Organization and Differential Expression of the Human Monocyte Chemoattractant Protein 1 Receptor Gene

EVIDENCE FOR THE ROLE OF THE CARBOXYL-TERMINAL TAIL IN RECEPTOR TRAFFICKING*

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Two forms of the monocyte chemoattractant protein-1 receptors (the type A monocyte chemoattractant protein 1 (MCP-1) receptor CCR2A and the type B MCP-1 receptor (CCR2B) have been recently cloned and found to differ only in their terminal carboxyl tails. Here, we report that the two isoforms are alternatively spliced variants of a single MCP-1 receptor gene. Sequencing of the gene revealed that the 47-amino acid carboxyl tail of CCR2B was located in the same exon as the seven transmembrane domains of the receptor, and the 61-amino acid tail of CCR2A was in a downstream exon. Examination of freshly isolated human monocytes by reverse transcriptase-polymerase chain reaction revealed that CCR2B was the predominant isoform and that message levels of both CCR2A and CCR2B decreased as the monocytes differentiated into macrophages. In stably transfected cell lines, CCR2B trafficked well to the cell surface, but CCR2A was found predominately in the cytoplasm. Equilibrium binding studies revealed that those CCR2A receptors that successfully trafficked to the cell surface bound MCP-1 with high affinity (Kd = 310 pM), similar to CCR2B. In signaling studies, both CCR2A and CCR2B mediated agonist-dependent calcium mobilization, as well as inhibition of adenylyl cyclase. Creation of chimeras between CCR2A and the human thombin receptor revealed that the cytoplasmic retention of CCR2A was due to its terminal carboxyl tail. Progressive truncation of the carboxyl tail indicated that a cytoplasmic retention signal(s) was located between residues 316 and 349. These data indicate that the alternatively spliced form of the human MCP-1 receptor (CCR2A) binds MCP-1 with high affinity and is a functional receptor and that expression at the cell surface is controlled by amino acid sequences located in the terminal carboxyl tail.

Chemokines are small (8–10 kDa), secreted basic peptides that are involved in the directed migration and activation of leukocytes (see Refs. 1–4 for recent reviews). They can be divided into two groups based on the arrangement of the first two of four conserved cysteines. The α- or “C-X-C” branch is characterized by the presence of a single amino acid between the first two cysteines and includes interleukin-8 (IL-8),1 GRO (α, β, and γ), NAP-2, and platelet factor 4. In the β- or “C-C” branch, the first two cysteines are adjacent. Members of the β-branch include monocyte chemoattractant protein 1, 2, and 3 (MCP-1, MCP-2, and MCP-3), RANTES (Regulated on Activation Normal T cell Expressed and Secreted), macrophage inflammatory proteins 1α and 1β (MIP-1α and MIP-1β), and eotaxin. In general, C-X-C chemokines are chemotactic for neutrophils, whereas the C-C chemokines are chemoattractants for monocytes and lymphocytes. The recently identified peptide eotaxin is specific for eosinophils (5). A novel peptide, lympho-tactin, which lacks the first and third cysteines, has been isolated from progenitor T cells (6) and may represent the first member of a third branch of the chemokine family.

We recently reported the cloning of two MCP-1 receptors, type A (CCR2A) and type B (CCR2B), that differed only in their terminal carboxyl tails, suggesting that they were derived from a single gene via alternative splicing (7). Inspection of the amino acid sequence of the type B carboxyl tail revealed a 32% identity with both the MIP-1α/RANTES receptor (CCR1 (8)) and the eotaxin receptor (CCR3 (9, 10)), as well as a 21% identity with CCR4 (11). In contrast, this region of CCR2A bore little resemblance to other chemokine receptors. In functional studies, both forms of the MCP-1 receptor conferred robust, agonist-dependent calcium mobilization in Xenopus oocytes (7). In human embryonic kidney (HEK)-293 cells, the type B receptor was shown to inhibit adenyl cyclase and rapidly mobilize intracellular calcium via coupling to Gαo (12). Signaling studies of the type A receptor, however, were hampered by its relatively poor surface expression in transfected cells.

To further elucidate the biology of CCR2A, we have cloned and sequenced the human MCP-1 receptor gene. We have examined the relative expression of CCR2A and CCR2B in monocyte cell lines and in human monocyte/macrophages and have established that the signal for cytoplasmic retention of CCR2A is found in its carboxyl tail. Finally, we report that type A recep-

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1 The abbreviations used are: IL-8, interleukin-8; CCR, C-C chemokine receptor; CCR2A, the type A MCP-1 receptor; CCR2B, the type B MCP-1 receptor; FITC, fluorescein isothiocyanate; HEK, human embryonic kidney; MCP-1, monocyte chemoattractant protein 1; MCP-1R, monocyte chemoattractant protein 1 receptor; MIP-1α, macrophage inflammatory protein 1α; RT-PCR, reverse transcriptase-polymerase chain reaction; PBS, phosphate-buffered saline; RANTES, regulated on activation, normal T expressed and secreted; UTR, untranslated region; ELISA, enzyme-linked immunosorbent assay; bp, base pair(s); kb, kilobase pair(s).
tors that successfully traffic to the cell surface bind MCP-1 with high affinity and couple to Gαo, to mediate signal transduction.

MATERIALS AND METHODS
Reagents—MCP-1 was obtained from R&D Systems, Inc. (Minneapolis, MN). Indo-1 AM was purchased from Molecular Probes, Inc. (Eugene, OR). Lipofectamine, G418, and minimal essential medium with Earle’s balanced salt solution were obtained from Life Technologies, Inc. M1 and M2 monoclonal antibodies to the Flag epitope were purchased from Kodak. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody was purchased from Calbiochem. Flow cytometry grade FITC-conjugated goat anti-mouse antibody was from Zymed (South San Francisco, CA). Ficoll/Hypaque was from Pharmacia Bio-Tech Inc. Horseradish peroxidase-conjugated goat anti-mouse antibody was obtained from Bio-Rad. Epitope-tagged thrombin receptor (13) was generously provided by Dr. Shaun Coughlin (University of California, San Francisco).

Isolation of Genomic Clones—A Lambda FIX II human genomic library (Stratagene, San Diego, CA) was screened with a full-length CCR2B cDNA probe under conditions of high stringency. A total of 1.3 million plaques were screened from which four independent clones were isolated. Hybridizations were carried out in 50 ml of 50% formamide, 0.1 mg/ml rat liver DNA, 0.1 mg/ml salmon sperm DNA, and 5 × 10⁶ cpm/ml radiolabeled probe at 42 °C for 14–20 h. The cDNA probe was labeled with [α-32P]dCTP by the random priming method (Prime-It kit, Stratagene) to a specific activity of 10⁸–10⁹ cpm/μg. Filters were prehybridized in the same solution at 42 °C for 1–2 h before addition of the probe. Membranes were washed by rinsing with 100 ml of a 0.2 × SSC, 0.1% SDS solution at room temperature for 30 min, followed by washing at 65 °C for 2 h. The positively hybridizing plaques were purified. From one clone (AFixII-2A), overlapping EcoRI and PstI fragments were subcloned into pBSSK and sequenced to generate a map of the gene. Restriction enzyme digests and sequence analysis of a second clone (AFixII-17A), as well as Southern hybridization analysis of all four genomic clone isolates, were performed to confirm our map of the MCP-1 receptor gene. The MCP-1 receptor genomic sequence has been deposited in GenBank (accession numbers U80923 and U80924).

Construction of Epitope-tagged MCP-1 Receptors, MCP-1 Receptor Mutants, and Chimeras—The sequence encoding the “Flag” epitope (DYKDDDD) preceded by the prolactin signal sequence (MDSKGS- SQKGSBRLLLLLVSNLLQCGVVS) (13) was ligated onto the 5′ end of the CCR2A and CCR2B cDNAs. These modified cDNAs were subcloned into the mammalian expression vector pEITN (Invitrogen, San Diego, CA). MCP-1RA receptor mutants with the cytoplasmic tail terminating at either position 316 or 349 were made using a two-step overlapping PCR mutagenesis procedure (15). The described (14). Thrombin/MCP-1 receptor chimeras were constructed using a two-step overlapping PCR mutagenesis procedure (15). The described (14). Thrombin/MCP-1 receptor chimeras were constructed using a two-step overlapping PCR mutagenesis procedure (15). The described (14). Thrombin/MCP-1 receptor chimeras were constructed using a two-step overlapping PCR mutagenesis procedure (15). The described (14). Thrombin/MCP-1 receptor chimeras were constructed using a two-step overlapping PCR mutagenesis procedure (15).

Isolation of Human Monocytes and Macrophages—Buffy coats were purchased from Irwin Memorial Blood Bank (San Francisco, CA). Human monocytes and macrophages were isolated and prepared as described (16). Cells were washed several times to remove the Percoll, and monocytes were quick frozen. The cells were greater than 80% monocytes, as determined by neutral red uptake. To obtain macrophages, monocytes were cultured in RPMI 1640 with 10% autologous serum for the indicated number of days.

mRNA Isolation, Reverse Transcription, and PCR—mRNAs from monocytes, macrophages, and cultured cell lines were isolated using Trizol reagent (Life Technologies, Inc.) following the manufacturer’s instructions. Total RNA was treated with RNase-free DNase (Boehringer Mannheim) at 0.1 units/μg total RNA at 37 °C for 15 min. RT-PCR was performed using a GeneAmp RNA PCR kit (Perkin-Elmer), according to the manufacturer’s instructions, and 1 μg of DNase-treated RNA. PCR conditions for first strand synthesis were 42 °C for 15 min, 99 °C for 5 min, and 5 °C for 5 min using the Perkin-Elmer GeneAmp PCR system 9600. Second step PCR conditions were incubation at 95 °C for 10 s, 95 °C for 15 s, and 60 °C for 30 s. After 34 cycles, the reaction was terminated by heating to 72 °C for 7 min. The resulting products were analyzed on a 2% agarose gel. For detection of CCR2A and CCR2B, a common 5′ primer was used in conjunction with two carboxyl tail-specific 3′-anti-sense primers. In control experiments using CCR2A and CCR2B plasmid DNA, we identified PCR primer pairs that amplified both receptors equally.

Differential Expression of the MCP-1 Receptor—We have previously identified two MCP-1 receptor cDNAs (CCR2A and CCR2B) that differed only in the predicted amino acid sequence of their terminal carboxyl tails (7). To test the hypothesis that these cDNAs represented alternatively spliced variants of a single gene and to elucidate potential splicing mechanisms, we sequenced 1.3 million plaques from which four independent clones were isolated. Hybridizations were carried out in 50 ml of 50% formamide, 0.1% Denhardt’s, 0.1 mg/ml salmon sperm DNA, and approximately 5 × 10⁶ cpm/ml radiolabeled probe at 42 °C for 14–20 h. The cDNA probe was labeled with [α-32P]dCTP by the random priming method (Prime-It kit, Stratagene) to a specific activity of 10⁸–10⁹ cpm/μg. Filters were prehybridized in the same solution at 42 °C for 1–2 h before addition of the probe. Membranes were washed by rinsing with 100 ml of a 0.2 × SSC, 0.1% SDS solution at room temperature for 30 min, followed by washing at 65 °C for 2 h. The positively hybridizing plaques were purified. From one clone (AFixII-2A), overlapping EcoRI and PstI fragments were subcloned into pBSSK and sequenced to generate a map of the gene. Restriction enzyme digests and sequence analysis of a second clone (AFixII-17A), as well as Southern hybridization analysis of all four genomic clone isolates, were performed to confirm our map of the MCP-1 receptor gene. The MCP-1 receptor genomic sequence has been deposited in GenBank (accession numbers U80923 and U80924).

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FIG. 2. Expression of CCR2A and CCR2B in human monocyte/macrophages. Total RNA isolated from human hematopoietic cell lines and monocyte/macrophages was assayed by RT-PCR. The sense (5′) primer was derived from the seventh transmembrane domain, and two antisense (3′) primers were derived from the coding region of the type A or type B carboxyl tails (see horizontal arrows in Fig. 1). PCR products of the size expected for the CCR2A and CCR2B cDNAs are indicated. Fully transcribed but unspliced mRNA was detected as a 1.4-kb PCR product (A). Untransfected HEK-293 cells (lane 1) were used as a negative control. HEK 293 cells stably transfected with CCR2B (lane 2) and CCR2A (lane 3) were used as positive controls. K562 cells (lane 4) are a human erythroleukemia cell line; THP-1 (lane 5) and MonoMac6 (lane 6) cells are human monocytic leukemia lines. Monocytes (lane 7) were prepared by centrifugation of freshly drawn blood over Ficoll-Hypaque, as described under “Materials and Methods.” Macrophages were obtained by maintaining human monocytes in culture for 3 days (lane 8) or 7 days (lane 9). DNA from a P1 lambda phage clone (lane 10) was used as a positive control for the migration of unspliced mRNA.

The 5′-untranslated region (UTR) of both isoforms is interrupted by a long intron (−3 kb) followed by a long exon (exon 2) of 1.9 kb that codes for the remaining 1.9-kb sequence of CCR2B mRNA. Exon 3 lies 260 bp downstream of the end of exon 2 and codes for the carboxyl-terminal tail and the entire 3′-UTR of the CCR2A isoform. Therefore, both forms of the receptor have identical 5′ ends composed of exon 1 and the 5′-half (981 bp) of exon 2. The remaining 3′-half of exon 2 is absent from the CCR2A cDNA, giving rise to unique carboxyl tails (and 3′-UTR) in CCR2A versus CCR2B (Fig. 1, bottom). Strong consensus sequences for canonical 5′ donor and 3′ acceptor splice site junctions are indicated. The restriction sites shown are PsI I (P); BgII (B); XbaI (X); EcoRI (E); and HindIII (H).

Expression of CCR2A and CCR2B mRNA in Monocytes and Macrophages—To determine the relative expression of type A and type B MCP-1 receptor mRNAs in human monocytes and macrophages, we performed RT-PCR using an upstream (sense) primer corresponding to the seventh transmembrane domain and a downstream (antisense) primer derived from either the type A or type B receptor carboxyl tails. Preliminary reactions were performed using plasmid cDNAs and P1 genomic clones as templates, and primer pairs were selected that gave robust, reproducible, and comparable amplification of CCR2A and CCR2B. As shown in Fig. 2, amplification of CCR2A or CCR2B cDNAs with these primers resulted in PCR products of the expected size (281 and 485 bp, respectively), without detectable cross-reactivity. CCR2B was amplified in the monocytic THP-1 and MonoMac6 cell lines but not in the K562 erythroleukemia cell line (Fig. 2B). Freshly isolated human monocytes were also strongly positive by PCR. Macrophages were obtained by maintaining human monocytes in culture for up to 7 days, during which time their scavenger receptor activity (i.e. internalization of acetylated low density lipoprotein, which is associated with monocyte to macrophage differentiation) increased (data not shown). Less PCR product was seen in the 7-day monocyte/macrophage cultures, suggesting that the CCR2B receptor mRNA levels had fallen. A similar expression pattern was seen for the type A MCP-1 receptor (Fig. 2A), although the PCR products were less abundant. Freshly isolated human monocytes and 3-day monocyte/macrophages were positive for CCR2A by PCR, and less product was seen in the 7-day macrophages. An additional band of approximately 1.4 kb was also detected using the primer specific for the type A receptor (Fig. 2A, top band). This size corresponds to mRNA from which the second intron has not been spliced, as evidenced by the identically sized band obtained in the P1 lambda phage clone (Fig. 2A, lane 9). This transcript would likely encode the type B MCP-1 receptor. The longer transcript was also seen in THP-1 and MonoMac6 cells but not in K562 cells. These data suggest that CCR2B is the predominant form of the MCP-1 receptor in both monocytic cell lines and human monocyte/macrophages and also indicate that receptor mRNA levels fall as monocytes mature into macrophages.
the cell surface (Fig. 3A). Similar results were obtained using immunofluorescence, which further revealed intense staining for CCR2A in permeabilized cells but not on the surface of intact cells (Fig. 3B). Western blot analysis indicated that CCR2A was at least as abundant as CCR2B in these cells (data not shown). These data indicated that whereas CCR2B was expressed at the cell surface and in the cytoplasm, CCR2A was found almost exclusively in the cytoplasm.

**Thrombin/MCP-1 Receptor Chimeras**—Since the two MCP-1 receptors differ only in their cytoplasmic tails, it was likely that sequences within the 61-amino acid carboxyl tail of the type A receptor were responsible for its low expression at the cell surface. To test this hypothesis, chimeras were created in which the cytoplasmic tails of either the type A or type B form of the MCP-1 receptor were ligated to the end of the seventh transmembrane domain of the human thrombin receptor. Trafficking studies of the thrombin receptor have previously shown that it is expressed at the cell surface, as well as in an intracellular pool (13, 20). cDNAs encoding the wild-type and chimeric receptors were transiently transfected into HEK-293 cells. Replacement of the carboxyl tail of the thrombin receptor with that of CCR2B resulted in a chimera that was expressed at the cell surface, as well as intracellularly (Fig. 4A). In contrast, when the cytoplasmic tail of CCR2A (amino acids 314–374) was used in place of the thrombin receptor tail, the chimera was not detected at the cell surface. Permeabilization of these cells revealed that this receptor was expressed at high levels in the cytoplasm (Fig. 4B) in a manner reminiscent of the distribution of wild-type CCR2A (see Fig. 3B). Cell surface expression of each of these constructs, quantitated by ELISA (Fig. 5), revealed that the CCR2A cytoplasmic tail reduced surface expression of the thrombin receptor to a level comparable with that of CCR2A. In contrast, replacement of the wild-type tail with that of CCR2B did not significantly reduce thrombin receptor expression at the cell surface. Similar results
were obtained in transfected COS-7 cells (data not shown). These data indicate that the poor surface expression of CCR2A is attributable to its cytoplasmic tail and further suggest that sequences in this region contain trafficking information applicable to other seven transmembrane domain receptors.

**CCR2A Cytoplasmic Tail Deletion and Mutation Analysis**—To determine the amino acid sequences responsible for intracellular retention of CCR2A, two additional constructs were made with the cytoplasmic tail truncated at either position 349 or 316. We also prepared a construct in which the two leucine residues at positions 350 and 351 were changed to alanine. Surface expression of these altered receptors in transiently transfected cells was quantitated by ELISA (Fig. 6). In both HEK-293 and COS-7 cells, the surface expression of CCR2A was significantly lower than CCR2B. Removal of the carboxyl 25 amino acids of the cytoplasmic tail (CCR2A-349) reduced expression of the receptor to below that of the wild-type CCR2A. However, deletion of virtually all of the cytoplasmic tail (CCR2A-316) resulted in a receptor whose surface expression approached that of CCR2B. Mutation of the Leu-Leu motif to Ala-Ala (CCR2A-L/A) did not affect surface expression. These results suggest that the cytoplasmic tail of the CCR2A contains both positive (between 349 and 374) and negative (316–349) signals for cell surface expression. Immunofluorescence studies confirmed these results and further revealed intense cytoplasmic staining of cells transfected with CCR2A, CCR2A-316, and CCR2A-L/A (data not shown).

The truncation mutants were examined further in signaling experiments. In transiently transfected COS-7 cells, little phosphatidylinositol turnover was detected with wild-type CCR2A or either of the two cytoplasmic tail truncation mutants (Fig. 7). As recently shown by our group (17), co-transfection with the cDNA for the chimeric G-protein G$_{q5}$ significantly enhanced agonist-dependent signaling of CCR2A. Signaling by CCR2A-316 was only comparable with that of CCR2A, despite the significantly higher surface expression of the truncated recep-

**FIG. 6.** Expression of carboxyl tail truncated CCR2A in HEK-293 cells. The carboxyl tail of CCR2A was truncated after amino acids 349 (CCR2A-349) or 316 (CCR2A-316). In a third construct, the two leucines at positions 350 and 351 of the full-length carboxyl tail were changed to alanines (CCR2A-L/A). Surface expression in transiently transfected HEK-293 and COS-7 cells was quantitated by ELISA as described in Fig. 5. Results are expressed as percentages of CCR2B expression. All data points were determined in triplicate, and error bars represent the standard deviations. Shown are the results of one of three similar experiments. The amino acid sequences of the carboxyl tails of CCR2A, CCR2A-349, and CCR2A-316 and the location of the LL-to-AA mutation are displayed below the graph.

**FIG. 7.** Agonist-dependent phosphatidylinositol turnover in transfected COS-7 cells. COS-7 cells were transiently transfected with cDNAs (1 µg/ml) encoding CCR2A, CCR2A-316, or CCR2A-349, in the presence or absence of the cDNA (0.5 µg/ml) for the chimeric G-protein G$_{q5}$. Cells were loaded for 24 h with myo-[H]inositol and incubated in the presence of 10 nM LiCl for 1 h at 37 °C with MCP-1 (100 nM). Total [H]inositol phosphate was measured as described under “Materials and Methods.” Each data point was determined in triplicate, and the data shown are the means (±S.D.) of five independent experiments.
tor. CCR2A-349, which was poorly expressed at the cell surface, did not signal as well as CCR2A. Given the robust surface expression of CCR2A-316 relative to wild-type CCR2A, these data suggest an important role for the receptor carboxyl tail in receptor coupling to G-proteins.

**Ligand Binding and Signaling Characteristics of CCR2A and CCR2B**—To further study signaling and ligand binding, we screened a number of stably transfected HEK-293 cell lines and identified those which had the highest levels of CCR2A (25–50% of CCR2B) at the cell surface. In equilibrium binding assays, 125I-labeled MCP-1 bound specifically to CCR2A-transfected cells (Fig. 8). Scatchard analysis of these data revealed a dissociation constant ($K_d$) of 310 pM, very similar to the $K_d$ of 260 pM determined previously for binding to CCR2B (12).

We have previously shown that the activation of type B MCP-1 receptor signals mobilization of intracellular Ca$^{2+}$ in an agonist-dependent manner, with an EC$_{50}$ value of approximately 3 nM (12). Agonist-dependent mobilization of intracellular calcium was also seen in CCR2A-transfected cells, although the magnitude of the calcium response was considerably reduced and the apparent EC$_{50}$ was higher (30 nM) (Fig. 9). As was the case for CCR2B (12), the calcium flux was blocked by pretreatment of the cells with pertussis toxin (data not shown). CCR2A also inhibited adenyl cyclase in response to MCP-1 binding (Fig. 10). As compared with CCR2B, the dose-response curve for inhibition of cyclase by CCR2A was shifted approximately 5-fold to the right, with IC$_{50}$ values of 0.8 and 1.4 nM, respectively.

**DISCUSSION**

We have previously reported the cloning of cDNAs encoding two functional human MCP-1 receptors, designated CCR2A and CCR2B (7). Because these two receptors differed only in their terminal carboxyl tails, we speculated that they arose via alternative splicing of a single gene. Here, we report the organization of the MCP-1 gene and the identification of exons encoding the alternative carboxyl tails of CCR2A and CCR2B. We found that both forms of the receptor were present in human monocytes and macrophages and that the poor cell-surface expression of CCR2A was due to its terminal carboxyl tail. Finally, we have provided evidence that CCR2A receptors that do reach the cell surface are functional in ligand binding and signaling assays.

Sequence analysis of human genomic clones indicated that the MCP-1 receptor genes spans at least 7 kb, comprises 3 exons bounded by canonical splice site consensus sequences, and contains multiple polyadenylation signals at the 3’ ends of exon 2 and exon 3. Whereas exon 1 encodes part of the 5’-UTR of both receptor isoforms, exon 2 encodes the entire open reading frame and 3’-UTR of CCR2B. Exon 3, on the other hand, codes for only the carboxyl tail and 3’-UTR of CCR2A. Near the center of exon 2 and at the beginning of exon 3 lie strong 5’
donor and 3' acceptor splice site consensus sequences, respectively, that define the region of the gene removed to form CCR2A. Therefore, both isoforms share a common 5' end composed of exon 1 and the 5'-half of exon 2 but differ in their carboxyl tails and 3'-UTRs.

Regulation of expression of the alternative MCP-1 receptor transcripts may be controlled by recognition of alternative polyadenylation signals located at the end of exon 2 in a mechanism similar to that shown for the regulation of membrane-bound versus secreted forms of immunoglobulin μ chains in B lymphocytes (21–23). In the MCP-1 receptor gene, if exon 2 polyadenylation signals are recognized, cleavage and polyadenylation would be expected to produce CCR2B transcripts. However, if these signals are not recognized, mRNA synthesis would continue through exon 3, thus providing the 3' acceptor site necessary for the splicing and synthesis of CCR2A mRNA. In this case, full-length pre-mRNA encoding both carboxyl tail and 3'-UTR isoforms would be synthesized, and any regulation of MCP-1 receptor isoform expression would be predominantly controlled by the degree of splicing of these pre-mRNAs. Interestingly, in our earlier Northern blot analysis of THP-1 and MonoMac6 cell RNAs, the major hybridizing band detected by carboxyl tail-specific probes to both CCR2A and CCR2B was 3.5 kb (7). This is the transcript size predicted if both exons 2 and 3, in their entirety, are included in the mRNA and may imply that the majority of the mRNA retains the potential to be spliced to yield CCR2A. Examination of these blots also revealed the presence of lower molecular weight forms in the CCR2A but not the CCR2B lanes, consistent with a small amount of splicing to produce fully processed CCR2A (7).

We have used RT-PCR to examine the relative expression of CCR2A and CCR2B in monocytic cell lines and freshly isolated human monocyte/macrophages at the mRNA level. Quantitative determination of the expression of CCR2A and CCR2B at the protein level is not feasible at the present time because high affinity, carboxyl tail-specific antibodies are not yet available. CCR2B was readily detected in monocytic cell lines, as well as in freshly isolated human monocytes, whereas CCR2A was less abundant. To examine MCP-1 receptor expression in macrophages, human monocytes were maintained in culture for up to 7 days, during which time they differentiate into cells that morphologically resembled macrophages (16). At the same time, scavenger receptor activity increased in the macrophages, compared with freshly isolated monocytes, as expected (24). In the case of both the type A and type B MCP-1 receptors, mRNA levels fell as the monocytes differentiated into macrophages. Taken together, these data are consistent with earlier studies in which radiolabeled MCP-1 failed to bind to macrophages (25–27) and suggest a possible mechanism for limiting the mobility of macrophages at sites of inflammation or injury.

Significant differences were observed in the cell-surface expression of CCR2A and CCR2B. In a number of different transfected cell types, including HEK-293 cells, RAT-1 fibroblasts, COS-7 cells, Jurkat T cells, and RBL-2H3 basophilic cells, CCR2A was expressed at relatively low levels at the cell surface, as compared with CCR2B. CCR2A was synthesized in these cells, as revealed by the very abundant staining of the cytoplasm after permeabilization. A similar intracellular localization of a G-protein-coupled receptor has been observed for the Mox2-4H receptor (28) and has been postulated to represent a reserve pool of receptors that could be rapidly transferred to the plasma membrane. A significant intracellular pool of thrombin receptors has also been noted in both transfected cells and endothelial cells, and evidence for translocation of these receptors to the cell surface has been reported (20). The physiological significance of the intracellular pool of CCR2A receptors remains to be determined.

Through the creation of receptor chimeras, we found that the poor trafficking of CCR2A to the cell surface was due to its terminal carboxyl tail and that this region of CCR2A could cause retention of other seven transmembrane domain receptors in the cytoplasm. Further support for this hypothesis came from truncation studies, which revealed that removal of the carboxyl tail significantly increased surface expression, as compared with wild-type CCR2A, and implicated amino acids 317–349 as containing the signal(s) for cytoplasmic retention. Examination of the amino acid sequence of the CCR2A tail did not reveal the presence of known retention signals (29) but did reveal the presence of two adjacent leucine residues at positions 350 and 351. Di-leucine motifs function as internalization signals for some receptors, as well as sorting signals in the endosome membrane (30, 31). For example, a di-leucine motif in the cytoplasmic tail contributes to lysosomal targeting and endocytosis of CD3 (32). Mutation of the leucines at positions 350 and 351 to alanines, however, had no effect on the cell-surface expression of CCR2A.

A limited number of transfected cell lines were identified in which CCR2A was expressed at the cell surface at 25–50% of...
the level of CCR2B. Permeabilization of these cells revealed extremely intense staining for CCR2A, and only in such lines was CCR2A detected on the cell surface at greater than 10% of the level of CCR2B. The type A receptors that were expressed on the cell surface bound MCP-1 with the same high affinity as CCR2B (12), mobilized intracellular calcium, and mediated inhibition of adenylyl cyclase in a dose-dependent manner. The reduced magnitude of the intracellular calcium response, as inhibition of adenyllylcyclase in a dose-dependent manner. The on the cell surface bound MCP-1 with the same high affinity as CCR2A detected on the cell surface at greater than 10% of extremely intense staining for CCR2A, and only in such lines the level of CCR2B. Permeabilization of these cells revealed that carboxyl tail splice variants of the G-protein-coupled EP3 receptor differed in their efficiency of coupling to Goi. In this regard, Hasegawa et al. (33) have reported that carboxyl tail splice variants of the G-protein-coupled EP3 receptor differed in their capacity to couple to Gαi. In our case, the disparity in the number of cell-surface receptors precludes a more definitive interpretation of the data. Despite extensive efforts, we have not been able to isolate a transfected CCR2A cell line with surface expression equal to that of CCR2B. Therefore, the question remains unanswered as to whether differences in the carboxyl tails of the two forms of the MCP-1 receptor cause qualitatively different G protein coupling.

The biological significance of the existence of two forms of the MCP-1 receptor is not clear at this time, in part because we have not identified a cell that expresses CCR2A at high levels. There is precedent, however, for multiple isoforms of chemokine receptors. The two forms of the IL-8 receptor differ in their tissue distributions. IL-8RB is the predominant form in monocytes and lymphocytes, whereas IL-8RA and IL-8RB are present in equal amounts in polymorphonuclear leukocytes (34). Recently reported differences in the affinity of IL-8 for the two forms of the receptor have led to the suggestion that IL-8RB may be relatively more important for the initiation of chemotaxis (34). In contrast, the lower affinity of IL-8 for IL-8RA indicates that this receptor would only become activated in the presence of high concentrations of IL-8 (e.g. at sites of acute inflammation). Similarly, two isoforms of the thromboxane A2 receptor with alternatively spliced carboxy tails have been found to differ in their tissue distribution (35). Our data suggest that CCR2B is the predominant form of the MCP-1 receptor in human monocytes and macrophages. It is possible that CCR2A is the major form of the receptor in other leukocytes or that its expression is increased upon activation of monocyte/macrophages. It is also possible that CCR2A will be found in non-hematopoietic cells. The elucidation of the structure of the MCP-1 receptor gene represents an important first step in understanding the role of this receptor in normal homeostasis and in response to infection or injury.

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