3 h and exposed to x-ray film with intensifying screens for 1–3 days at –80°C.

For Northern blots, 10 μg of total RNA was size-fractionated on a 1.0% agarose, 0.66 M formaldehyde gel, transferred to a nylon membrane (Hybond-N, Amersham), and stained with 0.03% methylene blue

Calcium Efflux Assay—The calcium efflux assay was performed as described (21). Briefly, cDNAs were cloned downstream of the SF6 promoter of pDE3, the gene encoding β-galactosidase RNA (cRNA) was transcribed using SP6 RNA polymerase. The size and concentration of the transcription product were confirmed by gel electrophoresis. Xenopus laevis oocytes were injected with 25 ng of mRNA in a total volume of 50 nl per oocyte 1 day after harvesting. After incubation in modified Barth’s medium (21) for 2 days at 16°C, the oocytes were incubated with 40 Ci/ml [35S]Methionine (Amersham) for 1 h, and placed in groups of seven into wells of a 24-well plate in a volume of 0.5 ml. Expression of recombinant receptors at the oocyte surface was confirmed using an enzyme-linked immunosorbent assay, as described (26). The 45Ca2+ efflux following addition of agonist was determined by collecting samples of the medium at 10-min intervals and counting β emissions in a liquid scintillation counter. Agonists were applied to the oocytes in Barth’s medium for 10 min. Uninjected oocytes were used as controls.

Chemokine Binding Assay—Binding studies were performed using membranes prepared from stable cell lines. Approximately 5 × 106 stably transfected HEK-293 cells were harvested by incubation with 1 ml EDTA in phosphate-buffered saline, washed once with phosphate-buffered saline, and resuspended in 20 ml of 10 mM HEPES, pH 7.2, 0.2 mM CaCl2, 1 mM MgCl2, 0.1% bovine serum albumin, 2 mM EDTA, 1 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml pepstatin, and 1 μg/ml DNase. The resuspended cells were homogenized using a Dounce homogenizer and sonicated for 45 s (Branson Model 450 sonicator, output level 5, Danbury, CT), centrifuged at 1000 × g for 10 min, and the supernatants collected. Membranes were obtained by centrifugation of the supernatants at 48,000 × g for 30 min at 4°C. The membrane pellet was resuspended in binding buffer (50 mM HEPES, pH 7.4, 1 mM CaCl2, 5 mM MgCl2, 0.5% bovine serum albumin). Approximately 2.5 μg of cell membranes in 0.3 ml of binding buffer were added to 12 × 75 mm polypropylene tubes. Membranes were washed with 1 ml of 0.5% bovine serum albumin in 10 mM HEPES, pH 7.2, 0.2 mM CaCl2, 1 mM MgCl2 for 10 min. Membranes were washed with 1 ml of 10 mM HEPES, pH 7.2, 1 mM CaCl2, 5 mM MgCl2 for 10 min, and placed in groups of seven into wells of a 24-well plate in a volume of 0.5 ml. Expression of recombinant receptors at the oocyte surface was confirmed using an enzyme-linked immunosorbent assay, as described (26). The 45Ca2+ efflux following addition of agonist was determined by collecting samples of the medium at 10-min intervals and counting β emissions in a liquid scintillation counter. Agonists were applied to the oocytes in Barth’s medium for 10 min. Uninjected oocytes were used as controls.
in 0.3 M sodium acetate, pH 5.2, to visualize ribosomal RNAs. The filter was sequentially hybridized with 32P-labeled receptor-specific probes described above. The probe concentration was 1.0 \times 10^6 \text{ cpm/ml}, and hybridizations were at 42 \degree C overnight in the above hybridization mixture, except that 5% dextran sulfate was included and PIPES (0.8 M NaCl, 20 mM PIPES, pH 6.5) replaced the SSC. The membrane was stripped by boiling for 10 min in water containing 0.5% SDS.

**P1 Clones**—Murine genomic bacteriophage P1 clones containing each of the two receptors were obtained using PCR primer pairs. A 129 ES mouse genomic library (average insert size of 85 kb) was screened by Genome Systems (St. Louis, MO) using primer pairs specific for each receptor. The mMIP-1α receptor (MIP-1α-R) primers were located at nucleotides 1015–1033 (sense) and 1144–1164 (antisense) of the cDNA and the JE receptor primers at nucleotides 1360–1378 (sense) and 1593–1612 (antisense) of the cDNA. Of five positive clones for the mMIP-1α receptor, four also produced a specific product using the JE receptor primer pair. We further characterized two of the double positive clones by PCR and confirmed that both primer pairs amplified a receptor primer pair. We further characterized two of the double positive clones by PCR and confirmed that both primer pairs amplified a receptor primer pair.

**Genome Localization**—The genes for mJE-R and mMIP-1α-R were mapped by linkage analysis of an interspecific backcross of (C57BL/6J × Mus musculus) F1 × C57BL/6J mice, constructed as described previously (28). The cross was previously typed for several hundred restriction fragment length variants (RFLVs) and simple sequence repeat length polymorphisms (28). To identify informative RFLVs, DNAs from the parental strains were digested with various restriction enzymes and subjected to Southern hybridization analysis as described above. A survey of restriction enzymes revealed an RFLV for the mJE receptor gene with the enzyme PvuII. Following digestion with PvuII, DNA from parental strain C57BL/6J exhibited a 6.2-kb hybridizing fragment, DNA from M. musculus exhibited a 3.8-kb and 1.6-kb fragments, and DNA from F1 hybrids exhibited all three fragments (not shown). A survey of restriction enzymes revealed an RFLV for the mMIP-1α receptor gene using the enzyme HindIII; thus, following digestion, DNA from strain C57BL/6J exhibited bands of 16 kb and 7.2 kb, DNA from M. musculus exhibited a band of 6.4 kb, and DNA from F1 mice exhibited all three bands. The RFLVs were then examined in a set of backcross mice, and the segregation patterns were compared with those of previously typed markers.

**Chimeric Receptors**—The amino termini of the two murine receptors were interchanged by taking advantage of a conserved EcoRV site located within the putative first intracellular loop of each receptor (residue 92 of the mJE-R and residue 69 of the mMIP-1α-R). These chimeric receptors, designated mJE/mMIP-1α-R and mMIP-1α/mJE-R, were expressed and assayed for signaling in Xenopus oocytes as described above.

**RESULTS**

**Cloning and Expression of Murine Chemokine Receptors**—To identify cell lines expressing the mJE receptor, we screened murine monocyte/macrophage cell lines for responsiveness to mJE and related C-C chemokines. As shown in Fig. 1, mJE and, to a lesser extent, hMCP-1 induced a transient intracellular calcium flux in WEHI 274.1 cells. Calcium fluxes were not observed in WEHI 265.1, WEHI 3, or P388D1 cells (data not shown). To clone the mJE receptor, we constructed a cDNA library from the WEHI 274.1 cells and screened this library with a probe complementary to a highly conserved region of the human MCP-1R. A 2.9-kb cDNA clone was obtained that conferred mJE- and hMCP-1-dependent signaling, when expressed in Xenopus oocytes, as assayed by 45Ca2+ efflux (Fig. 2). This receptor was specific for JE/MCP-1, as no calcium efflux was observed in response to the closely related C-C chemokines mMIP-1α, mMIP-1β, hRANTES, hMIP-1α, or hMIP-1β (Fig. 2A). In addition, HEK-293 cells stably expressing this cDNA also underwent a robust intracellular calcium flux in response to mJE (1–30 nM) (Fig. 3A), thus confirming that this receptor signals in mammalian cells. Human MCP-1 also elicited a calcium flux, although at higher concentrations than mJE (Fig. 3B). A small but reproducible signal was seen in response to mMIP-1α (100 nM), but not to hMIP-1α, hMIP-1β, or hRANTES (Fig. 3C). We therefore refer to this cDNA clone as the mJE receptor.

Screening of a mouse spleen library yielded a second cDNA clone that also hybridized strongly to the hMCP-1 receptor probe. In contrast to the mJE receptor, however, the receptor encoded by this cDNA signaled in response to mMIP-1α, hRANTES, and hMIP-1β, but did not respond to mJE, hMCP-1, mMIP-1β, or hMIP-1α (Fig. 4). We therefore refer to this receptor as the murine MIP-1α (mMIP-1α) receptor. The response of this receptor to murine, but not human MIP-1α, is intriguing, as the human MIP-1α/RANTES (C-C CKR-1) receptor.

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**Fig. 1.** Calcium mobilization in WEHI 274.1 cells by mJE and hMCP-1. WEHI 274.1 cells were loaded with Indo-1 AM and challenged with mJE (30 nM) or hMCP-1 (100 nM) at the time indicated by the arrow.

**Fig. 2.** Expression of the mJE receptor in Xenopus oocytes. A, specificity of the mJE receptor. All chemokines were used at a final concentration of 100 nM. B, dose-response curves for mJE and hMCP-1. All data points were determined in triplicate. The data shown are representative of three experiments.
responds equally well to both human and murine MIP-1α (19).

Binding of Radiolabeled Chemokines—Radiolabeled JE bound with high affinity to membranes prepared from HEK-293 cells expressing the putative JE receptor (Fig. 5A). Analysis of these binding data by the method of Scatchard revealed a dissociation constant \( K_d \) of 46 pM. In competition binding assays using 150 pM 125I-labeled JE, we observed very similar IC50 values for unlabeled JE (195 pM) and human MCP-1 (210 pM) (Fig. 5B). HEK-293 cells expressing the mMIP-1α receptor bound 125I-mMIP1α with high affinity \( K_d \) of 640 pM (Fig. 5C). In competition assays, we found that unlabeled hMIP-1β, as well as mMIP-1α, competed efficiently with 125I-mMIP-1α for binding to the receptor (data not shown).

Sequence Similarity of the Murine and Human C-C Chemokine Receptors—The murine JE receptor cDNA encoded a protein of 373 amino acids (Fig. 6). Hydropathy analysis of the predicted amino acid sequence revealed seven putative transmembrane domains and an extracellular amino terminus of 50 residues. The mJE receptor was closely related to the MCP-1 receptor, being 75% identical overall. However, the transmembrane (TM) domains and intracellular loops, particularly in the region of the second and third TMs, were almost identical. Conversely, the amino termini, the second and third extracellular loops, and the carboxyl-terminal tails were less well conserved between the two murine receptors (Fig. 6), suggesting that these regions may be involved in determining chemokine specificity.

Chromosomal Localization of mJE and mMIP-1α Receptors—Hybridization of murine genomic DNA with probes specific for the mJE and mMIP-1α receptors revealed single bands in each lane of the Southern blot, suggesting that each receptor is encoded by a single copy gene (Fig. 7). Rehybridization of the same blot with a probe derived from a highly conserved portion of the coding region of these two receptors (TM1 to TM3) failed to reveal additional bands, suggesting the absence of other closely related receptors (data not shown). The chromosomal locations of the two genes were determined by linkage analysis of an interspecific backcross involving the parental mouse strains C57BL/6J and M. spretus as described previously (28). Receptor-specific probes were used to identify informative RFLVs of the genes upon Southern hybridization. The segregation of the RFLV was examined in 65 (C57BL/6J × M. spretus) F1 × C57BL/6J backcrossed mice. DNA from these mice has been typed previously for over 200 genetic markers spanning all chromosomes except the Y chromosome (28). The mJE receptor RFLV exhibited linkage with a number of mark-

![Fig. 3. Calcium mobilization in 293 cells stably transfected with the mJE receptor cDNA. Dose-response curves to mJE (A), hMCP-1 (B), and other C-C chemokines (100 nM) (C).](image)

![Fig. 4. Expression of the murine MIP-1α receptor in Xenopus oocytes. A, specificity of the mMIP-1α receptor. The indicated chemokines were used at final concentrations of 100 nM. B, dose-response curves for mMIP-1α and hMIP-1β. All data points were determined in triplicate. The data shown are representative of three similar experiments.](image)
ers on the distal portion of mouse chromosome 9, the nearest proximal marker being the microsatellite marker D9Mit19 (1 recombinant out of 65 animals) and the nearest distal marker being the random cDNA RFLV D9Ucla3 (1 recombinant out of 65 animals) (Fig. 8). The linkage was highly significant, as both markers exhibited logarithm of the odds scores exceeding 17.3. Analysis of the segregation of an RFLV for the mMIP-1α receptor gene revealed complete co-segregation with the mJE receptor RFLV (no recombination out of 65 animals). These results indicate that the genes for the mJE receptor and the mMIP-1α receptor are tightly linked on mouse chromosome 9 (Fig. 8). The results indicate the following order of markers typed on distal chromosome 9, with distances given in centimorgans: centromere-(D9Ucla2, D9Mit9) (5.9 ± 3.3 centimorgans)-2Mit6 h2 (1.5 ± 1.5 centimorgans)-D9Mit19 (1.5 ± 1.5 centimorgans)-(Jer, Mip1ar) (1.5 ± 1.5 centimorgans)-D9Ucla5. We designate the symbols Jer and Mip1ar for the JE receptor and MIP-1α receptor, respectively. This region of murine chromosome 9 is syntenic to human chromosome 3p21 (29).

Further evidence that the two receptors are closely linked was obtained by screening a murine 129ES genomic library

Fig. 5. Binding of chemokines to the cloned receptors. A, radiolabeled mJE was added at the indicated concentrations to membranes prepared from HEK-293 cells stably expressing the mJE receptor. Nonspecific binding was determined by subtraction of the nonspecific binding from the total binding. The dissociation constant (Kd), determined by Scatchard analysis, was 46 ± 18 pM. Shown is one of three similar experiments. Very similar results were obtained using intact HEK-293 cells. B, competition of mJE and hMCP-1 for the mJE receptor. Radiolabeled mJE (150 pM) was added to membranes prepared from HEK-293 cells stably expressing the mJE receptor. Unlabeled mJE and hMCP-1 were added at the indicated concentrations. The IC50 values were 195 pM for mJE and 210 pM for hMCP-1. C, radiolabeled mMIP-1α was added at the indicated concentrations to HEK-293 cells stably expressing the mMIP-1α receptor. Nonspecific binding was determined by the addition of a 100-fold excess of unlabeled mMIP-1α. The apparent Kd was 640 pM.

Table 1

|          | mJE  | mMIP-1α | hMCP-1 | hMIP-1α/RANTES |
|----------|------|---------|--------|---------------|
| mJE      | 72   | 75      | 71     | 54            |
| mMIP-1α  | 72   | 75      | 71     | 54            |

Fig. 6. Predicted amino acid sequence of the mJE and mMIP-1α receptors. The murine receptors are shown aligned with the human C-C chemokine receptors. Gaps inserted to optimize the alignments are indicated by dashes. The seven predicted transmembrane domains are indicated by the horizontal bars and numbers.

Fig. 7. Southern blot analysis of murine chemokine receptor genes. Mouse genomic DNA (10 μg) was digested with HindIII (lane 1), EcoRI (lane 2), BamHI (lane 3), or XbaI (lane 4) and hybridized under conditions of high stringency with radiolabeled probes specific for the 3′-untranslated regions of the mJE and mMIP-1α receptors.
constructed in P1 bacteriophage clones (average insert size of 85 kb). Using PCR primer pairs specific for each receptor (see "Materials and Methods"), we obtained two independent P1 clones that amplified the predicted products for both receptors (Fig. 9). In addition, Southern analysis of one P1 clone (clone 5340) produced the same hybridization pattern with receptor-specific probes as that observed with total genomic DNA (data not shown). These data indicate, therefore, that the two receptors are closely linked on mouse chromosome 9.

Expression of the JE Receptor and the MIP-1α Receptor in Murine Monocytic Cell Lines—We examined a number of hematopoietic and non-hematopoietic murine cell lines for expression of the mJE receptor and the mMIP-1α receptor. As shown in Fig. 10, the mJE receptor mRNA was expressed by monocytic WEHI 274.1 cells and at lower levels by WEHI 265.1 and WEHI 3 cells. P388D1 cells, a macrophage-like line, and a variety of non-myeloid cell lines did not express detectable levels of the mJE receptor mRNA. Conversely, the mMIP-1α receptor message was expressed at high levels by P388D1 cells, at much lower levels by WEHI 265.1 cells, but was not detectable in the other cell lines tested (Fig. 10). These results are consistent with the observations that WEHI 274.1 cells respond to mJE (Fig. 1), and that P388D1 cells respond to hRANTES and mMIP-1α (Table II).

Receptor Chimeras—To investigate the role of the amino terminus in ligand recognition and signaling, we constructed chimeric receptors in which the amino termini, along with TM1, were interchanged between the mJE receptor and the mMIP-1α receptor. These chimeric receptors were expressed in Xenopus oocytes and assayed for signaling in response to various chemokines. As shown in Fig. 11, the chimeric mJE/mMIP-1α receptor (amino terminus and TM1 from the mJE receptor spliced onto the mMIP-1α receptor) signaled well in response to mJE receptor and the mMIP-1α receptor. WEHI 274.1 and P388D cells in response to C-C chemokines (100 nM) from the indicated cell lines were electrophoresed in a 1% agarose gel and hybridized with radiolabeled probes specific for the mJE receptor and the mMIP-1α receptor. WEHI 274.1 and 265.1 are monocytic cell lines. WEHI 3 and 293 are non-hematopoietic cells. The positions of 18S and 28S ribosomal RNAs are indicated to the left (data not shown). Methylene blue staining of the filter revealed intact ribosomal bands of approximately equal intensity in all lanes, except for the BALB/c lane, in which the amount of RNA was reduced (data not shown).

### Table II

| Chemokine | WEHI-274.1 | P388D |
|-----------|------------|-------|
| mJE       | ++         | –     |
| hMCP-1    | –          | –     |
| mMIP-1α   | –          | ++    |
| mMIP-1β   | –          | –     |
| hMIP-1α   | –          | –     |
| hMIP-1β   | –          | –     |
| hRANTES   | –          | –     |

*++, strong signal; +, weak signal; –, no detectable Ca2+ flux.

To investigate the role of the amino terminus in ligand recognition and signaling, we constructed chimeric receptors in which the amino termini, along with TM1, were interchanged between the mJE receptor and the mMIP-1α receptor. These chimeric receptors were expressed in Xenopus oocytes and assayed for signaling in response to various chemokines. As shown in Fig. 11, the chimeric mJE/mMIP-1α receptor (amino terminus and TM1 from the mJE receptor spliced onto the mMIP-1α receptor) signaled well in response to mJE receptor and the mMIP-1α receptor. In contrast, the complementary mMIP-1α/mJE receptor chimera failed to signal to any of the C-C chemokines (Fig. 11). Enzyme-linked immunosorbent assays confirmed that this chimera was expressed on the surface of microinjected oocytes at levels comparable to, or higher than, the wild-type receptors (data not shown). These data indicate, therefore, that the ami-
no-terminal domain of the mJE receptor, but not the mMIP-1α receptor, is critically involved in receptor activation and signal transduction.

**DISCUSSION**

Murine JE has been implicated in models of disease characterized by prominent monocyte/macrophage infiltrates, but the mechanisms of monocyte activation and directed migration induced by mJE are not well understood. To gain insight into this phenomenon, and as a first step in genetic modification of the mJE receptor gene, we have cloned its cDNA from a murine monocytic cell line. Several lines of evidence support the conclusion that this cDNA encodes a murine JE receptor. First, injection into Xenopus oocytes of cRNA obtained from this clone conferred mJE/hMCP-1-dependent activation at low nanomolar concentrations. We have confirmed these results in transfected mammalian cells. In both Xenopus oocytes and HEK-293 cells expressing the JE receptor, calcium is mobilized much more efficiently by mJE as compared to hMCP-1. Similar results were obtained using wild-type WEHI 274.1 murine monocytes. Second, these responses are specific for mJE/hMCP-1, as other closely related chemokines failed to induce signals. Third, 125I-labeled JE bound with high affinity to HEK-293 cells transfected with this cDNA. Fourth, Northern blot analysis revealed high levels of expression of the receptor mRNA in monocytic cell lines that responded to mJE and little or no mRNA in lines that failed to respond to mJE. Finally, sequencing of the cDNA revealed a putative seven-transmembrane domain receptor with a predicted amino acid sequence that was 72% identical with the hMCP-1 receptor. We conclude, therefore, that this cDNA encodes a murine JE receptor.

The second receptor cloned in this study signaled well in response to low nanomolar concentrations of murine MIP-1α and also bound this chemokine with high affinity. It is likely, therefore, that mMIP-1α is the natural ligand for this receptor. The mMIP-1α receptor also signaled in response to human MIP-1β and thus represents the first example of a cloned receptor activated by MIP-1β. In addition, hMIP-1β competed well with radiolabeled mMIP-1α in receptor binding assays. Whether or not MIP-1β is a natural ligand for this receptor remains unclear, however, because the murine form of MIP-1β was not efficient at receptor activation. Similarly, this receptor was activated by human RANTES, and it will be interesting to determine if murine RANTES is a functional ligand.

The mJE and mMIP-1α receptors are almost completely identical in the putative transmembrane domains, as well as in the first extracellular loop. In addition, the second and third intracellular loops are nearly identical, suggesting that both receptors may couple to the same or very similar G-proteins. Interestingly, the murine MIP-1α receptor is more closely related to the MCP-1 receptor than to C-C CRK-1, the human receptor that binds and signals in response to MIP-1α and RANTES (19). These data suggest that the MIP-1α receptor is a novel receptor and not simply a murine homolog of the human MIP-1α/RANTES receptor. Based on primary sequence identity, it may in fact represent the murine form of a human MCP-1 receptor homolog. This receptor does not, however, signal in response to human or murine MCP-1. The mJE receptor signals primarily in response to mJE and hMCP-1 and, in this regard, is very reminiscent of the ligand specificity of the hMCP-1 receptor. We have recently found that hMCP-3, but not hMCP-2, is a functional ligand for the human MCP-1 receptor (30). It remains to be determined if the MARC/fic protein (31), which appears to be the murine homolog of hMCP-3, activates the mJE receptor.

The cloned mJE receptor bound mJE and hMCP-1 in a comparable manner, yet signaled much more efficiently in response to mJE as compared to hMCP-1. High affinity binding of the ligand to the receptor thus appears to be necessary, but not sufficient, to initiate signaling. These data are consistent with a model in which one portion of the receptor binds the ligand with high affinity, while a second receptor domain interacts with the chemokine to initiate signaling. Recent work in our laboratory on the binding of hMCP-1 to its receptor supports such a model, as does published work on the C5a receptor (32). It should be noted that hMCP-1 and mJE have been found to be equipotent in inducing chemotaxis of human monocytes (9). Thus, unlike the murine receptor, the human MCP-1 receptor may not distinguish between human and murine MCP-1. Studies are currently in progress in our laboratory comparing the binding and signaling properties of the human and murine MCP-1 receptors.

The mJE and mMIP-1α receptors appear to have arisen by gene duplication and may represent the first two members of a family of receptor genes clustered on chromosome 9. The evidence for this hypothesis includes the high degree of identity between these two receptors at the DNA sequence level, their co-segregation in a genetic cross, and their co-localization on a P1 bacteriophage clone. This area of mouse chromosome 9 is syntenic to human chromosome 3p21, where the hMIP-1α/ RANTES (20) and hMCP-1 receptors are found in close proximity. This region does not correspond to any mutations with obvious relevance to these receptors. In addition, Gao and Murphy (24) have very recently identified a murine MIP-1α receptor distinct from the receptors described in this paper, as well as two additional closely related murine receptors without identified ligands, all of which map to mouse chromosome 9. Other chemokine receptors have been localized to human chromosome 2q34-q35 in the case of the human type A and B IL-8 receptors (33) and to chromosome 19q13.3 for the formyl peptide and C5α receptors (34).

The amino-terminal domains represent the areas of greatest sequence divergence between the mJE and mMIP-1α receptors. The amino termini of the receptors for thrombin (35), thryrotropin (36), C5a (37), and IL-8 (38) participate in the binding of their respective ligands. Taken together, these observations suggest that divergence of this domain in the mJE and mMIP-1α receptors may contribute to their different agonist specificities. To test this hypothesis, we constructed two chimeric receptors in which the amino-terminal domains were

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\(^{2}\) F. S. Monteclaro and I. F. Charo, unpublished observations.

\(^{3}\) I. F. Charo and A. J. Lusis, unpublished observations.
exchanged between the mJE receptor and the mMIP-1 receptor. Analysis of the signaling properties of these two chimeras in *Xenopus* oocytes indicated that the amino terminus of the mJE receptor, but not the mMIP-1 receptor, was critical for signaling. This result is in agreement with recent results obtained using the human MCP-1 receptor, and suggests that distinct mechanisms of ligand binding have evolved within the C-C chemokine receptor family. Since the first extracellular loop of the mMIP-1 receptor and mJE receptor are identical, it is likely that the second and third extracellular loops of the mMIP-1α-R will be found to mediate ligand binding and specificity.

In summary, we have cloned two novel murine receptors that appear to define a family of C-C chemokine receptors clustered on chromosome 9. Through the construction of receptor chimeras, we have demonstrated that signaling of the mJE receptor, but not the mMIP-1 receptor, is critically dependent upon ligand interaction with the receptor amino terminus. The identification of the murine JE receptor represents an important step in the creation of genetically modified mice to probe the role of JE/MCP-1 in models of human disease.

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REFERENCES

1. Oppenheim, J. J., Zachariae, C. O. C., Mukaida, N., and Matsushima, K. (1991) *Annu. Rev. Immunol.* 9, 617–648
2. Rollins, B. J. (1991) *Cancer Cells* 3, 517–524
3. Schall, T. J. (1994) in *The Cytokine Handbook* (Thomson, A. W., ed.) 2nd Ed, pp. 419–460, Academic Press, London
4. Murphy, P. M. (1994) *Annu. Rev. Immunol.* 12, 593–633
5. Jose, P. J., Griffiths-Johnson, D. A., Collins, P. D., Walsh, D. T., Moqbel, R., Griffiths-Johnson, D. A., Jenkins, N. A., Copeland, N. G., Bazan, J. F., Moore, K. W., Schall, T. J., and Zlotnik, A. (1994) *J. Biol. Chem.* 269, 19752–19756
6. Kehrl, G. H., McCluskey, S., Laidman, T. M., Finegold, M. J., and Schall, T. J. (1994) *J. Exp. Med.* 179, 881–887
7. Kehrl, G. H., McCluskey, S., Laidman, T. M., Finegold, M. J., and Schall, T. J. (1994) *J. Exp. Med.* 179, 881–887
8. Cochrane, B. H., Reffel, A. C., and Stiles, C. D. (1983) *Cell* 33, 939–947
9. Rollins, B. J., Morrison, E. D., and Stiles, C. D. (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85, 3738–3742
10. Ernst, C. A., Zhang, Y., Hancock, P. C., Roulstone, B. J., Corless, C. L., and Rollins, B. J. (1994) *J. Immunol.* 152, 3541–3549
11. Gillitzer, R., Wolf, K., Tong, D., Muller, C., Yoshimura, T., Hartmann, A. A., Stingl, G., and Berger, R. (1993) *J. Invest. Dermatol.* 101, 127–131
12. Antoniades, H. N., Neville-Golden, J., Galanopoulos, T., Kradin, R. L., Valente, A. J., and Graves, D. T. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 5371–5375
13. Koch, A. E., Kunkel, S. L., Harlow, L. A., Johnson, B., Ervavnoff, H. L., Haines, G. K., Burdick, M. D., Pope, R. M., and Strieter, R. M. (1992) *J. Clin. Invest.* 89, 772–779
14. Nelken, N. A., Coughlin, S. R., Gordon, D., and Wilcox, J. N. (1991) *J. Clin. Invest.* 88, 1121–1127
15. Yia-Herttuala, S., Lipton, B. A., Rosenfeld, M. E., Sarkinja, T., Yoshimura, T., Leonard, E. J., Witztum, J. L., and Steinberg, D. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 5252–5256
16. Liao, F., Berliner, J. A., Mebrahtu, M., Navab, M., Demer, L. L., Lusis, A. J., and Fogelman, A. M. (1991) *J. Clin. Invest.* 88, 1273–1280
17. Rollins, B. J., and Sunday, M. E. (1991) *Mol. Cell. Biol.* 11, 3125–3131
18. Holmes, W. E., Lee, J., Kuwajima, Y., Rimmer, E. C., and Wod, W. I. (1991) *Science* 253, 1273–1280
19. Murphy, P. M., and Tiffany, H. L. (1991) *Science* 253, 1280–1283
20. Neste, K., DiGregorio, D., Mak, J. Y., Horuk, R., and Schall, T. J. (1993) *Cell* 72, 415–425
21. Gao, J.-L., Kuhns, D. B., Tiffany, H. L., McDermott, D., Li, X., Francke, U., and Murphy, P. M. (1993) *J. Exp. Med.* 177, 1421–1427
22. Charo, I. F., Myers, S. J., Herman, A., Franci, C., Connolly, A. J., and Coughlin, S. R. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 2752–2756
23. Myers, S. J., Wong, L. M., and Charo, I. F. (1995) *J. Biol. Chem.* 270, 5786–5792
24. Casalano, G., Lee, J., Kikly, K., Ryan, A. M., Pitts-Meek, S., Hultgren, B., Wood, W. I., and Moore, W. M. (1994) *Science* 265, 682–684
25. Gao, J.-L., and Murphy, P. M. (1995) *J. Biol. Chem.* 270, 17494–17501
26. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463–5467
27. Ishii, K., Hein, L., Kohilkia, B., and Coughlin, S. R. (1993) *J. Biol. Chem.* 268, 9780–9786
28. Warden, C. H., Davis, R. C., Ueno, M.-Y., Hui, D. Y., Svenson, K., Xia, Y.-R., and Charo, I. F., (1994) *J. Lipid Res.* 35, 1415–1455
29. Copeland, N. G., Jenkins, N. A., Gilbert, D. J., Eppig, J. T., Maltais, L. M., Miller, J. C., Dietrich, W. F., Weaver, A., Lincoln, S. E., Steen, R. G., Stein, L. D., Nadeau, J. H., and Lander, E. S. (1993) *Science* 262, 57–66
30. Franci, C., Wong, L. M., Van Damme, J., Proost, P., and Charo, I. F. (1995) *Science* 265, 9780–9786
31. Thirion, S., Nys, G., Fiten, P., Masure, S., Van Damme, J., and Opdenakker, G. (1994) *Biochem. Biophys. Res. Commun.* 201, 493–499
32. Siciliano, S. J., Rollins, T. E., DeMartino, J., Konteatis, Z., Malkowitz, L., Van Riper, G., Bondy, S., Rosen, H., and Springer, M. S. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 1214–1218
33. Aduo, S. K., Orzechik, T., Milatovic, A., Francke, U., and Murphy, P. M. (1993) *Nat. Genet.* 4, 31–36
34. Gerard, N. P., Bao, L., Xiao-Ping, H., Eddy, R. L., Jr., Shows, T. B., and Gerard, C. (1995) *Biochemistry* 32, 1243–1250
35. Vu, T. K. H., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) *Cell* 64, 1057–1068
36. Wadsworth, H. L., Chazenbalk, G. D., Nagayama, Y., Russe, D., and Rappoport, B. (1990) *Science* 245, 1423–1425
37. DeMartino, J. A., Van Riper, G., Siciliano, S. J., Moulineaux, C., Konteatis, Z. D., Rosen, H., and Springer, M. S. (1994) *J. Biol. Chem.* 269, 14446–14450
38. Gayle, R. B., III, Sleath, P. R., Sirinivason, S., Birks, C. W., Weerawarna, K. S., Cerretti, D. P., Kozlowsky, C. J., Nelson, V., Van den Bosch, T., and Beckmann, M. P. (1993) *J. Biol. Chem.* 268, 7283–7289