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Cloning and chromosomal mapping of an orphan chemokine receptor: mouse RDC1

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Abstract Degenerate RT-PCR was used to identify a new seven-transmembrane-spanning receptor expressed in astrocytes. A receptor, termed RDC1, displaying the characteristic structural features of a chemokine receptor was cloned. The predicted 362-amino-acid sequence displayed 92% and 91% similarity to the human and dog orphan receptor RDC1, respectively. In addition, RDC1 shares 43% amino acid similarity to rabbit and mouse CXCR2. Transcripts of RDC1 were found in astrocytes, heart, kidney, the mesangial tumor line MES-13, spleen, and neutrophils by means of northern blot. Using linkage analysis of interspecies backcross mice, we localized to chromosome 1 the genes for mouse CXCR2, CXCR4, and RDC1. Mouse RDC1 is linked to and lies between the genes for the mouse CXC chemokine receptors CXCR2 and CXCR4. The combined data of chromosomal location and sequence similarity suggest that RDC1 is an orphan CXC chemokine receptor.

Key words Astrocytes · Receptors · Chemokines · Mouse chromosome 1

Introduction

Chemokines are a growing family of chemotactic cytokines (Lindley et al. 1993). Based on the spacing of the first two cysteine residues in a conserved four-cysteine motif, two chemokine subfamilies can be distinguished (Strieter et al. 1996): CC and CXC. Chemokines are important for the recruitment of lymphocytes, monocytes, eosinophils, and/or neutrophils to sites of inflammation (Ben-Baruch et al. 1995; Murphy 1996). Induction of chemokine production can occur under a variety of circumstances including septic shock, adult respiratory distress syndrome (ARDS), encephalomyelitis, asthma, and rheumatoid arthritis (Baggiolini and Dahinden 1994; Gura 1996; Strieter et al. 1996,).

The receptors for CXC as well as CC chemokines belong to the group of seven-transmembrane-spanning, G-protein-coupled receptors (Power and Wells 1996). So far, eight CC chemokine receptors (CCR) and four CXC chemokine receptors (CXCR) have been identified in humans (Baba et al. 1997; Tiffany et al. 1997; Yoshida et al. 1997). With the exception of CCR6, CCR7, CCR8, CXCR1, and CXCR3 homologues for the chemokine receptors have been cloned in mice. Most chemokine receptors are promiscuous, displaying specificity for multiple members of either the CC or CXC chemokine families, but usually do not bind ligands from both subfamilies. Thus, mouse CCR1 and CCR4 bind the CC chemokines MIP-1α and RANTES with high affinity (Hoogewerf et al. 1996; Post et al. 1995). Mouse CCR2 is a principle receptor for JE, the proposed mouse homologue of MCP-1 (Boring et al. 1996). The chemokine eotaxin is the primary ligand for CCR3, whereas CCR5 binds MIP-1α, MIP-1β, and RANTES (Boring et al. 1996; Daugherty et al. 1996; Post et al. 1995). Mouse CXCR2 binds the CXC chemokines MIP-2 and KC, the mouse homologue of GRO-α (Bozic et al. 1994). Mouse CXCR4 which has two alternate splice variants, A and B, is a receptor for the CXC chemokine SDF-1α (Heesen et al. 1997). Interest in chemokine receptors was also stimulated by the finding that CCR2B, CCR3, CCR5, and CXCR4 serve as co-receptors mediating infection by HIV-1 (Choe et al. 1996; Doranz et al. 1996; Feng et al. 1996) and HIV-2 (Endres et al. 1996).

Most of the chemokine receptors known to date were cloned from cDNA preparations derived from hematopoietic cell lines. However, there is increasing data documenting the effects of chemokines on non-hematopoietic cells. KC and MCP-1 induce the migration of astrocytes (Heesen et al. 1996b). Mesangial cells and vascular smooth muscle

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Table 1 Primer sequences, concentrations, and size of the PCR products used for chromosomal mapping

	Primer sequence	Concentration	Size of PCR product
mRDC1			
Sense	5′-CATGTTGCAAATGGGGCGGCTG-3′	0.750 μM	164
Antisense	5′-CTGTCAGGATGGGCATCCAG-3′		
mCXCR4			
Sense	5′-CCACACATTCTGGAATGTTC-3′	0.80 μM	238
Antisense	5′-GAGACTGACCAGTCTTGTAC-3′		
mCXCR2			
Sense	5′-GACTGTTCACCTAAACGGTG-3′	0.040 μM	194
Antisense	5′-CATACCAAGATGGAAGGGAGC-3′		

cells respond upon incubation with the CC chemokine TCA3 with chemotaxis, proliferation, and increased adhesiveness (Luo and Dorf 1996; Luo et al. 1996).

To further evaluate the interaction of chemokines with cells of non-hematopoietic origin we used RT-PCR with degenerate primers to identify potential chemokine receptors from astrocytes. We report the cloning, northern blot, and chromosomal mapping analysis of a seven-transmembrane-spanning receptor with similarity to an orphan human and dog chemokine receptor and to CXCR2.

Materials and methods

Cells and cell lines

Astrocytes from neonatal mice and neutrophils were prepared as described earlier (Devi et al. 1995; Hayashi et al. 1993). The T-cell lymphoma EL4, the B-cell lymphoma A20, the monocyte/macrophage cell line P388.D1, and the SV-40 transformed glomerular mesangial tumor cell line MES-13 were obtained from the American Type Culture Collection (Rockville, Md.). The mast cell clone MC9 was a gift from S. Galli (Beth Israel-Deaconess Hospital, Boston, Mass.).

Degenerate polymerase chain reaction on astrocyte cDNA

After isolation of total RNA according to a modification of the method of Chomczynski and Sacchi (1987), 1 μg total RNA from neonatal astrocytes was subjected to RT as described previously (Heesen et al. 1996b). Two degenerate primers were designed on the basis of conserved regions of the second and seventh transmembrane regions of known human and mouse chemokine as well as of orphan receptors. The sequences of the 5′ and the 3′ primer were A(C/T)II(C/T)T(A/T)CITIIT(C/G)AACCTG and TTCGAATTCTAGAT(G/T)A(C/T)IGG-GTT(G/C)A(G/T)GCAGC(A/T)(A/G)TG, respectively, with I for inosine.

One-twentieth of the RT reaction was used in a polymerase chain reaction (PCR) using the touch-down approach with annealing temperatures decreasing 1 °C per 2 cycles from 55 °C to 45 °C as detailed elsewhere (Heesen et al. 1996b). A PCR product of 700 base pairs (bp) was cloned into the pT7 blue cloning vector (Novagen, Madison, Wis.) and sequenced. A clone termed O1 revealed the classical features of parts of a seven-transmembrane-spanning receptor.

We hypothesized that chemokine receptors involved in cell migration and inflammation should also be expressed in hematopoietic cells. Therefore, a lamda bacteriophage gt22 cDNA library from mouse peritoneal exudate cells was prepared as detailed elsewhere (Bozic et al. 1994). To clone the missing 5′ and 3′ end of this cDNA, sense and antisense gene-specific primers were designed based on the sequence of clone O1. The two other primers, 5′ gt22 and 3′ gt22, matched the nucleotide sequences flanking all inserts in the peritoneal exudate cell cDNA library. A gene-specific antisense primer and 5′ gt22 were used to amplify the 5′ end, and a gene-specific sense primer and the 3′ end of the RNA, with the cDNA library as a template. The PCR products were cloned into pT7 blue and sequenced. These sequences served for the design of 5′ and 3′ primers which were used to amplify the full-length coding region in a PCR.

The nucleotide sequence of the full-length cDNA of mouse RDC1 was compared with known genes using the program BLAST (Altschul et al. 1990). Comparison of the amino acid sequences was performed with the program PileUp for multiple alignment and Gap for pairwise alignment using the BLOSUM 62 amino acid similarity table (Wisconsin Sequence Analysis Package; Genetics Computer Group, Inc., Madison, Wis.) based on the algorithm described by Needleman and Wunsch (1970).

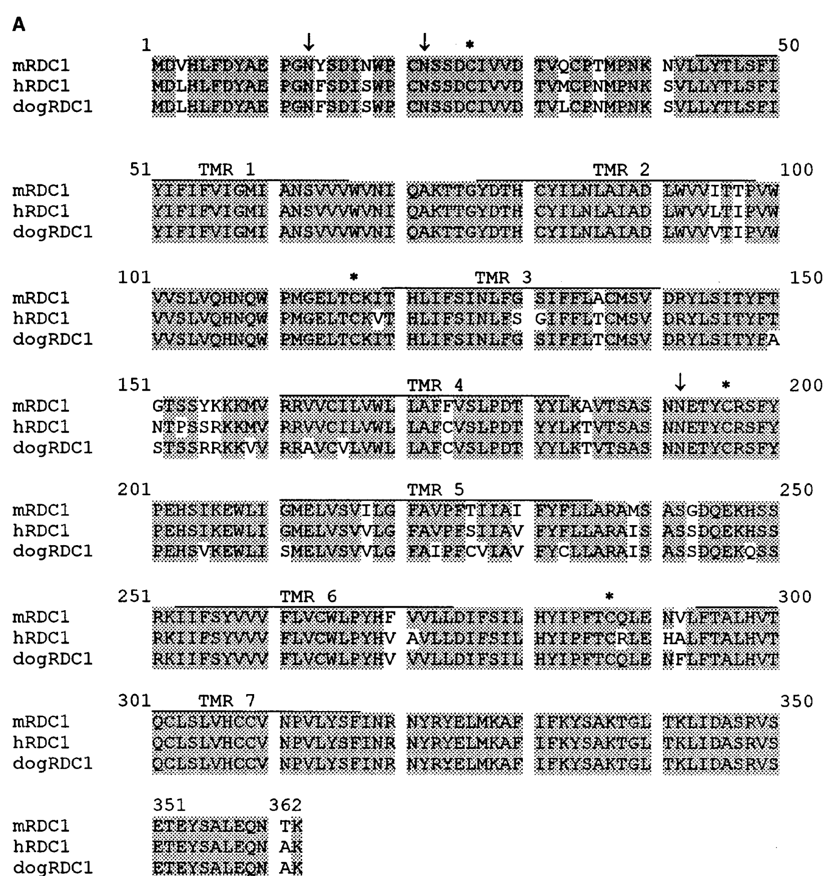
Northern blots

Electrophoresis and blotting of total RNA were carried out as described (Heesen et al. 1996a). A northern blot with kidney RNA was generously provided by J.-C. Gutierrez-Ramos (Center for Blood Research, Boston, Mass.). The cDNA of the receptor was ^{32}P-radiolabeled using the PCR method of Mertz and Rashtchian (1994). Briefly, 100 pg DNA was subjected to a PCR with 2.5 mM $MgCl_2$, 2.5 μM of the gene-specific primers, 3.3 μM dTTP, dATP, dGTP each, and 0.83 μM [α-^{32}P]dCTP (New England Nuclear, Boston, Mass.) over 30 cycles with 55 °C as annealing temperature. For radiolabeling of β-actin, a random primer-labeling method was applied (Random Primers DNA Labeling System; Life Technologies, Gaithersburg, Md.). Blots were hybridized for 16 h at 42 °C in 50% formamide, washed, and exposed on X-ray film as described previously (Heesen et al. 1996a).

Chromosomal localization

The genes for mouse RDC1, mouse CXCR2, and mouse CXCR4 were mapped by single-strand conformation polymorphism (SSCP) analysis of an interspecies backcross of (C57BL/6J X *Mus spretus*) F_1×C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Me.) as described elsewhere (Rowe et al. 1994). PCRs were carried out on the DNA samples using primer pairs covering regions of the 3′ untranslated area of each receptor gene. Primers for mCXCR4 and mCXCR2 were included in one PCR reaction. The primer sequences, concentrations used, and size of the predicted PCR products in 129/SvJ mice are given in Table 1. One-hundred nanograms of the sense primers were ^{32}P-radiolabeled in a 20 μl reaction with 10 units T4 polynucleotide kinase (New England Biolab, Beverly, Mass.) in 70 mM TRIS/HCl pH 7.6, 10 mM $MgCl_2$, 5 mM dithiotreitol, and 5 μl [γ-^{32}P]ATP 3000 Ci/mmol and 10 μCi/μl (New England Nuclear, Boston, Mass.) over 30 min at 37 °C. After the addition of 20 μl water the reaction was stored at –20 °C and 1.25 μl was used for PCR. The 25 μl PCR mixtures contained 25 ng genomic DNA, 50 mM KCl, 10 mM TRIS/HCl pH 8.4, 62.5 μM dNTPs, 1.5 mM $MgCl_2$, 1.25 units Ampli*Taq* DNA Polymerase (Perkin Elmer, Foster City, Calif.). After pre-heating for 5 min, PCR was carried out at 94 °C for 45 s, at 63 °C for 45 s, and 72 °C for 45 s over 35 cycles. Two microliters of the PCR products was mixed

Fig. 1 For continued legend see next page.



with 6 µl of formamide and heat denatured by boiling for 5 min and chilling on ice. Electrophoresis of the PCR products was performed on an 8% polyacrylamide gel (50:1, polyacrylamide:bisacrylamide) at 4 °C and 5 W for 16 h. SSCP results were analyzed with the MapManager computer program of Jackson Laboratories (Bar Harbor, Me.).

Results

Structural analysis of mouse RDC1

The sequenced cDNA had a size of 1861 bp (GenBank accession no. AF000236). An open reading frame between nucleotides 89 and 1177 was identified which encodes a protein containing 362 amino acids. A hydropathy plot showed the typical features of a G protein-coupled receptor with an extracellular N-terminus, seven-transmembrane-spanning regions, and an intracellular C-terminus. A search in the GenBank showed 88% similarity at the nucleotide level to a human orphan receptor termed RDC1 (Sreedharan et al. 1991). The similarity at the amino acid level was 92%. Similarity to the RDC1 dog receptor (Libert et al. 1989) was 85% and 91% at the nucleotide and amino acid level, respectively (Fig. 1A). There are two long (>50 residue) stretches of complete amino acid conservation (residues 42–94 and 293–360). In contrast to other chemokine receptors which preferentially display variation in the extracellular regions (Heesen et al. 1996a), the 39 amino acid residues of RDC1 which demonstrate species specific variations are evenly distributed among the ectodomains, transmembrane regions, and internal portions of RDC1.

The next highest similarity was between mouse RDC1 and the rabbit IL-8 receptor type B, termed rabCXCR2 (Prado et al. 1994), whose amino acid sequences share 32% identity and 43.3% similarity (Fig. 1B). Similarities involving ten or more consecutive amino acids were observed at four transmembrane regions including mRDC1 residues 82–91, 135–146, 258–269, and 309–318 of the second, third, sixth, and seventh transmembrane region, respectively. These are the domains which express the highest similarity in chemokine receptors (Charo et al. 1994; Power et al. 1995; Raport et al. 1996) . The similarity between mouse RDC1 and mouse CXCR2 is 43.2% at the amino acid level, further supporting the similarity between RDC1 and chemokine receptors.

Potential N-linked glycosylation sites are conserved in the N-terminus (amino acid residues 13 and 22) and in the second extracellular loop (residue 192) of mouse RDC1 (Fig. 1A). This orphan receptor also conserved four cysteine residues, one in each extracellular segment. The cysteines located in the first and second extracellular loop (residues 117 and 196) are present in most seven-transmembrane spanning receptors (Strader et al. 1994). They could build a disulfide bridge to stabilize the protein. The cysteines of the N-terminus and the third extracellular loop

Fig. 1 **A** Alignment of the predicted amino acid sequences of mouse RDC1, human RDC1, and dog RDC1. Areas of identity between mouse and human or dog RDC1 are *shaded*. Transmembrane regions (TMR) 1–7 are indicated by *horizontal bars*. Cysteine residues (*) and potential N-linked glycosylation sites (↓) are marked. **B** Comparison of the predicted amino acid sequences of mouse RDC1 and rabbit CXCR2. Amino acid residues with identity (|), high similarity (:), and moderate similarity (.) are marked

B

```
mRDC1        1 MDVHLFD.YA.EP..GNYSDINWPCNSSDC..IVVDTVQC..PTM.PNKN 41
               |     .: |. |   |.:|. .:    |.|     ..|.  |    .:  |
rabCXCR2     1 MQEFTWENYSYEDFFGDFSNYSY...STDLPPTLLDSAPCRSESLETNSY 47

mRDC1       42 VLLYTLSFIYIFIFVIGMIANSVVVWVNIQAKTTGYDTHCYILNLAIADL 91
               |.| |     || :|.: :: ||.|. | : .:.|    |   |:||||||||
rabCXCR2    48 VVLIT....YILVFLLSLLGNSLVMLVILYSRSTCSVTDVYLLNLAIADL 93

mRDC1       92 WVVITT.PVWVVSLVQHNQWPMGELTCKITHLIFSINLFGSIFFLACMSV 140
                   || |:| | | | |  |   ||: |: :| : |  |||.||
rabCXCR2    94 .LFATTLPIWAASKV.HG.WTFGTPLCKVVSLVKEVNFYSGILLLACISV 140

mRDC1      141 DRYLSITYFTGTSSYKKKMVRRVVCILVWLLAFFVSLPDTYYLKAVTSAS 190
               ||||.| : | |   |: :| : :|: .| ..  .|||   :  |:
rabCXCR2   141 DRYLAIVHATRTMIQKRHLV.KFICLSMWGVSLILSLPILLFRNAI.FPP 188

mRDC1      191 NNETYCRSFYPE..HSIKEW.LIGMELVSVILGFAVPFTIIAIF.Y.FLL 235
               |.   |   | :  .|  .| :: : :.    || .|  :: :| | | |
rabCXCR2   189 NSSPVC...YEDMGNSTAKWRMV.LRILPQTFGFILPL.LVMLFCYVFTL 233

mRDC1      236 ARAM.SA.SGDQEKHSSRKIIFSYVVVFLVCWLPYHFVVLLDIFSIL..H 281
                | :  |  |  :|| . ::||. |.:||.|||||. |.| |  .::  |
rabCXCR2   234 .RTLFQAHMG..QKHRAMRVIFAVVLIFLLCWLPYNLVLLTD..TLMRTH 278

mRDC1      282 YIPFTCQLENVLFTALHVTQCLSLVHCCVNPVLYSFINRNYRYELMKAFI 331
                |  ||: | :  ||  |: |  .| |.||::|.|| . :|| |:|  |
rabCXCR2   279 VIQETCERRNDIDRALDATEILGFLHSCLNPIIYAFIGQKFRYGLLK..I 326

mRDC1      332 FKYSAKTGL.TK..LIDASRVSETEYSALEQ.NTK... 362
                   |  || .| |  || |  : |   ||
rabCXCR2   327 L...AAHGLISKEFLAKESRPS...FVASSSGNTSTTL 358
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(residues 26 and 287) are commonly found among chemokine receptors and presumably form a second stabilizing disulfide bridge (Murphy 1994). The C-terminus contains several conserved serine residues (amino acids 335, 347, 350, and 355) which could be targets of phosphorylation during receptor desensitization (Strader et al. 1994).

Cell and tissue expression of mouse RDC1

Tissues and cell lines of different origins were screened for expression of mouse RDC1 RNA by northern blot (Fig. 2). One band of 2.4 kilobases (kb) was detected in heart, spleen, and kidney. The mesangial tumor line MES-13, astrocytes, and neutrophils were also positive for the RDC1 message. No signal was detected in RNA preparations of thymus, the T-cell lymphoma EL4, the B-cell lymphoma A20, the monocytic cell line WEHI-265.1, the mast-cell clone MC9, or the macrophage tumor P388.D1. A β-actin probe was used to evaluate comparable loading of RNA among samples.

Chromosomal mapping

PCR of genomic DNA of SV129/J mice with primers covering the 5′ and 3′ ends of the coding region gave a product of 1.1 kb identical to the product obtained with

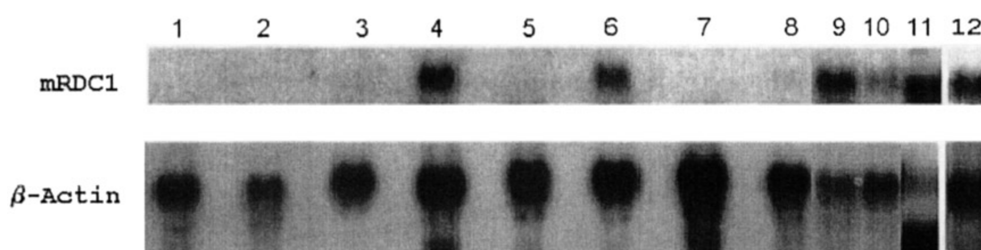

Fig. 2 Northern blot analysis of mouse RDC1 (*top*) and β-actin (*bottom*) expression. *Lanes 1* T cell lymphoma EL4, *2* B cell lymphoma A20, *3* monocytic tumor WEHI-265.1, *4* astrocytes, *5* mast cell clone MC9, *6* mesangial tumor MES-13, *7* macrophage tumor P388.D1, *8* thymocytes, *9* kidney, *10* spleen, *11* heart, and *12* neutrophils

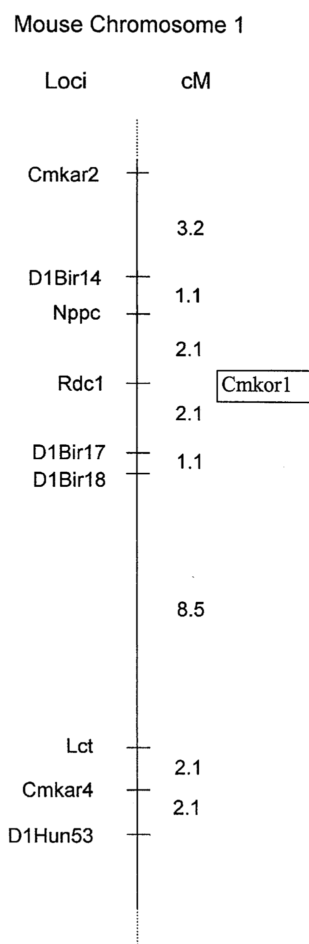

Fig. 3 Schematic representation of part of the map of mouse chromosome 1. The loci of the chemokine receptor genes CXCR2 (*Cmkar2*), CXCR4 (*Cmkar4*), RDC1 (*Rdc1* subsequently renamed *Cmkor1*), and of the flanking markers are indicated. The distance between the genes is given in cM

cDNA (data not shown). Thus, mRDC1 does not have an intron within the coding region. This result is consistent with findings on most other chemokine receptors which lack an intron (Murphy 1994).

A panel of 94 interspecies backcross mice was used to localize the chromosomal positions of the loci for mouse CXCR2 (*Cmkar2*), mouse CXCR4 (*Cmkar4*), and mouse RDC1 (*Rdc1/Cmkor1*). All three genes were found to reside on chromosome 1. The mapping results indicated that the mouse *Cmkor1* locus is situated between the genes for the two known mouse CXC chemokine receptors *Cmkar2* and *Cmkar4*. The recombination frequencies and genetic distances (cM ± SE) are *Cmkar2* to *Cmkor1* 6/87, 5.8 ± 2.5, and *Cmkor1* to *Cmkar4* 13/88, 14.8 ± 3.8. A more comprehensive map including other genes previously mapped to chromosome 1 in this backcross is provided in Fig. 3.

Discussion

The 92% amino acid similarity between mouse and human RDC1 suggests that these receptors are evolutionarily conserved (Sreedharan et al. 1991). This is the highest recorded similarity between human and mouse chemokine receptors. Moreover, a dog homologue of these orphan receptors has also been identified (Libert et al. 1989). The protein sequences of the mouse and dog receptors share 91% similarity. The receptors of all three species contain 362 amino acids contrasting with several other chemokine receptors which vary in length among species. The high level of species conservation especially in the ectodomains is uncommon among chemokine receptors and suggests an equally strong conservation of the ligand.

Sreedharan and co-workers (1991) initially reported human RDC1 to be a receptor for vasoactive intestinal peptide (VIP). However, these data were later retracted (Sreedharan et al. 1993). A ligand for the dog receptor also remains to be identified. The next closest relative of RDC1 is rabbit CXCR2, the IL-8 receptor type B (Prado et al. 1994) which shares 55% identity at the nucleic acid and 32% identity at the amino acid level, pointing to CXC chemokines as potential ligands. Mouse CXCR2 is also homologous to mRDC1, sharing 31.4% amino acid identity. Among the non-chemokine G-protein-coupled seven-transmembrane-spanning receptors, the rat adrenomedullin receptor (Kapas et al. 1995) displays the highest similarity, with 30.8% identity with mRDC1 at the amino acid level.

Another feature suggesting chemokines as the potential ligands of RDC1 comes from the chromosomal mapping of the locus for mouse RDC1 between the genes for the two defined mouse CXC chemokine receptors. In this report we present the first data for the mapping of mouse CXCR4, and RDC1. In addition we demonstrate the linkage of the genes for CXCR2, CXCR4 and RDC1 on chromosome 1. The mapping of mouse CXCR2 is consistent with a previous report (Cerretti et al. 1993) which localized this gene to chromosome 1. The loose linkage of these receptor genes mirrors the mapping of the human CXCR2 and CXCR4 genes on human chromosome 2. The gene for human CXCR2 was localized to chromosome 2q34-35 (Ahuja et al. 1992) while the human CXCR4 gene is also located on chromosome 2 at position q21 (Federsppiel et al. 1993). However, the gene encoding human RDC1 has not been mapped. The CC chemokine receptor genes of mice and humans display close linkage but are localized to different chromosomes: namely, mouse CC chemokine receptors are clustered on chromosome 9 and their human homologues reside on chromosome 3 (Kozak et al. 1995; Samson et al. 1996).

The mRNA for RDC1 was detected in heart, kidney, and spleen. The messages of the human and dog homologue were also found in these organs. RNA preparations of the mesangial cell line MES-13, astrocytes, and neutrophils were also positive for mouse RDC1 by northern blot. The finding that human RDC1 is expressed in brain is also in accord with our results (Sreedharan et al. 1991). RDC1 tissue expression appears to be unique in the family of chemokine receptors but is consistent with observations that chemokines also act on cells of parenchymal origin including astrocytes and mesangial cells (Heesen et al. 1996b; Luo and Dorf 1996; Luo et al. 1996).

The role of chemokines is not restricted to the induction of a migratory response. It was recently demonstrated that SDF-1, a CXC chemokine, has important functions for

embryogenesis (Nagasawa et al. 1996). Mice with a targeted disruption of this gene not only presented an impairment of B cell development and myelopoesis but also a ventricular septal defect. All SDF-1-deficient mice died before or hours after birth (Nagasawa et al. 1996). The potential role of RDC1 and its ligand in organ development remain to be determined.

In summary, RDC1 has structural similarity to CXCR2 and maps to a chromosomal region near mouse CXCR2, suggesting that mouse RDC1 may be a member of the CXC chemokine receptor family. This receptor is highly conserved among species, implying an important biological role. However, the ligands of mouse RDC1 as well as the human and dog homologues await identification.

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