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CCR9A and CCR9B: Two Receptors for the Chemokine CCL25/TECK/Ckβ-15 That Differ in Their Sensitivities to Ligand

Cheng-Rong Yu,* Keith W. C. Peden,† Marina B. Zaitseva, † Hana Golding, † and Joshua M. Farber1*†

We isolated cDNAs for a chemokine receptor-related protein having the database designation GPR-9-6. Two classes of cDNAs were identified from mRNAs that arose by alternative splicing and that encode receptors that we refer to as CCR9A and CCR9B. CCR9A is predicted to contain 12 additional amino acids at its N terminus as compared with CCR9B. Cells transfected with cDNAs for CCR9A and CCR9B responded to the chemokine CC chemokine ligand 25 (CCL25)/thymus-expressed chemokine (TECK)/chemokine β-15 (CKβ-15) in assays for both calcium flux and chemotaxis. No other chemokines tested produced responses specific for the cDNA-transfected cells. mRNA for CCR9A/B is expressed predominantly in the thymus, coincident with the expression of CCL25, and highest expression for CCR9A/B among thymocyte subsets was found in CD4+CD8+ cells. mRNAs encoding the A and B forms of the receptor were expressed at a ratio of ~10:1 in immortalized T cell lines, in PBMC, and in diverse populations of thymocytes. The EC50 of CCL25 for CCR9A was lower than that for CCR9B, and CCR9A was desensitized by doses of CCL25 that failed to silence CCR9B. CCR9 is the first example of a chemokine receptor in which alternative mRNA splicing leads to proteins of differing activities, providing a mechanism for extending the range of concentrations over which a cell can respond to increments in the concentration of ligand. The study of CCR9A and CCR9B should enhance our understanding of the role of the chemokine system in T cell biology, particularly during the stages of thymocyte development. The Journal of Immunology, 2000, 164: 1293–1305.

The chemokines are now known to number more than 30 human chemotactic cytokines distributed among CXC, CC, C, and CX3C subfamilies, which act through seven-transmembrane-domain G protein-coupled receptors, of which 15 have been described in humans (reviewed in Ref. 1). The chemokine system is increasingly recognized to play an important role in lymphocyte biology. The activity of chemokines as chemotactic factors for lymphocytes has been known for some time, along with selective effects of chemokines on T cell subsets (2). However, the recent identification of many additional lymphocyte-active chemokines has suggested broad involvement of the chemokine system in lymphocyte trafficking, which is integral to lymphocyte development and to the functions of mature cells in immunity and inflammation (reviewed in Refs. 1, 3, and 4). The discoveries that chemokine receptors function as obligatory coreceptors for HIV entry and that chemokines can act as inhibitors of HIV infection have increased the importance of understanding the roles of chemokines and their receptors in lymphocyte physiology and pathophysiology to develop effective therapies (reviewed in Ref. 5).

We have previously identified and characterized novel chemokine receptors and HIV/SIV coreceptors on lymphocytes (6–10), and work in our and other laboratories using immortalized CD4+ T cell lines have suggested that there might exist as yet unidentified HIV-1 coreceptor(s) (K.W.C.P., unpublished observations, and Ref. 11). In experiments to identify such receptors, we found that the SUP-T1 T cell lymphoma line expressed GPR-9-6, a gene entered in the database as encoding a chemokine receptor-related protein (GenBank accession number U45982). While our experiments to date with a limited number of HIV-1 strains have not revealed coreceptor activity for GPR-9-6, we found that GPR-9-6 encodes signaling receptors for the CC chemokine ligand 25 (CCL25)2/thymus-expressed chemokine (TECK)/Ckβ-15. In the remainder of this work, we will, for the sake of clarity, designate the GPR-9-6 gene CCR9, in accordance with the recommendations of the committee for chemokine receptor nomenclature (Philip Murphy and Craig Gerard, personal communication). Similarly, we will adopt the chemokine nomenclature proposed at the Keystone Symposium on Chemokines and Chemokine Receptors, January 18–23, 1999, and refer to the chemokine TECK/Ckβ-15 as CCL25.

CCL25 was discovered by sequencing cDNAs made from the thymuses of RAG-1-deficient mice and is an unusual CC chemokine in many respects (12). The sequence of CCL25 is less than 30% identical to any other CC chemokine, and the CCL25 gene is not found in any of the known CC chemokine gene clusters, but is instead on chromosome 8 in mice (12) and on chromosome 19 in humans (13). Expression of the CCL25 gene is restricted, with mRNA detected in thymus and small intestine, and in spleen after challenge with LPS; expression in the thymus was shown to be in

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2 Abbreviations used in this paper: CCL, CC chemokine ligand; Ckβ, chemokine β; CXCL, CXC chemokine ligand; GFP, green fluorescent protein; HEK, human embryonic kidney; SDF-1, stromal cell-derived factor-1; TECK, thymus-expressed chemokine; TMD, transmembrane domain.

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dendritic cells and, at least in the fetal thymus, in MHC II⁺ epithelium as well (12, 14). CCL25 has been reported to be a chemooattractant for mouse thymocytes and for thymocyte precursors (12, 14) as well as for mouse splenic dendritic cells and IFN-γ-activated macrophages (12). Based on these observations, CCL25 has been proposed to be important in T cell development, either by affecting recruitment of thymic precursors, migration of thymocytes within the thymus, or recruitment of thymic dendritic cells that are important for autoantigen-driven negative selection (12).

Although the large number of lymphocyte-targeting chemokines, together with ligand/receptor promiscuity, suggest extensive redundancy in the activities of the chemokine system in lymphocyte biology, it is becoming clear that particular ligand/receptor groups act preferentially on lymphocytes at specific stages of development, activation, and differentiation. As examples, CXCL12 (stromal cell-derived factor-1) is necessary for resting memory T cells (8, 19); and a number of chemokine receptors have been reported to show preferential expression depending on whether CD4 T cells have differentiated down Th1 vs Th2 pathways (reviewed in Ref. 20). With respect to thymocyte development, a good deal of circumstantial evidence based on expression data has suggested the importance of the chemokine system, but no definitive role for chemokines and their receptors has been established. CCL25 is of particular interest in this regard because of its restricted tissue and cell-type expression and its activity on thymocytes.

We have isolated cDNAs encoding receptors for CCL25. These cDNAs encode two proteins that we have designated CCR9A and CCR9B. As the result of differential splicing of the CCR9 mRNA, CCR9A is predicted to contain 12 additional amino acids at its N terminus as compared with CCR9B. This degree of polymorphism at the N terminus of a chemokine receptor is novel. Of greater interest, we demonstrate that CCR9A and CCR9B, while both active, are not functionally equivalent, with CCR9A signaling and being desensitized at lower concentrations of chemokine as compared with CCR9B. These data suggest that the two forms of CCR9 extend the range of CCL25 concentrations over which a cell can sense increments in the ligand. Consistent with the expression of CCL25, the CCR9 gene is highly expressed in thymus, with analysis of thymocyte subsets revealing highest levels in the CD4⁺CD8⁺ cells. In all lymphocytes examined, mRNAs for CCR9A and CCR9B were found at a ratio of ~10:1. Our work provides information and tools that are likely to lead to an increased understanding of the activities of the chemokine system, particularly in regard to thymocytes and T cell development, enhancing our appreciation of the breadth of involvement of chemokines and chemokine receptors in lymphocyte biology.

Materials and Methods

Cell culture

Jurkat-TAg cells, which had been transfected with the large T-Ag gene of SV40, were derived as described (21) and kindly provided by Lawrence Samelson, National Institutes of Health (Bethesda, MD). Jurkat clone E6-1, SUP-T1, U937, and human embryonic kidney (HEK) 293 cells were obtained from American Type Culture Collection (Manassas, VA). CEM x174 and MOLT-4 clone 8 cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, MD). MT4 cells were kindly provided by Malcolm Martin (National Institutes of Health). H9 cells were derived as described (22) and obtained from the Center for Biologics Evaluation and Research, Food and Drug Administration. Elutriated monocytes and PBMC were obtained from normal donors by the Department of Transfusion Medicine, National Institutes of Health. Macrophages were produced by culturing monocytes for 1 wk in Iscove’s medium containing 10% human serum type AB (Sigma, St. Louis, MO) plus pyruvate. PBMC were activated by culturing with 1 μg/ml PHA and 20 IU/ml of IL-2 for 3 days. HEK-293 cells were grown in MEM plus 10% horse serum. Other cell lines and PBMC were grown in RPMI 1640 with 10% FBS. For purification of thymocytes, thymus fragments were obtained during cardiac surgery from children (ages 1 mo-3 yr) with congenital heart disease. The tissue was minced, large aggregates were removed by passing through a nylon mesh, and thymocytes were separated by centrifugation on a Ficoll-Paque gradient (Pharmacia Biotech, Uppsala, Sweden). The total thymocyte suspension was separated into CD4⁺, CD4⁻CD8⁺, CD4⁺CD8⁻, and CD8⁻CD4⁻ thymocyte subsets using the CD4 MultiSort Kit and VarioMACS magnet separator according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). The phenotypes of separated thymocyte subsets were analyzed using PE-conjugated anti-CD4 and allophycocyanin-conjugated anti-CD8 mAbs from Pharmingen (San Diego, CA). The CD4⁺CD8⁻ preparation contained ~20% CD4⁺CD8⁻ cells, which are T cell progenitors. The CD4⁺CD8⁺ preparation contained, in addition to these cells, ~20% CD4⁺CD8⁻ cells and 10% CD4⁻CD8⁺ cells, and the CD4⁺CD8⁻ preparation contained, in addition to these cells, ~20% CD4⁺CD8⁻ cells, 20% CD4⁺CD8⁻ cells, and 10% CD4⁺CD8⁻ cells.

Cloning of CCR9A and CCR9B cDNAs

Total RNA was prepared from the SUP-T1 cells using TRIzol reagent (Life Technologies, Gaithersburg, MD); poly(A)⁺ RNA was selected using oligo(dT) cellulose (Collaborative Biomedical Products, Bedford, MA); and cDNA was synthesized using oligo(dT) primers and the SuperScript Pre-amplification System (Life Technologies), according to suppliers’ protocols. For amplification, pools of degenerate primers containing Not I sites (bolded below) were designed based on transmembrane domain (TMD) III amino acid sequences of 11 G protein-coupled receptors from the human sequences for CCR2B, CCR5, CCR3, CCR8, CXCR1, CXCR4, STRL33, GPR15, APJ, GPR1, and D6. The TMD III primers that yielded the amplified cDNAs described below were 5’-GGCTTGCCGC GCCGC(G/G)T(C/G/G)T(G/A/G)T(A/C/G/T)(A/G/T)(G/A/G)C/AGT(A/G/T)(G/A/G)T(A/C/G/T)I(A/G/G)T and the TMD VII primers were 5’-GGCTTGCCGC GCCGC(G/G)T(C/G/G)T(G/A/G)T(A/C/G/T)(A/G/T)(G/A/G)C/AGT(A/G/T)(G/A/G)T(A/C/G/T)I(A/G/G)T. PCR amplifications were done with poly(A)⁺ cDNA synthesized from CXCR4, which is highly expressed in SUP-T1 cells and which contains an internal BamHI site, and which was then sequenced. PCR was done using 20% CD4 dullCD8⁺ cells, 20% CD4⁺CD8⁻ cells, and 10% CD4⁺CD8⁻ cells.

Construction of CCR9A and CCR9B expression vectors

CCR9A cDNA clone 6 was cut at BamHI sites at positions 85 and 1351 (see GenBank sequence for CCR9A, accession number AF145439), ligated to a 5’ adapter containing an XhoI site and a 3’ adapter containing a SalI site, and then ligated into pCEP4 (Invitrogen, Carlsbad, CA) that had been cut with XhoI and BamHI. CCR9B cDNA clone 3
washed in 10–50% with the SuperScript PreAmplification System (Life Technologies) and treated as for CCR9A above. To place the CCR9AB cDNAs in a vector containing an SV40 origin of replication, they were excised from pCEP4 using XhoI and SalI and inserted into a similarly cut pCI-neo (Promega, Madison, WI). For transfections, plasmid DNAs were prepared by banding twice through CsCl-ethidium bromide gradients.

**Cellular transfections**

For all transfections of Jurkat-TAg cells, 400 μl of the cells suspended at 2.5 × 10⁶ cells/ml in RPMI 1640 medium containing 10 mM HEPES was transferred to a cuvette with a 0.4-cm gap (Bio-Rad Laboratories, Hercules, CA), and electroporation was performed in a Gene Pulser apparatus (Bio-Rad) at room temperature using 250 V and 960 μA, after which the cells were left at room temperature for 10 min, diluted to 5 ml with RPMI 1640/10% FBS, and cultured for ~36 h before harvesting for assays for calcium flux or chemotaxis. For studies on the flow cytometer, cells were transfected with 40 μg of pCI-neo or pCI-neo/CCR9A or pCI-neo/CCR9B each with 25 μg of pEGFP-F encoding a farnesylated green fluorescent protein and containing an SV40 origin of replication (Clontech, Palo Alto, CA). For preparing cells for chemotaxis assays and for using in the fluorescence spectrometer, the pEGFP-F DNA was omitted and the amounts of the other DNAs were as stated in the text. For transfections to produce stable cell lines, pCEP4/CCR9A and pCEP4/CCR9B were used to transfect HEK-293 cells, as described (9), and colonies were selected and grown using 200 μg/ml hygromycin B (Sigma).

**Northern blot analysis**

Human tissue blots of poly(A)⁺ RNA (Clontech) were hybridized and washed according to the supplier’s protocols. For the dot blot not shown, the nonhybridizing samples included poly(A)⁺ RNA from whole brain, amygdala, caudate nucleus, cerebellum, cerebral cortex, frontal lobe, hippocampus, medulla oblongata, occipital lobe, putamen, substantia nigra, temporal lobe, thalamus, thymus, and tongue, as well as liver, fetal spleen, fetal thymus, and fetal lung. For analysis of RNA in cell lines and leukocytes, total RNA was prepared using Trizol (Life Technologies), separated by electrophoresis in a formaldehyde–agarose gel, and transferred onto a nylon membrane (Bio-Rad). Hybridizations were done as described (24), and the probe was used as described (9), along with a GAPDH oligonucleotide probe (accession number AF145439, using the random primer-based Megaprime screen).

**Determination of expression of mRNA**

For CCR9A and CCR9B using PCR

Using 1–5 μg of total RNA, prepared as above, cDNA was synthesized with the SuperScript PreAmplification System (Life Technologies) and diluted in 10–50 μl of water. Sequences of interest were amplified by PCR, using the Takara Taq PCR kit (Takara Shuzo, Otsu, Japan) in reactions containing various amounts of cDNA and 1 μM of each primer in a volume of 20 μl. Amplifications were done using 34 cycles of denaturation at 95°C for 45 s, annealing at 65°C for 1 min, and elongation at 72°C for 1 min. The primers used for amplifying CCR9 sequences were 5'-GTCGAGGTCATCTGCATCCAG (sense) and 5'-GCTGGAATTCTGCTAGAGC (antisense), and the predicted products were 500 bp for CCR9A and 451 bp for CCR9B. The primers used for GAPDH were 5'-ACCACCATGAGGAGGCTTGAG (sense) and 5'-CTCAGTGATGCGCCGATGATC (antisense), as previously described (26). PCR products were resolved by electrophoresis on a 2% agarose gel, and the DNA was transferred to Zeta-Probe nylon membranes (Bio-Rad) after base denaturation and neutralization, according to the manufacturer’s protocol. The membrane was prehydrized in 6× SSC, 10× Denhardt’s solution, 20 μg/ml yeast RNA, 50 μg/ml salmon sperm DNA, and 1% SDS at 50°C for 2 h. Hybridizations were done in 6× SSC and 1% SDS at 50°C overnight with 1–2 million cpm/ml using an oligonucleotide containing sequences common to CCR9A and -B, 5'-GCTGATGACTATGGCCTGAACTCCATCATC (or using a GAPDH oligonucleotide 5'-GTGAAACTTCAGTGCACCAGCACCTCACTCC (which had been 32P end-labeled using polynucleotide kinase (New England Biolabs, Beverly, MA), according to the manufacturer’s protocol. Membranes were washed at 55°C three times in 6× SSC and 1% SDS for 20 min, followed by one wash in 1× SSC and 1% SDS. Signal intensities were measured using a Phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). GAPDH signals were normalized to internal standards, with which there were linear relationships between input cDNA and signal from the PCR products. For quantitative comparisons among samples, the CCR9 signals were normalized to the signals for GAPDH. Using equal amounts of input, CCR9A and CCR9B cDNAs gave equal amounts of the 500 bp (CCR9A) and the 451 bp (CCR9B) products, demonstrating equal efficiencies in amplifying the two products.

**Southern blot analysis of genomic DNA**

Twenty micrograms of genomic DNA was isolated from flow-sorted fetal blood leukocytes and digested separately with the enzymes HindIII, BamHI, and PstI. The digested fragments were separated by agarose gel electrophoresis and transferred to a Zeta-Page nylon membrane (Bio-Rad) that had been hybridized overnight in 0.25 M sodium phosphate (pH 7.2), 7% SDS at 65°C; washed three times with 20 mM sodium phosphate (pH 7.2), 5% SDS at 65°C for 60 min; and finally washed twice for 60 min in 20 mM sodium phosphate (pH 7.2), 1% SDS, per the supplier’s recommendations. Preparation of probe and autoradiography was done as described above for Northern blotting.

**Measurements of calcium flux**

Assays on the flow cytometer for measurement of calcium fluxes in transfected cells and cell lines were done as described (18). Briefly, cells were washed with HBSS containing calcium and magnesium, 10 mM HEPES, and 1% FBS (HBSS/FBS) resuspended at 2 × 10⁶ cells/ml, loaded with the fluorescent calcium probe Indo-1 acetoxymethylester and the detergent Pluronic (Molecular Probes, Eugene, OR) at concentrations of 10 μM and 300 μg/ml, respectively, for 45 min at 37°C with occasional shaking, followed by two washes in HBSS/FBS buffer. Measurements of calcium flux were performed on a FACS Vantage flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with an argon laser tuned to 488 nm and a krypton laser tuned to 360 nm with fluorescence analyzed at 390/20 and 530/20 for Ca²⁺-bound and free probe, respectively. For each stimulation, the cells were kept at room temperature and using a sample of 0.5 μl of cells, 50 μl of HBSS/FBS was added at 30 s as a sham injection, followed by injection of 50 μl of HBSS/FBS containing various concentrations of chemokines. Data were analyzed using the FlowJo software (Treestar, Inc., Ashland, OR). For the data shown in Fig. 5, CCL25 was a preparation of Ckβ-15 obtained from Human Genome Sciences (Rockville, MD). This recombinant Ckβ-15 contained the N-terminal sequence MRGSHHHHHHGSVES, with a histidine tag, fused to the CCL25 sequence beginning, according to the published sequence (12), at Val31 and extending to the C terminus at Leu196 (24). All other chemokines were used in the other experiments for which data are shown, and all other chemo- kines used in calcium flux and/or chemotaxis assays were obtained from Peprotech (Rocky Hill, NJ), except for CCL21 (Ckβ-9/SLC) and CCL13 (Ckβ-10/MCP-4), which were also obtained from Human Genome Sciences. The CCL25 provided by Peprotech begins at Gln30 and extends to the C terminus at Leu196 (24), according to the supplier’s specifications, and this protein was more active in our assays than the preparation from Human Genome Sciences.

Assays of calcium flux on a ratio fluorescence spectrometer (model RF-M2001; Photon Technology International, South Brunswick, NJ) were done as described (27). Briefly, cells were suspended at 2 × 10⁶/ml and loaded with 2 μM fura-2 acetoxymethyl ester (Molecular Probes), as described above for indo-1. Measurements were made at 37°C in cuvettes containing 2 ml of cells at 0.5 × 10⁶ cells/ml. Excitation was alternated at 340 and 380 nm with emission measured at 510 nm. Using an integration time of 0.1 s, the ratios of the signals obtained at the two excitation wavelengths were plotted as a function of time. For quantifying responses, maximal increases in ratio fluorescence were measured.

**Chemotaxis assays**

Chemotaxis assays were done as described (28), with modifications, using 6.5-mm Transwell tissue culture inserts with a 5 μm pore size (Corning,
Corning, NY). Transfected cells and cell lines were suspended at $1 \times 10^7$ cells/ml in RPMI 1640 with 0.5% BSA, and 100 μl of cell suspension was added to an insert in a well with 600 μl of medium. After equilibration at 37°C for 2 h, chemokine was added to the wells and the plates were incubated for an additional 90 min before cells were harvested, collected by centrifugation, and counted. Duplicate wells were used for each condition.

Testing an N-terminal peptide from CCR9A as a CCL25 antagonist

A peptide consisting of the N-terminal 12 aa of CCR9A was synthesized (Commonwealth Biotechnologies, Richmond, VA) and dissolved in 1% acetic acid. A total of 3.5 μM CCL25 with and without 3.5 mM peptide was incubated in 0.5% acetic acid at room temperature for 1 h and tested on fura-2-AM-loaded, CCR9A-transfected HEK-293 cells at a concentration of 70 nM CCL25 using a calcium flux assay, as described above.

Results

Isolation and characterization of CCR9 cDNAs

To identify novel HIV-1 coreceptors and/or chemokine receptors expressed in the immortalized, CD4+ T cell line, SUP-T1, we performed RT-PCR with poly(A)+ RNA prepared from these cells using pools of degenerate primers based on conserved amino acid sequences in TMD VII and in, and immediately carboxyl-terminal to, TMD III. Among the initial ~100 PCR products sequenced, in addition to cDNA fragments for CXCR4, which has been shown to be expressed in SUP-T1 cells (9), we also found a cDNA fragment corresponding to the gene whose sequence had been deposited in the database as GPR-9-6 (GenBank accession number U45982) and that encodes a chemokine receptor-like, orphan G protein-coupled receptor. As noted above and based on the data presented below, we will refer to this gene as CCR9 in the remainder of the manuscript.

After Northern analysis verified the expression of the CCR9 mRNA in SUP-T1 cells (see below), we screened a cDNA library that we had prepared from these cells using a CCR9 probe to obtain cDNA clones for functional studies. Nine cDNAs were isolated and four were analyzed in detail, revealing two types of CCR9 cDNAs that differed in their predicted coding sequences. Two representative cDNAs, clone 6 and clone 3, were sequenced in their entireties and contained 2544 and 2462 bp, respectively, as depicted in Fig. 1A. The sequences have been deposited in GenBank.
Bank with accession number AF145439 for clone 6 and AF145440 for clone 3. These cDNAs are likely to be close to full length, since their lengths corresponded to the approximate size of the major species of CCR9 mRNA, as revealed by Northern analysis (see below).

Clone 6, as well as two additional cDNAs, encoded a predicted seven-transmembrane-domain protein of 369 aa, while clone 3 encoded a protein of 357 aa that was identical to the 369-aa protein, except that the clone 3 open reading frame began at Met13 and therefore lacked the first 12-aa predicted by the other three cDNAs. For both clone 6 and clone 3, the predicted initiator codons are the first ATGs in the sequences and are in the reading frame predicted by comparison with other G protein-coupled receptors. Each is in a favorable context for initiation of translation according to the empirical rules of Kozak (29). cDNA clone 6, but not clone 3, contains an in-frame stop codon 5′ to the predicted initiating ATG.

The differences between the open reading frames in clone 6 and clone 3 were due to a 49-bp insertion in clone 6 with respect to clone 3. Comparisons of clone 6 and clone 3 sequences with sequences in the database from an extended region of human chromosome 3 (GenBank accession number AC005669) revealed that the 49-bp fragment found in clone 6 is bounded by the canonical intronic splice acceptor and splice donor dinucleotides. In addition to the 369-codon open reading frame, clone 6 contained a 5′ nontranslated region of 157 bp and a 3′ nontranslated region of 1280 bp, absent the poly(A) tail. In addition to the 357-codon open reading frame, clone 3 contained a 5′ nontranslated region of 112 bp and a 3′ nontranslated region, absent the poly(A) tail, of 1279 bp.

We refer to the protein encoded by clone 6 as CCR9A and that encoded by clone 3 as CCR9B, and refer to them collectively, when appropriate, as CCR9A/B. Comparisons between CCR9A/B and related chemokine receptors and orphan receptors are shown in Fig. 1B. Like other chemokine receptors, CCR9A/B has an N-terminal region with a high number of acidic residues, a short and basic third intracellular loop, and cysteine residues in the N-terminal region (Cys38 in CCR9A) and the third extracellular loop (Cys289 in CCR9A) (30). Like many other G protein-coupled receptors, CCR9A/B has a site for N-linked glycosylation in the N-terminal region. Asn32 in CCR9A/B, conserved cysteines in extracellular loops 1 (Cys119 in CCR9A) and 2 (Cys198 in CCR9A), and the DRY sequence following TMD III, which mutagenesis studies have found to be important for G protein coupling (reviewed in Ref. 31). CCR9B/B also contains a cysteine residue in the carboxyl-terminal region (Cys337 in CCR9A) that, based on the data for rhodopsin, the β2-adrenergic receptor, and related receptors, is likely to be palmitoylated (reviewed in Ref. 32).

**Expression of the CCR9 gene**

Fig. 2 shows that there is a single human gene for CCR9A/B. As noted above, the CCR9 gene was found within 176,968 bp sequenced from chromosome 3 and entered in GenBank accession number AC005669. Our cDNAs were included within positions (3′) 3,666–5′ 20,337 of that GenBank entry. None of the other chemokine receptor or chemokine receptor-related orphan receptor genes that are located on chromosome 3 was found within this sequence.

Hybridization of CCR9 probe to a dot blot containing mRNAs from 50 human tissues produced a signal only with the sample from thymus (see Materials and Methods; data not shown). Similarly, Fig. 3A shows high level expression of the CCR9 gene limited to the thymus, with relatively low level expression also in spleen. The major species of CCR9 mRNA is ~2.6 kb. On a longer exposure of the blot in Fig. 3A and on other blots not shown, faint signals could be detected in PBL or PBMC, which were not increased after activation with PHA and IL-2. Fig. 3B demonstrates, as noted above, that CCR9 is expressed in the CD4+ T cell lines SUP-T1 and MOLT-4 clone 8 without detectable expression in several other T cell or monocyteic lines or in monocyte-derived macrophages. CCR9 expression was also not detected in the NK cell lines NK3.3 and YT, or in a line of EBV-transformed B cells, or in cord blood T cells that had been differentiated into Th1 or Th2 cell lines (not shown). Of note, thymus contains minor CCR9 mRNA species of ~6 and 8.5 kb, not well seen in Fig. 3B, that are not found in MOLT-4 clone 8 cells.

The high expression of CCR9 in thymus led us to investigate levels of CCR9 mRNA in thymocyte subsets using RT-PCR. As shown in Fig. 4A, CD4+CD8+ thymocytes expressed significantly higher levels of CCR9 as compared with other thymocyte subpopulations. As detailed in Materials and Methods, while the preparation of CD4+CD8+ cells was extremely pure, the preparations of single-positive cells contained up to 20% CD4+CD8- cells as well, so that the levels shown for single-positive cells are most likely overestimates. To analyze the relative expression of mRNA encoding CCR9A vs CCR9B in thymocytes and other cells, we designed PCR primers that would yield products of 500 and 451 bp from CCR9A and CCR9B cDNAs, respectively. As shown in Fig. 4B, CCR9A mRNA was expressed uniformly at ~10-fold higher levels as compared with CCR9B cDNA in thymocyte subsets as well as in PBMC and T cell lines.

**Signaling by receptors CCR9A/B**

cDNA fragments encoding CCR9A and CCR9B were inserted downstream of the CMV immediate-early promoter in the vector...
hybridized as in with 1 monocytes by culturing for 1 wk, and the PBMC, which were activated except for MDM, which were macrophages derived from peripheral blood are indicated. Samples are from the immortalized cell lines, as indicated, bridized to an oligonucleotide probe for the 18S rRNA. RNA size markers

[Image 104x388 to 224x734]

FIGURE 3. The CCR9 gene is expressed predominately in the thymus and in cell lines derived from T cell malignancies. A, A commercially supplied Northern blot containing ~2 μg of poly(A) RNA per lane was hybridized as in A. RNA size markers are indicated. B, Twenty micrograms of total RNA per lane were electrophoresed on a 1.2% agarose-formaldehyde gel, transferred to reinforced nitrocellulose, hybridized to the TMD 3–7 CCR9A/B cDNA32P-radiolabeled probe, and then stripped and hy-

bried to an oligonucleotide probe for the 18S rRNA. RNA size markers are indicated. Samples are from the immortalized cell lines, as indicated, except for MDM, which were macrophages derived from peripheral blood monocytes by culturing for 1 wk, and the PBMC, which were activated with 1 μg/ml PHA and 20 IU/ml IL-2 for 3 days.

pCI-neo, which contains an SV40 origin of replication. pCI-neo, pCI-neo encoding CCR9A, and pCI-neo encoding CCR9B were each cotransfected with the plasmid pEGFP-F, which contains an SV40 origin of replication and sequences encoding a farnesylated and codon-optimized green fluorescent protein (33), into Jurkat-TAg cells, which express the SV40 large T Ag (21). pEGFP-F DNA was included to identify cells expressing protein from the transfected DNAs on the flow cytometer. Approximately 36 h after transfection, the cells were loaded with the ratiometric fluorescent calcium probe indo-1 acetoxyethyl ester and tested for responses to a collection of chemokines using a flow cytometer-based calcium flux assay (18). As shown in Fig. 5, the chemokine CCL25 produced a calcium flux on the CCR9A and, to a lesser extent, on the CCR9B-transfected cells, but not on the cells transfected with the unmodified pCI-neo vector. Calcium responses to CCL25 were seen only in the GFP-bright cells, consistent with detectable responses being confined to those cells likely to be expressing the highest levels of CCR9A and CCR9B from the cotransfected DNAs. CXCL8 (IL-8) was used as a negative control and produced no signals. As expected from the known expression of CXCR4 in Jurkat cells (9, 34), CXCL12 (SDF-1) produced signals on the pCI-neo-transfected control cells and equally on both the GFP-negative/low and the GFP-high cells. Similarly, responses to ligands for CCR4, CCR7, and CXCR3 were seen that were not specific for, or enhanced, on the CCR9A or the CCR9B-transfect- ed cells (not shown).

In addition to experiments on the flow cytometer with cells co-

transfected with CCR9A/B and GFP plasmids, Jurkat-TAg cells were also transfected with CCR9A and CCR9B plasmids alone and analyzed for calcium fluxes on a ratio fluorescence spectrometer after the cells had been loaded with fura-2 acetoxyethyl ester. Transfected cells analyzed on the fluorometer responded to CCL25, and as with the experiments on the flow cytometer, there were no specific responses on the CCR9A- or CCR9B-transfected cells to chemokines other than CCL25. CCR9A- and CCR9B-med-

iated responses to chemokines were also analyzed using CCR9A- and CCR9B-transfected HEK-293 cell lines. Besides CXCL12 (SDF-1), which signals on the parent HEK-293 cells, only CCL25, and not CCR4, CCR7, or CXCR3 ligands, which gave responses on the control-transfected Jurkat-TAg cells, produced signals on the CCR9A- or CCR9B-expressing lines (not shown). The results from testing the transfected Jurkat-TAg cells, which were done using the largest number of chemokines, are shown in Table I.

The fluorometer was used for dose-response analysis to deter-

mine the EC50 of CCL25 on CCR9A- and CCR9B-expressing cells. In comparing the EC50 on CCR9A- vs CCR9B-transfected cells, we considered the possibility that receptor expression within the population of cells might reach levels at which further increases in receptor expression would not result in further signal enhancement. Under such conditions, differences in the EC50 between the CCR9A- and CCR9B-expressing cells might be influ-

enced by levels of receptor expression, with the EC50 falling at highest levels of expression. To overcome this possibly confound-

ing effect, the Jurkat-TAg cells were transfected with a range of DNA amounts and the cells were then analyzed for calcium fluxes in response to varying concentrations of CCL25. As shown in Fig. 6, the levels of the maximal calcium signals were dependent on the amounts of DNA used for the transfections. Also as shown, inde-

pendent of the amount of DNA used and the level of maximal signals, the EC50 for CCL25 was consistently lower on the CCR9A-expressing cells compared with the CCR9B-expressing cells, even though at the highest concentrations of CCL25 we could demonstrate equal, maximal responses for the two receptors, all suggesting that the receptors showed differences in signaling that were not due to differences in receptor expression. Our con-

cern that the EC50 might fall at higher levels of receptor expression was unfounded, since in fact, the EC50 with both CCR9A and CCR9B tended to rise slightly with transfections done using greater amounts of DNA. Fig. 6E demonstrates that, as expected from the mRNA expression data, the MOLT-4 clone 8 cells responded to CCL25. The EC50 for CCL25 on MOLT-4 clone 8 cells was ~25 ng/ml, close to that found for the CCR9A-transfected Jurkat-TAg cells, consistent with the predominant expression of CCR9A on these cells (Fig. 4B).

The lower EC50 of CCL25 on the CCR9A-expressing cells sug-

gested that CCR9A would be desensitized at lower concentrations of ligand as compared with CCR9B. Using the receptor-transfected HEK-293 cells, as shown in Fig. 7, we demonstrated that this was the case. For example, following stimulation of cells expressing CCR9A with 100 ng/ml CCL25, the subsequent addition of 1000 ng/ml did not produce a detectable signal. In contrast, CCR9B-expressing cells responded comparably to CCL25 after both the initial stimulation with 100 ng/ml and the subsequent stimulation
with 1000 ng/ml. Of note, just as for the transiently transfected Jurkat cells, the CCR9A-expressing HEK-293 cells responded to lower concentrations of CCL25 than did the cells expressing CCR9B. Because the above results suggested that the N terminus of CCR9A might be important in interacting with CCL25, we tested a peptide containing the 12 N-terminal residues of CCR9A as a CCL25 antagonist (see Materials and Methods). The peptide did not inhibit the signaling of CCL25 on CCR9A-expressing HEK-293 cells (not shown).

**Migration of CCR9A- and CCR9B-expressing cells to CCL25**

We analyzed the migration of Jurkat-TAg cells transfected with pCI-neo and pCI-neo encoding CCR9A and CCR9B, as well as MOLT-4 clone 8 and SUP-T1 cells in response to varying concentrations of CCL25, using porous Transwell tissue culture inserts to separate the cells in the upper chambers from the chemokine-containing medium in the lower chambers. As shown in Fig. 8, the CCR9A- and CCR9B-transfected cells as well as MOLT-4 clone 8 and SUP-T1 cells, but not the control-transfected Jurkat-TAg cells, migrated in response to CCL25 in a dose-dependent fashion, with decreased migration at the highest concentration of chemokine, producing the expected bell-shaped curve. Consistent with our calcium flux data shown in Figs. 5–7, CCR9A-expressing cells were more responsive to CCL25 than cells expressing CCR9B, responding at lower concentrations of CCL25 and showing a more dramatic falloff in migration at a CCL25 concentration of 2000 ng/ml. The CCR9A-transfected cells showed dose responses for chemotaxis to CCL25 that were close to those seen for the MOLT-4 clone 8 and SUP-T1 cell lines. Chemotaxis experiments using cells transfected with varying amounts of DNA showed that approximately twice as much CCR9B as compared with CCR9A DNA was required to produce cells migrating in equivalent numbers (not shown).

**Discussion**

We have isolated CCR9 cDNAs that differ due to alternative splicing and that encode two proteins, CCR9A and CCR9B, which function as receptors for CCL25 in assays for both calcium flux and chemotaxis. CCL25 has been shown to be expressed in the thymus by dendritic cells, perhaps by the lymphoid-derived dendritic cells, as well as in the small intestine (12), and has recently been shown to be expressed in MHC II+ epithelium from mouse fetal thymus (14). CCL25 was reported to be chemotactic for thymocytes, splenic dendritic cells, activated peritoneal macrophages (12), and precursors from fetal day 14 thymus (14) from mice, and for IFN-γ-activated THP-1 cells (12). Consistent with the expression pattern for CCL25 and its activity on thymocytes, we have found the CCR9 gene to be highly expressed in thymus, with detectable but much lower expression in spleen and PBL. We did not detect expression of CCR9 mRNA in human intestine or appendix by dot blot (Fig. 3A), or in IFN-γ-activated monocyte-derived macrophages, or in monocyte-derived dendritic cells by RNase protection assay (not shown). Because of possible differences between mice and humans and between the origins and culture conditions for primary cells, and because of the limits of detection in our probing for mRNA, our data do not definitively demonstrate any discrepancies between published reports of CCL25 responsiveness and CCR9A/B expression that would suggest another CCL25 receptor(s); and despite testing an extensive collection of chemokines, we have no indication that there are additional ligands for CCR9A and/or CCR9B.

The high and relatively selective expression of the CCR9 gene and CCL25 in the thymus raises the obvious question as to whether this receptor/ligand pair is involved in stem cell recruitment to, or thymocyte migration within or out of, the thymus. There is evidence that stem cell recruitment to the thymus and migration of...
FIGURE 5. Jurkat-TAg cells transfected with cDNAs encoding CCR9A and CCR9B responded to CCL25. A, Forty micrograms of pCI-neo containing sequences of CCR9A cDNA together with 25 μg of pEGFP-F DNA were used per transfection of 10⁷ Jurkat-TAg cells. After −36 h, the cells were loaded with indo-1 acetoxymethyl ester and tested for calcium flux in response to added chemokines. The scattergrams across the top row show responses to CCL25; those across the middle row show responses to CXCL8 (IL-8); and those across the bottom row show responses to CXCL12 (SDF-1). In these scattergrams and those that follow, the y-axis shows channel numbers that reflect the fluorescence ratio of the indo-1 calcium probe. For each sample, a sham injection of buffer was done at 30 s and the chemokines as indicated were injected at 60 s to a final concentration of 2 μg/ml. For analysis, cells from individual samples were separated into GFP−low and GFPhigh, as shown. B, Forty micrograms of pCI-neo containing sequences of CCR9B cDNA together with 25 μg of pEGFP-F DNA were used per transfection of 10⁷ Jurkat-TAg cells, and the cells were treated and analyzed as in A. C, Forty micrograms of pCI-neo without cDNA sequences together with 25 μg of pEGFP-F DNA were used per transfection of 10⁷ Jurkat-TAg cells, and the cells were treated and analyzed as in A. Responses of the CCR9A- and CCR9B-transfected cells to CCL25 were also seen in three other experiments.
thymocytes within and possibly out of the thymus require G protein activation. Pertussis toxin, which inactivates chemokine receptor-coupled G proteins of the G family, can block T cell precursor migration across a filter to repopulate precursor-depleted mouse embryonic thymic lobes (14); and transgenic mice express the pertussis toxin transgene to emigrate. These results implicating Gi proteins in thymocyte migration suggest that the CCR9 gene has been amplified in these cells and that the expression of the receptor, through autocrine activities of CCL25 or otherwise, might confer some growth advantage.

MOLT-4 clone 8 and SUP-T1 cells have been used in HIV research, since they readily form syncytia with many isolates. It is possible that the abilities of some HIV-1 (and/or SIV) variants to infect SUP-T1 and MOLT-4 clone 8 cells are due to the cells’ expression of CCR9/B, and analysis of coreceptor function for these receptors is ongoing.

Table 1. Responses of transfected Jurkat-TAg cells to chemokines

| Chemokines<sup>a</sup> | pCI-neo | pCI-neo/CCR9A | pCI-neo/CCR9B |
|------------------------|---------|---------------|---------------|
| CCL1 (I-309)           | −       | −             | −             |
| CCL2 (MCP-1)           | −       | −             | −             |
| CCL3 (MIP-1α)          | −       | −             | −             |
| CCL4 (MIP-1β)          | −       | −             | −             |
| CCL5 (RANTES)          | −       | −             | −             |
| CCL7 (MCP-3)           | −       | −             | −             |
| CCL8 (MCP-2)           | −       | −             | −             |
| CCL11 (Eotaxin)        | −       | −             | −             |
| CCL13 (MCP-4)          | −       | −             | −             |
| CCL14 (HCC-1)          | −       | −             | −             |
| CCL15 (Leukotactin-1)  | −       | −             | −             |
| CCL16 (LEC-4)          | −       | −             | −             |
| CCL17 (TARC)           | +       | +             | +             |
| CCL19 (MIP-3β)         | +       | +             | +             |
| CCL20 (MIP-3α)         | +       | +             | +             |
| CCL21 (SLC)            | +       | +             | +             |
| CCL22 (MDC)            | +       | +             | +             |
| CCL23 (MPIF-1)         | −       | −             | −             |
| CCL24 (MPIF-2)         | −       | −             | −             |
| CCL25 (TECK, Ckβ-15)   | +       | +             | +             |
| CXCL2 (Groβ)           | +       | +             | +             |
| CXCL4 (PF4)            | +       | +             | +             |
| CXCL5 (ENA-78)         | −       | −             | −             |
| CXCL6 (GCP-2)          | +       | +             | +             |
| CXCL8 (IL-8)           | −       | −             | −             |
| CXCL9 (MIG)            | +       | +             | +             |
| CXCL10 (IP-10)         | +       | +             | +             |
| CXCL11 (I-TAC)         | +       | +             | +             |
| CXCL12 (SDF-1)         | +       | +             | +             |
| CXCL13 (Fractalkine)   | +       | +             | +             |

<sup>a</sup> Chemokines are designated according to the nomenclature proposed at the Keystone Symposium on Chemokines and Chemokine Receptors, January 18–23, 1999, and common names are in parentheses. MCP, monocyte chemoattractant protein; MIP, macrophage-inflammatory protein; TARC, thymus and activation-regulated chemokine; PF4, platelet factor 4; IP-10, interferon-γ-inducible protein-10; LEC, liver-expressed chemokine; SLC, secondary lymphoid-tissue chemokine; MDC, macrophage-derived chemokine; MPIF, myeloid progenitor inhibitory factor; ENA, epithelial cell-derived neutrophil activator; GCP, granulocyte chemotactic protein; MIG, monokine induced by IFN-γ; I-TAC, IFN-inducible T cell alpha-chemotactant.

<sup>b</sup> Responses of transfected cells to chemokines are designated with − for negative, + for positive.

CCR7 and responsiveness to CCR7 ligands are acquired as thymocytes become fully mature (36, 43), suggesting that these receptors might be important in migration of thymocytes across the corticomедullary junction.

Responsiveness to CCL25 has been found to extend from double-positive cells through the single-positive, CD69<sup>+</sup> subset, disappearing on the mature single-positive cells (43). In a separate analysis of chemokine mRNA expression in MHC II<sup>+</sup> epithelium from mouse fetal thymus as compared with nonthymic tissue, CCL25 was unusual in being preferentially expressed in thymic epithelium, and CCL25 was a chemoattractant for fetal day 14 thymic precursors (14). Nonetheless, in these same studies, Abs to CCL25 failed to block trans-filter migration of precursor cells to repopulate precursor-depleted mouse embryonic thymic lobes (14). These results do not, however, rule out a role for CCR9A/B in stem cell recruitment, since there may be an as yet unidentified CCR9A/B ligand(s).

Our data on CCR9A/B expression on thymocyte subsets are consistent with the published descriptions of subset responses to CCL25. We found expression on double-negative/CD4<sup>+</cd>CD8<sup>+</sup> cells, which represent the precursors of the double-positive thymocytes (23), consistent with the responses to TECK seen for immature mouse thymocytes (14). However, we found the highest expression of CCR9A/B on CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (Fig. 4A). This suggests that CCR9 and its ligand are involved in events subsequent to recruitment of cells to the thymus and is consistent with the observations that CCL25 is expressed not only by fetal thymic epithelial cells, but also by medullary dendritic cells. Together, the data on receptor and ligand expression raise the possibility that CCR9 promotes the movement of double-positive thymocytes to contact epithelial and dendritic cells during migration from cortex to medulla, and is therefore important for that migration and/or for the processes of positive and negative selection, which depend on these interactions. Because single-positive thymocytes show much-diminished expression of CCR9 and PBL express little CCR9 on average, it is also possible that CCL25/CCR9 are involved in retaining cells in the thymus until maturation is complete. Finally, it is worth considering that CCL25/CCR9 may be involved in providing signals for thymocyte maturation in ways not limited to effects on cell movement.

We have shown that both SUP-T1 and MOLT-4 clone 8 cells express the CCR9 gene and respond to CCL25. The SUP-T1 line was derived from a child with T cell non-Hodgkin’s lymphoma (44), and the MOLT-4 line was derived from a young adult with acute lymphoblastic leukemia (45). The very high level of expression of CCR9 mRNA and CCR9A/B function on MOLT-4 clone 8 cells raises the possibility that the CCR9 gene has been amplified in these cells and that the expression of the receptor, through autocrine activities of CCL25 or otherwise, might confer some growth advantage.

MOLT-4 clone 8 and SUP-T1 cells have been used in HIV research, since they readily form syncytia with many isolates. It is possible that the abilities of some HIV-1 (and/or SIV) variants to infect SUP-T1 and MOLT-4 clone 8 cells are due to the cells’ expression of CCR9/B, and analysis of coreceptor function for these receptors is ongoing.

Of particular interest is our cloning of cDNAs that reflect alternative splicing of CCR9 mRNA, giving rise to two forms of the receptor that differ by 12 aa in the N-terminal region. Although the coding regions of chemokine receptor genes are often found on single exons, there are now many examples of exceptions to this rule, including, for example, the genes for CCR2 (46), CCR6 (7), CCR7 (47), CXCR4 (37, 48), and CXCR5 (49). Alternative splicing is well described as a mechanism of generating diversity.

<sup>2</sup> Transfected DNA

<sup>3</sup> Transient transfection of Jurkat-TAg cells with expression vectors.

<sup>4</sup> Expression of CCR9A/B ligand(s).

<sup>5</sup> Responses of transfected cells to chemokines are designated with − for negative, + for positive.

<sup>a</sup> Chemokines are designated according to the nomenclature proposed at the Keystone Symposium on Chemokines and Chemokine Receptors, January 18–23, 1999, and common names are in parentheses. MCP, monocyte chemoattractant protein; MIP, macrophage-inflammatory protein; TARC, thymus and activation-regulated chemokine; PF4, platelet factor 4; IP-10, interferon-γ-inducible protein-10; LEC, liver-expressed chemokine; SLC, secondary lymphoid-tissue chemokine; MDC, macrophage-derived chemokine; MPIF, myeloid progenitor inhibitory factor; ENA, epithelial cell-derived neutrophil activator; GCP, granulocyte chemotactic protein; MIG, monokine induced by IFN-γ; I-TAC, IFN-inducible T cell alpha-chemotactant.

<sup>b</sup> Responses of transfected cells to chemokines are designated with − for negative, + for positive.
among G protein-coupled receptors, resulting in changes in the C termini and intracellular loops as well as truncated receptors, sometimes resulting in changes in receptor activities (reviewed in Ref. 50). Among chemokine receptors, alternative splicing produces two forms of human CCR2, CCR2A and CCR2B, which differ in their C-terminal regions (51), and two forms of mouse CXCR4, which differ by 2 aa within the N-terminal region (52, 53). However, with the exception of a recently described serotonin receptor in Caenorhabditis elegans (54), we are not aware of other examples of alternative splicing producing variants of a G protein-coupled receptor with the magnitude of the N-terminal difference found for CCR9A and CCR9B.

While this manuscript was under revision, a report appeared describing a human CXCR4 RNA of \( 4 \text{ kb} \) that was predicted to encode a novel receptor that differs at its N terminus as compared with that encoded by the major 1.7-kb mRNA species (55). This novel form of CXCR4, termed CXCR4-Lo, is predicted to lack the five N-terminal residues found in the standard sequence and to contain nine other residues in their places, and was reported to have an EC\(_{50} \approx 3\)-fold that of the standard receptor. The CXCR4-Lo mRNA does not represent an alternatively spliced form, but an mRNA with a retained intron, producing a species with more than 2 kb of additional 5' nontranslated sequence as compared with the mRNA for CXCR4. The presence of both multiple upstream AUGs in the CXCR4-Lo RNA as well as a particularly unfavorable context for the postulated start codon for CXCR4-Lo according to the rules described by Kozak (29) suggest that CXCR4-Lo will not be translated efficiently from the 4-kb RNA, and raise questions about its biological significance. Kozak has noted that such incompletely processed mRNAs are particularly common in lymphocytes and that mRNA processing might therefore be a mechanism used in lymphocytes to regulate translation (56).

With regard to activities for chemokine receptor variants arising from alternative splicing, functional studies have not revealed differences in the responses to ligands between the two forms of
we have not yet measured the affinity directly. Because receptor in the longer N-terminal region on CCR9A as compared with CCR9B taxis, although the analysis here was less extensive. It is likely that CCR9A also appeared superior to CCR9B in mediating chemoreceptor. Consistent with our findings in the calcium flux assays, compared with CCR9B, indicating that CCR9A is the more efficient CCR2 (46, 51) or the variants of mouse CXCR4 (48, 52). It is therefore of particular note that we have demonstrated functional differences between CCR9A and CCR9B. Our experiments using calcium flux assays with both transiently transfected Jurkat cells and stably transfected HEK-293 cell lines showed that the EC_{50} of CCL25 for CCR9A is lower than for CCR9B and similarly that CCR9A is desensitized at lower concentrations of CCL25 as compared with CCR9B, indicating that CCR9A is the more efficient receptor. Consistent with our findings in the calcium flux assays, CCR9A also appeared superior to CCR9B in mediating chemotaxis, although the analysis here was less extensive. It is likely that the longer N-terminal region on CCR9A as compared with CCR9B produces a receptor with enhanced affinity for CCL25, although we have not yet measured the affinity directly. Because receptor in the active conformation is the target for the kinases that inactivate G protein-coupled receptors (57), lower affinity of CCR9B for CCL25 would be a straightforward explanation for the relative insensitivity of CCR9B to homologous desensitization (Fig. 7).

We have analyzed the relative levels of mRNAs for CCR9A and CCR9B on thymocyte subsets, cell lines, and PBMC. As shown in Fig. 4B, both forms were expressed in the primary cells as well as in the cell lines, and the ratios of these alternatively spliced forms were \( \sim 10:1 \) in all samples tested. The expression of mRNAs for both CCR9A and CCR9B in cell lines and the invariant ratio of the two forms in preparations of primary cells suggest that both forms are coexpressed on individual cells. These data raise the question as to the advantage of a cell expressing both the A and the B forms of the receptor. Although we have no evidence for a ligand for CCR9A/B other than CCL25, it is possible that there are other ligands for these receptors, or perhaps alternative forms of CCL25, that show greater differences in their activities on CCR9A and CCR9B than we have documented using our preparation of rCCL25. It is also possible that there are qualitative differences in signaling pathways activated by CCL25 on CCR9A vs CCR9B. Of course, it may be that the two receptors are only distinguished functionally by the quantitative differences that we have documented.

Although the advantage of a sensitive receptor would seem obvious in allowing a cell to respond to ligand gradients at a distance from the ligand source, the role of a coexpressed insensitive receptor is less clear. In this regard, our data on receptor desensitization may be particularly informative, since it demonstrates that CCR9B could enable a cell to respond to a change in ligand concentration at concentrations in which CCR9A is inactive. Together, the two receptors would extend the conditions under which a cell could respond to CCL25 beyond what could be achieved with a single receptor. This might be advantageous for trafficking as a cell moves up a gradient of CCL25, or it might enable a cell that has come to rest at a site with a high CCL25 concentration to react in other ways to a local release of CCL25. Our data suggest a novel mechanism for expanding the range of concentrations over which a cell can respond to increments in the concentration of chemokine.

While this manuscript was under review, Zaballos et al. (58) reported that GPR-9-6 is a receptor for TECK/CCL25 and designated the receptor CCR9. The sequence of the cDNA that they reported and used in their studies corresponds to our CCR9A. The major additional data provided by our work are the identification of CCR9B and the characterization of expression and activity of CCR9B as compared with CCR9A, demonstrating the existence of CCL25 receptors that differ in their sensitivities to ligand and showing expression of both forms in human thymocytes, with highest levels in CD4+CD8+ cells. It is of interest that the mouse sequence reported by Zaballos et al. (58), which shows 86% identity with the human receptor, contains the CCR9A Met^{11}, suggesting that CCR9B may exist in mice as well as in humans.

Data accumulating from many studies suggest that chemokine receptors expressed on lymphocytes function preferentially at particular stages in the life of a T cell (3, 18). The information that has accumulated to date has been primarily related to mature T cells, both for homeostatic trafficking of naive cells and for the trafficking of memory/effector cells, particularly those activated and differentiated down Th1 and Th2 pathways. Although circumstantial evidence also suggests that the chemokine system has roles in the earlier stages of T cell development, there is as yet no proof of this supposition. The description and characterization of CCR9A and CCR9B as receptors for CCL25 will provide tools to address the question directly, since the high and preferential expression of
CCL25 and CCR9A/B in the thymus suggest that these particular components of the chemokine system are likely to play important roles in thymocyte migration and/or in T cell development. If this speculation is borne out, it will expand our understanding of the breadth of the involvement of the chemokine system in T cell biology and contribute to the emerging appreciation that, rather than being a collection of molecules with redundant activities, individual chemokines and their receptors play dedicated roles in establishment and maintenance of immune functions.

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References

1. Zlotnik, A., J. Morales, and J. A. Hedrick. 1999. Recent advances in chemokines and chemokine receptors. Annu. Rev. Immunol. 17:657.
2. Schall, T. J., K. Bacon, K. J. Toy, and D. V. Goeddel. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature 347:669.
3. Kim, C. H., and H. E. Broxmeyer. 1999. Chemokines: signal lamps for trafficking of T and B cells for development and effector function. J. Leukocyte Biol. 65:6-9.
4. Yoshie, O., T. Imai, and H. Nomiyama. 1997. Novel lymphocyte-specific CC chemokines and their receptors. J. Leukocyte Biol. 62:634.
5. Berger, E. A., P. M. Murphy, and J. M. Farber. 1999. Chemokine receptors as HIV coreceptors: roles in viral entry, tropism, and disease. Annu. Rev. Immunol. 17:657.
6. Liao, F.-H. Lee, J. J. Chang, and J. M. Farber. 1996. Cloning of STRL12, a new human gene encoding a G protein-coupled receptor related to chemokine receptors and CXC receptors on chemotactic platelet-activating factor receptors. J. Biol. Chem. 271:28253-28260.
7. Liao, F., R. P. Alderson, J. S. Ullrich, B. L. Kreider, and J. M. Farber. 1997. STRL12 is a receptor for the CC chemokine MIP-3α. Biochem. Biophys. Res. Commun. 236:212.
8. Liao, F., R. J. Rabkin, C. S. Smith, G. Sharma, T. B. Nutman, and J. M. Farber. 1999. CC-chemokine receptor 6 is expressed on diverse memory subsets of T lymphocytes. J. Exp. Med. 189:9999.
9. VanRuths, S. L., D. Shiver, T. A. Dyer, and D. H. Sather. 1993. Identification of CCR8 as the specific receptor for the human CC-chemokine I-309: implications for human T lymphocyte activation. J. Clin. Invest. 96:129.
10. Liao, F., R. J. Rabkin, C. S. Smith, G. Sharma, T. B. Nutman, and J. M. Farber. 1999. CC-chemokine receptor 6 is expressed on diverse memory subsets of T lymphocytes. J. Exp. Med. 189:9999.
11. Civin, A. P., D. J. Figueroa, J. A. Hedrick, J. S. Foster, K. P. Singh, S. Menon, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, K. B. Bacon, and A. Zlotnik. 1997. TECK, a novel CC chemokine specifically expressed by thymic dendritic cells and potentially involved in T cell development. Immunity 7:291.
12. Nomiyama, H., K. Amano, J. Kusuda, T. Imai, R. Miura, O. Yoshie, and Y. Matsuda. 1998. The human CC chemokine TECK (SCYA25) maps to chromosome 7q15.1. Genomics 46:311.
13. Wilkinson, B. J., E. J. Owen, and J. J. Jenkinson. 1999. Factors regulating stem cell recruitment to the fetal thymus. J. Immunol. 162:3873.
14. Nogata, K., S. Hiraoka, T. Kinosaki, Y. Toyoda, Y. Horiuchi, Y. Horiuchi, K. Nomura, and M. Kikutani. 1999. Identification of two distinct CC-chemokine receptors on thymocytes. Nature 402:635.
15. Gokhman, D., S. K. Lee, R. L. Rabin, K. H. Tiffeny, J. M. Farber, K. B. C. Peden, P. M. Murphy, and M. G. Kaldner. 1998. Identification of CCR5 as the specific receptor for the human CD4+ T lymphocyte CC chemokine I-309. J. Biol. Chem. 273:22108.
16. Kadowaki, T., M. Watanabe, and M. Ueda. 1999. Molecular cloning of the mouse chemokine receptor CCR5. J. Leukocyte Biol. 66:272.
17. Kawasaki, K., S. K. Lee, R. L. Rabin, K. H. Tiffeny, J. M. Farber, K. B. C. Peden, P. M. Murphy, and M. G. Kaldner. 1998. Identification of CCR5 as the specific receptor for the human CD4+ T lymphocyte CC chemokine I-309. J. Biol. Chem. 273:22108.
18. Kadowaki, T., M. Watanabe, and M. Ueda. 1999. Molecular cloning of the mouse chemokine receptor CCR5. J. Leukocyte Biol. 66:272.
19. Kawasaki, K., S. K. Lee, R. L. Rabin, K. H. Tiffeny, J. M. Farber, K. B. C. Peden, P. M. Murphy, and M. G. Kaldner. 1998. Identification of CCR5 as the specific receptor for the human CD4+ T lymphocyte CC chemokine I-309. J. Biol. Chem. 273:22108.
20. Kadowaki, T., M. Watanabe, and M. Ueda. 1999. Molecular cloning of the mouse chemokine receptor CCR5. J. Leukocyte Biol. 66:272.
21. Kawasaki, K., S. K. Lee, R. L. Rabin, K. H. Tiffeny, J. M. Farber, K. B. C. Peden, P. M. Murphy, and M. G. Kaldner. 1998. Identification of CCR5 as the specific receptor for the human CD4+ T lymphocyte CC chemokine I-309. J. Biol. Chem. 273:22108.
chemokine receptor CXCR4, a major entry co-receptor for human immunodeficiency virus type 1. J. Biol. Chem. 273:4754.

49. Dobner, T., I. Wolf, T. Emrich, and M. Lipp. 1992. Differentiation-specific expression of a novel G protein-coupled receptor from Burkitt’s lymphoma. Eur. J. Immunol. 22:2795.

50. Journot, L., D. Spengler, C. Pantaloni, A. Dumuis, M. Sebben, and J. Bockaert. 1994. The PACAP receptor: generation by alternative splicing of functional diversity among G protein-coupled receptors in nerve cells. Semin. Cell. Biol. 5:263.

51. Charo, I. F., S. J. Myers, A. Herman, C. Franci, A. J. Connolly, and S. R. Coughlin. 1994. Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. Proc. Natl. Acad. Sci. USA 91:2752.

52. Heesen, M., M. A. Berman, U. E. Hopken, N. P. Gerard, and M. E. Dorf. 1997. Alternate splicing of mouse fusin/CXC chemokine receptor-4: stromal cell-derived factor-1α is a ligand for both CXC chemokine receptor-4 isoforms. J. Immunol. 158:3561.

53. Moepps, B., R. Frodl, H. R. Rodewald, M. Buggiolini, and P. Gierschik. 1997. Two marine homologues of the human chemokine receptor CXCR4 mediating stromal cell-derived factor 1α activation of G12 are differentially expressed in vivo. Eur. J. Immunol. 27:2102.

54. Hamdan, F. F., M. D. Ungrin, M. Abramovitz, and P. Ribeiro. 1999. Characterization of a novel serotonin receptor from Caenorhabditis elegans: cloning and expression of two splice variants. J. Neurochem. 72:1372.

55. Gupta, S. K., and K. Pillarisetti. 1999. Cutting edge: CXCR4-L0: molecular cloning and functional expression of a novel human CXCR4 splice variant. J. Immunol. 163:2368.

56. Kozak, M. 1996. Interpreting cDNA sequences: some insights from studies on translation. Mamm. Genome 7:563.

57. Palczewski, K., and J. L. Benovic. 1991. G-protein-coupled receptor kinases. Trends Biochem. Sci. 16:387.

58. Zaballos, A., J. Gutierrez, R. Varona, C. Ardavin, and G. Marquez. 1999. Cutting edge: identification of the orphan chemokine receptor GPR-9-6 as CCR9, the receptor for the chemokine TECK. J. Immunol. 162:5671.