Cutting Edge: Identification of the Orphan Chemokine Receptor GPR-9-6 as CCR9, the Receptor for the Chemokine TECK

Ángel Zaballos, Julio Gutiérrez, Rosa Varona, Carlos Ardavín and Gabriel Márquez

*J Immunol* 1999; 162:5671-5675; ;
http://www.jimmunol.org/content/162/10/5671

This information is current as of May 12, 2017.

References
This article cites 20 articles, 10 of which you can access for free at:
http://www.jimmunol.org/content/162/10/5671.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: Identification of the Orphan Chemokine Receptor GPR-9-6 as CCR9, the Receptor for the Chemokine TECK

Angel Zaballos,* Julio Gutiérrez,* Rosa Varona,* Carlos Ardavín,† and Gabriel Márquez2*

Thymus-expressed chemokine (TECK) has been reported to chemoattract dendritic cells, thymocytes, and activated macrophages. Here, we show that TECK is a specific agonist for a human orphan receptor called GPR-9-6. We have determined the cDNA sequence of human GPR-9-6 and cloned the corresponding murine cDNA. Human and murine GPR-9-6 expression is very high in the thymus and low in lymph nodes and spleen. RT-PCR analysis of murine GPR-9-6 expression on murine FACS-sorted thymocyte subpopulations showed that this gene is expressed in both immature and mature T cells. Additions of human or murine TECK to HEK 293/human GPR-9-6 and HEK 293/murine GPR-9-6 transfectants provoked intracytoplasmic calcium mobilization. Human TECK also induced the in vitro migration of HEK 293/human GPR-9-6 cells. These results confirm that GPR-9-6 is a specific receptor for TECK. According to the established nomenclature system, we propose to rename GPR-9-6 as CC chemokine receptor 9 (CCR9). The Journal of Immunology, 1999, 162: 5671–5675.

Chemokines are a family of small, structurally related proteins that attract and activate leukocytes. The positioning of two conserved cysteines in the N-terminal region of the proteins defines four chemokine subfamilies: CXC, CC, C, and CX3C (1, 2). Chemokine receptors are G-protein-coupled polypeptides with seven transmembrane domains. To date, a number of human receptors specific for CXC (CXCR1–CXCR5), CC (CCR1–CCR8), C (XCR1), or CX3C (CX3CR1) chemokines have been described (1–5).

Up to now, nearly 50 chemokines have been described. Trying to dissect the chemoattractant properties of so many similar proteins, a very active field of research has been, and still is, the identification of their receptor binding specificity. Indeed, binding preferences have already been established for most chemokines. Nevertheless, the receptors for some CC chemokines such as NCC-4 (6), pulmonary- and activation-regulating chemokine (7), or TECK (8) are not known yet. Similarly, some putative CC chemokine receptors like STRL33/Bonzo (9), CKRX/HCR (10), and GPR-9-6 (EMBL accession number U45982) remain orphan. We have analyzed the phylogenetic relationships between CC chemokine receptors and orphan receptors. Our data indicate the existence of a separate group the members of which are CCR6, CCR7, STRL33/Bonzo, and GPR-9-6. Besides their structural similarities, CCR6, CCR7, and STRL33/Bonzo also share an expression pattern that is mainly restricted to lymphoid organs (9, 11, 12). Data on the expression of GPR-9-6 were not available. Here we report that the CC chemokine TECK is a specific ligand for GPR-9-6. We have also cloned the mGPR-9-6 cDNA and found that it is activated by the murine version of TECK. In addition, the constitutive RNA expression of both genes in tissues, cell lines, and thymocyte subpopulations is also reported. Following current rules for chemokine receptor nomenclature, we propose to designate GPR-9-6 as CCR9.

Materials and Methods

Chemokines and cells

Chemokines were purchased from Peprotech, Rocky Hill, NJ, or R&D Systems, Minneapolis, MN. The HEK 293 and MOLT-4 cell lines were obtained from the American Type Culture Collection, Manassas, VA. Human and mGPR-9-6 cDNAs were cloned in pCIneo (Promega, Madison, WI), and stable transfectants were obtained after G418 selection of cells transfected with the resulting plasmids by the calcium phosphate method, as described (13). Thymocytes were purified from 5–7-wk-old female BALB/c mice using a FACSort cytometer (Becton Dickinson, Mountain View, CA) as described (14). Briefly, double-positive thymocytes were sorted as CD4+CD8+ cells. CD4+ single-positive thymocytes were sorted as CD4+ cells after depletion of CD8+ thymocytes. CD8+ single-positive thymocytes were sorted as CD8+ cells. Pre-T cells were sorted as CD4+CD8+ cells after depletion of CD4+ and CD8+ thymocytes. After reanalysis, the sorted cell populations had a purity of >98%.

Abbreviations used in this paper: CCR, CC chemokine receptor; TECK, thymus-expressed chemokine; GPR, G-protein-coupled receptor; m, murine; h, human; SDF-1α, stromal cell-derived factor-1α; RACE, rapid amplification of cDNA end.
effect of pertussis or cholera toxins on the TECK-mediated GPR-9-6 signaling was analyzed by performing the assays on cells cultured for 16 h in 0.1 μg/ml pertussis toxin or 0.4 μg/ml cholera toxin.

Sequencing of mGPR-9-6

Based on the hGPR-9-6 sequence, a pair of oligonucleotides was designed (5’-AARTTYCARACTTATGYTGAAYA-3’ and 5’-GRTGHTATIATITGRTRTARCARCA-3’) and assayed in PCR amplifications using 129/SvJ mouse genomic DNA as template. A DNA fragment with the expected size was produced, and the sequencing of this material showed an open reading frame with a translated sequence that was 82% identical with that of the corresponding hGPR-9-6 sequence. Then, specific oligonucleotides were designed and used for 5’- and 3’-RACE of adapted murine thymus cDNA (Marathon cDNA amplification kit, Clontech Laboratories, Palo Alto, CA). DNA fragments corresponding to both the 5’ and 3’ mGPR-9-6 ends were obtained and sequenced. Finally, a DNA fragment containing the complete mGPR-9-6 coding sequence was synthesized by PCR from murine thymus cDNA.

Analysis of gene expression

Multiple tissue Northern blots (Clontech) were probed with a 32P-labeled 1120-bp DNA fragment containing most of the hGPR-9-6 coding sequence, as recommended by the supplier. Total RNA samples from mouse tissues or FACS-sorted cell subsets were extracted with Tri-reagent (Sigma Chemical, St. Louis, MO). RNA from tissues was electrophoresed on a denaturing formaldehyde-agarose gel and blotted onto nylon Hybond membranes. Prehybridization of the membranes and hybridization with a 32 P-labeled DNA fragment containing the coding sequence of mGPR-9-6 were conducted in Rapid Hyb buffer (Amersham, Arlington Heights, IL) as recommended by the supplier. For RT-PCR analysis, total RNA from FACS-sorted cell subpopulations was reverse-transcribed as described (14). Equal amounts of cDNA were subjected to PCR amplification of mGPR-9-6 using specific forward (5’-ACGTGCTAGCCGCCATGATGCCACAGAACTC-3’) and reverse (5’-TGACCTTCAGGATCAAGACAATG-3’) primers under the following conditions: 15 s at 94°C; 15 s at 59°C; 45 s at 72°C.

Calcium mobilization and chemotaxis studies

Calcium fluorometry on HEK 293 transfectant cells was conducted with indo-1-AM (Molecular Probes, Leiden, The Netherlands)-loaded cells as described (15). Each measure was done on 1-ml aliquots containing 3 × 106 cells. Additions of chemokines were done in 10 μl. Cell migration assays with stable HEK 293/hGPR-9-6 or HEK 293/pCIneo transfectants
Results and Discussion

Analysis of the phylogenetic relationships between CC chemokine receptors and orphan chemokine receptors indicates the existence of two major branches of receptors. One of them is formed by CCR6, CCR7, and the orphan receptors STRL33/Bonzo and GPR-9-6 (Fig. 1A). CCR6, CCR7, and STRL33/Bonzo also shared a restricted expression pattern, mainly limited to lymphoid organs. Moreover, unlike most CC receptors, CCR6 and CCR7 genes have coding sequences interrupted by introns (12, 16), and their ligands, liver- and activation-regulated chemokine/macroage-inflammatory protein-3α and secondary lymphoid-tissue chemokine, respectively, are also sequence related (14). These facts prompted us to characterize in detail GPR-9-6, the other member of this group of receptors for which the only information available was the genomic sequence deposited in data banks.

Identification of the hGPR-9-6 cDNA sequence and cloning of mGPR-9-6

We followed a 5′-RACE procedure on a human thymus Agt11 cDNA library and found that the protein encoded by the GPR-9-6 mRNA was 12 amino acids longer than that encoded by the genomic coding sequence previously reported. This indicates that an intron interrupts the N-terminal coding sequence, as in CCR6 and CCR7. Recently, the sequence of the region encompassing hGPR-9-6 gene has been reported (EMBL accession number AC005669). Comparison of this sequence with that of the hGPR-9-6 mRNA we report here revealed the presence of at least three exons in the hGPR-9-6 gene (Fig. 1B).

A degenerate PCR procedure with oligonucleotides based on the human sequence, combined with 5′- and 3′-RACE allowed us to clone the murine homologue of GPR-9-6 from mouse thymus cDNA. Sequencing of the PCR products obtained detected an open reading frame the predicted amino acid sequence of which was 86% identical with that of hGPR-9-6. Particularly, the 78 C-terminal residues are the same in both species; this identity is unique in the chemokine receptor family. Fig. 1C shows the alignment of the predicted 369 amino acid sequences of both hGPR-9-6 and mGPR-9-6 proteins. N32 is a potential site for N-glycosylation in both polypeptides; N38 is an additional potential site present only in the murine protein.

Analysis of hGPR-9-6 and mGPR-9-6 expression

Tissue distribution of hGPR-9-6 and mGPR-9-6 was studied by Northern blot analysis of human poly(A)+ RNA or mouse total RNA samples (Fig. 2). Both genes showed a high expression level in thymus; weak mRNA expression in spleen and lymph nodes was also detected. Transcript sizes were 2.7 kb for hGPR-9-6 and 3.3 kb for mGPR-9-6. Among several cell lines tested only MOLT4 showed expression of hGPR-9-6 (Fig. 2A). Because thymus was the tissue showing maximal expression of mGPR-9-6, an RT-PCR approach was used to analyze the expression of this gene in FACS-sorted thymocyte subpopulations. Murine GPR-9-6 expression was found in CD25+CD4+ CD8− pre-T cells, CD4+CD8− immature thymocytes, as well as in single positive CD4+ and CD8+ T cells (Fig. 2C).

TECK-induced calcium mobilization and migration through the GPR-9-6 receptor

The ligand specificity of GPR-9-6 was investigated by monitoring changes in the intracellular calcium concentration of HEK 293/hGPR-9-6 stable transfected cells (293/hGPR-9-6), after sequential addition of samples of chemokines. Among 36 recombinant human chemokines tested (Fig. 3A), only TECK elicited a response. The addition of 200 nM hTECK to 293/hGPR-9-6 cells resulted in significant calcium flux (Fig. 3B, upper panel), and the cell response was correlated to the chemokine dose down to 20 nM. HEK 293/mGPR-9-6 transfecant cells were also stimulated to mobilize intracellular calcium by mTECK (Fig. 3B, middle panel). Addition of a high concentration of hTECK to 293/hGPR-9-6 cells resulted in complete desensitization to a second addition of the same stimulus (Fig. 3C, upper tracing). Both human and mouse TECK were able to stimulate the human receptor in the transfecant cells, and this resulted in desensitization to a subsequent stimulus with hTECK (Fig. 3C, lower tracing). This interspecies cross-reactivity is consistent with the report by Vicari et al. (8) on mTECK-induced migration of human THP-1 cells and with the high degree of homology between the amino acid sequences of both receptors.
Calcium mobilization induced by hTECK was decreased but not abolished by pertussis toxin (Fig. 3D). In contrast, the cell response to hSDF-1α, a chemokine for which HEK 293 cells express an endogenous receptor, was completely abolished by this toxin. Cholera toxin did not affect the hTECK-induced calcium response of 293/hGPR-9-6 cells (not shown). These results suggest that hGPR-9-6 is partially but not exclusively coupled to the Gαi class of Gq subunits (17). A similar situation has also been described for other chemokine receptors (14, 18, 19). The functionality of hGPR-9-6 is partially but not exclusively coupled to the Gαi class of Gq subunits (17). A similar situation has also been described for other chemokine receptors (14, 18, 19). The functionality of hGPR-9-6 upon hTECK stimulation in HEK 293 transfectants was also confirmed by chemotaxis assays (Fig. 3E). Maximal migration of the transfectant cells occurred at 100 nM hTECK. Control 293/pCIneo cells were not induced to migrate by hTECK (not shown).

hTECK-induced calcium mobilization in MOLT4 cells

Northern analysis revealed that the MOLT4 cell line expresses hGPR-9-6 RNA. This is consistent with the thymic origin of these CD4+ lymphoblastic T cells. Indo-1-AM-loaded MOLT4 cells showed a high increase of intracellular calcium after stimulation with 200 nM hTECK (Fig. 4A). The cell response was correlated to the chemokine dose. MOLT4 cells were slightly more sensitive to the transfectants at low doses of the chemokine, given that 6 nM hTECK induced a detectable response. Pertussis toxin reduced but did not abolish the MOLT4 response to hTECK (Fig. 4B), while cholera toxin did not affect that response (not shown). These results are essentially identical with those obtained with the 293/hGPR-9-6 transfectants and suggest that hTECK-induced stimulation of MOLT4 cells is mediated, mainly if not exclusively, by hGPR-9-6.

All of these data agree with the reported behavior of other chemokine-chemokine receptor pairs and support the conclusion that TECK is a functional ligand for GPR-9-6, both in the human and the murine systems. According to the current guidelines for chemokine receptor nomenclature, a new name should be assigned to GPR-9-6. Only eight CC receptors have been unambiguously identified as functional receptors. Although another receptor has been reported independently as D6/CCR9 (20) and CCR10 (21), the ability of this molecule to transduce a signal remains controversial. Therefore, in agreement with the committee appointed at the 1997 Gordon Conference on Chemokines, we propose GPR-9-6 to be designated as CCR9. According to its phylogenetic relationship to CCR6 and CCR7, CCR9 does not show ligand binding promiscuity, as happens with CC receptors of the other phylogenetic group. CCR9 binds only one chemokine among 36 tested. This indicates a remarkable specificity, although we cannot exclude that new CCR9 agonists may appear. In addition, the CCR9 ligand, TECK, is also phylogenetically close to macrophage-inflammatory protein-3α and secondary lymphoid-tissue chemokine, the ligands for CCR6 and CCR7, respectively, sharing unique structural features, as the Asp-Cys-Cys-Leu motif (14). Also interesting is the fact that CCR9 has an intron interrupting its coding sequence, as is the case for CCR6 and CCR7.

The fact that both immature and mature thymocytes express mCCR9 suggests that this receptor might play a role during the whole process of thymocyte development. Because thymic dendritic cells are producers of TECK (8), the TECK-mediated chemotaxis might be a mechanism contributing to thymocyte confinement in the thymus until completion of their development. The identification of CCR9 as the functional receptor for TECK will help to unravel the important role these proteins seem to play in thymus.

Acknowledgments

We thank C. Martínez-A. for comments and support, P. Martin and M. Navarrete for excellent technical assistance, J. Sarrustegui and F. Gavilanes for their assistance in the fluorometric assays, and P. Murphy and C. Gerard for their advice on chemokine receptor nomenclature.

References