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Cloning of the Murine Interferon-Inducible Protein 10 (IP-10) Receptor and Its Specific Expression in Lymphoid Organs

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To isolate the interferon-inducible protein 10 (IP-10) receptor gene, we searched for cells that respond to IP-10. Among several human and murine T cell lines, only CTLL2 cells (a murine cytotoxic T cell line) responded to IP-10 with transient elevation of intracellular Ca^{2+}. The murine IP-10 receptor gene has been cloned from cDNA derived from CTLL2 cells using the reverse transcriptase-polymerase chain reaction protocol with two degenerate primers corresponding to conserved regions of chemokine receptors. The cDNA encoding the murine IP-10 receptor has an open reading frame of 1101 bp corresponding to a protein of 367 amino acids that exhibits 86 % identity with the human IP-10 receptor. It mediates Ca^{2+} mobilization in response to IP-10, but does not recognize other rodent chemokines, including GRO, RANTES, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1α (MIP-1α). Northern blot analysis revealed that murine IP-10 and its receptor mRNA were constitutively expressed in the spleen and thymus from normal mouse, while IP-10 and its receptor mRNA were derived from stromal cells and lymphocytes in both tissues, respectively. *In vivo* treatment with concanavalin A (Con A) for 12 hrs revealed that splenocytes significantly induce IP-10 receptor mRNA expression and show a good chemotactic response to IP-10. Therefore, it is supposed that IP-10 and its receptor are important for lymphocyte trafficking to lymphoid organs and that the IP-10 receptor on lymphocytes is rapidly inducible on inflammation or in immunological events. © 1998 Academic Press

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The chemokine family comprises more than 30 proteins, and now the biological function of each chemokine has been studied (reviewed in 1–3). There are two major subfamilies of chemokines, termed CXC and CC according to the arrangement of the first two of four conserved cysteines that are separated by one amino acid and adjacent, respectively. The CXC chemokine family includes interleukin-8 (IL-8), GRO/melanoma growth-stimulating activity, ENA-78, interferon-inducible protein-10 (IP-10), and monokine induced by interferon-gamma (Mig). The CC chemokine family includes monocyte chemoattractant protein-1 (MCP-1), RANTES, and macrophage inflammatory protein-1 (MIP-1) α and β. Most CXC chemokines have a chemoattractant activity for neutrophils except IP-10 and Mig, while CC chemokines are chemoattractants for a variety of cells, such as monocytes, lymphocytes, basophils and eosinophils. IP-10 and mig seemed to be distinctive chemokines in the CXC subfamily, and have recently been shown to be more selective chemoattractants for activated T lymphocytes (4). Indeed, our early experiments (unpublished data) showed that human IP-10 has a very low or no chemoattractive effect on human peripheral blood lymphocytes (PBL), and even on $CD45RO^+$ memory T cells, although MCP-1 and RANTES cause the chemotaxis of $CD45RO^+$ T cells, to which many of the known chemokine receptors were demonstrated to be restricted (5–7). This finding suggests that IP-10 attracts a specific subset of T lymphocytes which is different from that attracted by other chemokines.

We have demonstrated that IP-10 is induced selectively in the liver and kidney following systemic treatment with proinflammatory stimulants (8, 9), and further that IP-10 is produced in hepatocytes surrounded by infiltrating mononuclear cells in chronic hepatitis (10). These results led us to examine the attraction by IP-10 of liver-infiltrating lymphocytes. Then we clari-

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fied the differential effect which IP-10 has on infiltrating lymphocytes in the liver in hepatitis, but which other chemokines, including MCP-1, RANTES and MIP-1α, do not have (manuscript in preparation). The results allow the prediction that liver-infiltrating lymphocytes, at least in part, possess the IP-10-specific receptor, which other chemokines cannot recognize, which led us to clone and characterize it.

Although the human IP-10 receptor named as CXCR3 was cloned (11), while we were identifying the murine IP-10 receptor, we have demonstrated in this study that both IP-10 and its receptor mRNA are constitutively expressed in lymphoid organs. In addition, *in vivo* treatment with concanavalin A (Con A) significantly induced the expression of IP-10 receptor mRNA and chemotactic activity toward IP-10 in splenocytes. These results suggest that IP-10 and its receptor play roles in lymphocyte migration not only to specific inflamed tissues, but also to lymphoid organs with or without immunological events.

MATERIALS AND METHODS

Chemokines. Recombinant rat IP-10 was obtained from transiently transfected COS cells (manuscript in preparation). Purified recombinant chemokines were obtained from the following sources: human IP-10, rat GRO, rat MCP-1 and rat RANTES from PeproTech.; murine MIP-1α from R&D Systems.

Cell culture. CTLL2 cells were grown in RPMI 1640 supplemented with 10 % FCS, 50 U/ml penicillin, 50 μg/ml streptomycin and 5 U/ml human IL-2 (GIBCO BRL). CHO-K1 cells were cultured in DMEM supplemented with 10 % FCS and the antibiotics mentioned above.

Intracellular calcium measurements. Cells (10^7/ml) were incubated in a loading buffer comprising 136 mM NaCl, 48 mM KCl, 1 mM $CaCl_2$, 1 mg/ml glucose and 20 mM HEPES, pH7.4, containing 5 μM Fura-2/AM (Molecular Probes) for 20 min at 37 °C in the dark. Cells were washed twice with the loading buffer and then resuspended at 4×10^6 cells/ml. 0.5 ml of the suspension was placed in a continuously stirred cuvette at 37 °C in a fluorimeter (Japan Spectroscopic Co.). Chemokines were added to a final concentration of 1 μg/ml. Fluorescence was monitored at 340 nm (λ_{ex1}), 380 nm (λ_{ex2}), and 510 nm (λ_{em}). The data were recorded as the relative ratio of fluorescence with excitation at 340 nm to that at 380 nm.

Cloning of chemokine receptor cDNA. Total RNA was isolated from CTLL2 cells using guanidine isothiocyanate cesium chloride according to the previously published method (12). Poly $(A)^+$ RNA was prepared using a mRNA Purification Kit (Pharmacia), and 5 μg of the purified poly $(A)^+$ RNA was used for cDNA synthesis with a Superscript Lambda System (GIBCO BRL) according to the supplier's protocol. The synthesized cDNA was subjected to 30 cycles of polymerase chain reaction (PCR) (95 °C for 2 min, 37 °C for 2 min, and 72 °C for 2 min) in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 2.5 units of Taq DNA polymerase (Takara), using 1 μM each of two degenerate oligonucleotide primers corresponding to conserved regions of chemokine receptors (sense, 5′- GITACCTGGCIATIGTICA(C/T)GC; antisense, 5′-TAGA(G/T)(C/G)AIIGG(A/G)TTIAI(A/G)CA), in a DNA Thermal Cycler (Perkin-Elmer Cetus). The PCR products were visualized on 1 % agarose gels containing 0.5 mg/ml ethidium bromide. The reaction products migrating to the predicted positions (500–550 bp) were gel-purified and then directly ligated into the pCRII vector (Invitrogen) as recommended by the supplier. Plasmid DNA was isolated using a Quiagen kit (Quiagen) according to the supplier's protocol, and then subjected to DNA sequence analysis with an AutoRead Sequencing kit and an A.L.F. DNA Sequencer II (Pharmacia). The cDNA, identified as orphan receptors, was labeled with [α^{32}P] dCTP using Ready To Go DNA-labeling Beads (Pharmacia), and then used to screen a CTLL2 cDNA library ligated into the λ phage vector, λ ZipLox (GIBCO BRL), whose average size was approximately 1.4 kb. For plaque-lift hybridization screening, about 4×10^5 plaques were transferred to Hybond N nylon membranes (Amersham International) and then probed with the 500–550 bp insert DNA of the orphan receptors. Hybridization was performed in 6 × SSC and 1 × Denhardt's solution (0.02 % Ficoll, 0.02 % bovine serum albumin and 0.02 % polyvinylpyrrolidone) with 10^6 cpm/ml denatured probe at 68 °C for 16 hrs. The filters were washed once in 2 × SSC-0.1 % SDS at room temperature for 10 min, 1 × SSC-0.1 % SDS at 65 °C for 30 min, and 0.1 × SSC-0.1 % SDS at 65 °C for 30 min. The isolated cDNA clones were inserted into the plasmid vector, pZL1 (GIBCO BRL), and then sequenced as described above.

Transfection. Murine IP-10 receptor cDNA was ligated into the EcoRI-NotI site of the pME18S expression vector with the neomycin resistance gene (13). CHO-K1 cells were transfected by electroporation using a Gene Pulser (Bio-Rad). The transfected CHO cells were continuously selected with 700 μg/ml G 418 (GIBCO BRL), and G 418-resistant clones were picked up and expanded.

Treatment of mice. Female C57Bl/6 mice (7 weeks old) were purchased from Charles River Japan Inc. The mice were maintained in microisolater cages with autoclaved food and bedding in order to minimize exposure to viral and microbial pathogens. For determination of the distribution of IP-10 receptor mRNA, mice were injected with 10,000 U of murine IFNγ (Genzyme) or PBS (vehicle) intravenously (*i.v.*) via a tail vein. Two hours later, the mice were sacrificed and tissues were removed. For Northern analysis and the chemotaxis assay, female C57Bl/6 mice were treated with 0.5 mg/mouse of Con A (Sigma), *i.v.*, and 12 hrs later splenocytes were isolated as described below.

Preparation of splenocytes and thymocytes. Spleens and thymuses from C57Bl/6 mice were pressed through a 150- μm stainless steel mesh and then a 70 μm nylon mesh (Becton Dickinson). After washing, the pellet was resuspended in RBC lysis solution (155 mM NH_4CL, 10 mM $KHCO_3$, 1 mM EDTA and 170 mM Tris, pH 7.3), and then washed twice with PBS. After 2 hrs culture in RPMI 1640 medium containing 10 % FCS, the non-adherent cells were used as splenocytes or thymocytes. For the chemotaxis assay, splenocytes were further cultured in RPMI 1640 supplemented with 10 % FCS, 50 U/ml penicillin, 50 μg/ml streptomycin and 50 U/ml human IL-2 (GIBCO BRL) for 2 days.

Chemotaxis assay. Splenocytes were resuspended at 7.5×10^6 cells per ml in the assay medium (a 1:1 mixture of RPMI medium 1640 and M199 (GIBCO BRL) plus 0.25 % bovine serum albumin). Seven hundred μl of the assay medium with or without chemokines was added to a 24-well tissue culture plate. A murine collagen type 4-coated cell culture insert (Becton Dickinson) of 3-μm pore size was put into each well and splenocytes (1.5×10^6 / 200 μl) were placed on each insert. The assay plates were incubated for 4 hrs at 37 °C under air containing 5 % CO_2. After 4 hrs incubation, the inserts were removed, and the cell number in each bottom well was determined with a hemocytometer. The statistical significance of these results was determined using an unpaired student's *t* test. $p < 0.05$ was considered significant.

Northern hybridization. Total RNA was extracted from whole tissues or isolated lymphocytes by homogenization with a Polytron sonicator/homogenizer for 2 min in the presence of guanidine isothiocyanate cesium chloride. Equal amounts of RNA were denatured and subjected to electrophoresis in a 1 % agarose-formaldehyde gel. The RNA was then blotted by capillary transfer onto nylon membranes (Boehringer Mannheim). The blots were prehybridized for 6 hrs at 42

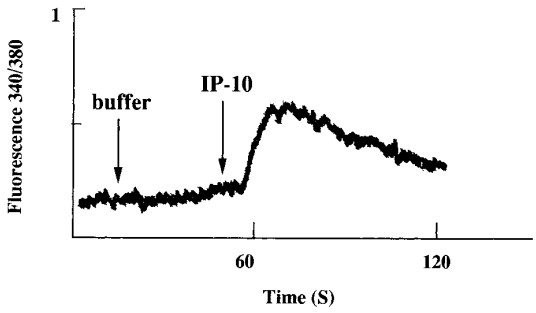

FIG. 1. Recombinant rat IP-10 elicits transient elevation of $[Ca^{2+}]_i$ in the murine cytotoxic T cell line, CTLL2. CTLL2 cells were loaded with Fura-2/AM and then stimulated at the indicated times with the buffer or 1 μg/ml rat IP-10. The data were recorded as the relative ratio of fluorescence with excitation at 340 and 380 nm.

°C in 50 % formamide, 1 % SDS, 5 × SSC, 1 × Denhardt's solution, 0.25 mg/ml denatured herring testes DNA and 50 mM sodium phosphate buffer, pH6.5. Hybridization was carried out at 42 °C for 12 to 18 hrs with 7×10^5 cpm/ml of a denatured probe. The filters were washed for 30 min at room temperature in 2 × SSC-0.1 % SDS, and then for 15–30 min at 55 °C in the same solution. The blots were then exposed to XAR-5 X-ray film (Eastman Kodak) with Dupont Cronex Lightening Plus intensifying screens at −70 °C. Expression of α-tubulin was used as an internal control.

RESULTS AND DISCUSSION

Activation of CTLL2 Cells by Recombinant Rat IP-10

Chemokines have been shown to mobilize intracellular calcium through G-protein coupled receptors (3). To clone the IP-10 receptor gene, we searched for cells that respond to IP-10 through the detection of calcium mobilization. Since IP-10 was recently shown to be a chemoattractant for T lymphocytes (4, 14), we have examined whether or not several human and murine T cell lines respond to IP-10. While Jurkat, MOLT-4, HPB-ALL, WR19L and HT-2 cells (the first three and last two being human and murine T cell lines, respectively) did not respond to IP-10 (data not shown), CTLL2 cells (a murine cytotoxic T cell line) responded to recombinant rat IP-10 with an increase in intracellular Ca^{2+} (Fig. 1). Murine MIP-1α and rat RANTES caused calcium mobilization in CTLL2 cells as well as rat IP-10, but rat MCP-1 did not. MIP-1α was the most potent stimulant, followed by IP-10 and RANTES in that order (data not shown). These data demonstrate that CTLL2 cells express some kinds of receptors for chemokines including murine IP-10.

Molecular Cloning of the Murine IP-10 Receptor

To isolate murine IP-10 receptor cDNA from the CTLL2 cDNA library, novel DNA fragments coding for putative chemokine receptors were generated using the following PCR protocol. Two degenerate oligonucleotide primers corresponding to highly conserved motifs, i.e. RYLAIVHA in intracellular loop-2 and C(I/L/M/V)NP(I/L/V)IY in transmembrane domain-7, were designed based on the published sequences of human and murine chemokine receptors. These regions are well conserved in chemokine receptors. Reverse transcription (RT)-PCR was carried out using poly $(A)^+$ RNA derived from CTLL2 cells. The resultant PCR products of the predicted size (500–550 bp) were cloned and partially sequenced. Most of the sequences determined encode murine CCR-5 (15), which is in consistent with the finding that MIP-1α and RANTES caused calcium mobilization in CTLL2 cells, since both chemokines have been found to be agonists for CCR5 (15). We also detected a number of clones corresponding to murine CCR4, to which MIP-1α and RANTES could bind but MCP-1 could not (16). Additionally, several murine orphan receptor genes were amplified, including one clone designated as T126. A clone corresponding to T126 of full length was isolated by screening the cDNA library derived from CTLL2 cells. This clone (T126F) has an open reading frame of 1,101 bp, encoding a

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GCAAGTTCCC AACCACAAGT GCCAAAGGCA GAGAAGCAGG CAGCACGAGA CCTGACCCCA GCAGCCACAG CCGGA   75

GCACCAGCCA AGCCATGTAC CTTGAGGTTA GTGAACGTCA AGTGCTAGAT GCCTCGGACT TTGCCTTTCT TCTGG  150
            M  Y  L  E  V  S  E  R  Q  V  L  D  A  S  D  F  A  F  L  L  E
AAAACAGCAC CTCTCCCTAC GATTATGGGG AAAACGAGAG CGACTTCTCT GACTCCCCGC CCTGCCCACA GGATT  225
  N  S  T  S  P  Y  D  Y  G  E  N  E  S  D  F  S  D  S  P  P  C  P  Q  D  F
TCAGCCTGAA CTTTGACAGA ACCTTCCTGC CAGCCCTCTA CAGCCTCCTC TTCTTGCTGG GGCTGCTAGG CAATG  300
  S  L  N  F  D  R  T  F  L  P  A  L  Y  S  L  L  F  L  L  G  L  L  G  N  G
GGGCCGTGGC TGCTGTGCTA CTGAGTCAGC GCACTGCCCT GAGCAGCACG GACACCTTCC TGCTCCACCT GGCTG  375
  A  V  A  A  V  L  L  S  Q  R  T  A  L  S  S  T  D  T  F  L  L  H  L  A  V
TAGCCGATGT TCTGCTGGTG TTAACTCTTC CATTGTGGGC AGTGGATGCT GCTGTCCAGT GGGTTTTCGG CCCTG  450
  A  D  V  L  L  V  L  T  L  P  L  W  A  V  D  A  A  V  Q  W  V  F  G  P  G
GCCTCTGCAA AGTGGCAGGC GCCTTGTTCA ACATCAACTT CTATGCAGGG GCCTTCCTGC TGGCTTGTAT AAGCT  525
  L  C  K  V  A  G  A  L  F  N  I  N  F  Y  A  G  A  F  L  L  A  C  I  S  F
TCGACAGATA TCTGAGCATA GTGCACGCCA CCCAGATCTA CCGCAGGGAC CCCCGGGTAC GTGTAGCCCT CACCT  600
  D  R  Y  L  S  I  V  H  A  T  Q  I  Y  R  R  D  P  R  V  R  V  A  L  T  C
GCATAGTTGT ATGGGGTCTC TGTCTGCTCT TTGCCCTCCC AGATTTCATC TACCTATCAG CCAACTACGA TCAGC  675
  I  V  V  W  G  L  C  L  L  F  A  L  P  D  F  I  Y  L  S  A  N  Y  D  Q  R
GCCTCAATGC CACCCATTGC CAGTACAACT TCCCACAGGT GGGTCGCACT GCTCTGCGTG TACTGCAGCT AGTGG  750
  L  N  A  T  H  C  Q  Y  N  F  P  Q  V  G  R  T  A  L  R  V  L  Q  L  V  A
CTGGTTTCCT GCTGCCCCTT CTGGTCATGG CCTACTGCTA TGCCCATATC CTAGCTGTTC TGCTGGTCTC CAGAG  825
  G  F  L  L  P  L  L  V  M  A  Y  C  Y  A  H  I  L  A  V  L  L  V  S  R  G
GCCAGAGGCG TTTTCGAGCT ATGAGGCTAG TGGTAGTGGT GGTGGCAGCC TTTGCTGTCT GCTGGACCCC CTATC  900
  Q  R  R  F  R  A  M  R  L  V  V  V  V  A  A  F  A  V  C  W  T  P  Y  H
ACCTGGTGGT GCTAGTGGAT ATCCTCATGG ATGTGGGAGT TTTGGCCCGC AACTGTGGTC GAGAAAGCCA CGTGG  975
  L  V  V  L  V  D  I  L  M  D  V  G  V  L  A  R  N  C  G  R  E  S  H  V  D
ATGTGGCCAA GTCAGTCACC TCGGGCATGG GGTACATGCA CTGCTGCCTC AATCCGCTGC TCTATGCCTT TGTGG 1050
  V  A  K  S  V  T  S  G  M  G  Y  M  H  C  C  L  N  P  L  L  Y  A  F  V  G
GAGTGAAGTT CAGAGAGCAA ATGTGGATGT TGTTCACGCG CCTGGGCCGC TCTGACCAGA GAGGGCCCCA GCGGC 1125
  V  K  F  R  E  Q  M  W  M  L  F  T  R  L  G  R  S  D  Q  R  G  P  Q  R  Q
AGCCGTCATC TTCACGGAGA GAATCATCCT GGTCTGAGAC AACTGAGGCC TCCTACCTGG GCTTGTAATT CTGGA 1200
  P  S  S  S  R  R  E  S  S  W  S  E  T  T  E  A  S  Y  L  G  L  .
CTGGAACTGT AGCCTGCGCA GCCCAAGTCC TAACACACTC CAAGTGCTTG TCCTCCTGGT AGTTGGGCTA GCTCG 1275

AACTTACCCG TAACTTTGCT GCCAGGATGC ACTGACAGCT CAGCATATAT CCAGCTCTCC TGAGAATCAA TCTCA 1350

GCAACAAGGA CAACACCATT ACTGTGCCTT AGCTGCCATC CCCTATCTTG CTGTTTTAGA ACTAGCTGCC TGGAG 1425

CCCCACCGCC CTACTAAATT AGCAAGTAGA ACTCAGCCAT CCCTGTGTGA GAAGAGGGAG AGGCAAATAG CACAG 1500

AGGGCCAGGC GTTGTCAGCA CTGAATGTGC CCATCTCAGT ATCTCAATAT TTGCCCAATT TTATTTCTAG AAACC 1575

TCACTTAAAC TTTCAATAAA CAAGGTAATG AGGAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAA 1650

AAAAAAAAA                                                                        1659
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FIG. 2. Nucleotide sequence of cDNA for murine IP-10 receptor clone T126F. The predicted open reading frame is indicated below the nucleotide sequence. The putative polyadenylation signal is underlined. The nucleotide sequence of cDNA for murine IP-10 receptor clone T126F is available in the Genbank/EMBL databases under the accession number, AB003174.

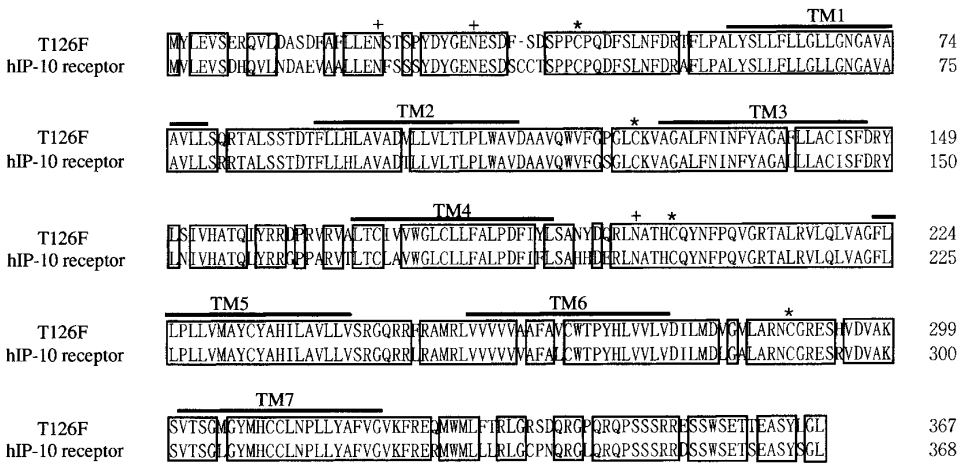

FIG. 3. Alignment of the deduced T126F and human IP-10 receptor (CXCR3) protein sequences. The boxed letters indicate identical residues. The dash indicates a gap that was inserted to optimize the alignment. The putative transmembrane segments (TM1-TM7) are overlined. The conserved residues, potential N-linked glycosylation sites (+) and cysteines (*), are indicated.

protein of 367 amino acids with a predicted molecular mass of 40,970 daltons (Fig. 2). While we were identifying the murine IP-10 receptor, the human IP-10 receptor (CXCR3) was cloned (11). Amino acid sequence alignment of the protein encoded by T126F with the human IP-10 receptor is shown in Figure 3. This protein is highly homologous to the human IP-10 receptor, exhibiting 86 % sequence identity. In particular, all the three potential N-linked glycosylation sites are entirely conserved. The presence of N-linked glycosylation sites at the N terminus and/or in the second extracellular loop is a common feature of chemokine receptors (3, 17). The T126F product and the human IP-10 receptor possess four conserved cysteine residues at the N terminus and in extracellular loops-1, 2 and 3, which may form disulfide bridges, indicating another common feature associated with chemokine receptors (3, 17), while the human IP-10 receptor has been reported to have two additional cysteine residues at its N terminus (11). These similarities between the T126F product and the human IP-10 receptor suggest that T126F encodes the murine IP-10 receptor. The T126F product exhibits 29–32 % identity with murine CC chemokine receptors (CCR1-5), and 33 % identity with a murine chemokine receptor homologous to human IL-8 receptor B (CXCR2) (15, 16, 18, 19). The sequence homology between murine chemokine receptors and the human homologs has been reported to be between 60–80 %, although CCR4 and CXCR4 (fusin) in the two species exhibit 85 % and 91 % identity, respectively (15, 16, 18–22). Therefore, it was demonstrated that the IP-10 receptor is one of the well conserved chemokine receptors in humans and mice.

Calcium Mobilization in Transfected Cells

To determine whether or not the product of the T126F gene is the murine IP-10 receptor, the T126F gene was stably expressed in CHO-K1 cells and then ligand-stimulated calcium mobilization was examined. Untransfected CHO-K1 cells never responded to rodent chemokines including, IP-10, GRO, RANTES, MCP-1 and MIP-1α, at the dose of 1 μg/ml (data not shown). In contrast, rat IP-10 (1 μg/ml) induced transient elevation of intracellular Ca^{2+} in T126F transfectants (Fig. 4, top panel). Human IP-10 also induced calcium mobilization in the transfectants although much less potently than rat IP-10 did (data not shown). However, the transfectants did not respond to 1 μg/ml of rodent GRO, RANTES, MCP-1 or MIP-1α (Fig. 4, second to fourth panels). It has commonly been observed that chemokine receptors show altered sensitivity to repeated stimulation due to desensitization. While pretreatment with rodent GRO, RANTES, MCP-1 or MIP-1α had no effect on the transient elevation of intracellular Ca^{2+} induced by sequential stimulation with IP-10 (Fig. 4, second to fourth panels), repeated IP-10 treatment caused desensitization (data not shown), suggesting that the T126F gene encodes the functional murine IP-10 receptor and that IP-10 specifically binds to the receptor. It has been reported that the human CXC chemokine, Mig (23), also functions through the IP-10 receptor (CXCR3) (11), although we did not examine the response to murine Mig in this study.

Tissue-Specific Expression of the Murine IP-10 Receptor

IP-10 mRNA expression has been demonstrated to be induced drastically in a tissue-specific manner following systemic treatment with proinflammatory stimulants including IFNγ in mice (8, 9). Since human IP-10 receptor (CXCR3) mRNA expression has been reported to be inducible *in vitro* (11), we have wanted to clarify whether or not the gene expression of the

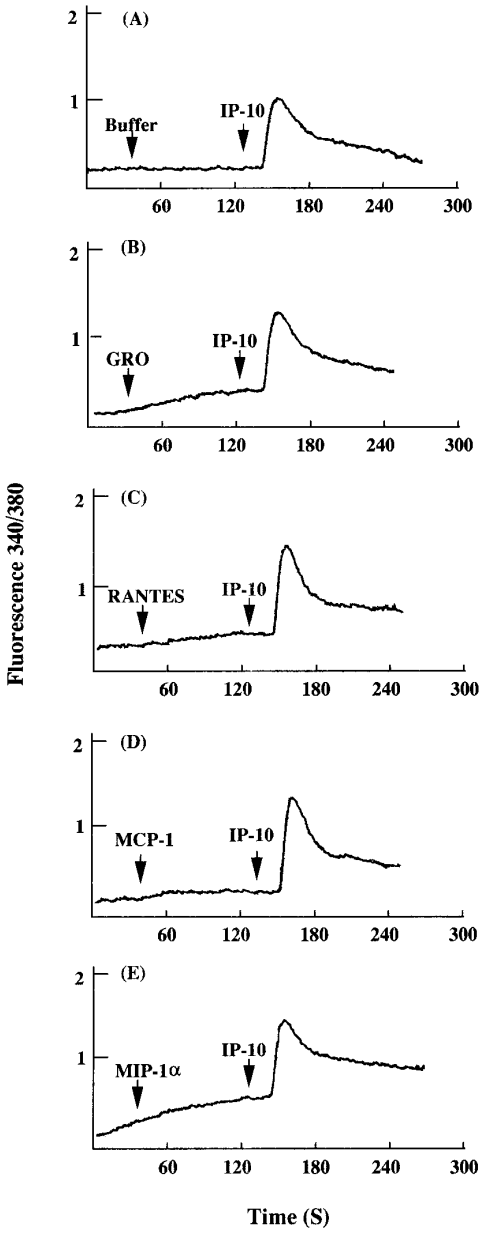

FIG. 4. Calcium mobilization in CHO cells stably expressing the T126F product. T126F-transfected CHO cells were first stimulated with buffer or 1 μg/ml of rat GRO, rat RANTES, rat MCP-1 or murine MIP-1α, and then the response to rat IP-10 (1 μg/ml) was examined. The data were recorded as the relative ratio of fluorescence with excitation at 340 and 380 nm.

murine IP-10 receptor is similar to that of IP-10. C57Bl/6 female mice were injected *i.v.* with IFNγ (10,000 U/mouse) or PBS (vehicle). Two hours later, the animals were sacrificed, and total RNA from the liver, kidney, heart, lung, spleen and thymus was prepared. As shown in Fig. 5A, IP-10 receptor mRNA was expressed in the spleen and thymus, but not at all in the other organs examined. The IP-10 receptor mRNA expression was not inducible on *in vivo* treatment with IFNγ for 2 hrs, differing from IP-10 expression. Next, to determine the sources of mRNA in the two tissues, we isolated splenocytes and thymocytes, and then the expression of the mRNA for IP-10 and the IP-10 receptor were analysed (Fig. 5B). IP-10 mRNA was shown to be constitutively expressed in the whole spleen and thymus, but not in splenocytes or thymocytes, as reported previously (24). On the other hand, IP-10 receptor mRNA was constitutively expressed not only in the whole tissues but also in lymphocytes derived from both tissues. These data suggest that IP-10 is constitutively expressed in the splenic and thymic stroma, and that, in contrast, splenocytes and thymocytes express the IP-10 receptor in mice. In addition, IP-10 and its receptor mRNA were also expressed in enlarged popliteal lymph nodes from sensitized rats injected into their foot pads (24, and our unpublished data). Therefore, IP-10 and its receptor are supposed to be important mediators of lymphocyte trafficking into lymphoid organs. Recently, it was reported that newly cloned lymphocyte-specific chemokines such as TARC (thymus and activation-regulated chemokine) (25), MDC (macrophage-derived chemokine) (26), ELC (Epstein-Barr virus induced gene 1-ligand chemokine) (27), SLC (secondary lymphoid tissue chemokine), and PARC (pulmonary and activation-regulated chemokine) (28, 29) are expressed differentially in lymphoid organs. Therefore, each of these chemokines and IP-10 are supposed to attract different subgroups of lymphocytes into different lymphoid organs.

Induction of IP-10 Receptor mRNA and Chemotactic Activity Toward IP-10 in Splenocytes of Con A-Treated Mice

It has been reported that the expression of human IP-10 receptor (CXCR3) mRNA in peripheral blood lymphocytes was induced by culture with IL-2 for 10 days (11). Also, the expression of murine IP-10 receptor mRNA in splenocytes was only slightly or not increased by treatment with IL-1β, IL-2, IL-4, IL-6, IL-12, IFNγ or TNFα for 4 hrs *in vitro* (data not shown). These data suggest that *in vitro* treatment with a cytokine is not enough to induce IP-10 receptor mRNA expression rapidly and significantly in lymphocytes. Because multiple cytokines may be produced and stimulate lymphocytes simultaneously on inflammation or in immunological events, we examined whether or not the expression of IP-10 receptor mRNA in lymphocytes is induced more significantly in an inflammatory condition *in vivo.* Concanavalin A (Con A) is commonly used as a potent agent that stimulates the *in vitro* release of a broad spectrum of lymphokines from lymphocytes and other mononuclear cells (30). In addition, systemic injection of Con A into mice was reported to cause the release of several cytokines, including IL-1, IL-2, IL-6 and TNF, into the plasma, and to activate T

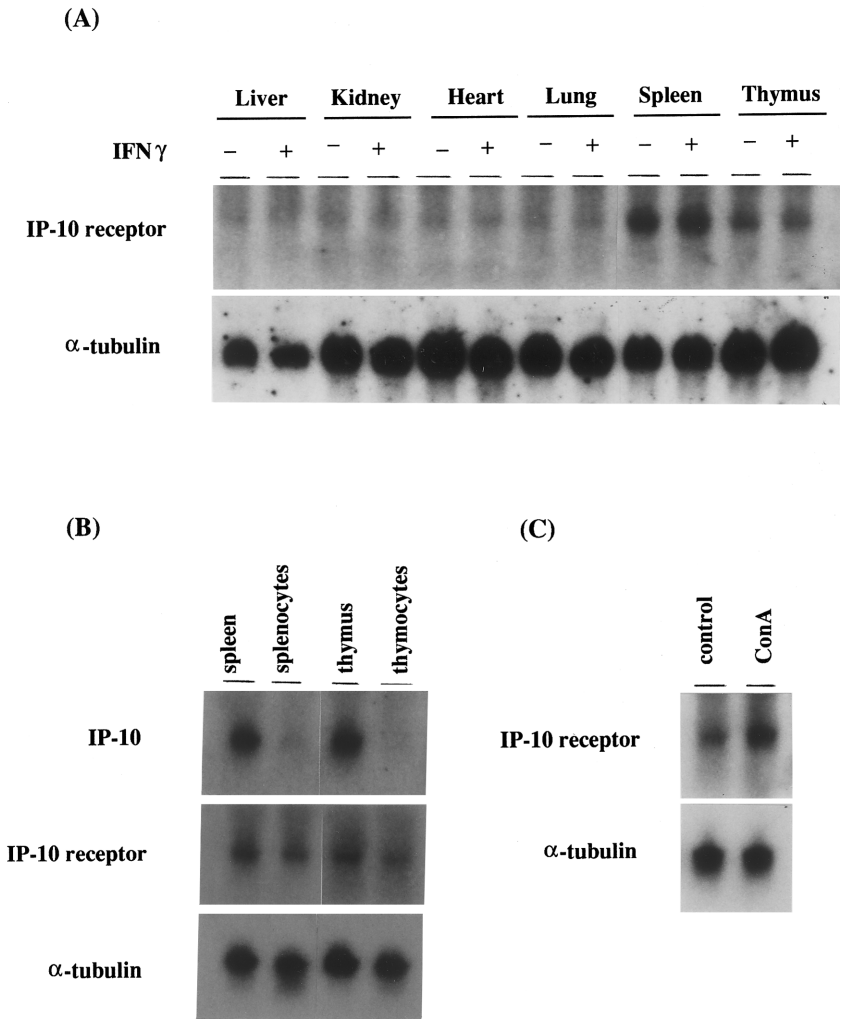

FIG. 5. Expression of murine IP-10 receptor mRNA. (A) C57Bl/6 mice were injected with 10,000 units of murine IFNγ or the vehicle via a tail vein. At 2 hrs later, total RNA (30 μg) was prepared from each indicated tissue and subjected to Northern blot analysis. (B) Whole spleen, splenocytes, whole thymus and thymocytes were isolated, and total RNA (15 μg) was used for Northern analysis using the murine IP-10, IP-10 receptor or α-tubulin as a probe. (C) Con A (0.5 mg/mouse) or the vehicle was injected in mice with a tail vein. Twelve hrs later, their spleens were removed and splenocytes were prepared. Total RNA (15 μg) from non-stimulated or Con A-stimulated splenocytes was used for Northern analysis for IP-10 receptor mRNA.

lymphocytes, resulting in T lymphocyte-dependent hepatic injury (31, 32). So we investigated the expression of IP-10 receptor mRNA and the chemotactic response to IP-10 of splenocytes systemically activated with Con A. Con A (0.5 mg/mouse) or the vehicle was injected into a tail vein. Twelve hours later, the spleen was removed, and splenocytes were prepared for Northern analysis and the chemotaxis assay. Northern blot analysis revealed that the expression of IP-10 receptor mRNA is significantly induced on *in vivo* treatment with Con A for 12 hrs (Fig. 5C). Next, we examined the chemotactic activity of splenocytes systemically activated with Con A. The splenocytes were found to migrate in response to IP-10 in a dose-dependent manner (Fig. 6A). In the chemotaxis assay with rat IP-10, MCP-1 and RANTES, only IP-10 significantly induced the chemotaxis of splenocytes treated with Con A (Fig. 6B). Also, murine MIP-1α had no effect on the chemotaxis of splenocytes treated with Con A (data not shown). The cells migrating in response to IP-10 were both $CD4^+$ and $CD8^+$ T lymphocytes (manuscript in preparation). This finding is consistent with the report that human IP-10 receptor (CXCR3) mRNA is expressed on both $CD4^+$ and $CD8^+$ T cell clones (11). In addition, $CD4^+$ cells migrating in response to IP-10 produced both T helper cell type 1 (Th1) and Th2 cytokines (manuscript in preparation). With regard to this, it was recently reported that human CXCR3 was preferentially expressed in Th1 lines generated *in vitro* (33), or highly in both Th1 and Th2 clones (34). From these data, it is supposed that the expression of the IP-10 receptor and chemotactic activity toward IP-10

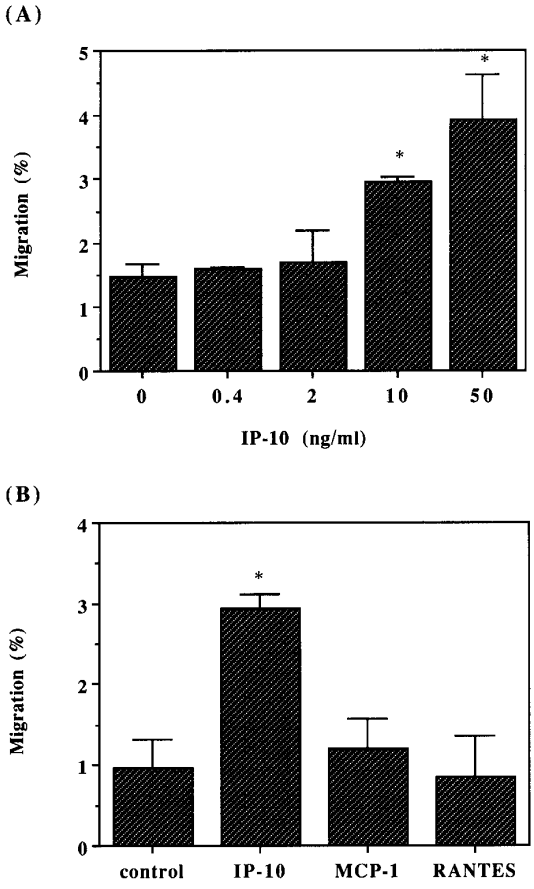

FIG. 6. Chemotaxis assay with splenocytes from Con A-treated mice. Mice were treated with 0.5 mg of Con A for 12 hrs and then splenocytes were prepared as described under Materials and Methods. Chemokines were added to the bottom chambers and splenocytes (1.5×10^6 cells) were added to the top chambers. After 4 hrs incubation at 37 °C, the cells in each bottom chamber were determined with a hemocytometer and migration (%) was calculated. (A) Dose response to IP-10 of Con A-treated splenocytes. 0.4-50 ng/ml of purified rat IP-10 was used for the chemotaxis assay. (B) Chemotaxis assay with 100 ng/ml of rat IP-10, rat MCP-1 and rat RANTES. The results in (A) and (B) are the means ± S.D. of triplicate samples, and each experiment was repeated several times with similar results. *: $p < 0.05$, compared to medium control.

are rapidly induced in T lymphocytes through activation by multiple cytokines expressed on inflammation or in immunological events.

Although IP-10 receptor mRNA is constitutively expressed in splenocytes from normal mice (Fig. 5B, C), we did not detect a predominant chemoattractive effect of IP-10 on resting splenocytes (data not shown). The sensitivity of our assay system may not be enough to detect the effect of IP-10 on resting splenocytes. Alternatively, there is a possibility that only the signals through the IP-10 receptor are probably not enough to induce chemotaxis in resting splenocytes. The activation of splenocytes induces expression of the IP-10 receptor and simultaneously may be needed for the signaling for chemotaxis. After trafficking to lymphoid organs through IP-10 and its receptor system, T lymphocytes may remain at the organs with low or nonfunctional IP-10 receptors. Further studies are necessary to clarify the importance of IP-10 and its receptor system for lymphocyte trafficking to lymphoid organs, and the effect of activation on the function of the IP-10 receptor.

We have shown here that the murine IP-10 receptor is highly homologous with the human IP-10 receptor. This high homology between the human and murine IP-10 receptor genes indicates that this gene has been conserved during evolution, and suggests that the important function of the gene product in mouse models of human diseases is considered to be similar to that in humans.

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