Protein Science (1997), *6*:1643–1652. Cambridge University Press. Printed in the USA.

Functional and receptor binding characterization of recombinant murine macrophage inflammatory protein 2: Sequence analysis and mutagenesis identify receptor binding epitopes

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(RECEIVED February 3, 1997; ACCEPTED May 7, 1997)

Abstract

Murine macrophage inflammatory protein-2 (MIP-2), a member of the α-chemokine family, is one of several proteins secreted by cells in response to lipopolysaccharide. Many of the α-chemokines, such as interleukin-8, gro-α/MGSA, and neutrophil activating peptide-2 (NAP-2), are associated with neutrophil activation and chemotaxis. We describe the expression, purification, and characterization of murine MIP-2 from *Pichia pastoris*. Circular dichroism spectroscopy reveals that MIP-2 exhibits a highly ordered secondary structure consistent with the α/β structures of other chemokines. Recombinant MIP-2 is chemotactic for human and murine neutrophils and up-regulates cell surface expression of Mac-1. MIP-2 binds to human and murine neutrophils with dissociation constants of 6.4 nM and 2.9 nM, respectively. We further characterize the binding of MIP-2 to the human types A and B IL-8 receptors and the murine homologue of the IL-8 receptor. MIP-2 displays low-affinity binding to the type A IL-8 receptor (K_d >120 nM) and high-affinity binding to the type B IL-8 receptor (K_d 5.7 nM) and the murine receptor (K_d 6.8 nM). The three-dimensional structure of IL-8 and sequence analysis of six chemokines (IL-8, gro-α, NAP-2, ENA-78, KC, and MIP-2) that display high-affinity binding to the IL-8 type B receptor are used to identify an extended N-terminal surface that interacts with this receptor. Two mutants of MIP-2 establish that this region is also involved in binding and activating the murine homologue of the IL-8 receptor. Differences in the sequence between IL-8 and related chemokines identify a unique hydrophobic/aromatic region surrounded by charged residues that is likely to impart specificity to IL-8 for binding to the type A receptor.

Keywords: chemokine; MIP-2; mutagenesis; receptor binding site

Macrophage inflammatory protein-2 (MIP-2) is a member of the chemokine superfamily, a group of structurally related proteins ranging in size from 8 to 10 kDa and containing four conserved cysteines (Oppenheim et al., 1991). Many of the proteins in this superfamily mediate inflammation by directing the migration of leukocytes to sites of infection or tissue injury. The proteins in the superfamily have been separated into two groups based on whether the first two cysteines are separated by a single intervening amino acid (α-chemokines) or are adjacent to each other (β-chemokines). The segregation of the proteins into two families has genetic and functional significance. Many of the genes for the α-chemokine family cluster on human chromosome 4q12-q21 and those for β-chemokines are found on chromosome 17 (Murphy, 1994). In general, α-chemokines are involved in the recruitment and activation of neutrophils, while β-chemokines are involved in the recruitment and activation of monocytes, T cells, basophils, and eosinophils.

MIP-2 was first isolated from the conditioned media of lipopolysaccharide-activated murine macrophage cell line RAW 264.7 (Wolpe et al., 1989). A number of reports indicate that MIP-2 is involved in acute and chronic inflammatory responses. MIP-2 causes a localized inflammatory reaction characterized by neutrophil infiltration upon injection into the footpads of mice (Wolpe et al., 1989) or intratracheal installation of rats (Frevert et al., 1995). MIP-2 expression in mice has also been correlated with a variety of inflammatory disorders, including type II collagen-induced arthritis (Kasama et al., 1995), glomerulonephritis (Feng et al., 1995), and endotoxemia (Standiford et al., 1995). Inhibition of MIP-2 by treatment with anti-MIP-2 antibodies delays the onset and severity of arthritis (Kasama et al., 1995) and increases survival in

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endotoxemic mice (Standiford et al., 1995). In addition to its pro-inflammatory activities, MIP-2 also regulates cell growth. It enhances the proliferation of granulocyte-macrophage colony forming units (GM-CFU) stimulated by granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) (Broxmeyer et al., 1990).

The actions of chemokines are mediated by G protein-coupled receptors. A number of these receptors, including two human interleukin-8 receptors (Holmes et al., 1991; Murphy & Tiffany, 1991), the SDF-1 receptor/fusin HIV-1 co-receptor (Bleul et al., 1996; Feng et al., 1996; Oberlin et al., 1996), five β-chemokine receptors (Neote et al., 1993; Charro et al., 1994; Combadiere et al., 1995; Power et al., 1995; Samson et al., 1996), an erythrocyte chemokine receptor (Chaudhuri et al., 1994), and various murine homologues (Gao & Murphy, 1995; Lee et al., 1995; Hoogewerf et al., 1996) have been cloned and characterized. These receptors share 30–77% sequence identity and exhibit an interesting pattern of ligand specificities. With the exception of binding to the erythrocyte chemokine receptor, only chemokines *within* families compete with each other for binding to individual receptors. For example, none of the β-chemokines binds to the IL-8 receptors. Only IL-8 displays high-affinity binding to the type A IL-8 receptor. IL-8, gro-α, NAP-2, and murine KC compete for high-affinity binding to the type B IL-8 receptor, which shares 77% sequence identity with the type A IL-8 receptor (Gayle et al., 1993; Bozic et al., 1995). Several studies indicate that the Glu-Leu-Arg motif at the amino-terminal region of these chemokines is essential for IL-8 receptor binding and activation (Clark-Lewis et al., 1991; Hebert et al., 1991; Hesselgesser et al., 1995). Other regions important for receptor binding, as well as the source of receptor specificity, are under active investigation.

Recombinant murine MIP-2 has been crystallized (Lolis et al., 1992), but a full description of its physical, receptor binding, and biological properties has not been reported. In this study we describe the expression and purification of recombinant MIP-2 from *Pichia pastoris*, report its *in vitro* activities associated with chemotaxis, and characterize its binding to human and murine neutrophils and to the IL-8 receptors. Sequence analysis of the proteins that bind to the human IL-8 receptors reveals structural epitopes involved in receptor binding and specificity. Site-directed mutagenesis reveals that the N terminus of MIP-2 is critical for binding and activation of the murine receptor and extends structure–function relationships among members of the α-chemokine family.

Results

Expression, mutagenesis, and purification of MIP-2

The cDNA for the mature form of MIP-2 and two N-terminal mutants were cloned into the *P. pastoris* expression plasmid pPIC9. The GS115 strain of *P. pastoris* carries a mutant HIS4 (histidinol dehydrogenase) gene and requires histidine-containing media for growth and survival. Transformation of *P. pastoris* spheroplasts with *Sal* I-linearized plasmid favors vector integration into the genome at the HIS4 locus. Growth for 4–6 days on histidine deficient RD plates was used as an initial selection screen for cells that incorporated the vector into their genome. Twenty colonies from these plates were screened for MIP-2 expression and secretion as judged by SDS/PAGE and western blot analysis. A single high-expressing clone for each construct was chosen for further work. Each clone was grown to saturation in 200 ml of BMGY media in a 2-liter flask at 30 °C with 250–300 rpm agitation. Protein synthesis was induced with methanol containing media (BMMY). Protein expression was maintained with daily additions of methanol to a final concentration of 1%. Aliquots were collected at regular intervals to monitor levels of secreted protein. After 72 h, the major protein in the supernatant was MIP-2, which was purified using heparin affinity chromatography. Eight milligrams of wild-type MIP-2 per 200 mL of culture could be purified using this protocol. An N-terminal deletion mutant (MIP-2:5-72) and a double mutant in which Glu-6 and Arg-8 are substituted by alanine (MIP-2:E6A/R8A) were expressed at slightly lower levels.

Physical characterization of MIP-2

Amino acid analysis of recombinant MIP-2 was consistent with the amino acid composition of the native protein. The molecular weight of MIP-2 as estimated by SDS/PAGE was 8 kDa. A molecular weight of 7,845 Da was determined by mass spectrometry. This agrees exactly with the predicted molecular weight based on the amino acid sequence and the loss of four hydrogen atoms upon formation of two disulfide bonds. These data rule out any post-translational modification of the protein and indicate efficient cleavage of the α-factor signal sequence.

Recombinant MIP-2 migrates as a dimer in gel-filtration chromatography (data not shown). IL-8, gro-α, and MIP-1α have also been shown to form dimers, whereas platelet factor 4 (PF-4) and NAP-2 form tetramers (St. Charles et al., 1989; Clore et al., 1990; Fairbrother, 1994; Lodi et al., 1994; Malkowski, 1995). Far UV-CD (Fig. 1) indicates that MIP-2 has an α/β structure resembling other chemokines (Villanueva et al., 1988).

Biological activity of MIP-2

Recombinant MIP-2 is chemotactic for human and murine neutrophils in a dose-dependent manner (Fig. 2A). The maximal chemotactic response is obtained at 10 nM for murine neutrophils and declines as the concentration of MIP-2 is raised up to 1 μM. The N-terminal deletion mutant MIP-2:5-72 also shows a dose-dependent chemotactic response for murine neutrophils, but 100 nM is required to achieve a maximal response. The MIP-2:E6A/R8A double mutant is slightly chemotactic at only the highest concentration

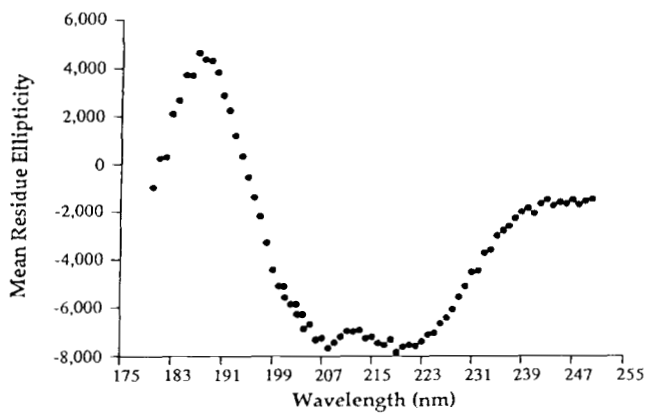

Fig. 1. Cicular dichroism spectrum of MIP-2. The spectrum of MIP-2 (20 μM) was measured from 250 to 180 nm in 10 mM phosphate buffer, pH 6.8, in a 0.5-mm path-length cuvette.

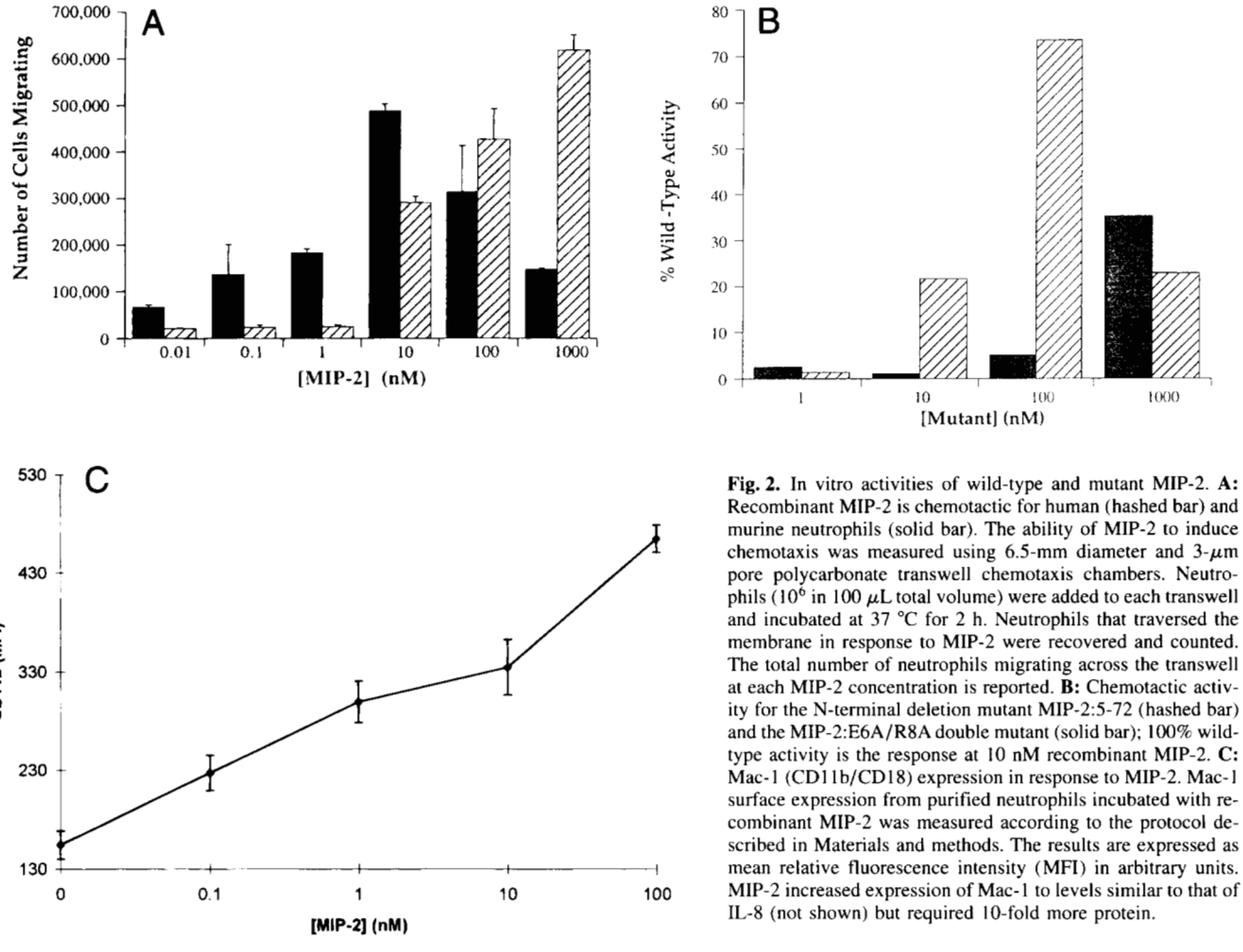

Fig. 2. In vitro activities of wild-type and mutant MIP-2. **A:** Recombinant MIP-2 is chemotactic for human (hashed bar) and murine neutrophils (solid bar). The ability of MIP-2 to induce chemotaxis was measured using 6.5-mm diameter and 3-μm pore polycarbonate transwell chemotaxis chambers. Neutrophils (10^6 in 100 μL total volume) were added to each transwell and incubated at 37 °C for 2 h. Neutrophils that traversed the membrane in response to MIP-2 were recovered and counted. The total number of neutrophils migrating across the transwell at each MIP-2 concentration is reported. **B:** Chemotactic activity for the N-terminal deletion mutant MIP-2:5-72 (hashed bar) and the MIP-2:E6A/R8A double mutant (solid bar); 100% wild-type activity is the response at 10 nM recombinant MIP-2. **C:** Mac-1 (CD11b/CD18) expression in response to MIP-2. Mac-1 surface expression from purified neutrophils incubated with recombinant MIP-2 was measured according to the protocol described in Materials and methods. The results are expressed as mean relative fluorescence intensity (MFI) in arbitrary units. MIP-2 increased expression of Mac-1 to levels similar to that of IL-8 (not shown) but required 10-fold more protein.

(Fig. 2B). We find that physiological concentrations of MIP-2 increase the surface expression of the β_2-integrin Mac-1 (Fig. 2C) to levels similar to those achieved with interleukin-8 (data not shown).

Neutrophil and IL-8 receptor binding

Direct binding experiments were used to assess the affinity of MIP-2 for murine neutrophils and the murine IL-8 receptor. Saturation binding of [^{125}I]-MIP-2 to murine neutrophils indicates specific high-affinity binding with a K_d of 2.9 nM to approximately 8,000 receptor sites per cell (Fig. 3A). A clonal HEK-293 cell line expressing the murine IL-8 receptor was incubated with increasing concentrations of [^{125}I]-MIP-2. Similar to the neutrophil binding experiments, specific binding saturated in a hyperbolic manner. The transfected cells expressed 30,000 receptor sites per cell and bound MIP-2 with a dissociation constant of 6.8 nM (Fig. 3B).

Competitive displacement experiments were used to study the binding of (1) MIP-2 to human neutrophils, (2) MIP-2 to the two human IL-8 receptors, and (3) MIP-2 mutants to the murine IL-8 receptor (Table 1). Varying concentrations of IL-8 or MIP-2 in the presence of 0.25 nM [^{125}I]-IL-8 reveal dissociation constants for human neutrophils of 5.0 and 6.4 nM for IL-8 and MIP-2, respectively. To delineate the affinity of MIP-2 for each of the human IL-8 receptors, the type A and B receptors were expressed in HEK-293 cells. The dissociation constant of IL-8 for HEK-293 cells expressing each human IL-8 receptor separately was in agreement with published values of 1–2 nM (Lee et al., 1992; Cerretti et al., 1993; Ahuja et al., 1996). MIP-2 could also displace radiolabeled interleukin-8 from these receptors, but the affinity for each receptor was different. To the type B IL-8 receptor, MIP-2 bound tightly with a K_d of 5.7 nM. In contrast, binding of MIP-2 to the type A IL-8 receptor was much weaker with a K_d greater than 120 nM. Competitive displacement experiments were also used to study the binding of the two N-terminal mutants to the murine IL-8 receptor expressed in HEK-293 cells. Mutants MIP-2:5-72 and MIP-2:E6A/R8A displaced radiolabeled MIP-2 from the murine IL-8 receptor with dissociation constants of 2.2 nM and >200 nM, respectively.

Sequence analysis

Sequence alignment of six chemokines (IL-8, gro-α, NAP-2, ENA-78, murine KC, and MIP-2) that interact with the type B IL-8 receptor reveals 18 positions that are invariant (Fig. 4A). Many of these strictly conserved residues are therefore likely to contribute to the receptor binding site of the proteins. The availability of

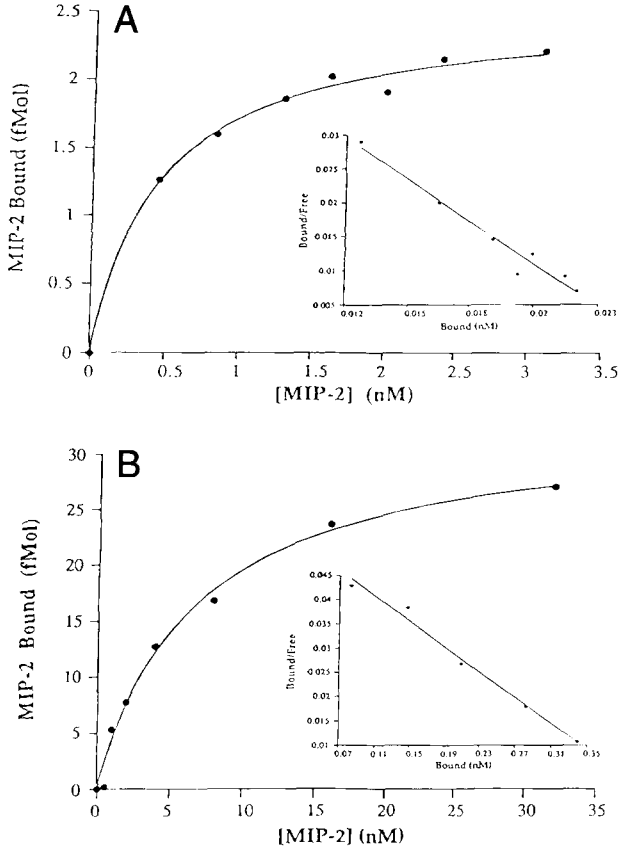

Fig. 3. Cell and receptor binding properties of MIP-2. Saturation binding studies of MIP-2 to (**A**) murine neutrophils and (**B**) stable HEK-293 cells expressing the murine homologue of the IL-8 receptor. Cells (4×10^5) were incubated with increasing amounts of [^{125}I]-MIP-2 for 2 h at 4 °C. For neutrophil experiments, the binding mixtures were centrifuged through 500 μL sucrose cushion (20% sucrose + 0.1% BSA in PBS), and cell pellets counted in a γ-counter. Nonspecific binding was determined as that which remained in the presence of 500× unlabeled MIP-2. For experiments with HEK-293 cells, the free ligand was removed and the adherent cells were washed with PBS. Cells were solubilized with 0.1 N NaOH and radioactivity measured with a γ-counter. *Inset*: Scatchard transformation of the binding data.

three-dimensional structures for IL-8 (Clore et al., 1990), NAP-2 (Malkowski, 1995), and gro-α (Fairbrother, 1994) allows this analysis to be put in a structural context. The display of the identical residues in the IL-8 monomer reveals five distinct regions of strict conservation (Fig. 4B). The most prominent region is a large solvent-accessible surface of about 600 Å^2 consisting of Glu-4, Leu-5, Arg-6, Cys-7, Cys-9, Thr-12, Gly-31, Cys-34, Glu-38, Cys-50, and Pro-53 and is termed the N-terminal surface. At the opposite end of the molecule, residues Lys-20 and Lys-64, together with the basic residue at position 60 (which is not strictly conserved as it is an arginine in IL-8 and a lysine in the other chemokines), form a positively charged region that may interact with negatively charged moieties on the receptor or with the sulfate groups in heparin sulfate proteoglycans. Two other conserved residues, Leu-43 and Gly-46, are positioned at the ends of a protruding loop, but they do not form a continuous surface because their side chains extend in opposite directions. Leu-66 projects from the C-terminal α-helix. In the dimer, this residue interacts with the α-helix of the other subunit (not shown). Finally, Ile-22 and Leu-51 are virtually inaccessible to solvent and probably contribute to the hydrophobic core of the protein.

An alignment of chemokines that bind to the type A IL-8 receptor is not possible because IL-8 is the only identified chemokine with high-affinity binding to this receptor. Nonetheless, sequence differences between IL-8 and the other five chemokines must account for receptor specificity. There are 26 residues that are present in IL-8 but not in NAP-2, gro-α, ENA-78, murine KC, or murine MIP-2 (Fig. 5A). A display of these residues on the three-dimensional structure of IL-8 illustrates they occupy many different regions of the protein (Fig. 5B). Therefore, the specificity determining region cannot be distinguished from residues that have undergone neutral drift during evolution. To overcome this problem, the characteristics of the residues at the 26 positions were examined in greater detail. Reasoning that dramatic changes in the properties of residues are more likely to confer specificity than conservative substitutions, the 26 positions were reduced to 14. These 14 residues have differences in charge, aromaticity, and geometric constraints (e.g., amino acids involving glycine or proline). The most striking observation from the display of these residues on the three-dimensional structure is that three of four

Table 1. *Competitive binding of IL-8, MIP-2, and MIP-2 mutants to neutrophils and IL-8 receptors*[a]

Competing ligand	Radioligand	Transformed receptor/cells	K_d (nM)
IL-8	IL-8	Human neutrophils	5.0
MIP-2	IL-8	Human neutrophils	6.4
MIP-2:5-72	MIP-2	Murine IL-8 receptor/HEK-293	2.2
MIP-2:E6A/R8A	MIP-2	Murine IL-8 receptor/HEK-293	>200
MIP-2	IL-8	Human type A IL-8 receptor/HEK-293	>120
MIP-2	IL-8	Human type B IL-8 receptor/HEK-293	5.7

[a]Human neutrophils or stably transfected HEK-293 cells expressing either receptor-A, receptor-B, or the murine IL-8 receptor were used in competition binding assays with [^{125}I]-labeled IL-8 or MIP-2. MIP-2:5-72 and MIP-2:E6A/R8A designate the N-terminal truncation mutant consisting of residues 5–72 and the alanine double mutant, respectively. The K_d values represent fits from Scatchard analysis using LIGAND (Munson & Rodbard, 1980).

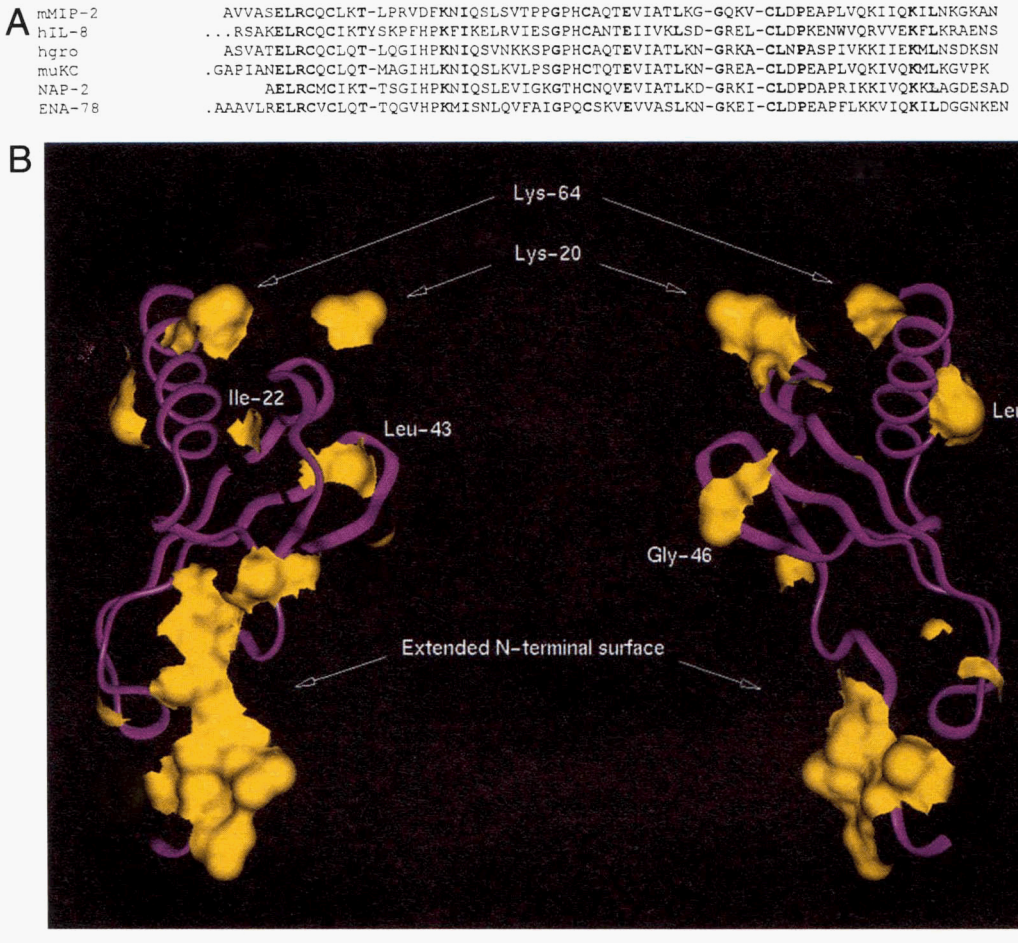

Fig. 4. Receptor binding epitope for the type B IL-8 receptor based on sequence analysis. **A:** The aligned sequences of IL-8 type B receptor binding chemokines MIP-2, human IL-8, gro-α, NAP-2, ENA-78, and murine KC. Residues that are identical among these chemokines are shown in bold. **B:** Two views of the solvent-accessible surface of the identical residues shown in the context of the IL-8 ribbon diagram. The two views are related by 180°.

aromatic residues (Tyr-13, Phe-17, and Phe-21) form a large hydrophobic surface (Fig. 5C). This hydrophobic surface is bordered by Lys-15 and Ser-44. According to the alignment shown in Figure 4A, Lys-15 is an insert relative to the other chemokine sequences, and Ser-44 in IL-8 replaces a charged lysine in each of the other five chemokines. Ser-44 forms a bridge between the hydrophobic surface and three other dramatically different residues: Lys-23, Glu-24, and Lys-42. For each of these residues, the corresponding amino acids in NAP-2, gro-α, ENA-78, KC, and MIP-2 are hydrophilic but uncharged. Other residues that are dramatically different in IL-8 include Pro-16, Glu-29, Lys-54, Glu-55, and Asn-56. Glu-29 is found near the N-terminal surface, but is not near any of the other dramatically different residues. Residues 54 and 55 are located a few Ångstroms from Lys-15 and may contribute to an electrostatic potential gradient that is complementary to the type A IL-8 receptor.

Discussion

Purification and characterization of MIP-2

A major aim of this study was to develop an overexpression system for MIP-2 that could generate milligram quantities of easily purified, bioactive protein for structural characterization and mutational analysis. The methylotropic yeast *P. pastoris* has been developed as a host for eukaryotic protein overexpression (White et al., 1994). The expression plasmid pPIC9 directs proteins to be secreted from *P. pastoris* into the medium using the α-factor secretion signal. This is an appropriate system to use for chemokines because these proteins are normally secreted *in vivo*. Furthermore, expression in yeast instead of *Escherichia coli* significantly reduces the possibility of endotoxin contamination. In light of the recent discoveries that the chemokines RANTES, MIP-1α, MIP-1β, and PBSF/SDF-1 inhibit the binding of HIV-1 to human cells, the *P. pastoris* expression system may be suitable for the production of large quantities of these chemokines and their analogues (Cocchi et al., 1995; Bleul et al., 1996; Choe et al., 1996; Deng et al., 1996; Oberlin et al., 1996).

We find that MIP-2 is expressed at high levels, is easily purified, properly folded, and biologically active. Recombinant MIP-2 stimulates up-regulation of the adhesion molecule Mac-1 and induces neutrophil chemotaxis. Mac-1 participates in the attachment of neutrophils to cells of the vascular endothelium expressing intercellular adhesion molecule-1 (ICAM-1). The effects on Mac-1 surface expression and neutrophil chemotaxis suggest that MIP-2 is involved in the extravasation of neutrophils from the vasculature and their migration to sites of tissue injury or infection.

A
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hIL-8     RSAKELRCQCIKTYSKPFHPKFIKELRVIESGPHCANTEIIVKLSD-GRELCLDPKENWVQRVVEKFLKRAENS
mMIP-2    AVVASELRCQCLKTLP-RVDFKNIQSLSVTPPGPHCAQTEVIATLKG-GQKVCLDPEAPLVQKIIQKILNKGKAN
hgro      ASVATELRCQCLQTLQ-GIHPKNIQSVNKKSPGPHCAQTEVIATLKN-GRKACLNPASPIVKKIIEKMLNSDKSN
muKC      GAPIANELRCQCLQTMA-GIHLKNIQSLKVLPSGPHCTQTEVIATLKN-GREACLDPEAPLVQKIVQKMLKGVPK
NAP-2       AELRCMCIKTTS-GIHPKNIQSLEVIGKGTHCNQVEVIATLKD-GRKICLDPDAPRIKKIVQKKLAGDESAD
ENA-78    AAAVLRELRCVCLQTTQ-GVHPKMISNLQVFAIGPQCSKVEVVASLKN-GKEICLDPEAPFLKKVIQKILDGGNKEN
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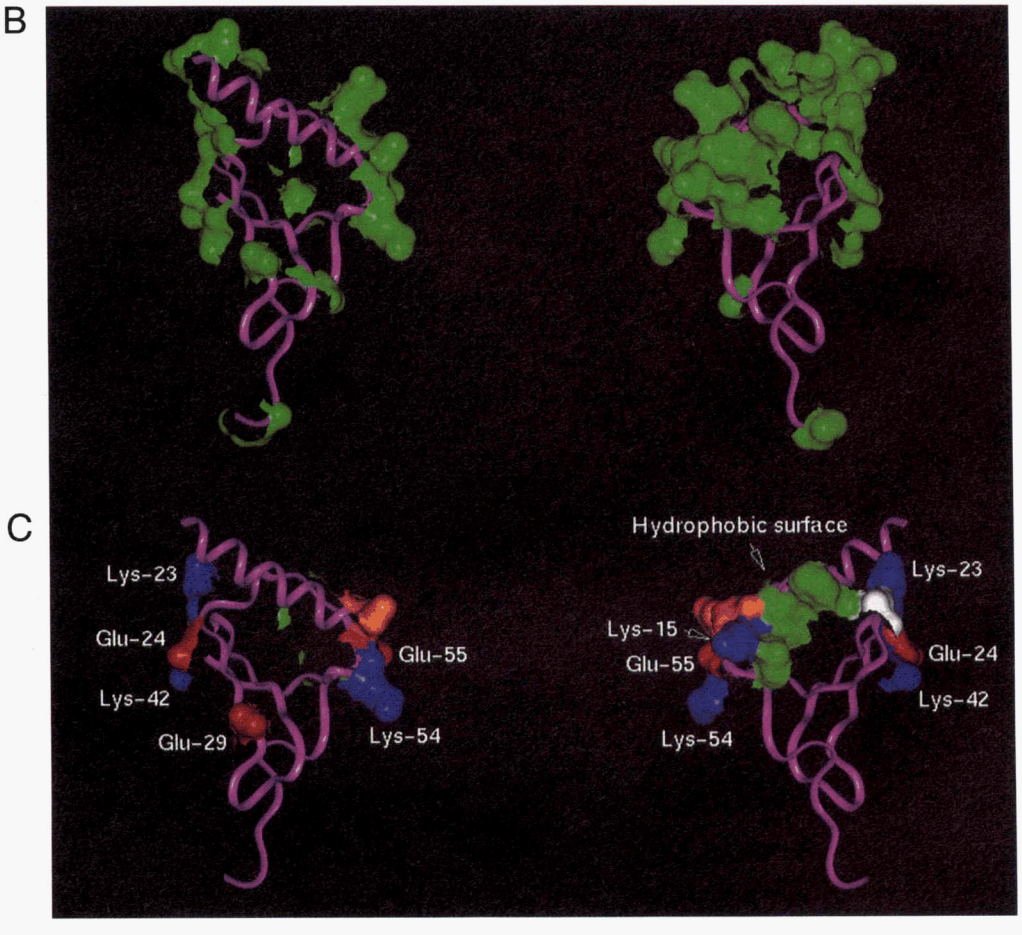

Fig. 5. Receptor binding epitope for the type A IL-8 receptor based on sequence analysis. **A:** Differences in the sequence between IL-8 and the other chemokines (human gro-α, NAP-2, ENA-78, murine KC, and MIP-2) that bind to the type B IL-8 receptor. IL-8 residues that are not present in any of the other chemokines are shown in bold. Residues that have dramatic differences such as ionic charge, aromaticity, or geometric constraints (Pro or Gly) are also underlined. **B:** Two views of the solvent-accessible surface of the IL-8 residues that are not present in human gro-α, NAP-2, ENA-78, murine KC, and MIP-2. These residues are shown in bold in Figure 5A. The two views are related to each other by 180 ° and are related to the view in Figure 4B by 90°. **C:** Two views of the solvent-accessible surface of the underlined residues in A shown in the context of the IL-8 ribbon diagram. The surface shown in green is of Tyr-13, Phe-17, and Phe-21. The blue and red surfaces correspond to lysine (15, 23, 42, and 54) and glutamate (24, 29, and 55) residues, respectively. The white surface is from Ser-44, and the gold surface is from Pro-16 and Asn-56.

The binding of MIP-2 to neutrophils and cell lines expressing the IL-8 type A receptor, type B receptor, or the murine homologue of the IL-8 receptor is also characterized. Murine MIP-2 binds with high affinity to murine neutrophils, having a dissociation constant of 2.9 nM. Only one binding site for MIP-2 on murine neutrophils and the murine homologue of the IL-8 receptor is identified. The related murine α-chemokine, KC, is reported to have two binding sites on murine neutrophils, but interacts with a single site on the murine IL-8 receptor (Bozic et al., 1995). The molecular identity of the second binding site for KC on murine neutrophils remains to be determined.

Human neutrophils express two IL-8 receptors. Equilibrium binding experiments with stable cell lines expressing each receptor indicate that MIP-2 has high affinity for the type B receptor, but low affinity for the type A receptor. This observation is similar to results of binding studies with gro-α, NAP-2, ENA-78, and murine KC (Bozic et al., 1995, 1996; Ahuja et al., 1996). MIP-2 and KC are the only murine chemokines that act on neutrophils that have been identified. They are 68% identical in amino acid sequence and share many biological activities. They each up-regulate Mac-1 expression, are chemotactic for murine neutrophils, and display high-affinity binding to the type B IL-8 receptor and the murine IL-8 receptor homologue. Their chemokine activity on human neutrophils, however, is different. MIP-2 is chemotactic for human neutrophils, whereas KC does not induce the migration of human neutrophils despite the high affinity binding to the IL-8 type B receptor (Bozic et al., 1994).

Receptor binding epitopes

Regions of α-chemokines responsible for receptor binding and activation have been studied by structure–activity relationships utilizing chemically synthesized analogues or site-directed mutants. Results from these studies indicate that the monomer is sufficient for receptor binding and activation and that the Glu-Leu-Arg motif at the amino terminus is essential for biological activity (Clark-Lewis et al., 1991, 1993, 1994; Hebert et al., 1991; Rajarathnam et al., 1994). In the current study, a mutant in which the first four residues of MIP-2 are deleted behaves as a partial agonist: the mutant exhibits high-affinity binding to the murine homologue of the IL-8 receptor, yet requires a 10-fold increase in concentration relative to wild-type MIP-2 to achieve a maximal chemotactic response. This observation suggests that the four deleted residues do not participate in receptor binding, but are involved in the activation of the receptor. A second mutant in which Glu-6 and Arg-8 are each mutated to alanine is only chemotactic at 1 μM. Displacement binding experiments indicate that the E6A/R8A double mutant binds to the receptor weakly, if at all, and demonstrates that the murine receptor also requires the ELR motif for receptor binding and activation.

Although residues in the ELR motif are necessary for receptor binding, studies have also shown they are insufficient for achieving maximum binding and biological activity (Clark-Lewis et al., 1991, 1993, 1994; Hebert et al., 1991). Mutational analysis may not always be the best approach for identifying the entire receptor binding surface because only those residues that contribute strongly to the overall free energy of binding will be identified (Clackson & Wells, 1995). Consequently, the receptor binding surface derived solely from mutational analysis will underestimate the actual contact area between chemokines and receptors. Indeed, NMR studies of [^{15}N]-labeled IL-8 and a peptide comprising part of the IL-8 type A receptor identifies a large number of residues that experience chemical shifts upon complex formation (Clubb et al., 1994). Here we complement previous approaches to define the IL-8 receptor binding sites by analyzing the sequences of α-chemokines that bind to these receptors.

The existence of six chemokines (IL-8, gro-α, NAP-2, ENA-78, murine KC, and MIP-2) that bind to the IL-8 type B receptor suggests they arose from a common ancestor. The pairwise sequence identity of these proteins ranges from 35% to 65%. Because receptor binding sites are under evolutionary pressure to maintain a precise structural arrangement, residues at these sites can be expected to have far less sequence variability than other positions in the protein structure. The alignment of the sequences reveals 18 positions that are identical for all six chemokines (Fig. 4A). Our analysis of these positions in the context of the three-dimensional structure of IL-8 indicates that the N-terminal surface, which includes residues up to Pro-53, is most likely to be involved in receptor binding.

The 18 identical residues are also present in a number of other α-chemokines, including precursors of NAP-2 (platelet basic protein, CTAP-III, β-thromboglobulin), gro-β, gro-γ, cytokine-induced neutrophil chemoattractant (CINC)-1, and CINC-2. Some of these cytokines have already been shown to display high-affinity interactions with neutrophils. In neutrophil binding studies and cross-desensitization experiments, gro-β and gro-γ display properties consistent with low- and high-affinity binding to the types A and B IL-8 receptors, respectively (Geiser et al., 1993). The precursors of NAP-2, however, do not bind neutrophils, presumably due to an extended amino terminus that interferes with receptor binding (Walz et al., 1989; Moser et al., 1991). Hybrid proteins between IL-8 and the α-chemokines PF-4 or IP-10, two proteins that do not bind neutrophils, underscore the importance of the N-terminal surface in receptor binding. One IL-8/IP-10 hybrid containing all of the identical residues of the N-terminal surface possesses the potency and neutrophil binding properties of wild-type IL-8 (Clark-Lewis et al., 1994). The IL-8/PF-4 hybrid protein with greatest neutrophil activity displays 1/15th the affinity of IL-8 for neutrophils (Clark-Lewis et al., 1993). This hybrid protein has a leucine instead of a proline at position 53, suggesting that a subtle change in the N-terminal surface can compromise binding to neutrophils.

A similar type of sequence analysis to identify residues that impart specificity for binding to the IL-8 type A receptor is not possible because IL-8 is the only chemokine with high-affinity binding to this receptor. However, an analysis of differences between IL-8 and the five other chemokines reveals a cluster of hydrophobic and aromatic residues present in IL-8, but not in the other chemokines. This hydrophobic surface is surrounded by a number of residues that exhibit charge differences with corresponding residues from the other chemokines. The distribution of hydrophobic and charged residues at this region of the structure is likely to promote binding of IL-8 to the type A IL-8 receptor and preclude the other chemokines from binding to this receptor.

The results of this analysis are consistent with recent experiments to identify important residues for binding to the IL-8 type A receptor. Tyr-13 and Lys-15 were shown to impart IL-8 type A receptor binding to rabbit IL-8 (Scraufstatter et al., 1995). A gro-γ/IL-8 chimeric chemokine possessing IL-8 residues 1–18 and 46–53 confers high-affinity binding to both IL-8 receptors (Hammond et al., 1996). The region consisting of residues 18–32 but not 32–46 was also found to be involved in binding to both receptors. In a similar experiment involving gro-α/IL-8 chimeric chemokines, IL-8 residues 10, 11, 13–17, and 49 imparted IL-8 type A receptor binding properties to gro-α (Lowman et al., 1996). Phe-21 was also found to improve binding to this receptor. An NMR study identifies a much larger number of residues (including Gln-8, Thr-12, Lys-15, Phe-17, His-18, Lys-20, Phe-21, Ser-44, Gln-48, Leu-49, Cys-50, and Val-61) that interact with a peptide corresponding to the amino terminus of the type A IL-8 receptor (Clubb et al., 1994) .

The present sequence analysis and mutagenesis study supports a critical role for the N-terminus of α-chemokines and suggests that the receptor binding region is much larger than anticipated previously based solely on mutagenesis studies. In addition to the extended N-terminal surface, which includes residues up to Pro-53, a region of IL-8 that is adjacent to the N-terminus and is composed of a hydrophobic surface surrounded by charged residues appears to confer specificity to IL-8 for high-affinity binding to the type A IL-8 receptor.

Materials and methods

Reagents, strains, and media

Oligonucleotides for PCR were synthesized by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Restriction endonucleases and Vent polymerase were purchased from New England Biolabs (Beverly, Massachusetts). Plasmids pPIC-9, pRc/RSV, and pRc/CMV were purchased from Invitrogen (San Diego, California). Rabbit anti-MIP-2 antibodies

were a kind gift of Dr. Barbara Sherry (Picower Institute, Manhasset, New York). [^{125}I]-IL-8, Bolton-Hunter iodination reagent, and Sequenase 2.0 were obtained from Amersham (Arlington Heights, Illinois). The strains used in this study were *P. pastoris* strain GS115 from Phillips Petroleum Company (Bartlesville, Oklahoma), *E. coli* NM522 from Promega (Madison, Wisconsin), and HEK-293 cells from the American Type Culture Collection (Rockville, Maryland). Media for cell growth and tissue culture were obtained from DIFCO (Detroit, Michigan) and GIBCO BRL (Gaithersburg, Maryland). *E. coli* was grown in LB media, and competent cells were prepared according to the $CaCl_2$ method (Sambrook et al., 1989). Reagents for the preparation and transformation of *P. pastoris* spheroplasts were obtained from Invitrogen. Buffered glycerol-complex media (BMGY) containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base (without amino acids), 1.6 μM biotin, and 1% glycerol was used for *P. pastoris* growth. The buffered methanol-complex media (BMMY) for inducing protein expression in *P. pastoris* replaced the 1% glycerol in BMGY with 1% methanol. For selecting *P. pastoris* transformants, RD plates containing 1 M sorbitol, 1% dextrose, 1.34% yeast nitrogen base, 1.6 μM biotin, 0.005% of L-glutamic acid, L-methionine, L-lysine, L-leucine, and L-isoleucine were used. Neutrophil isolation media contained 2 mL PMN medium (Accurate Scientific Westbury, New York), 1 mL mono-poly resolving medium (ICN Irvine, California), and 1 mL neutrophil isolating medium (Cardinal Associates, Santa Fe, New Mexico). All other chemicals were purchased from Sigma (St. Louis, Missouri).

Expression, mutagenesis, and purification of MIP-2

The coding sequence of the mature form of murine MIP-2 was amplified by PCR. The sequence of the sense primer (5′-AA *CTC GAG* AAA AGA GAG GCT GAA GCT **GCT GTT GTG GCC AGT GAA**-3′) contained an *Xho* I site (italicized), the coding sequence for the final six amino acids of the yeast α-factor signal sequence (underlined), and the coding sequence for the first six amino acid residues of the mature form of MIP-2 (bolded). Two additional sense primers were used for producing an N-terminal deletion mutant (MIP-2:5-72) and a double mutant in which Glu-6 and Arg-8 were replaced by alanine (MIP-2:E6A/R8A). The sequence of the anti-sense primer (5′-G *GAATTC* **TCA GTT AGC CTT GCC TTT GTT**-3′) annealed to the region corresponding to the codons for the last six amino acid residues and the TGA stop codon (bolded), and also contained an *Eco*R I site (italicized). The template for each reaction was pUC/MIP-2 (a gift of Dr. Barbara Sherry, Picower Institute, Manhasset, New York), a plasmid containing the full-length MIP-2 cDNA. The PCR products of the wild-type and mutant MIP-2 were purified, digested with *Xho* I and *Eco*R I, and ligated into pPIC-9. Transformed *E. coli* were selected on LB plates containing 100 μg/mL ampicillin. Plasmid from a single colony of each clone was purified and the correct DNA sequence for the insert was verified. Twenty micrograms of plasmid DNA were linearized with *Sal* I and used to transform spheroplasts of the GS115 *P. pastoris* strain. Transformants were selected on histidine deficient RD plates and incubated at 30 °C for 4–6 days. Ten milliliters of BMGY media were inoculated with individual colonies and grown for 24–48 h. Protein expression was induced by replacing the media with 10 mL BMMY. Every 24 h, an aliquot of 25 μL was removed for analysis of protein expression, and methanol was resupplied to the culture at a final concentration of 1% to maintain induction. Expression levels were assessed at 24, 48, and 72 h by 15% PAGE and western blotting. For large scale growth and purification, a single, high-expressing, immunoreactive clone for wild-type MIP-2 was used to inoculate 200 mL of BMGY. After 48 h of growth at 30 °C with vigorous shaking, the culture was pelleted by centrifugation and resuspended in 200 mL of BMMY to initiate induction. Methanol was resupplied every 24 h to a final concentration of 1%. After 72 h, the culture was pelleted at 10,000 rpm and the supernatant was decanted and passed over a 0.2-μm filter prior to loading onto a Sepharose 6B heparin affinity column (Pharmacia) equilibrated with 20 mM Tris pH 7.4, 20 mM NaCl, and 1 mM EDTA. Following a 5 column volume wash, the protein was eluted with a 0.02–2.0 M linear NaCl gradient. Wild-type and mutant MIP-2 eluted at approximately 600–700 mM NaCl and was >98% pure as judged by SDS-PAGE.

Amino acid sequence, mass spectrometry analysis

Amino acid analysis was performed at the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University). Mass spectrometry was performed at Bayer Corporation (West Haven, Connecticut).

Gel-filtration

FPLC was performed using a Sephacryl S-100 HR column (Pharmacia). The column was calibrated with 250 μg of gel-filtration calibration standards (Sigma). For determination of the size of MIP-2, 0.2 mL of a 1.8 mg/mL MIP-2 solution was injected and eluted at a flow rate of 0.6 mL/min. Fractions were monitored at 254 nm.

Circular dichroism

CD spectra were recorded at room temperature on an Aviv (Lakewood, New Jersey) model 62D spectrometer. Measurements were obtained in a 0.5-mm circular cuvette at a protein concentration of 20 μM in 10 mM sodium phosphate buffer, pH 6.8. Baseline corrections were done by subtracting the buffer spectrum from the sample spectrum. The spectrum was analyzed with the program PROSEC (Aviv) to determine secondary structure content.

Iodination of MIP-2

Iodination was performed with the Bolton-Hunter di-iodo reagent according to instructions supplied by Amersham. Five micrograms were iodinated to a specific activity of 140 Ci/mmol.

Receptor cloning and expression

Plasmids containing the cDNA for interleukin-8 receptor types A and B, pcDNA/IL-8RA and IL-8RB, were provided by Dr. Urs Widmer (Picower Institute, Manhasset, New York) and used to transform bacteria. Plasmids were purified, the cDNA for individual receptors was excised with *Hin*D III and *Xba* I, and purified fragments were ligated into pRc/RSV. The murine homologue of the interleukin-8 receptor type B (the MIP-2 receptor) was obtained by PCR from murine genomic DNA (a gift of Dr. Martina Brueckner, Yale University). The 5′ sense primer (5′-GGCC *AAGCTT* ATG GGA GAA TTC AAG GTG GAT-3′) contained a

*Hin*D III site (italicized) followed by the first 21 nucleotides of the coding sequence (underlined). The 3′ antisense primer (5′-GC *TCTAGA* CTA GAG GGT AGT AGA GGT GT-3′) annealed to 20 nucleotides which ended at the stop codon (underlined), and coded for an *Xba* I site (italicized). HEK-293 cells grown in Dulbecco's Modified Eagles Media supplemented with 10% fetal calf serum (FCS) were transfected with pRc/RSV-IL8RA, pRc/RSV-IL8RB, or pRc/CMV-MIP2R using the lipofectamine method (GIBCO BRL). Stable clones were selected in the presence of 900 μg/mL geneticin for 14 days. Single cells were isolated by limiting dilution, grown, and used in subsequent assays.

Neutrophil isolation and purification

Neutrophils were purified by differential centrifugation from human venous blood collected aseptically in the presence of 10 U/mL heparin (Sigma). Ten milliliters whole blood was layered onto 4 mL neutrophil purification media and centrifuged at 300 × *g* for 20 min. The neutrophil layer was removed, washed in Hanks Balanced Salt Solution (HBSS), and residual erythrocytes lysed with hypotonic saline. Murine neutrophils were obtained by peritoneal lavage 5 h after injection of 3% fluid thioglycollate medium in Balb/C mice (Baron and Proctor, 1982). Both human and murine neutrophils were washed with HBSS and counted. Neutrophils were suspended at 10^7 cells/mL in HBSS for chemotaxis assays or binding mixture (HBSS containing 20 mM HEPES, pH 7.5, and 0.5% BSA) for binding assays.

Neutrophil chemotaxis assay

10^6 neutrophils in 100 μL total volume were placed in 3 μm pore polycarbonate transwell (Corning/Costar) chemotaxis chambers. The transwells were placed in the wells of a 24-well tissue culture plate containing 600 μL of RPMI, 2.5% FCS, and varying concentrations of MIP-2. The cells were incubated in a 37 °C humidified incubator with 5% CO_2 for 2 h. Cells that migrated through the transwells were recovered and counted with a Coulter counter.

Mac-1 expression assay

Neutrophils (10^6/mL in HBSS containing 0.1% human serum albumin) were incubated with varying concentrations of MIP-2 for 10 min at 37 °C and stained with 1.25 μg/mL FITC-conjugated anti-CD11b (AMAC Inc., Westbrook, Maine) or FITC-conjugated isotype matched control antibody (DAKO Corp., Carpenteria, California) for 30 min at 4 °C. The samples were washed and the fluorescence was measured with a FACScan fluorescence activated cell sorter (Beckton-Dickenson, San Jose, California) at an excitation wavelength of 488 nm and emission of 530 nm.

Neutrophil binding assays

For saturation binding experiments, 4×10^5 neutrophils were incubated with increasing concentrations of [^{125}I]-MIP-2 in a total volume of 200 μL for 2 h at 4 °C. The binding mixture was centrifuged over a 0.5 mL sucrose cushion (20% sucrose and 0.1% BSA in PBS) and the cell pellet was counted in a γ-counter. Nonspecific binding was determined for each data point as the binding that remained in the presence of a 500-fold excess of unlabeled MIP-2. Dissociation constants and the number of receptor sites were calculated using Scatchard analysis with the program LIGAND (Munson & Rodbard, 1980). For competition assays, 4×10^5 neutrophils were incubated with either 0.25 nM [^{125}I]-IL-8 or 1 nM [^{125}I]-MIP-2 and unlabeled ligand at concentrations ranging from 1×10^{-12} M to 1×10^{-6} M.

HEK-293 binding assays

Cells expressing transfected receptors were seeded in 6 well culture dishes at 2×10^5 cells/well 24 h prior to assaying for binding. Cells were washed twice with PBS, incubated in 0.6 mL binding mixture, and assayed as described above in saturation and displacement assays. After incubation, the binding mixture was removed and the cells were washed twice with PBS. These solutions were saved as the free ligand. The cells were solubilized with 1 mL 0.1 N NaOH. Bound and free ligand was measured with a γ-counter, and the dissociation constants and receptor numbers were calculated by the program LIGAND (Munson & Rodbard, 1980).

Sequence analysis

The PILEUP program in the GCG sequence analysis package (Devereux et al., 1984) was used to align protein sequences. The positions of the identical residues were viewed with the IL-8 structure (Clore et al., 1990) using INSIGHT (Biosym Technologies, San Diego, California) on a Silicon Graphics workstation.

Acknowledgments

This work was supported by the Arthritis Foundation, the Patrick and Catherine Weldon Donaghue Medical Research Foundation, and the Critical Technologies Program of Connecticut Innovations, Inc. We thank Bob Tilton and Jay Prendergast (Bayer Corp., West Haven, Connecticut) for use of their circular dichroism spectrometer, Anthony Paiva and Lee Huang (Bayer Corp., West Haven, Connecticut) for mass spectrometry, and William Ross (University of Virginia) for assistance with the FACS analysis. We also thank Drs. Barbara Sherry (Picower Institute), Martina Brueckner (Yale University), and Urs Widmer (Picower Institute) for biological reagents.

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