Reverse Transcription-PCR Cloning of Macrophage Inflammatory Protein 1α Receptor and C-X-C Chemokine Receptor

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The chemokines are a superfamily of 8-10 KDa soluble proteins that have been implicated in chemotaxis and a wide range of inflammatory and immune reactions. They are classified into two branches C-C and C-X-C superfamily according to their primary structure. MIP-1α is a member of C-C chemokine superfamily. For the cloning of murine(m) MIP-1α receptor(R), we used synthetic oligonucleotides corresponding to transmembrane(TM) conserved sequences of already cloned human(h)IL-8-R as primers, and performed RT-PCR amplification using murine macrophage cell mRNA. Among 5' RT-PCR products, we isolated a homologous cDNA to hIL-8-R that presumably is a putative mIL-8-R cDNA. While this study was being undertaken, cloning of hMIP-1α-R has been done in other laboratory. We, therefore, synthesized oligonucleotides corresponding to hMIP-1α-R TM sequences and performed RT-PCR cloning of mMIP-1α-R from murine cytolytic T lymphocyte mRNA, using the oligonucleotides as primers. One putative mMIP-1α-R cDNA was isolated. cDNA library of CTL-L8 cell was screened with this cDNA as a probe, and one positive clone was identified.

Key words: chemokine, receptor, macrophage inflammatory protein 1α receptor, RT-PCR cloning

Introduction

The chemokines (for chemoattractant and cytokine activity) are a superfamily of 8-10 KDa soluble proteins that have been implicated in chemotaxis and a wide range of inflammatory and immune reactions (Oppenheim et al., 1991). The chemokines can be divided into two subfamilies (α or C-X-C and β or C-C) based on overall sequence homology, the disposition of the first two of four conserved cysteine residues, and the chromosomal location of the corresponding genes. Most C-X-C chemokines, such as interleukin 8 (IL-8), melanocyte growth-stimulatory activity (MGSA), neutrophil-activating peptide 2, neutrophil-activating protein derived from epithelial cells (ENA-78), attract neutrophils but not monocytes (Baggioiini et al., 1989; Matsushima and Oppenheim, 1989; Walz et al., 1992; Yoshimura et al., 1987). whereas C-C chemokines, including such molecules as monocyte chemotactic protein 1 (MCP-1), RANTES, and macrophage inflammatory protein 1α and 1β (MIP-1α and MIP-1β), attract monocyte but not neutrophils (Leonard and Yoshimura, 1990; Matsushima et al., 1989; Schall et al., 1990). Certain chemokines have chemoattractant properties for T lymphocytes: MIP-1α is a chemoattractant for CD8+ T cells (Taub et al., 1993), and RANTES for memory T cells in vitro (Schall et al., 1990), and IL-8 has been reported to attract T cells in vivo and in vitro (Larsen et al., 1989). C-C chemokines, particularly MIP-1α and RANTES, also have effects on other blood leukocytes. MIP-1α activates macrophage (Fahey et al., 1992). RANTES and MIP-1α are direct mediators of the release of histamine from basophils and mast cells (Kuna et al., 1992a, 1992b; Bischoff et al., 1992; Alam et al., 1992), RANTES and MIP-1α are chemoattractants and activators of eosinophils (Kameyoshi et al., 1992; Rot et al., 1992). Also MIP-1α has been reported to suppress the proliferation of bone marrow stem cell (Graham et al., 1990; Broxmeyer et al., 1990), and cytolytic T lymphocyte (Oh et al., 1991).

Two distinct IL-8 receptors(Rs) exhibiting a 77% amino acid identity have been molecularly cloned from human neutrophils (Holmes et al., 1991; Murphy and Tiffany, 1991). This structural feature is characteristic to the superfamily of guanine nucleotide-binding protein (G-protein)-coupled receptors that have seven transmembrane(TM)

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domains. The receptor sequence of IL-8 is sufficiently similar to what had been identified as an isoform of the bacterial formylated tripeptide fMet-Leu-Phel(MLP)-R and C5α-R (Holmes et al., 1991; Murphy and Tiffany, 1991). Based on competition binding assay using $^{125}$I-IL-8, the receptors for IL-8 are clearly different from the receptors for other cytokines, and only three of seven tested chemokines compete in $^{125}$I-IL-8 binding, suggesting that the receptors for the various chemokines are not all the same but individually specific (Samanta et al., 1989). Human MCP-1 has been reported to bind to monocytes with affinity of 2 nM (Valente et al., 1991; Yoshimura and Leonard, 1990). The detection of the receptors for ACT-2 (Napolitano et al., 1990) and LD-78 (Nakao et al., 1990) on blood leukocytes has also been reported. MIP-1α-R was identified on a mouse T cell and a macrophage cell line with an affinity of 1.5 and 0.9 nM, respectively (Oh et al., 1991).

While chemokines have probably evolved to alert immune system to microbial invasions of host, there has been, very recently, new information based on the study of chemokine receptors which have indicated that microbes may use the chemokine system to the disadvantage of the host through molecular mimicry (Ahuja et al., 1994). However, since the biological activities of these cytokines are not yet established, the functional consequences of ligand binding to these receptors remains to be defined. Recently, the authors have detected MIP-1α-R on a various immune and inflammatory cell lines (Oh et al., 1993). In the present study, for the cloning of murine(m)MIP-1α-R, we used synthetic oligonucleotides corresponding to conserved TM sequences of already cloned human(h) IL-8-R as primers, and performed RT-PCR amplification using murine macrophage cell mRNA. While this study was being undertaken, cloning of hMIP-1α-R has been done by Neote and colleagues (Neote et al., 1993). We, therefore, took advantage of this and synthesized oligonucleotides corresponding to hMIP-1α-R TM sequences and performed an independent study to clone mMIP-1α-R. We now report here a partial characterization of cDNA clones encoding the mMIP-1α-R and a novel C-X-C-R that might be a putative mIL-8-R.

Materials and Methods

Cells

The murine cytolytic T lymphocyte (CTL) line CTL-LR8 was grown in RPMI 1640 (GIBCO Laboratories) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin plus 100 μg/ml streptomycin. The murine CTL line CTL-L2 was grown in same condition with additional supplement of 100 units/ml recombinant IL-2. The murine macrophage cell line RAW 264.7 and human promyelocytic cell line HL-60 were grown in DMEM containing 10% FBS and antibiotics as described above. CTL-LR8 cells were stimulated with 5 μg/ml Con A (Sigma), for 16 h. RAW 264.7 cells were stimulated with 1 μg/ml LPS from Escherichia coli 0127:B8 (Sigma), for 18 h.

Reverse Transcription (RT)-PCR, subcloning, and sequencing

Total RNA from CTL-L2, CTL-LR8, HL-60, and RAW 264.7 was prepared as described previously (Chomczynski and Sacchi, 1987) and used for poly(A)’ RNA selection which were used as substrates in reverse transcription. Degenerate 24-mer oligonucleotides corresponding to conserved TM region 6 (TM6) and TM7 of hIL-8-R were used as primers in the RT-PCR of RAW 264.7 mRNA. Base sequences of synthetic oligonucleotides were as follows.

Oligomer-IL-8-R-A :
5′-CATCTTCGCGTACGATCT-3′

Oligomer-IL-8-R-B :
5′-AAGTTTGGCAATGAAGCCTGAG-3′

Degenerate 20-mer and 36-mer oligonucleotides corresponding to TM3-adjacent region (388-407) and TM7-adjacent region (877-912) of hMIP-1α-R were used as primers in the RT-PCR of CTL-L2 mRNA. The same experiment was duplicated with human promyelocytic cell line HL-60 as an hMIP-1α-R-positive control. Base sequences of synthetic oligonucleotides were as follows.

Oligomer-MIP-A :
5′-GACAGTGACCTGGCCTCTG-3′

Oligomer-MIP-B :
5′-AAGAAAGCGTAGATACCTGGTCTGACG-3′

Oligonucleotides and poly(A)’ RNA were reacted with Superscript as recommended by the manufacturer (BRL) and incubated with RNase H (Epicenter Technologies) at 37°C for 20 min. PCR conditions were as follows.
3 cycles: 94°C-1 min 37°C-1 min 72°C-1 min
15 cycles: 94°C-1 min 50°C-1 min 72°C-1 min
15 cycles: 94°C-1 min 50°C-1 min 72°C-2 min
1 cycle: 72°C-10 min

PCR products were gel purified and subcloned into pGEM3 vector. Plasmid DNA was isolated using the Qiagen kit (Qiagen Inc.) as recommended by the manufacturer. Sequencing was performed with the Sequenase kit (US Biochemicals) by dye-deoxy chain termination method (Sanger et al., 1977).

**Screening of cDNA library**

First and second strand cDNA were synthesized from Con A stimulated CTLT-R8 poly(A)⁺ RNA by using reverse transcriptase and DNA polymerase sequentially as recommended by manufacturer (Pharmacia). cDNAs were ligated into λgt11/EcoRI site and packaged by addition of packaging extract recommended by manufacturer (Stratagene). λgt11 cDNA library was plating on 150 mm agar plates (3x10⁵ plaques/plate) and in situ hybridization was performed with cloned RT-PCR product as a probe. Hybridized plaque and adjacent 10 plaques were separated and amplified. Then, phage DNA was isolated and analyzed by Southern blot with cloned RT-PCR product as a probe.

**Results**

**Isolation of C-X-C chemokine receptor (putative mIL-8-R) cDNA from RAW 264.7**

Originally we have tried to do RT-PCR cloning of novel chemokine receptor, especially MIP-1α-R, using synthetic oligonucleotides of conserved sequences of already cloned hIL-8-R as primers. We used RAW 264.7 and CTLT-R8 cells which had been shown to have a single class of high affinity MIP-1α-R on their surfaces with 380 and 1200 binding sites per cell, respectively (Oh et al., 1991). However we got the RT-PCR products only from RAW 264.7 cell. As shown in Fig. 1, electrophoresis of RT-PCR products from RAW 264.7 mRNA demonstrated five bands migrated to the position of approximately 500, 400, 330, 200, and 150 base pairs(bp). Next, we purified each bands from acrylamide gel, subcloned, and analyzed their base sequences. Among five products, only

150 bp cDNA showed 85% sequence identity with optimally aligned base sequence of hIL-8-R and, presumably, is a partial cDNA of putative mIL-8-R (Fig. 2).

**Isolation of C-C chemokine receptor (putative mMIP-1α-R) cDNA from CTLT-2**

Results of our previous receptor binding study of CTLT-2 with ³⁵S]-MIP-1α indicated the presence of approximately 3000-4000 MIP-1α binding sites per cell (Oh et al., 1993). So we decided to use CTLT-2 for mMIP-1α-R RT-PCR cloning instead of RAW264.7. Furthermore, CTL line CTLT-2 was thought to be preferable to RAW264.7 for MIP-1α-R cloning, because activated macrophage cells are known to express many different kinds of chemokine receptors which would hamper the finding of scarce message of MIP-1α-R. As a hMIP-1α-R-positive con-
Fig. 3. RT-PCR product of putative C-C chemokine receptor mRNA. RNA was extracted from murine cytolytic T cell CTLL-2. Oligonucleotides corresponding to 388-407 and 877-912 bps of hMIP-1α-R sequence were used as primers. Arrow indicates partial length cDNA of putative mMIP-1α-R.

*Oligo dT: commercial oligo(dT) was used as primer for reverse transcription.

Fig. 4. Comparison of base sequences of hMIP-1α-R and putative mMIP-1α-R cDNA.

Fig. 5. Southern hybridization of phage DNA from one positive plaque (lane 3) and the adjacent plaques. Lane 1 λ/HindIII marker, Lane 2-11 phage DNA.

Screening of cDNA library

λgt11/CTLL-R8 cDNA library was screened by in situ hybridization with 200 bp RT-PCR product (putative mMIP-1α-R cDNA) as a probe. One plaque was hybridized with 200 bp cDNA as a result of screening total 210,000 plaques. A positive plaque and the adjacent 10 plaques were separated and analyzed in Southern blot. Only one phage DNA (Fig. 5, lane 3) was hybridized with a probe and this could be a mMIP-1α-R cDNA.

Discussion

Various receptors have been cloned by cDNA expression or RT-PCR amplification method. Among the cytokine receptors IL-1-R and IL-8-R (Sims et al., 1988; Yamasaki et al., 1988) were the first whose cDNAs were cloned by expression cloning technique. However, cDNA expression cloning is not a proper method for cloning the receptor that has low expression message (Taga and Kishimoto, 1992). Recently, much attention has been focused on cloning the various cytokine receptor cDNAs by using the RT-PCR amplification method (Kawasaki, 1990). Two distinct IL-8-Rs exhibiting a 77% amino acid identity have been molecularly cloned from neutrophils (Holmes et al., 1991; Murphy and Tiffany, 1991). The primary sequences show
that IL-8Rs contain a seven-pass motif consisting of seven hydrophobic membrane-spanning domains. This structural feature is characteristic of the superfamily of guanine nucleotide-binding protein (G protein)-coupled receptors, indicating that IL-8Rs may constitute a subfamily of this large supergene family. The cDNA of only one C-C chemokine receptor (hMIP-1α/RANTES-R) has been isolated (Neote et al., 1993) and this also belongs to the G protein-coupled receptor superfamily, a feature consistent with the fact that many of the functions of C-C chemokines can be blocked by pertussis toxin (Kuna et al., 1992b). The known identity of amino acid sequence of hMIP-1α-R and IL-8-R is ~30% (Holmes et al., 1991; Murphy and Tiffany, 1991, Neote et al., 1993). The sequence similarity among various chemokines and chemoattractant molecule receptors is higher in the TM regions, especially TM2, TM6, and TM7, and the greatest divergence is seen in the second intracellular loop (Neote et al., 1993). The highly conserved motif is at the end of TM3, DRYLAIVHA, and could represent a functionally important domain necessary for receptor function. We utilized this domain to design mMIP-1α-R cloning strategy, that is, oligomer-MIP-A synthesis. The chemoattractant receptors for anaphylatoxin C5a (Gerard and Gerard, 1991) and fMLP (Boulay et al., 1990) do not have the conserved motif DRYLAIVHA, although both of them are G protein-coupled seven transmembrane spanning receptor. In the present study, for the prevention of amplification of various chemokines and other chemoattractant receptor mRNAs, we used relatively less conserved domain (TM7 adjacent region) to design another oligonucleotide primer oligomer-MIP-B that enabled us to isolate only two cDNA products from CTL mRNA.

The distribution of MIP-1α-R RNA is consistent with a functional role for the cloned MIP-1α-R in neutrophil, monocyte, and B and T lymphocytes. Northern blot data from cell lines suggest that the gene may also be expressed in myeloid precursor cells (Neote et al., 1993). If so, the receptor could mediate the reported regulatory effects of MIP-1α on myelopoiesis (Oppenheim et al., 1991). Human and murine MIP-1α have been shown to have a number of activities. Besides proinflammatory effects, MIP-1α has also been described as an endogenous pyrogen that acts in a prostaglandin-independent manner (Davatellis et al., 1989); more recently, it has been shown to activate basophils and mast cells (Alam et al., 1992). MIP-1α and homologous protein MIP-1β have very complicated relationship in their biological activities. Brossmeyer et al. (1990) has shown that MIP-1β can block the inhibitory effects of MIP-1α on stem cell proliferation. And MIP-1β, when present in excess compared with MIP-1α, block the activating effects of MIP-1α on macrophages (Fahey et al., 1992). Furthermore, our previous study indicated two molecules compete the same binding sites (Oh et al., 1993). Thus, we might envisage MIP-1α-R playing a role since it binds to MIP-1β but transmits a poor Ca2+ signal. In the case of inhibitory effect on stem cell proliferation, it is also possible that MIP-1α-R is coupled to both adenylyl cyclase and phospholipase C, as it is known that cAMP can act as a growth-inhibitory signal in some hematopoietic cells (Neote et al., 1993).

Accumulating evidence suggest that monocyte express a shared MIP-1α/MIP-1β receptor and a shared RANTES/MIP-1α/MCP-1 receptor, and a restricted MCP-1 receptor (Ahuja et al., 1994). And MIP-1α-R transduces a strong Ca2+ signal in response to low concentration of RANTES as well as MIP-1α. Several observations suggest that RANTES interact differently with MIP-1α-R than does MIP-1α, although precise interaction between ligand and receptor is not known.

A striking homology between the open reading frame in cytomegalovirus (CMV) and hMIP-1α-R was noted (Neote et al., 1993). There is nearly 50% amino acid identity and this observation suggests that CMV-infected cells could bind to C-C chemokines as part of strategy to evade immune antiviral responses. If the value of chemokines to CMV can be defined, perhaps unsuspected physiological functions may be discovered for them. Moreover, the copied immunomodulatory genes of viruses may serve as useful starting point for developing new treatment for inflammation and viral infections. In addition to this, information of multiple ligand specificity of C-C receptors and agonist/antagonist profiles of each of the ligands enable us to get deeper understanding of interaction of chemokines and immune cells.

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