The Murine Interleukin 8 Type B Receptor Homologue and Its Ligands

EXPRESSION AND BIOLOGICAL CHARACTERIZATION*

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KC, the product of an immediate early gene induced in mouse fibroblasts by platelet-derived growth factor, was synthesized as a recombinant protein in Escherichia coli and binds with 0.8 ns affinity to mouse neutrophils. Human neutrophils also bind recombinant KC at a site competitive with human interleukin (IL8) and Gro-a/MGSA, consistent with binding at the IL8 type B receptor (IL8RB).

The cDNA corresponding to human IL8RB hybridizes strongly with two restriction fragments in murine genomic DNA, representing candidate receptor genes for KC. Molecular cloning of both mouse genomic DNA and neutrophil exudate cell cDNA libraries yielded a receptor with ~68% sequence identity to both the human IL8 type A and B receptors. Transient expression of the murine receptor cDNA in COS cells conferred binding ability to KC and a related gene product, macrophage inflammatory protein-2 (MIP-2) with high affinity (~5 ns). Human IL8 was a poor agonist for this expressed receptor (Kₐ = ~400 ns). The potent activity of human IL8 on mouse polymorphonuclear neutrophils is not consistent with binding on the cloned receptor and suggests that murine homologues of IL8 and an IL8 type A receptor remain to be identified. Our data indicate that KC is the murine homologue of human Gro-a, and the KC receptor is an IL8 type B receptor homologue capable of binding both KC and macrophage inflammatory protein-2 with high affinity.

The emerging picture concerning the biology of the chemokines places these molecules centrally in the trafficking of leukocytes. The most consistently observed biologic property of these peptides is their ability to cause chemotaxis in vitro. The α chemokines, typified by IL8, are potent chemoattractants for PMNs, while β chemokines, such as MCP-1 and RANTES, are chemoattractants for mononuclear cells (1–3). Eosinophils appear to be highly sensitive to the β chemokines as well (4, 5). More complex functions of these molecules have been reported, including growth stimulation (MGSA) (6), and inhibition of stem cell differentiation (MIP-1a) (7). All chemokines appear to interact with G-protein-coupled receptors that transduce the transient intracellular signaling events (1, 8, 9).

An unusual feature of the chemokine systems is the redundancy of agonists that interact at common receptor sites. The human interleukin 8 type B receptor binds at least four distinct gene products; IL8 and Gro-a/MGSA bind with high affinity (10), and desensitization studies indicate the type B receptor also binds ENA-78 and NAP-2 (neutrophil-activating peptide 2) (11) Similarly, the C-C CKR1 receptor binds both MIP-1a and RANTES (12). Thus, a limited repertoire of G-protein-coupled receptors binds an array of chemokines. Within an organ, multiple unique chemokines may be produced in response to similar stimuli (for example ENA-78 and IL-8 both derive from lung epithelia and endothelia) (11).

At present, no rodent homologue for human interleukin 8/NAP-1 has been identified. Endotoxin-stimulated macrophages were the initial source of a protein identified as MIP-2 (macrophage inflammatory protein-2) (13, 14). This protein has been characterized as a neutrophil-selective chemoattractant and has sequence homology with Gro-a/MGSA. A platelet-derived growth factor-inducible immediate early gene known as KC was initially identified as a growth factor-inducible gene (15, 16). By cDNA sequence homology, it has been suggested that KC is a homologue of human Gro-a/MGSA. At present, however, the protein encoded by this gene has not been characterized. A rat cytokine-induced neutrophil chemoattractant, CINC, has been purified and chemically synthesized (17, 18). This protein has 91% sequence identity to the predicted KC gene product.

A murine IL8 type B receptor homologue was reported by Cerretti et al. (19); however, the nature of its interaction with XCK ligands was not determined. In the present work, we report the biochemical characterization of the murine KC and its cellular receptor.

EXPERIMENTAL PROCEDURES

KC Protein Expression and Radiolabeling

Oligonucleotide primers based on the KC cDNA sequence (18) sense, 5'-GGATTCGCGGCACCCCGTGG-3'; antisense, 5'-GGTCTAGAGTTCTCCGTTACTGGA-3') were used to generate the 252-bp KC cDNA by polymerase chain reaction. Mouse genomic DNA was denatured at 94°C for 1 min, annealed at 45°C for 2 min, and extended with Taq polymerase (Perkin-Elmer) for 2 min at 72°C through 25 cycles of polymerase chain reaction. The cDNA generated was ligated to pMAL (New England Biolabs) and the protein expressed in Escherichia coli XLI-blue (Stratagene) following induction with isopropyl-1-thio-β-D-
galactopyranoside. The resulting maltase-binding protein-KC fusion protein was purified by anylose affinity chromatography, digested with factor Xa to release the KC, and purified by ion exchange and high performance liquid chromatography following denaturation and renaturation as described previously (20). Since recombinant KC has no tyrosyl residues, the expression construct was mutagenized to contain a tyrosine residue at position 2 following the factor Xa cleavage site. KC-Tyr was labeled with $^{125}$I to a specific activity of 74-276 Ci/mmol as described previously (21).

**KC Binding on Human PMN**

Peripheral blood neutrophils were obtained from normal human volunteers and purified (22). Cells were suspended at a density of 3 x 10$^8$/ml in Hank's balanced salt solution, 25 mM HEPES, pH 7.4, containing 0.1% bovine serum albumin and incubated at 24°C with 1 nM $^{125}$I-KC-Tyr or 0.25 nM $^{125}$I-IL8 (DuPont NEN) and competing ligand in the concentrations indicated, for 90 min. Cells were separated from unbound ligand by centrifugation through 20% sucrose and the amount of bound ligand determined by scintillation counting. All estimations of the binding affinities was performed using the Ligand program (23).

**KC Binding on Mouse Peritoneal Exudate Cells**

SV 129 mice at 6-8 weeks were injected intraperitoneally with 1 ml of sterile saline containing 0.1% oyster glycogen. After 4-6 h, mice were sacrificed and peritoneal cells lavaged and purified as described (24). Cell populations were determined by morphology following Wright's stain, indicating 3-5 x 10$^6$/cells/mouse consisting of approximately 60% neutrophils. Control mice yielded 2-3 x 10$^6$/cells/animal, of which <5% were neutrophils. Ligand binding experiments were performed as described for human PMN.

**Functional Activity of KC on Murine PMN**

Chemoattractant and measurement of Mac-1 up-regulation on murine peripheral blood neutrophils were performed as described previously (25, 26).

**Molecular Biological Methods**

**Southern and Northern Analyses**—Genomic DNA from SV 129 and BALB/c mice digested with PstI and BamHI was hybridized with either the 32P-labeled 361-bp 5' fragment of the human IL8 receptor B, generated by digestion with BglII and BamHI or the 773-bp 3' fragment derived from digestion with BamHI and SacI, washed in 2 x SSC at 65°C and exposed as described (27). Total RNA was isolated (28) from SV 129 mouse tissues, resident peritoneal cells, and peritoneal cells elicited 4 h after i.p. injection with 0.1% oyster glycogen and was hybridized with 5'-labeled 1081-bp murine KC receptor coding sequence under the same conditions as used for the Southern blot.

**cDNA Library Construction**—SV 129 mice injected intraperitoneally with sterile saline containing 0.1% oyster glycogen were sacrificed after 7 h and peritoneal cells isolated. Poly(A$^+$) RNA was isolated as described (29) and used for synthesis of double-stranded cDNA (30) and packaged into λgt22A (Superscript Lambda System, Life Technologies, Inc.).

**Isolation of KC Receptor cDNA and Genomic DNA Clones**—Candidate KC receptor gene and genomic DNA probes were isolated by screening a mouse SV 129 genomic library in λ FIX and the glycogen-elicited peritoneal exudate cell cDNA library described above using the 32P-labeled 1081-bp 5' BglII fragment of the human IL8RB fragment and 20 x SSC at 42°C. Plate-purified phage DNAs were purified, the inserts were subcloned into λBluescript SK$^+$ (Stratagene), and the DNA sequence was determined (31). KC receptor cDNAs were additionally cloned into the mammalian expression vector pcDNA1 (Invitrogen) for transfection studies.

**Binding Studies on Transfected COS Cells**

COS cells were maintained and transfected using DEAE-dextran as described previously (32). Transfected cells were incubated in the buffer used for neutrophil binding (above) with 2 nM $^{125}$I-KC-Tyr and increasing concentrations of unlabeled ligand for 90 min at 23°C. Cells were washed three times with ice-cold phosphate-buffered saline, 0.1% bovine serum albumin, trypsinized, and subjected to scintillation counting. Rat MIP-2, human Gro-α, rat CINC, and human IL8 were obtained from Peprotech.

**RESULTS AND DISCUSSION**

**Characterization of Recombinant Murine KC**—Recombinant KC was biologically active in the nanomolar range for both chemotaxis (Fig. 1, Table I) and the up-regulation of the cell surface expression of the integrin Mac-1 on mouse peripheral blood PMN (Table I). KC was ~10-30 times more potent than human IL8 or Gro-α or the highly homologous rat CINC in both of these assays.

**Binding of Recombinant KC to PMN**—$^{125}$I-KC-Tyr binds to glycogen-elicited mouse PMN with an apparent $K_D$ of 0.8 nM (Table I, Fig. 3). The related chemokine MIP-2 was equally effective in competition binding with $^{125}$I-KC-Tyr on these cells, with an $EC_{50}$ of 0.5 nM. Gro-α and CINC competed much less effectively, and IL8 appears to interact weakly, if at all, at the KC binding site, with an $EC_{50}$ of ~300 nM. On human PMN, $^{125}$I-KC-Tyr demonstrates similar high affinity binding (Table I). In contrast to the result with mouse PMN, however, human IL8 was effective in competition binding.

Humans have two receptors for IL8 (10, 33), and only IL8 binds with high affinity to the type A receptor. In contrast, multiple α chemokines interact with the IL8 type B receptor. The degeneracy of the ligand pool for a given receptor is a new paradigm for the chemokine receptors. MIP-1α and RANTES binding to C-C chemokine receptor 1 (C-C CR1) (12) and MCP-1 and MCP-3$^*$ binding to the C-C CR2K (34) are corollary examples emerging from studies of β chemokine receptors. Although the biological advantage for this is not yet apparent, it is conceivable that a major role of the chemokines is to provide tissue or cell-type specific signals for migration of leukocytes. In this case it might be advantageous to have the ligands derive from unique genes with unique regulatory controls, while sharing the common receptor. Both IL8 and Gro-α competed for binding of labeled KC to human PMN, with $EC_{50}$ values of 0.3 and 0.25 nM, respectively, suggesting that KC interacts with the IL8 type B receptor (10).

**Molecular Cloning of a Candidate Mouse KC Receptor**—Southern analysis was performed under conditions of moderate stringency with the human type B receptor cDNA as probe. Mouse genomic DNA from SV 129 and BALB/c mice was digested with PstI or BamHI and probed with either a 5'-half fragment or a 3'-half fragment of the human IL8RB receptor coding sequence as described under “Experimental Procedures.” As shown in Fig. 2A, both fragments recognize two

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2 I. F. Charo, personal communication.
distinct species in both inbred strains. When human genomic DNA is probed under similar conditions, three hybridizing species are seen, representing the IL8RA and B genes, as well as a pseudogene (35). These data demonstrate that a second murine gene exists that has structural homology to the human IL8 receptor. We have not yet identified this receptor by molecular cloning. Interestingly, when the isolated KC receptor is used as the Southern probe, the second gene is only weakly hybridizing (data not shown). Therefore, it appears that the second IL8 receptor-like species in the mouse will be more divergent from the presently reported molecule than from human IL8R.

A murine genomic DNA library was probed with the human IL8RB cDNA, and a single class of candidate clones was obtained. Sequence analysis revealed a unique coding sequence, which correlated best with the IL8 type B receptor cloned from man and rabbit (rabbit IL8RB (GenBank accession no. L24445) is 74.6% identical; human IL8RB (M73969) is 71.8% identical). The human and rabbit IL8 type A receptors have slightly less overall identity (rabbit IL8RA (M74240) is 66.2% identical; human IL8RA (M68932) is 68.9% identical).

When this isolated clone was used to reprobe the Southern blot, only the 6-kb PstI species and the 7-kb BamHI species hybridized significantly; consistent with the restriction map of the isolated genomic clone. Analysis of the intensities of the original blot suggested that the second murine genomic species (i.e., the 5.8-kb PstI and the 6.5-kb BamHI fragments) are more divergent from the presently reported molecule than from human IL8R.

Northern analysis of mouse tissue RNAs using the isolated cDNA as probe revealed a single site of expression in the cells from the inflamed peritoneum (Fig. 2B). A low level of hybridizing signal is seen on prolonged exposure of the spleen sample (data not shown). The presence of the KC receptor on the exudate cells correlates with the presence of neutrophils.

In an effort to confirm that the mRNA observed in peritoneal exudate cells derives from the isolated gene and not a related homologue, a cDNA library was constructed and screened with the human IL8RB cDNA. Two classes of cDNA clones were obtained, which differed only in the length of the 3'-untranslated segment (3.0 versus 1.6 kb total). The entire coding sequence in both cases was identical to the isolated genomic clone. Comparison of the 5'-untranslated sequence with the genomic clone sequence revealed divergence at nucleotide -23 relative to the initiating methionine, consistent with an intron/exon splice junction.

**Ligand Specificity of the Cloned Murine IL8 Receptor Homologue**—When transfected into COS cells, the murine IL8RB homologue confers high affinity binding for $^{125}$I-KC-Tyr (Fig. 3B). The $K_d$ was determined by Scatchard analysis to be 4.5 nM (Table I). This value is compared with 0.8 nM value on glycojen-elicited mouse PMN. The COS cell-expressed receptor demonstrated displacement characteristics similar to those for the mouse PMN; both MIP-2 and Gro-α were effective in displacing $^{125}$I-KC-Tyr (Fig. 3B). The selectivity for KC relative to related chemokines seen on mouse PMN was similar for the transfected receptor. In particular, human IL8 was a relatively ineffective agonist at the transfected KC receptor.

Given that the two chemokines, KC and MIP-2, are binding to the isolated receptor and that these ligands are most closely related to gro genes in man, the isolated receptor is biochemically behaving as a murine IL8 type B receptor homologue. Thus, the present study establishes the mouse neutrophil KC receptor as the murine homologue of the human IL8 receptor type B. The related chemokine mouse MIP-2 shares the KC receptor as its neutrophil binding site.

Inflammatory stimuli that release IL8 in the human system yield KC and MIP-2 in the rodent (13, 17), as detected by direct purification of chemoattractant activity. Direct cDNA cloning efforts based on homology with human IL8 or by subtractive techniques have not yielded molecules other than KC and MIP-2. There is some evidence, however, that an IL8-like molecule exists in rodents. Antisera against human IL8 have been reported to limit murine immune complex (24), carrageenan (36), and mast cell-dependent chemoattractant activities.

Data bearing on this issue could be interpreted in the recent work by Cacalano et al. (37), who created a gene knockout of a...
chemokines in the mouse are capable of binding the targeted receptor. The phenotype that may have multiple layers of complexity depending on which function (KC or MIP-2 or another unknown α chemokine) relies on a particular ligand. For example, is KC or MIP-2 (or both) the relevant chemokine with respect to regulation of myelopoiesis or lymphopoiesis?

In sum, we have characterized a murine IL8 type B receptor homologue and determined its ligand specificity as recognizing the KC and MIP-2 proteins. The cloned receptor is present in highest abundance on exudate neutrophils but may be present at low levels on other leukocytes. An additional murine gene with homology to the human IL8 receptor remains to be cloned.

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As a separate issue relating to the IL8RB homologue-negative mice, the present data clearly indicate that at least two α

C R Bozic and C Gerard, unpublished observations.