BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **236,** 212–217 (1997)
ARTICLE NO. RC976936

STRL22 Is a Receptor for the CC Chemokine MIP-3α

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Received June 5, 1997

STRL22 is a human seven transmembrane domain orphan receptor related to known chemokine receptors and expressed in peripheral blood lymphocytes, tumor infiltrating lymphocytes and lymphoid tissues. MIP-3α/LARC/Exodus is a CC chemokine that is chemotactic for lymphocytes and that is expressed in activated cells, including monocytes, T cells, endothelial cells, and fibroblasts, and in liver, lung, and some lymphoid tissues. We report here that *STRL22*-transfected human embryonic kidney 293 cells demonstrated specific binding for MIP-3α and that MIP-3α, but no other chemokines, produced a calcium flux in the *STRL22*-transfected cells. We show that MIP-3α, unlike other chemokines, produced a calcium flux in freshly-isolated peripheral blood lymphocytes and we show that MIP-3α also produced a signal in tumor infiltrating lymphocytes that express *STRL22*. Since STRL22 is the sixth functional CC chemokine receptor identified, it should be re-named CCR6.

The superfamily of G protein-coupled receptors includes receptors for the chemokines, a family of more than thirty human cytokines whose best-described activities are as chemotactic factors for leukocytes (1). Most chemokines fall into one of two subfamilies, either CC or CXC, depending on the arrangement of conserved cysteine residues. Recently, there has been increasing interest in the role of chemokines in the complex and poorly-understood process of lymphocyte trafficking (2,3). The discoveries that chemokine receptors function along with CD4 as obligate cofactors for HIV-1 entry into cells (4–9) have emphasized the medical importance of understanding the roles for chemokines and their receptors in T cell physiology.

As part of studies to identify new chemokine receptors on activated T cells, we discovered *STRL22,* a human gene located on chromosome 6q27 and encoding a chemokine receptor-related seven transmembrane domain protein (10). The *STRL22* gene was also reported by Zaballos et al., who used the designation *CKR-L3* (GenBank accession number Z79784 (11), and two additional groups have deposited similar sequences in the data bases with GenBank accession numbers U45984 and U60000.

The *STRL22* gene is expressed prominently in lymphoid tissue such as spleen, lymph node and appendix (10), in peripheral blood lymphocytes, including $CD4^+$ and $CD8^+$ T cells and $CD19^+$ B cells (10–12), in $CD4^+$ and $CD8^+$ tumor infiltrating T cells (TIL)[1] and in EBV-transformed B cells (12). Previous attempts by us (10) and by others (11) to identify an agonist(s) for STRL22 were unsuccessful.

As part of a large-scale project to isolate and sequence human expressed sequence tags (ESTs) (13,14), we discovered ESTs encoding a protein that we designated Chemokine β-4 (Ckβ-4) based on similarities to known CC chemokines (B. K. et al., unpublished results). Analysis of ESTs by several other groups led to the identification and characterization earlier this year of the same CC chemokine, published under the names MIP-3α (15), LARC (16) and Exodus (17). The protein that we have studied matches the sequence of MIP-3α and LARC and for the sake of clarity and brevity, we will use the MIP-3α designation in the remainder of this paper.

The *MIP-3α* cDNA encodes a predicted precursor protein of 96 amino acids and a predicted secreted protein of 70 amino acids, Mr 8,029. While clearly a CC chemo-

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Abbreviations: TIL, tumor infiltrating lymphocytes; EST, expressed sequence tag; HEK, human embryonic kidney; MEM, minimum essential medium; PBL, peripheral blood lymphocytes; HBSS, Hanks' balanced salt solution; HEPES, N-2-Hydroxyethylpiperazine-N′-2-Ethane Sulfonic Acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

0006-291X/97 $25.00

kine, MIP-3α is not closely related to the other members of the subfamily. The *MIP-3α* gene is not in the CC chemokine cluster at chromosome 17q11.2, but has been mapped instead to chromosome 2q33-q37 (16) and the MIP-3α amino acid sequence matches other CC chemokine sequences at only 20–28% of residues. By Northern analysis, the *MIP-3α* gene is expressed in liver, lung and lymphoid tissue, although not in spleen, and is induced by activation of endothelial cells and monocytic cells (15–17). Induction in monocytes was found to be inhibited by IL-10 (15). Our EST data revealed *MIP-3α* expression also in activated T cells and fibroblasts (B. K. et al., unpublished results). The MIP-3α protein is chemotactic for lymphocytes (16 and B. K. et al., unpublished results) and can inhibit colony formation by hematopoietic progenitors in vitro (17). Specific binding sites for MIP-3α have been demonstrated on lymphocytes, but the specific receptor for MIP-3α was not identified (16). Here we report that STRL22 is a receptor for MIP-3α.

MATERIALS AND METHODS

Cell culture and preparation of peripheral blood lymphocytes. Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection, and were grown in MEM (Life Technologies, Gaithersburg, MD) plus 10% horse serum. The R8 tumor infiltrating lymphocytes (TIL) were derived as described (18), and were grown in AIM-V medium (Life Technologies) containing 500 U/ml of IL-2. Elutriated peripheral blood lymphocytes (PBL) were collected from normal donors by the Department of Transfusion Medicine, NIH. The PBL were further purified by Ficoll-paque banding.

Production of STRL22-transfected cell lines. An *Msp*A1 I-*Nsi* I *STRL22* genomic fragment containing the complete *STRL22* coding sequence was isolated, and the 5′ and 3′ ends were modified with adapters containing *Hin*d III and *Bam*H I restriction sites, respectively. The fragment was then inserted into pCEP4 (Invitrogen, San Diego, CA). The pCEP4/*STRL22* DNA was transfected into HEK 293 cells by calcium phosphate precipitation. Selection was done using 200 μg/ml hygromycin B (Sigma, St. Louis, Mo.). Hygromycin B resistant colonies were selected and expanded. Lines expressing the highest levels of *STRL22* mRNA were used to test responses to chemokines and in competition binding studies.

Library construction and expressed sequence tag (EST) analysis. Poly-adenylylated RNA was purified from a variety of tissues or cell lines using purification kits according to the manufacturer's recommendations (Life Technologies; Biotecx Laboratories, Houston, TX; Qiagen, Chatsworth, CA; Invitrogen). cDNA libraries were made using the ZAP-cDNA or ZAP-Express kit (Stratagene, La Jolla, CA) according to the manufacturer's protocols. This portion of the project was supported in part by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute.

Bacterial colonies containing plasmids with inserts were selected and lysed, and the cDNA inserts were amplified by PCR. EST clones were identified and analyzed using established EST methods (13,14). The initial EST clones used in this study were discovered as part of a collaboration between scientists at The Institute for Genomic Research (Gaithersburg, MD) and at Human Genome Sciences, Inc. For sequence analysis and assembly, Sequencher version 2.1.1 software (Gene Codes Corp., Ann Arbor, MI) was used.

Production and purification of Chemokine β-4/MIP-3α. Using a clone isolated from a gall bladder cDNA library, the coding sequence for Chemokine β-4 (Ckβ-4), which matched the sequence of MIP-3α and LARC, was amplified by PCR with introduction of *BamH* I and *Asp* I sites at the fragment termini. The PCR product was inserted into the A2 baculovirus transfer vector (Human Genome Sciences), which contains the *E. coli Lac Z* gene to facilitate the identification of recombinant viruses. Homologous recombination was used to introduce the *MIP-3α* sequences into *Autographa californica* and recombinant viruses were isolated.

The Sf9 *Spodoptera frugiperda* cell line was obtained from the American Type Culture Collection (ATCC CRL1711) and was grown at 27° C in EX-CELL 400 medium (JRH Biosciences, Lenexa, KS) containing 1% heat-inactivated FBS. Cells were infected at a multiplicity of infection of 2 and after 72 h the cells were removed by centrifugation. The supernatant was loaded onto a high throughput cation exchange column (POROS HS 50, PerSeptive Biosystems, Houston, TX), and protein was eluted with 40 mM sodium acetate plus 750 mM NaCl, pH 5.5. The fractions containing MIP-3α were pooled and then brought to a final concentration of 40 mM sodium acetate plus 100 mM NaCl, pH 5.5, before loading on a weak cation exchange column (POROS CM20, PerSeptive Biosystems), which was eluted with a linear gradient of 0.35 M to 2 M NaCl. The fractions containing MIP-3α were applied to a size exclusion column, Sephacryl S-75 (Pharmacia Biotech, Piscataway, NJ), yielding a MIP-3α preparation estimated to be greater than 95% pure. The protein concentration was determined by the BCA method (Pierce, Rockford, IL) using a BSA standard, according to the supplier's protocol.

For N-terminal analysis, protein was subjected to SDS-PAGE and then transferred onto a ProBlott membrane (Perkin-Elmer/Applied Biosystems, Inc., Foster City, CA). After staining with Ponceau S, the protein band was excised and analyzed using an ABI-494 sequencer (Perkin-Elmer/Applied Biosystems, Inc.). The N-terminal sequence, ASNFD, matched the predicted sequence of MIP-3α (15) and the N-terminal sequence that had been determined for recombinant LARC (16).

Measurement of calcium flux. For analysis of calcium flux by ratio fluorescence spectrometry, the *STRL22*-transfected HEK 293 cells were trypsinized and washed with HBSS containing 1% fetal bovine serum and 10 mM HEPES. The R8 TIL and the PBL were harvested and washed with the same buffer. The cells were incubated at 2×10^6/ml with 2 μM fura-2 AM (Molecular Probes Inc., Eugene, OR) for 1 h at 30°C with occasional shaking. Loaded cells were washed twice, and transferred to a cuvette in a temperature-controlled holder at 37°C with continuous stirring. Calcium measurements were done as previously described (19). Chemokines were prepared at Human Genome Sciences except for macrophage-derived chemokine (MDC), which was a gift from Ronald Godiska and Patrick Gray (ICOS, Bothell, WA) and fractalkine, which was a gift from Elizabeth Oldham and Thomas Schall (DNAX, Palo Alto, CA).

Iodination of MIP-3α. MIP-3α was iodinated with Iodobeads (Pierce) according to the manufacturer's protocol. Briefly, 10 μg of MIP-3α, diluted in 100 mM sodium phosphate buffer, pH 7.0, was reacted with 1 mCi (3.7 MBq) Na ^{125}I (Amersham, Arlington Heights, IL) and 2 Iodobeads for 10 min at ambient temperature. The labeled MIP-3α was separated from the free Na ^{125}I by Sephadex G-25 chromatography. The specific activity of the ^{125}I-MIP-3α was 19 Ci/mmol. The ^{125}I-MIP-3α was analyzed by SDS-PAGE to confirm that the label was associated only with protein of the correct size. Preparations of ^{125}I-MIP-3α and unlabelled MIP-3α had equivalent activities in a calcium flux assay using *STRL22*-transfected HEK 293 cells.

^{125}I-MIP-3α binding assay. Competition binding studies were done on *STRL22*-transfected HEK 293 cells. Unlabelled chemokines were obtained from Peprotech (Rocky Hill, NJ). The *STRL22*-transfected cells were resuspended in binding buffer (MEM containing 1% BSA and 20 mM HEPES), and 2×10^6 cells (50 μl) was then transferred to each well of a 96 well U bottom plate (Costar, Cambridge, MA). 1 nM ^{125}I-MIP-3α, and where appropriate 100 or 1000 fold

excess of cold competitor chemokines, were then added in 50 μl of binding buffer. After incubation for 2 h at 4°C with gentle agitation, the cells were pelleted by centrifugation at 1000 rpm for 5 min. Supernatants were removed and the cells were washed twice in cold PBS and transferred to a 24-well filtered-bottom plate (UniFilter GF/B, Packard, Dowers Grove, IL) that had been blocked with 1% BSA and 1% polyethylenimine in PBS. Following binding of cells, the filters were rinsed with 0.5 ml of cold PBS and dried. 250 μl of Microscint 20 (Packard) was added to each well, and the plates were counted in a Packard TopCount.

RESULTS

STRL22-transfected cells respond to and bind MIP-3α. A fragment of human genomic DNA containing the complete coding sequence of *STRL22* was inserted into the pCEP4 plasmid and the resulting DNA was used to transfect human embryonic kidney (HEK) 293 cells. Individual colonies of cells that survived selection with hygromycin B were expanded and analyzed for expression of *STRL22* RNA. Using cDNA from 7 of the transfected cell lines and PCR with primers flanking the intron that interrupts the *STRL22* coding sequence (10), we demonstrated, based on the size of the amplified fragment, that the RNA encoding STRL22 had been appropriately processed in the transfected cells (not shown).

Cell lines expressing the highest levels of *STRL22* RNA were analyzed for responses to a panel of chemokines using an assay for agonist-dependent changes in intracellular calcium, $[Ca]_i$. We had reported previously that no responses were found with HuMig, IP-10, Platelet factor 4, MCP-1, MCP-2, MCP-3, MCP-4, MIP-1α, MIP-1β, RANTES, I309, and lymphotactin (10), and testing done with macrophage-derived chemokine (MDC), soluble fractalkine, HCC-1, MIP-3β and nine additional, novel chemokines produced by Human Genome Sciences, Inc. was also negative (not shown). A transient rise in $[Ca]_i$ was seen, however, in response to MIP-3α. MIP-3α produced a calcium flux in four independent *STRL22*-transfected lines and responses of one of these lines to various doses of MIP-3α are shown in FIG. 1. No response to MIP-3α was seen with three independent HEK 293 lines transfected with DNA encoding a related receptor, STRL33 (12). The HEK 293 cells responded to MIP-3α with an EC_{50} of approximately 25 ng/ml. FIG. 1 shows that maximal activation of STRL22 made cells unresponsive to a second addition of ligand, demonstrating the desensitization phenomenon typical for G protein coupled receptors.

Consistent with the signalling data, FIG. 2 shows that while binding by 1 nM of radiolabelled MIP-3α to *STRL22*-transfected HEK 293 cells could be specifically inhibited with increasing concentrations of unlabelled MIP-3α, four other CC chemokines, even at μM concentrations, had no effect on MIP-3α binding. No specific binding of ^{125}I-MIP-3α could be detected on a

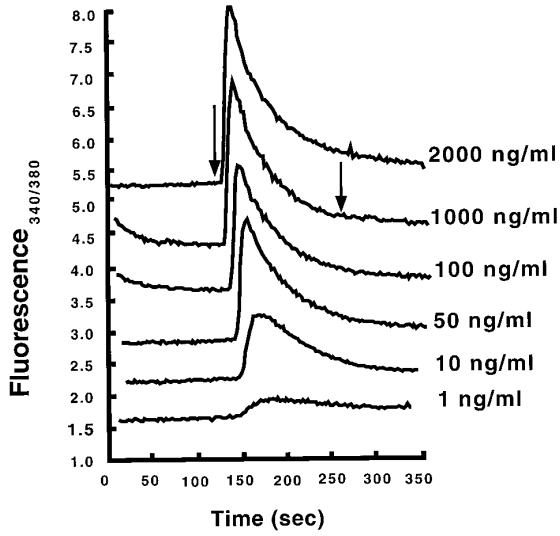

FIG. 1. *STRL22*-transfected cells respond to MIP-3α. At the times indicated by the arrow on the left, MIP-3α was added at concentrations of 1-2000 ng/ml to cuvettes containing 10^6 fura-2 AM-loaded *STRL22*-transfected HEK 293 cells and ratio fluorescence was recorded. In the study using 1000 ng/ml MIP-3α, a second addition of 1000 ng/ml was made as indicated by the arrow on the right.

control, vector-transfected line of HEK 293 cells (not shown).

Lymphocytes flux calcium in response to MIP-3α. Demonstrations that the *STRL22* gene is expressed in PBL (10–12), together with data that MIP-3α is chemotactic for PBL (16 and B. K. et al., unpublished results) led us to test lymphocytes for responses to MIP-3α in the calcium flux assay. As shown in FIG. 3, freshly-isolated PBL produced a rise in $[Ca]_i$ in response to MIP-3α. As anticipated for a bona fide response, the cells did not respond to a second addition of ligand.

The TIL R8 cells, $CD8^+$ T cells isolated from a human melanoma and maintained in vitro as described (18,19), had been shown to express easily-detectable levels of *STRL22* mRNA (12). As shown in FIG. 4A, the TIL responded to MIP-3α in a dose-dependent fashion with an EC_{50} of approximately 0.8ng/ml. The B10 TIL, in which the *STRL22* mRNA is not detectable (12), had no response to MIP-3α (not shown). FIG. 4B shows that the MIP-3α-dependent rise in $[Ca]_i$ could be blocked in the TIL by pertussis toxin, demonstrating that STRL22 was signalling through $G\alpha_i$.

DISCUSSION

We have demonstrated that *STRL22*-transfected cells respond to the CC chemokine MIP-3α and not to 26 other chemokines tested, and likewise that binding of MIP-3α to *STRL22*-transfected cells is specific and cannot be inhibited by several other CC chemokines.

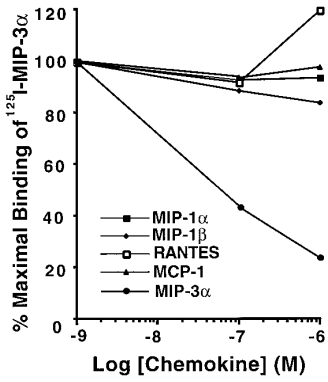

FIG. 2. Other CC chemokines do not compete with MIP-3α for binding to *STRL22*-transfected HEK 293 cells. 2×10^6 STRL22-transfected HEK 293 cells were incubated with 1 nM ^{125}I-MIP-3α together with 0, 100, or 1000 nM unlabelled chemokines for 2 h at 4°C before cells were transferred to filters, washed, and counted. Approximately 900 cpm were detected on cells incubated without unlabelled competitors.

While recognizing that additional ligands for STRL22 may yet be discovered, the specific relationship between MIP-3α and STRL22 is unusual among chemokines and their receptors. CC chemokine receptors CCR1 through CCR5 have each been reported to signal in response to more than one chemokine ligand (20). Conversely, our findings (not shown) that MIP-3α does not signal on the B10 TIL, which express *CCR1*, *CCR2*, *CCR4*, and *CCR5* (12) and which respond to ligands for all these receptors (R.L. Rabin, F.L. and J.F., unpublished results), are consistent with MIP-3α signalling exclusively through STRL22. Our previous demonstration that, among the major populations of leukocytes, expression of *STRL22* was limited to lymphocytes (12), together with the findings by Hieshima et al. (16) and our unpublished results (B. K. et al., unpublished results) that MIP-3α targets lymphocytes but not other leukocytes, supports this supposition.

Our observation that MIP-3α can cause calcium mobilization in freshly-isolated lymphocytes is of interest. The literature contains discrepant data on responses of freshly-isolated lymphocytes to chemokines, which may be at least partly the result of differences in the methods used for lymphocyte purification. While multiple CC chemokines (21), including MIP-3α (16), have been reported to be active as chemotactic factors for freshly-isolated lymphocytes, recent studies by Loetscher et al. (22) found that lymphocytes responded to CC chemokines only after culture in vitro with IL-2, which led to a dramatic induction of mRNAs for CCR1 and CCR2. Consistent with these latter findings, in studies with a broad panel of both CC and CXC chemokines, we have not seen calcium responses in PBL without first activating the cells in vitro (R.L. Rabin and JF, unpublished results). Our finding that freshly-isolated, non-activated lymphocytes mobilize calcium with MIP-3α sets MIP-3α apart from other chemokines, and suggests that MIP-3α may target a more diverse population of lymphocytes in vivo as compared with other chemokines, particularly other chemokines in the CC subfamily.

The concentrations of MIP-3α that we found effective

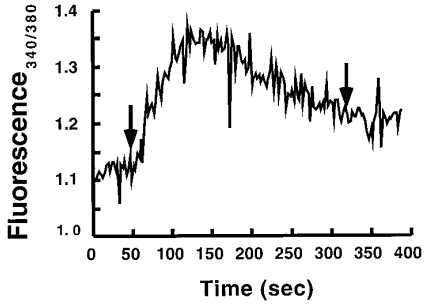

FIG. 3. Freshly-isolated peripheral blood lymphocytes respond to MIP-3α. Elutriated peripheral blood lymphocytes were loaded with fura-2 AM and ratio fluorescence was recorded using 10^6 cells and MIP-3α added at 1 μg/ml at the times indicated by the arrows.

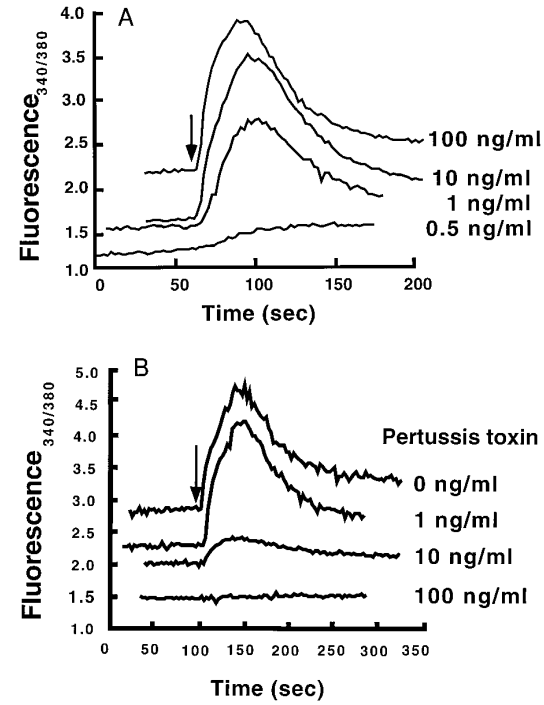

FIG. 4. Tumor infiltrating lymphocytes respond to MIP-3α and the response is inhibited by pertussis toxin. A, At the times indicated by the arrow, MIP-3α was added at concentrations of 0.5-100 ng/ml to cuvettes containing 10^6 fura-2 AM-loaded R8 TIL cells and ratio fluorescence was recorded. B, Following incubation with 0-100 ng/ml pertussis toxin at 37°C for 2.5 h, R8 TIL were washed and loaded with fura-2 AM and ratio fluorescence was recorded using 10^6 cells. MIP-3α was added at 1 μg/ml at the times indicated by the arrow.

for signalling on the *STRL22*-transfected HEK 293 cells are in the range of concentrations at which we (B. K. et al., unpublished results) and others (16,17) have found MIP-3α to be active as a lymphocyte chemotactic factor. In contrast, the EC_{50} for calcium mobilization in the R8 TIL was approximately 1/30 of the value for the transfected HEK cells. A similar discrepancy between the EC_{50} on natural target cells as compared to receptor gene-transfected cells has been observed for MIP-1α (23). The basis for these discrepancies is not known. Notwithstanding our previous discussion, one possibility is that the R8 TIL express a MIP-3α receptor in addition to STRL22. It is also possible that differences in EC_{50}'s reflect uses of different G proteins. In this regard, we have shown that in the R8 TIL, pertussis toxin can block calcium mobilization in response to MIP-3α, similar to findings with most chemokines (20), and demonstrating that the signal in these cells was dependent on $G\alpha_i$ (24). In contrast, in data not shown, 100 ng/ml pertussis toxin had no effect on MIP-3α signalling in the transfected HEK 293 cells, suggesting that signalling in these cells is independent of $G\alpha_i$. Similar cell type-dependent use of G proteins has been described for chemokine receptor CCR2 (25).

Our demonstration that STRL22 can signal in response to and bind MIP-3α establishes STRL22 as a CC chemokine receptor and STRL22 should, therefore, be re-named CCR6. Given these new functional data, it is appropriate to clarify the relationships among the *STRL22/CCR6*-related sequences in the literature and the sequence data bases. In our earlier work, we analyzed both genomic (GenBank accession number U68031) and cDNA (GenBank accession numbers U68030 and U68032) sequences for *STRL22/CCR6* (10). As noted above, the sequence data bases contain, in addition to our *STRL22* sequences, submissions of similar sequences from three other laboratories. The *CKR-L3* and U45984 sequences were obtained from genomic DNA, and in each case the open reading frame initiator was improperly assigned due to the presence of an intron interrupting the coding sequence, an uncommon finding among chemokine receptor genes (26). The U60000 sequence was obtained from cDNA from a hepatoma cell line and while the translation of the N-terminus of the predicted protein agrees with ours, the U60000 protein contains multiple discrepancies as compared with the STRL22. Of particular note, the carboxy-terminal sequence of the U60000 protein differs from that of STRL22 due to a frame shift resulting from what we believe to be the erroneous insertion of a guanosine residue at position 1108 of the U60000 cDNA sequence.

Our data suggest a specific and dedicated relationship between MIP-3α and STRL22/CCR6. Moreover, the data from our laboratories and others suggest that MIP-3α and STRL22/CCR6 play a role specifically in the physiology of lymphocytes. In contrast with other CC chemokine/receptor pairs, MIP-3α and STRL22/CCR6 appear to function on both non-activated as well as activated lymphocytes, the latter including T cells derived from human tumors. Additional studies will focus on characterizing MIP-3α responses and STRL22/CCR6 activation in lymphocyte subsets in order to understand more fully the role of this novel receptor/ligand pair in lymphocyte biology.

ACKNOWLEDGMENTS

Thanks are due to the Department of Sequencing and Bioinformatics, Human Genome Sciences, Inc., and to The Institute for Genome Research, Rockville, MD, for contributions to EST cloning and analysis, to John R. Yannelli, NCI, NIH for providing TIL, to Wei-lin Liu for help with the binding assays, to Ronald Godiska and Patrick Gray of ICOS for providing MDC, to Elizabeth Oldham and Thomas Schall of DNAX for providing fractalkine, and to Ronald Rabin for reviewing the manuscript.

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