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Molecular cloning, functional characterization and mRNA expression analysis of the murine chemokine receptor CCR6 and its specific ligand MIP-3α

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Abstract We have cloned the murine CCR6 receptor and its ligand, the β-chemokine mMIP-3α. Calcium mobilization assays performed with mCCR6 transfectants showed significant responses upon addition of mMIP-3α. Murine MIP-3α RNA is expressed in thymus, small intestine and colon, whereas mCCR6 RNA is expressed in spleen and lymph nodes. RT-PCR analysis of FACS-sorted lymphoid and antigen presenting cell subsets showed mCCR6 expression mainly in B cells, $CD8^-$ splenic dendritic cells and $CD4^+$ T cells. The cloning and functional characterization of the mCCR6 and mMIP-3α will allow the study of the role of these proteins in mouse models of inflammation and immunity.

Key words: Receptor cloning; Chemokine cloning; Dendritic cell

1. Introduction

Chemokines are a group of small chemoattractant cytokines that play a key role in the trafficking of inflammatory cells [1,2]. The presence or absence of an intervening amino acid residue between two conserved cysteines in the N-terminal region defines the two main subfamilies of chemokines that are called α (or CXC) and β (or CC), respectively. The number of described chemokines is continually increasing and recently the existence of two more subfamilies of chemokines (C and CX_3C) has been postulated [3–5]. A specific subset of the superfamily of seven transmembrane, G protein-coupled receptors are the mediators of the actions of chemokines on their target cells. Many human chemokine receptors have already been identified and their ligand binding characteristics have been established. Five of these receptors (CXCR1–CXCR5) are specific for α-chemokines [6,7], while receptors CCR1–CCR10 bind β-chemokines [1,2,8]. The Duffy red blood antigen, also known as Duffy antigen receptor for chemokines (DARC), is able to bind both α- and β-chemokines [9]. In addition, very recently CX_3CR1, the receptor for fractalkine [10] and XCR1, the receptor for lymphotactin [11] have been reported.

With few exceptions, the chemokine system of receptors and ligands is characterized by promiscuity. Most chemokine receptors are able to bind several chemokines and different chemokines interact with more than one receptor. One of these exceptions is CCR6, a receptor that we and others initially described as orphan [12,13], and which was then reported to be the specific receptor for a β-chemokine independently identified under the names MIP-3α/LARC/Exodus [14–20]. Besides this high specificity for a single ligand, two other features make CCR6 different from other β-chemokine receptors. First, its expression is quite restricted to lymphocytes and dendritic cells (DC), and secondly, the gene encoding CCR6 is located on chromosome 6 [12–17]; all the other mapped β-chemokine receptor genes are clustered on chromosome 3 [8,21,22]. Concerning MIP-3α, no other interaction with chemokine receptors has been found so far, apart from that with CCR6. Also, the MIP-3α gene is placed on chromosome 2 instead of chromosome 17, where most CC chemokine genes are located [14]. The singularity of CCR6 and its ligand prompted us to clone their murine counterparts, with the purpose of taking advantage of murine models to study the role of this unique receptor/ligand pair as potential regulators of the migration and recruitment of antigen presenting and immunocompetent cells during inflammatory and immunological responses.

In this paper we report the cloning of two murine genes whose predicted amino acid sequences are highly homologous to those of hCCR6 or hMIP-3α, respectively. Data showing the activation of mCCR6 transfected cells upon binding of mMIP-3α or hMIP-3α, and the constitutive RNA expression of these genes in tissues as well as in lymphoid and antigen presenting cell subsets are also reported.

2. Materials and methods

2.1. Cells

Thymic and splenic DCs were purified as described [23]. An enriched DC preparation was obtained from a low density cell fraction by immunomagnetic depletion after incubation with anti-CD3, anti-CD4, anti-IL-2Rα, anti-B220, anti-macrophage antigen F4/80, and anti-granulocyte antigen Gr1. $CD8^+$ and $CD8^-$ DC subsets were sorted as $CD8^+$ $CD11c^+$ and $CD8^-$ $CD11c^+$ cells, respectively, using a FACSort cytometer (Becton Dickinson, Mountain View, CA, USA). Langerhans cells were purified as described [24]. Ears were split into dorsal and ventral halves, and incubated with 0.5% trypsin for 30 min at 37°C to allow the separation of the epidermal sheets, which were cultured for 24 h with 100 ng/ml GM-CSF. After this culture period most LCs were released to the culture medium and were subsequently sorted as MHC class II^+ $CD11c^+$ cells. Peritoneal macrophages were sorted as Mac-1^+ F4/80^+ cells from a peritoneal cell preparation. Thymic B cells were isolated from a highly enriched thymic B cell preparation obtained from a low density cell fraction by immunomag-

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The nucleotide sequences of the genes reported in this paper have been submitted to the EMBL database with accession numbers AJ222714 (mCCR6) and AJ222694 (mMIP-3α).

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netic depletion after incubation with anti-CD3, anti-CD4, anti-IL-2Rα, anti-macrophage antigen F4/80, and anti-granulocyte antigen Gr1. B cells were then sorted as $B220^+$ MHC class II^+ cells. B cells, $CD4^+$ T cells and $CD8^+$ T cells were sorted from mesenteric lymph node cell suspensions as $B220^+$ $CD4^-$ $CD8^-$, $CD4^+$ $CD8^-$ $B220^-$ and $CD8^+$ $CD4^-$ $B220^-$ cells, respectively. Double positive thymocytes were sorted as $CD4^+$ $CD8^+$ cells. $CD4^+$ single positive thymocytes were sorted as $CD4^+$ cells after depletion of $CD8^+$ thymocytes. $CD8^+$ single positive thymocytes were sorted as $CD8^+$ cells. Pre-T cells were sorted as $CD4^-$ $CD8^-$ $CD25^+$ cells after depletion of $CD4^+$ and/or $CD8^+$ thymocytes. All the cell subsets were purified from 5–7 weeks old female BALB/c mice. After reanalysis the sorted cell populations had a purity of $>98\%$. Human embryonic kidney 293 cells were obtained from the ATCC and maintained in DMEM supplemented with 10% FCS (Seralab) and antibiotics. Human and murine CCR6 genes were cloned in pCIneo (Promega) and stable transfectants were obtained after G418 selection of cells transfected with the resulting plasmids by the calcium phosphate method, as described [25].

2.2. Cloning of mCCR6

A set of degenerate oligonucleotides was designed based on the conserved sequences in transmembrane domains (TM) 3 and 7 of known chemokine receptors, and used to perform PCR amplifications as described elsewhere [12]. Using mouse genomic DNA as template, PCR products obtained using primers (TM3) 5′-CTCCTGGCCTGY-ATNTCNGTNGAYMGNTAY-3′ and (TM7) 5′-GCCGATRAAN-GCRTANATNATNGGRTTNASRCARCA-3′ gave positive signals in Southern blots with a TM3–TM7 hCCR6 probe. Based on the sequence of this mouse DNA fragment, highly similar to the sequence of the corresponding region in hCCR6, two specific oligonucleotide primers were designed (5′-ATCGTCCAGGCAACCAAATCTTTCC-3′ and 5′-CCGCAGTCACGAGGAGGACCATG-3′) and used to PCR-screen a mouse genomic library in phage P1 (Genome Systems) to obtain the complete coding sequence of mCCR6, that was found to be contained within a 3.5-kbp *Hin*dIII-*Sca*I fragment from the positive clones obtained. To determine the 5′-end cDNA sequence of mCCR6, a DNA sample from a mouse spleen cDNA library (Stratagene) was used as template in PCR amplification experiments with mCCR6-specific and vector primers. An expression plasmid was finally constructed using the pCIneo vector and a DNA fragment containing the mCCR6 coding sequence 5′-flanked by an optimal Kozak motif.

2.3. Cloning of mMIP-3α

One μg of thymus poly(A)$^+$ RNA was reverse transcribed in 25 μl, using the SuperScript II enzyme (Gibco-BRL) as indicated by the supplier. One-tenth of this reaction was added to a 100-μl PCR mixture and amplified for 36 cycles (30 s/94°C; 40 s/50°C; 45 s/72°C) with 5 units of Expand polymerase (Boehringer Mannheim) and the following oligonucleotides: 5′-ACTGGGTACCCAGCACTGAGCA-GAT-3′ and 5′-ATATGAATTCTTGGGCTGTGTCCAATTCCAT-3′. The resulting DNA was treated with *Kpn*I and *Eco*RI, cloned in pBluescript KS and sequenced.

2.4. Chemokines

Recombinant human MIP-3α was purchased from PeproTech. Recombinant mouse TECK and 6Ckine were from R&D. The thrombin receptor agonist peptide SFLLR-amide was purchased from Bachem. Based on the sequence predicted for the mature protein, murine MIP-3α was synthesized on a fully automated peptide synthesizer (Applied Biosystems 430) by Fmoc-chemistry according to standard protocols. Purity and composition of folded and unfolded polypeptides has been confirmed by HPLC on a C-18 Nucleosil 120 analytical column (Tracer), by amino acid analysis using a Beckman 6300 amino acid analyzer, and by mass spectrometry using an ion-trap mass spectrometer model LCQ, with an electrospray interface (Finnigan, ThermoQuest, USA). Folding and disulfide bridges formation of the HPLC products has been carried out according to Clark-Lewis et al. [26].

2.5. Calcium mobilization studies

Calcium fluorometry on 293 transfectant cells was carried out with indo-1 AM-loaded cells as described elsewhere [27]. Each measure was done on 1-ml aliquots containing 3×10^6 cells. Additions of chemokines were done in 10 μl.

2.6. Analysis of gene expression

Total RNA from mouse tissues was extracted with Tri-reagent (Sigma) following the instructions of the supplier. The RNA samples were electrophoresed on a denaturing formaldehyde-agarose gel and blotted onto Nylon Hybond N$^+$ membranes. Pre-hybridization of the membranes and hybridization with ^{32}P-labeled probes (DNA fragments containing the TM3–TM7 region of mCCR6, or the complete coding sequence of mMIP-3α) were carried out in Rapid Hyb buffer (Amersham) as recommended by the supplier. For RT-PCR analysis, total RNA from FACS-sorted cell subpopulations was extracted and reverse-transcribed as indicated above. Equal amounts of cDNA were subjected to PCR amplification of mCCR6 using a forward primer specific to the untranslated 5′ region (5′-CTGCAGTTCGAAGT-CATC-3′) and a reverse primer specific to the mCCR6 open reading frame (5′-GTCATCACCACCATAATGTTG-3′) under the following conditions: 15 s/94°C; 15 s/59°C; 45 s/72°C.

3. Results

3.1. Cloning of mCCR6

We followed a degenerate PCR-based approach for the cloning of the murine CCR6 gene, using oligonucleotide pairs designed according to the conserved sequences in the transmembrane regions 3 and 7 of β-chemokine receptors. The complete open reading frame of the gene was first established by screening a mouse genomic DNA library in phage P1, and then confirmed on a spleen cDNA library. Similarly to what has been described for other members of the family, the complete open reading frame of mCCR6 is contained within a single exon of the gene. The situation is different in the hCCR6 gene, where an intron interrupts the N-terminal coding sequence [15]. Nevertheless, when mCCR6 cDNA was PCR-amplified with oligonucleotides covering the 5′-untranslated region of the cDNA, DNA fragments of different size were synthesized. The nucleotide sequence of some of these DNA fragments was determined, showing that alternate splicing at the 5′ non-coding region had occurred; at least three exons were identified in that DNA region (data not shown). Similar mRNA variants have also been identified in CXCR1, CXCR2, CXCR4, CCR2 and CCR5 [28–30]. The coding region of mCCR6 codes for a protein of 367 amino acid residues, whose predicted sequence is shown in Fig. 1, aligned with those of hCCR6 and six murine β-chemokine receptors. The similarity between the human and murine CCR6 sequences is 73.8% over the entire sequence, clearly higher than that existing between mCCR6 and other murine β-chemokine receptors. In this regard, mCCR6 is more similar to mCCR4 (33.6%), as is also the case when hCCR6 is compared with the other human β-chemokine receptors. The mCCR6 predicted protein is 7 amino acid residues shorter than its human counterpart, as a result of lacking 8 amino acids in the N-terminal region and having one extra amino acid in the C-terminal portion of the molecule. Murine CCR6 has two Asn residues at positions 2 and 35 that are potential substrates for *N*-linked glycosylation. In the case of hCCR6 two extra Asn residues located in the first and third extracellular loops are also potential sites for *N*-linked glycosylation [12].

3.2. Cloning of mMIP-3α

To clone the murine MIP-3α cDNA a PCR-based strategy was followed. An oligonucleotide pair designed to amplify the complete rat MIP-3α coding sequence (GenBank accession number U90447) was used in a reaction in which murine thymus cDNA was used as template. A DNA fragment with

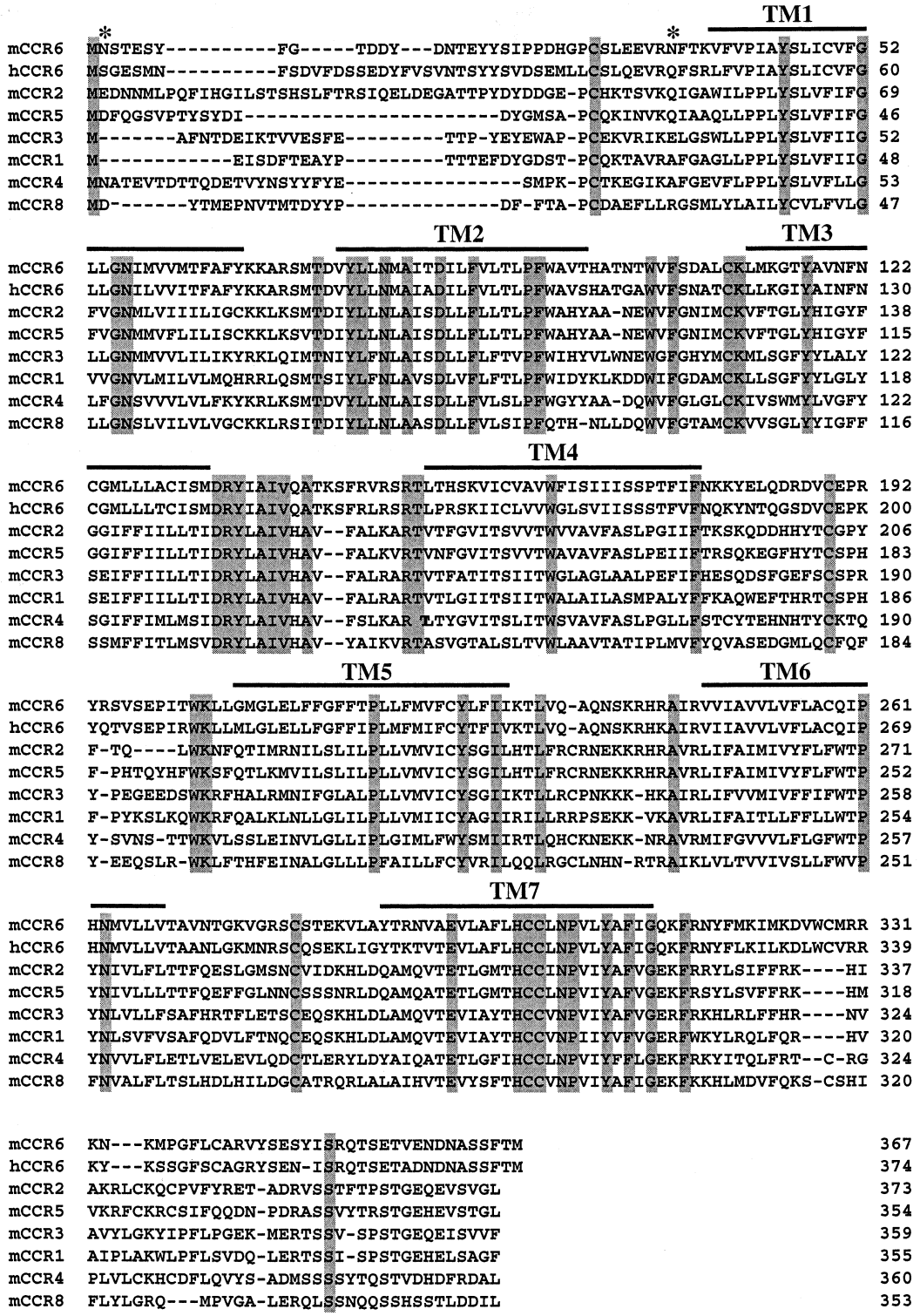

Fig. 1. Amino acid sequence alignment of mCCR6 with hCCR6 and other murine β-chemokine receptors. Identical residues are marked with grey boxes. Gaps are marked by dashes. TM, transmembrane domains. Potential *N*-glycosylation sites in the mCCR6 sequence are marked by an asterisk.

the expected size for a potential murine MIP-3α coding sequence was obtained and cloned. An open reading frame coding for a protein of 97 amino acids was identified, the first 27 residues corresponding to the predicted signal peptide (MACGGKRLLFLALAWVLLAHLCSQAEA↓ASNYDCC-L...). Sequencing of additional clones showed that a 3-bp deletion in the 5′-region of the coding sequence was present in 50% of them, thus predicting the absence of the Ala-28 residue. This indicates that such a variation may correspond to the existence of an allelic polymorphism rather than a change introduced in the PCR process. The sequence of the predicted 97 amino acid murine protein is highly similar to

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mMIP-3α            ASNYDCCLSYIQTPL-PSRAIV-GFTRQMADEACDIN-AIIFHTKK--RKSVCADPKQNWVKRAVNLLSLRVKKM
rMIP-3α            ASNFDCCLTYTKNVYHHARNFV-GFTTQMADEACDIN-AIIFHLKS--KRSVCADPKQIWVKRILHLLSLRTKKM
hMIP-3α            ASNFDCCLGYTDRILHP-KFIV-GFTRQLANEGCDIN-AIIFHTKK--KLSVCANPKQTWVKYIVRLLSKKVKNM
mTECK           QGAFEDCCLGYQHRIKWNVLRHARNYHQQEVSGSCNLR-AVRFYFR---QKVVCGNPEDMNVKRAIRILTARKRLVHWKSA*
mEXODUS-2       SDGGGQDCCLKYSQKKIPYSIVFTRGYRKQEPSLGCPIP-AILFLPRKHSKPELCANPEEGWVQNLMRRLDQPPAPGKQSP*
mC10-like    *HMGFQD-SSDCCLSYNSRI--QCSRFI-GYFP--TSGGCTRP-GIIFISKR--GFQVCANPSDRRVQRCIERLEQNSQPRTYKQ
mC10         *HQGFQDTSSDCCFSYATQI--PCKRFI-YYFP--TSGGCIKP-GIIFISRR--GTQVCADPSDRRVQRCLSTLKQGPRSGNKVIA
mTCA-3        KS-MLTVSNSCCLNTLKKEL-PLKFIQ-CYRK--MGSSCPDPPAVVFRLNK--GRESCASTNKTWVQNHLKKVNPC
mRANTES       SPYGSDTT-PCCFAYLSLAL-PRAHVK-EY-F-YTSSKCSNL-AVVFVTRR--NRQVCANPEKKWVQEYINYLEMS
mMIP-1α       APYGADTPTACCFSYSRKI--PRQFIV-DYFE--TSSLCSQP-GVIFLTKR--NRQICADSKETWVQEYITDLELNA
mFIC          QPDGPNAS-TCCY-VKKQKI-PKRNLK-SYRR-ITSSRCPWE-AVIFKTKK--GMEVCAEAHQKWVEEAIAYLDMKTPTPKP
mMCP-5        GPDAVSTPVTCCYNVVKQKI-HVRKLK-SYRR-ITSSQCPRE-AVIFRTIL--DKEICADPKEKWVKNSINHLDKTSQTFILEPSCLG
mJE           QPDAVNAPLTCCYSFTSKMI-PMSRLE-SYKR-ITSSRCPKE-AVVFVTKL--KREVCADPKKEWVQTYIKNLDRNQMR--SEPTTLF*
mEOTAXIN      HPGSI--PTSCCFIMTSKKI-PNTLLK-SYKR-ITNNRCTLK-AIVFKTRL--GKEICADPKKKWVQDATKHLDQKLQTPKP
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Fig. 2. Amino acid sequence alignment of mMIP-3α with related β-chemokines. The sequences of the predicted mature proteins are shown. Identical residues are marked with grey boxes. Gaps are marked by dashes. m, murine; h, human; r, rat. (*) The N-terminal extensions of mC10-like (QITHATETKEVQSSLKAQQGLEIEM) and mC10 (GLIQEMEKEDRRYNPPII), and the C-terminal extensions of mTECK (SDSQTERKKSNHMKSKVENPNSTSVRSATLGHPRMVMMPRKTNN), mEXODUS-2 (GCRKNRGTSKSGKKGKGSKGCKRTEQTQPS-RG), and mJE (KTASALRSSAPLNVKLTRKSEANASTTFSTTTSSTSVGVTSVTVN) have been omitted in the alignment for clarity.

that of rat (65.8%) and human (65.3%) MIP-3α, thus suggesting that we had indeed cloned the murine MIP-3α cDNA. Fig. 2 shows a comparison of the predicted mature amino acid sequences of the murine, rat and human MIP-3α proteins, along with those of other murine β-chemokines. The similarity of mMIP-3α to other murine chemokines is relatively low, ranging from 28% to mExodus-2 (without considering the long C-terminal extension of mExodus) to 23% to mRANTES. As hMIP-3α, mMIP-3α has the majority of the conserved features of β-chemokines, including the four characteristic cysteine residues, and amino acids Phe-39, Ala-49, Trp-55 and Val-56. As predicted for the human and rat proteins, mMIP-3α has Phe-23 and Ala-28, two positions that in most β-chemokines are occupied by Tyr and Thr residues, respectively.

3.3. *MIP-3α-induced calcium mobilization in 293/CCR6 cells*

For the functional characterization of mCCR6, murine MIP-3α was chemically synthesized as described in Section 2. The interaction of mMIP-3α with mCCR6 was studied by measuring changes in the intracellular calcium concentration of 293 cells stably transfected with an mCCR6 expression plasmid (293/mCCR6). As shown in Fig. 3A (upper panel), the addition of mTECK or m6Ckine, two recombinant murine β-chemokines, to 293/mCCR6 cells did not result in detectable calcium mobilization whereas subsequent addition of 100 nM mMIP-3α induced a significant wave of calcium mobilization. Murine MIP-3α did not induce a calcium wave in control 293 cells transfected with the void pCIneo vector (293/neo), whereas these cells were shown to be able to respond to a different, non-chemokine peptide such as SFLLR-amide, for which they express an endogenous receptor [31] (Fig. 3A, lower panel). The ability of mCCR6 to bind other mouse chemokines such as mC10 and mMCP-5 was also tested with negative results (data not shown). The calcium response of 293/mCCR6 cells to mMIP-3α was detectable at a chemokine concentration as low as 0.1 nM, and was dose-dependent (Fig. 3B), with a half-maximal effect at 10 nM. The 293/mCCR6 cells also responded to the addition of human MIP-3α, and this stimulus partially reduced the ability of

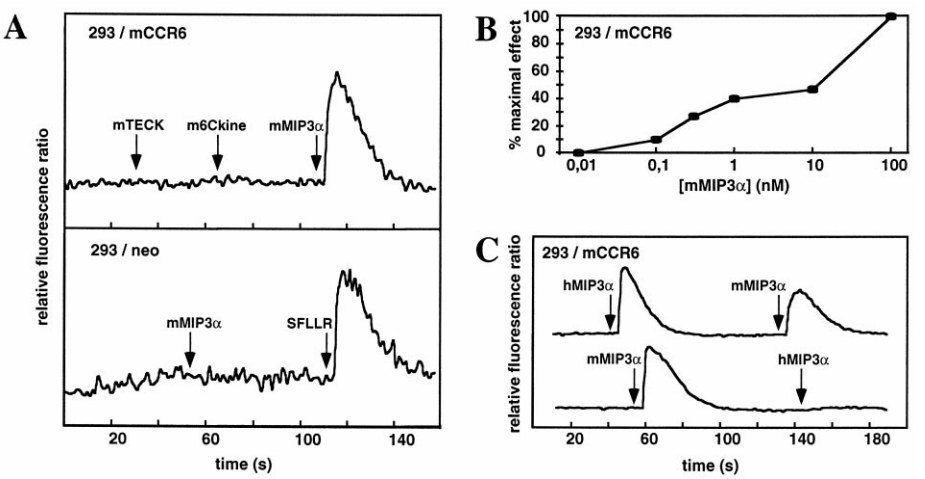

Fig. 3. MIP-3α-induced calcium mobilization on indo-1 AM-loaded 293 transfectant cells. A: Upper panel: 293/mCCR6 cells were stimulated with 72 nM mTECK followed by 84 nM m6Ckine and 100 nM mMIP-3α. Lower panel: Control pCIneo-transfected 293 cells loaded similarly with indo-1 AM were stimulated with 100 nM mMIP-3α followed by 27 μM SFLLR-amide, a molecule for which 293 cells express endogenous receptors. B: Dose-response of 293/mCCR6 cells to mMIP-3α. C: 293/mCCR6 cells were sequentially stimulated with 10 nM hMIP-3α and 10 nM mMIP-3α as indicated. Arrows indicate the time of the additions. Variations in the relative fluorescence ratios reflect changes in intracellular free $[Ca^{2+}]$.

the cells to respond to a subsequent addition of mMIP-3α; conversely, addition of mMIP-3α completely desensitized the cells to a subsequent addition of hMIP-3α (Fig. 3C). 293 cells stably transfected with a human CCR6 expression plasmid also responded to mMIP-3α, although less efficiently than to hMIP-3α (data not shown). All these assays were also performed using conditioned medium from 293 cells transfected with an expression plasmid encoding mMIP-3α as a source of natural mMIP-3α. The results (not shown) were essentially identical to those obtained with synthetic mMIP-3α.

3.4. Tissue distribution of mCCR6 and mMIP-3α

The distribution of the mCCR6 and mMIP-3α messages was studied by Northern blot of total RNA samples obtained from different mouse tissues. As shown in Fig. 4, the expression of both genes is quite restricted. mCCR6 is mainly expressed in spleen and lymph nodes, where a transcript of about 3.5 kb could be detected. Concerning the mMIP-3α gene, a transcript of about 1 kb was detected in thymus, colon and small intestine.

3.5. RT-PCR analysis of mCCR6 mRNA expression by lymphoid and antigen presenting cell subsets

Fig. 5 shows the expression of mCCR6 analyzed by RT-PCR of FACS-sorted lymphoid and antigen presenting cell subsets. mCCR6 expression was found in B cells isolated from the thymus, spleen and lymph nodes. Among thymocytes mCCR6 appears to be expressed solely by $CD4^+$ single positive cells, but not by $CD25^+$ $CD4^-$ $CD8^-$ pre-T cells, $CD4^+$ $CD8^+$ double positive immature thymocytes or $CD8^+$ single positive cells. Correspondingly, mCCR6 mRNA was found in $CD4^+$ but not $CD8^+$ peripheral T cells isolated from the spleen or lymph nodes. With regard to DC, in addition to epidermal Langerhans cells, in the murine system two main DC subtypes, differing in their phenotype and origin, have been defined: $CD8^+$ lymphoid DC and $CD8^-$ DC [32]. Interestingly, whereas no mCCR6 mRNA was found in epidermal Langerhans cells or in $CD8^+$ DC isolated from the thymus or spleen, splenic $CD8^-$ DC were positive for this chemokine receptor. Finally, no CCR6 expression was detected in macrophages purified from the peritoneal cavity.

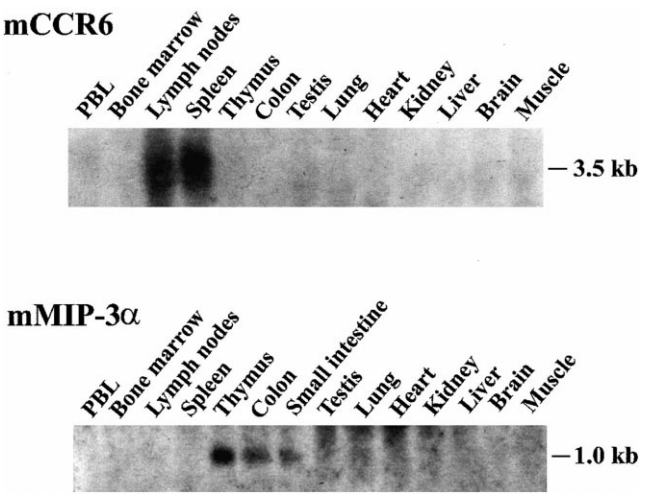

Fig. 4. Tissue distribution of mCCR6 and mMIP-3α RNAs. Northern blotting experiments were performed with 10 μg of total RNA obtained from adult mouse tissues, and probed as described in Section 2. Transcript size in kilobases is shown on the right. After the transfer, methylene blue staining of the membranes was performed to estimate the amount of RNA present in each lane (not shown).

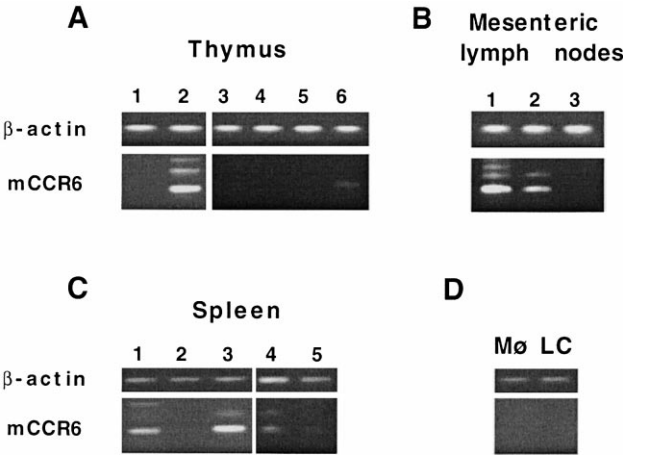

Fig. 5. RT-PCR analysis of mCCR6 expression in FACS-sorted lymphoid and antigen presenting cell subpopulations. Total RNA was isolated from the indicated FACS-sorted cell subsets, reverse-transcribed and subjected to PCR amplification using the mCCR6 specific primers described in Section 2. In addition, amplifications of β-actin were performed with the different samples as an internal control. A: Thymus cell types: 1, $CD8^+$ DC; 2, B cells; 3, $CD4^-$ $CD8^-$ double negative cells; 4, $CD4^+$ $CD8^+$ double positive cells; 5, $CD8^+$ $CD4^-$ single positive cells; 6, $CD8^-$ $CD4^+$ single positive cells. B: Mesenteric lymph node cell types: 1, B cells; 2, $CD4^+$ T cells; 3, $CD8^+$ T cells. C: Spleen cell types: 1, $CD8^-$ DC; 2, $CD8^+$ DC; 3, B cells; 4, $CD4^+$ T cells; 5, $CD8^+$ T cells. D: Peritoneal macrophages (Mø), and Langerhans cells (LC).

4. Discussion

We have isolated and characterized mCCR6 and mMIP-3α, two new members of the murine family of β-chemokine receptors and ligands. The predicted amino acid sequence of mCCR6 corresponds to a protein of 367 amino acids, with many of the conserved motifs of this family of receptors. Concerning mMIP-3α, it is interesting to point out the existence of a unique Asp-Cys-Cys-Leu motif that is also shared by hMIP-3α, hExodus-2 and mExodus-2, mTECK and the mC10-like protein [33]. Actually, in computer-generated phylogenetic trees these chemokines are grouped together (not shown). Presently it is not known whether this singularity reflects some common functional properties for this small group of β-chemokines. We have detected the existence of a mMIP-3α allele in which a 3-bp deletion is present, thus predicting the absence of one alanine residue located in the region around the signal peptide cleavage site. Since N-terminal sequence variations in chemokines have been reported to have important effects in their activities and receptor binding properties [34–37], it would be of interest to know whether the different mMIP-3α polypeptides behave differently. Interestingly, a similar situation has also been reported for the hMIP-3α gene, and, indeed, the long version of hMIP-3α has been shown to be a better chemoattractant for T cells than a short version of the protein, lacking the N-terminal Ala residue [16]. The fact that this structural dimorphism is conserved among two evolutionarily distant species as mice and humans suggests that it may have a relevant physiological role.

For the functional characterization of mCCR6 and mMIP-3α, calcium fluorometry studies were performed on stable 293/mCCR6 transfectant cells. The results showed that mCCR6 was able to transduce a strong calcium signal upon specific stimulation with chemically synthesized mMIP-3α. These data support the conclusion that the cloned mCCR6 and mMIP-3α indeed are both structural and functional homologs for hCCR6 and hMIP-3α. Human MIP-3α was also able to stimulate 293/mCCR6 cells, an interspecies crossreactivity that is consistent with the high degree of homology between the amino acid sequences of both chemokines (65.3%). Accordingly, the stimulation of 293/mCCR6 cells with either murine or human MIP-3α resulted in desensitization to a subsequent stimulus with the other one. However, mMIP-3α was a much more efficient desensitizer to hMIP-3α than the converse, suggesting that the crossreactivity of MIP-3α is limited. Studies on hCCR6 transfected 293 cells also indicated a preference for its homolog ligand (data not shown). The study of the potential role played by G proteins in the signal transduction pathway of mCCR6 was approached by incubating 293/mCCR6 cells with or without pertussis or choleric toxins prior to calcium fluorometry. No significant effects were noticed (data not shown). This contrasts with the behavior of most chemokine receptors, which are sensitive to inhibition of calcium signalling by pertussis toxin. However, the result is consistent with the reported lack of effect of pertussis toxin on the hMIP-3α-induced stimulation of hCCR6-transfected 293 cells [15].

The constitutive tissue distribution of the mCCR6 RNA seems to be restricted to spleen and lymph nodes, where a transcript of about 3.5 kb is expressed. This pattern of tissue expression is very similar to the one that we and others described for hCCR6 [12–14]. Semi-quantitative RT-PCR analysis of mCCR6 expression revealed that this chemokine receptor is mainly expressed by B cells, $CD8^-$ splenic DC and to a lesser extent by $CD4^+$ T cells. This restricted expression of mCCR6 is in accordance with a recent report by Greaves et al. [17] showing that hCCR6 was expressed by human DC derived from $CD34^+$ cord blood precursors, but not by monocyte-derived DC. With regard to the selective expression of mCCR6 by $CD8^-$ DC and B cells, these data suggest a possible role of CCR6 in the recruitment and positioning of defined subsets of lymphocytes and antigen presenting cells within lymphoid organs. In this sense, it has been reported that in the spleen $CD8^-$ DC are mainly located in the marginal zone [38], where they co-localize with a subset of specialized marginal zone B cells. In addition, a lineage relationship between B cells and $CD8^-$ DC has been proposed on the basis of the phenotype of mice homozygous for an Ikaros null mutation, in which T cells, $CD8^+$ DC and myeloid cells are produced, but neither B cells, $CD8^-$ DC, nor NK cells are produced [39].

In summary, we have cloned and functionally characterized the mouse homologs of the human CCR6 and MIP-3α genes. Based on its restricted mRNA expression and its selectivity for ligand binding, CCR6 is presumed to be an important target for modulating the immune response by controlling the recruitment and positioning of lymphocytes and specialized antigen presenting cells within secondary lymphoid organs. Our data show that the mouse and human genes have similar characteristics, thus making the murine model a suitable one for studying the biological functions of this receptor and its ligand. CCR6 as well as MIP-3α knockout mice are being generated in our lab to help in these studies.

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References

[1] Rollins, B.J. (1997) Blood 90, 909–928.
[2] Luster, A.D. (1998) New Engl. J. Med. 338, 436–445.
[3] Kelner, G.S., Kennedy, J., Bacon, K.B., Kleyensteuber, S., Largaespada, D.A., Jenkins, N.A., Copeland, N.G., Bazan, J.F., Moore, K.W., Schall, T.J. and Zlotnik, A. (1994) Science 266, 1395–1399.
[4] Pan, Y., Lloyd, C., Zhou, H., Dolich, S., Deeds, J., Gonzalo, J.-A., Vath, J., Gosselin, M., Ma, J., Dussault, B., Woolf, E., Alperin, G., Culpepper, J., Gutiérrez-Ramos, J.C. and Gearing, D. (1997) Nature 387, 611–617.
[5] Bazan, J.F., Bacon, K.B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D.R., Zlotnik, A. and Schall, T.J. (1997) Nature 385, 640–644.
[6] Gunn, M.D., Ngo, V.N., Ansel, K.M., Ekland, E.H., Cyster, J.G. and Williams, L.T. (1998) Nature 391, 799–803.
[7] Legler, D.F., Loetscher, M., Roos, R.S., Clark, L.I., Baggiolini, M. and Moser, B. (1998) J. Exp. Med. 187, 655–660.
[8] Bonini, J.A., Martin, S.K., Dralyuk, F., Roe, M.W., Philipson, L.H. and Steiner, D.F. (1997) DNA Cell Biol. 16, 1249–1256.
[9] Szabo, M.C., Soo, K.S., Zlotnik, A. and Schall, T.J. (1995) J. Biol. Chem. 270, 25348–25351.
[10] Imai, T., Hieshima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., Kakizaki, M., Takagi, S., Nomiyama, H., Schall, T.J. and Yoshie, O. (1997) Cell 91, 521–530.
[11] Yoshida, T., Imai, T., Kakizaki, M., Nishimura, M., Takagi, S. and Yoshie, O. (1998) J. Biol. Chem. 273, 16551–16554.
[12] Zaballos, A., Varona, R., Gutierrez, J., Lind, P. and Marquez, G. (1996) Biochem. Biophys. Res. Commun. 227, 846–853.
[13] Liao, F., Lee, H.H. and Farber, J.M. (1997) Genomics 40, 175–180.
[14] Baba, M., Imai, T., Nishimura, M., Kakizaki, M., Takagi, S., Hieshima, K., Nomiyama, H. and Yoshie, O. (1997) J. Biol. Chem. 272, 14893–14898.
[15] Liao, F., Alderson, R., Su, J., Ullrich, S.J., Kreider, B.L. and Farber, J.M. (1997) Biochem. Biophys. Res. Commun. 236, 212–217.
[16] Power, C.A., Church, D.J., Meyer, A., Alouani, S., Proudfoot, A.E., Clark-Lewis, I., Sozzani, S., Mantovani, A. and Wells, T.N.C. (1997) J. Exp. Med. 186, 825–835.
[17] Greaves, D.R., Wang, W., Dairaghi, D.J., Dieu, M.C., de Saint-Vis, B., Franz- Bacon, K., Rossi, D., Caux, C., McClanahan, T., Gordon, S., Zlotnik, A. and Schall, T.J. (1997) J. Exp. Med. 186, 837–844.
[18] Rossi, D.L., Vicari, A.P., Franz, B.K., McClanahan, T.K. and Zlotnik, A. (1997) J. Immunol. 158, 1033–1036.
[19] Hieshima, K., Imai, T., Opdenakker, G., Van Damme, J., Kusuda, J., Tei, H., Sakaki, Y., Takatsuki, K., Miura, R., Yoshie, O. and Nomiyama, H. (1997) J. Biol. Chem. 272, 5846–5853.
[20] Hromas, R., Gray, P.W., Chantry, D., Godiska, R., Krathwohl, M., Fife, K., Bell, G.I., Takeda, J., Aronica, S., Gordon, M., Cooper, S., Broxmeyer, H.E. and Klemsz, M.J. (1997) Blood 89, 3315–3322.
[21] Samson, M., Stordeur, P., Labbe, O., Soularue, P., Vassart, G. and Parmentier, M. (1996) Eur. J. Immunol. 26, 3021–3028.
[22] Samson, M., Soularue, P., Vassart, G. and Parmentier, M. (1996) Genomics 36, 522–526.
[23] Ardavin, C., Waanders, G., Ferrero, I., Anjuere, F., Acha, O.H. and MacDonald, H.R. (1996) J. Immunol. 157, 2789–2794.
[24] Lenz, A., Heine, M., Schuler, G. and Romani, N. (1993) J. Clin. Invest. 92, 2587–2596.

[25] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1989) in: Current Protocols in Molecular Biology, Vol. 2, Green, New York, NY.
[26] Clark-Lewis, I., Vo, L., Owen, P. and Anderson, J. (1997) Methods Enzymol. 287, 233–250.
[27] Goya, I., Gutierrez, J., Varona, R., Kremer, L., Zaballos, A. and Marquez, G. (1998) J. Immunol. 160, 1975–1981.
[28] Ahuja, S.K., Shetty, A., Tiffany, H.L. and Murphy, P.M. (1994) J. Biol. Chem. 269, 26381–26389.
[29] Heesen, M., Berman, M.A., Hopken, U.E., Gerard, N.P. and Dorf, M.E. (1997) J. Immunol. 158, 3561–3564.
[30] Raport, C.J., Gosling, J., Schweickart, V.L., Gray, P.W. and Charo, I.F. (1996) J. Biol. Chem. 271, 17161–17166.
[31] Hollenberg, M.D., Saifeddine, M., Al, A.B. and Kawabata, A. (1997) Can. J. Physiol. Pharmacol. 75, 832–841.
[32] Shortman, K. and Caux, C. (1997) Stem Cells 15, 409–419.
[33] Hromas, R., Kim, C.H., Klemsz, M., Krathwohl, M., Fife, K., Cooper, S., Schnizlein, B.C. and Broxmeyer, H.E. (1997) J. Immunol. 159, 2554–2558.
[34] Zhang, Y.J., Rutledge, J. and Rollins, B.J. (1994) J. Biol. Chem. 269, 15918–15924.
[35] Clark-Lewis, I., Dewald, B., Loetscher, M., Moser, B. and Baggiolini, M. (1994) J. Biol. Chem. 269, 16075–16081.
[36] Gong, J.-H. and Clark-Lewis, I. (1995) J. Exp. Med. 181, 631–640.
[37] Proudfoot, A.E., Power, C.A., Hoogewerf, A.J., Montjovent, M.O., Borlat, F., Offord, R.E. and Wells, T.N. (1996) J. Biol. Chem. 271, 2599–2603.
[38] Pulendran, B., Lingappa, J., Kennedy, M.K., Smith, J., Teepe, M., Rudensky, A., Maliszewski, C.R. and Maraskovsky, E. (1997) J. Immunol. 159, 2222–2231.
[39] Wu, L., Nichogiannopoulou, A., Shortman, K. and Georgopoulos, K. (1997) Immunity 7, 483–492.