Molecular Cloning of a Novel Human CC Chemokine Liver and Activation-regulated Chemokine (LARC) Expressed in Liver

CHEMOTACTIC ACTIVITY FOR LYMPHOCYTES AND GENE LOCALIZATION ON CHROMOSOME 2*

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Chemokines are a large family of small cytokines involved in a variety of immunoregulatory and proinflammatory responses, primarily by virtue of their leukocyte chemotaxis activity (1–3). Based on whether the first two of the four conserved cysteine residues are juxtaposed or separated by a single amino acid residue, this family is subdivided into two groups, the CXC and the CC chemokines. Recently, a new chemokine-like molecule, lymphotactin/SCM-1, which lacks the first and the third conserved cysteine residues, has been isolated and may represent a third group C (4–6). In general, CXC chemokines are potent chemoattractants for neutrophils, whereas CC chemokines are chemotactic mainly for monocytes and also for basophils, eosinophils, and lymphocytes with variable selectivity. Lymphotactin/SCM-1 has been shown to attract lymphocytes. In addition to chemotactic activities, some chemokines have regulatory roles in hematopoiesis (7, 8). Recently, three members of the CC chemokines, MIP-1α/LD78α, MIP-1β, and RANTES, have been shown to block entry of the human immunodeficiency virus type 1 into macrophages (9–12). The genes of the CXC and CC chemokines are clustered on human chromosomes 4 and 17, respectively (1), except for the CXC chemokine SDF-1/PBSF whose gene has been mapped to human chromosome 10 (13). The lymphotactin/SCM-1 gene has been localized to human chromosome 1 (5, 14).

Until recently, chemokines have been purified according to their chemotactic activities or have been cloned by subtraction or differential hybridization. The cell types used in such studies have been mainly blood lymphocytes or tumor cell lines. However, as shown by recent Northern hybridization analyses, chemokines are often expressed constitutively in some normal tissues (6, 13, 15–19). For example, the mRNA of eotaxin, which is a novel CC chemokine selectively chemotactic for eosinophils, is expressed at high levels in small intestine, colon, and heart but at low levels in peripheral blood leukocytes, spleen, and thymus (15–17). Therefore, there might still exist uncovered chemokines expressed in other cell types and tissues. Currently, world-wide efforts to find all human genes, approximately ~100,000, are in progress as part of the human genome project. Accordingly, cDNAs from libraries of various human tissues are sequenced from both 5′- and 3′-ends, and their partial, “single pass” cDNA sequences are deposited as expressed sequence tags (ESTs)1 in the public data bases. Re-

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1 The abbreviations used are: ESTs, expressed sequence tags; YAC, yeast artificial chromosome; dbEST, expressed sequence tag database;
cently we found four ESTs encoding CC chemokine-like proteins in the EST data base (dbEST), a division of GenBank (20). The genes of these four ESTs have been mapped in a yeast artificial chromosome (YAC) contig covering the CC chemokine gene cluster on human chromosome 17q11.2 (20). This demonstrates that the dbEST is a good source for new chemokines. Furthermore, the YAC contig may be useful to quickly assign new chemokine genes within the cluster.

Here we report the identification of a fifth new CC chemokine, termed LARC (Liver and Activation-Regulated Chemokine) from the dbEST. This new CC chemokine is different from other CC chemokine members in several respects. 1) LARC is only distantly related to other CC chemokines, and its gene is located on a different chromosome than other CC chemokines. 2) Unlike other CC chemokines, LARC is not chemotactic for monocytes but is primarily a lymphocyte chemotaxtractant. 3) Lymphocytes display a single class of receptors for LARC that is not shared by other CC or CXC chemokines.

EXPERIMENTAL PROCEDURES

**EST Data Base Search**—The dbEST (21) was searched with various CC chemokine nucleotide sequences or amino acid sequences as queries using the AnglEx search and analysis service Search Launcher (22), available on the World Wide Web. The program used was Basic Local Alignment Search Tool (23).

**Isolation and Characterization of cDNA Clones**—Human histiocytic lymphoma cell line U937 was grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and stimulated with phorbol 12-myristate 13-acetate (PMA) at a concentration of 50 ng/ml for 6 h. Poly(A)+ RNA was prepared using the QuickPrep mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). cDNA was synthesized by the rapid amplification of cDNA ends (RACE) method (24) using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA). Briefly, the double-stranded cDNA was synthesized from poly(A)+ RNA using a cDNA synthesis primer supplied in the kit. The cDNA adaptors were then ligated at both ends of the cDNA. The adaptor-ligated cDNA was then amplified by polymerase chain reaction (PCR) with one of the gene-specific primers derived from an EST sequence (GenBank accession number D31065) (5’t oligomer: GTACTCAACACTGAGCAGATCT and 3’t oligomer: AGGTGAGCTAGGCGACT) and an A1 primer which is complementary to part of the cDNA adaptor and supplied in the kit. PCR was performed in a 50-μl reaction mixture containing 0.25 mM each of the dNTPs, 50 pmol of each of the primers, 2.5 units of TAKARA Taq DNA polymerase in 50 mM Tris-hydrochloride, 72°C, 1 min, in a 5-μl reaction buffer containing 0.25 mM of each of the dNTPs, 50 pmol of each of the primers, and 0.125 units of TAKARA Taq (Takara). Products were electrophoresed on 2% agarose gels. Using these primers, PCR is expected to generate a 100-base pair fragment. The polymerase chain reaction data were analyzed using 50 ng of these DNAs. The PCR conditions were as described above.

**Preparation of Recombinant LARC Protein**—LARC protein was prepared using a baculovirus expression system. The LARC cDNA fragment containing the entire coding region was excised with BglII (5’-noncoding region) and NorI (cDNA synthesis primer sequence) from the 3’-RACE cDNA and subcloned into the BomHI-NorI sites of the pVL1393 baculovirus transfer vector (Invitrogen, San Diego, CA), downstream of the polyhedrin gene promoter. The resulting recombinant plasmid, termed pVL-LARC, was identified by restriction mapping.

**Northern Blot Analysis**—cDNA was prepared from U937, K562, an erythroleukemia cell line, Jurkat, a T cell line, and Bowes melanoma cells. Messenger RNAs were extracted from cells without or with PMA treatment (50 ng/ml, 6 h). After electrophoresis in a 1% formaldehyde-agarose gel (2 μg per lane), RNAs were blotted onto a nylon membrane. Northern blot filters containing human poly(A)+ RNA from various tissues (2 μg per lane) were purchased from Clontech. Filters were hybridized with the 32P-labeled LARC cDNA probe in a solution containing 5 × SSPE, 10 × Denhardt’s solution, 100 μg/ml denatured salmon sperm DNA, 2% sodium dodecyl sulfate, and 50 mM formamide at 42°C overnight and washed at 50°C for 20 min in 0.1 × SSC, 0.1% SDS. After autoradiography, filters were reprobed with a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. The LARC and GAPDH probes were prepared by labeling the 3’-RACE cDNA fragment (Fig. 1) and the RsaIII-XbaI fragment (0.5 kb) of the human GAPDH (25) to a high specific activity using Marathon cDNA amplification kit (Clontech, Palo Alto, CA), for chromosomal mapping and radiation hybrid mapping, respectively. The PCR conditions for amplification of the LARC-specific sequence were cycles of 94°C, 30 s, 60°C, 30 s, 68°C, 3 min, for 30 cycles. The products were inserted into a pGEM-T vector (Promega, Madison, WI) and sequenced on both strands using gene-specific and commercial primers.

**Human liver cDNA library, constructed with random hexamers and cloned in the Agt11 vector, was screened using the LARC 3’-RACE (fragment 1) as a probe according to standard methods (25). The library was kindly provided by Dr. M. Takiguchi of Kumamoto University Medical School. The cDNA fragments of the positive clones were excised with EcoRI and inserted into pBluescript II KS+ (Stratagene, La Jolla, CA) and sequenced.**

**Northern Blot Analysis—Poly(A)+ RNA was prepared from U937, K562, an erythroleukemia cell line, Jurkat, a T cell line, and Bowes melanoma cells. Messenger RNAs were extracted from cells without or with PMA treatment (50 ng/ml, 6 h). After electrophoresis in a 1% formaldehyde-agarose gel (2 μg per lane), RNAs were blotted onto a nylon membrane. Northern blot filters containing human poly(A)+ RNA from various tissues (2 μg per lane) were purchased from Clontech. Filters were hybridized with the 32P-labeled LARC cDNA probe in a solution containing 5 × SSPE, 10 × Denhardt’s solution, 100 μg/ml denatured salmon sperm DNA, 2% sodium dodecyl sulfate, and 50 mM formamide at 42°C overnight and washed at 50°C for 20 min in 0.1 × SSC, 0.1% SDS. After autoradiography, filters were reprobed with a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. The LARC and GAPDH probes were prepared by labeling the 3’-RACE cDNA fragment (Fig. 1) and the RsaIII-XbaI fragment (0.5 kb) of the human GAPDH (25) to a high specific activity using Marathon cDNA amplification kit (Clontech, Palo Alto, CA), for chromosomal mapping and radiation hybrid mapping, respectively. The PCR conditions for amplification of the LARC-specific sequence were cycles of 94°C, 30 s, 60°C, 30 s, 68°C, 3 min, for 30 cycles. The products were inserted into a pGEM-T vector (Promega, Madison, WI) and sequenced on both strands using gene-specific and commercial primers.**

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s). The total mononuclear cell fraction was used as a source for monocytes and lymphocytes. Blood lymphocytes were further purified by incubating mononuclear cells for 30 min at 4 °C with paramagnetic microbeads conjugated with monoclonal antibody against CD14 expressed on monocytes. The cell suspension was passed over a column plated with magnetic field (VarimoBACS, Miltenyi Biotec, Bergisch, Germany). Alternatively, monocytes and lymphocytes were isolated by magnetic cell sorting (VarimoBACS) using positive selection with anti-CD14 or anti-CD3, respectively. After positive or negative magnetic cell sorting, a cell purity (analyzed by fluorescence-activated cell sorter) of more than 90% was reached for monocytes (CD14+) and lymphocytes (CD3+). Purified granulocytes, mononuclear cells, lymphocytes, and THP-1 cells were washed, counted, and resuspended at 1 × 10^6, 2 × 10^6, 1 × 10^5, and 0.5 × 10^6 cells/mL, respectively, in HBSS (Life Technologies, Inc.) supplemented with pyrogen-free human plasma protein (1 mg/mL albumin, Cohn fraction V). The monocyctic THP-1 cells, grown in Dulbecco’s modified Eagle’s medium with 10% FCS (Life Technologies, Inc.), were used in the chemotaxis assay as an alternative for fresh monocytes.

Migration of cells was assessed in a microchamber (NeuroProbe, Cabin John, MD) as described previously (29). Briefly, the lower compartments of the microchamber were filled with dilutions of chemokine (27 μl) or with control buffer and the upper compartments with 50 μl of cell suspension. The two compartments were separated by a 5-μm pore size polycarbonate filter (Nuclepore, Pleasanton, CA). Polyvinylpyrrolidone-free filters were used for neutrophils and lymphocytes and polyvinylpyrrolidone-containing filters for monocytes and THP-1 cells. For lymphocyte chemotaxis, the membranes were coated with 20 μg/mL fibronectin (Life Technologies, Inc.) for 24 h at 4 °C. After incubation at 37 °C for 45 min (neutrophils), and 2 h (monocytes, THP-1 cells, or 4 h (lymphocytes), the filters were removed from the chambers, fixed, and stained with Diff-Quick (Harleco, Gibbstown, NJ). Finally, the cells of 10 oil immersion fields were counted. The chemotactic index was calculated from the number of cells migrated to the test sample divided by the number of cells migrated to the control. Synthetic human MCP-3 and natural IL-8, purified to homogeneity (29), were used as positive controls.

Production of LARC-SEAP Fusion Protein—LARC was expressed as a soluble fusion protein with secreted alkaline phosphatase (SEAP) containing a (His)6 COOH-terminal tag. For this purpose, a SEAP(His)6 tag vector, termed pDREF-SEAP(His)6-Hyg, was constructed as follows. Using the pSEAP-Enhancer (Clontech) as a template, the (His)6 coding sequence was introduced at the COOH-terminal region of SEAP by PCR using the 5′ XbaI-AP primer (5′-CGGCTCTAGAAGCTCCGGACGGTCGCGGCCGCTCAGTGATGGTGATGGTGATGACCCG-3′) and the 3′ I-AP primer (5′-CGCTCTAGAAGCTCCGGACGGTCGCGGCCGCTCAGTGATGGTGATGGTGATGACCCG-3′). The PCR product containing the coding sequence was introduced at the COOH-terminal region of SEAP tag vector, termed pDREF-SEAP(His)6-Hyg, was constructed as follows. Using the pSEAP-Enhancer (Clontech) as a template, the (His)6 coding sequence was introduced at the COOH-terminal region of SEAP by PCR using the 5′ XbaI-AP primer (5′-CGGCTCTAGAAGCTCCGGACGGTCGCGGCCGCTCAGTGATGGTGATGGTGATGACCCG-3′) and the 3′ I-AP primer (5′-CGCTCTAGAAGCTCCGGACGGTCGCGGCCGCTCAGTGATGGTGATGGTGATGACCCG-3′). The PCR product containing the coding sequence was inserted into the pDREF-SEAP(His)6 sequence was ligated with XbaI and NotI and inserted into the XbaI-NotI sites of pDREF-Hyg (18). Prior to subcloning of the LARC cDNA into this vector, the 5′-RACE LARC cDNA was amplified by PCR using the 5′ SauI-LARC primer (5′-CGGCGGCCCCATCTCTCTCTCTACAC) and the 3′ AP LARC primer (5′-CGGCTCTAGAAGCTCCGGACGGTCGCGGCCGCTCAGTGATGGTGATGGTGATGACCCG-3′) and the 3′ AP LARC primer (5′-CGGCTCTAGAAGCTCCGGACGGTCGCGGCCGCTCAGTGATGGTGATGGTGATGACCCG-3′). After digestion with SauI and XbaI, the product was ligated into the SauI-XbaI sites of the pDREF-SEAP(His)6 vector. The vector is a derivative of the secreted alkaline phosphatase (SEAP) vector pDREF-CD4ST (18), and the signal-deleted CD4 coding sequence was replaced with SEAP(His)6. This subcloning engineered a five-amino acid linker (Ser-Arg-Ser-Ser-Gly) between the NCC-7 and the SEAP(His)6.

To produce the LARC-SEAP fusion protein, 293/EBNA-1 cells (Invitrogen) were transfected with the expression vector using lipo- fectamin (Life Technologies, Inc.). The 293/EBNA-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS. The transfected cells were incubated for 3–4 days in Dulbecco’s modified Eagle’s medium containing 10% FCS. The culture supernatant was centrifuged, filtered (0.45 μm), and stored at 4 °C with 20 mM HEPEs (pH 7.4) and 0.02% sodium azide. For NH2-terminal sequence analysis, the fusion protein was purified by affinity chromatography on nickel agarose (QIAGEN, Hilden, Germany).

The concentration of LARC-SEAP was estimated by sandwich enzyme-linked immunosorbent assay. Briefly, 96-well microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with anti-idiotype alkaline phosphatase (anti-PLAP) monoclonal antibody (Medix Biotech, Foster City, CA) (2 μg/mL in 50 mM Tris-HCl (pH 9.5)). After blocking nonspecific binding sites with 1 mg/mL bovine serum albumin in phosphate-buffered saline (PBS), the samples were titrated in T-PBS (0.02% Tween 20 in PBS). After incubation for 1 h at room temperature, the plates were washed with T-PBS, incubated with biotinylated rabbit anti-PLAP antibody diluted 1:500 for 1 h at room temperature, washed again, and incubated with peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA) for 30 min. After washing, bound peroxidase was detected by 3,3′,5,5′-tetramethylbenzidine. The reaction was stopped by adding 1 N H2SO4 and the absorbance was read at 450 nm.

Alkaline phosphatase (AP) activities were determined by a chemiluminescent assay using the Great Esape Detection Kit (Clontech). Purified PLAP (Cosmo Bio) was used to generate standard curves. AP activities are expressed here as relative light units (1 pmol of SEAP (His)6, and LARC-SEAP (His)6 corresponds to approximately 8.7 × 10^4 and 1.7 × 10^6 relative light units, respectively).

Binding Assay—Peripheral blood mononuclear cells were isolated from venous blood obtained from healthy adult donors using Ficoll-Paque (Pharmacia Biotech). Monocytes and lymphocytes were incubated with fluorescein isothiocyanate-conjugated anti-CD14 antibody and separated by MACS (Miltenyi Biotec, Bergisch, Germany). The purity of the cells was >98% as determined by flow cytometry on a FACStar Plus (Beckton Dickinson, Mountain View, CA).

For displacement experiments, 2 × 10^5 cells were incubated for 1 h at room temperature with diluted supernatant containing 1 nm of SEAP (His)6, or LARC-SEAP (His)6 in the presence of increasing concentrations of unlabeled chemokines in 200 μl of RPMI 1640 containing 20 mM HEPEs (pH 7.4), 1% bovine serum albumin, and 0.02% sodium azide. MCP-1 and TARC were prepared as described (18). MIP-1α/LD78α, RANTES, and interleukin-8 (IL-8) were purchased from PeproTech (Rocky Hill, NJ). For saturation experiments, cells were incubated for 1 h at 15 °C with increasing concentrations of LARC-SEAP (His)6 in the presence of absence of 1 μM unlabeled LARC. After incubation, cells were washed five times and lysed in 50 μl of 10 mM Tris-HCl (pH 8.0), 1% Triton X-100. Samples were heated at 65 °C for 10 min to inactivate cellular phosphatase and then centrifuged. Bound AP activity in 25 μl of the lysate was determined by the chemiluminescence assay as described above. All samples were assayed in duplicate. The binding data were analyzed by the LIGAND program.

RESULTS

Cloning of LARC cDNAs—To find new members of the human CC chemokine family, we have searched the dbEST with various CC chemokine amino acid sequences and nucleotide sequences. This search identified five novel CC chemokine-like molecules encoded by single-pass EST sequences. Four of these were mapped within a YAC contig containing the human CC chemokine gene cluster on chromosome 1q74–11.2, providing additional evidence that they are indeed CC chemokine members (20). Another molecule, later termed LARC (Liver and Activation-Regulated Chemokine (see below)), was encoded by EST sequences, D17181, D31065, D82589, T27336, and T27433 (GenBank accession numbers), but its gene was not present in the YAC contig (data not shown). Sequence length, orientation, and the cDNA library of each EST clone are shown in Fig. 1.
To clone the full-length cDNA of LARC, we first utilized the RACE method. Since a preliminary reverse transcriptase-PCR analysis showed that the LARC mRNA is expressed in PMA-stimulated macrophage-like U937 cells, we used the cDNA prepared from the PMA-stimulated U937 mRNA for the RACE reaction. Two LARC gene-specific primers, one for 5’-RACE and one for 3’-RACE, were designed from one of the EST sequences, D31065. The RACE products were cloned and sequenced (Fig. 2A). To exclude the base substitutions that might be incorporated during PCR, three cDNA clones were further isolated from a human liver cDNA library with the 5’-RACE cDNA fragment as a probe. The three cDNA clones were identical. The cDNA sequence was also identical to those of the RACE products in overlapping regions and in agreement with the ESTs. Fig. 1 schematically shows the alignment of the RACE products, the phage three identical cDNA clones, and the five ESTs. The 5’-RACE product extended 7 nucleotides beyond the 5’-end of D31065 that was the longest of the five ESTs. The 5’-ends of the three phage cDNA clones were identical with the 5’-RACE product. Interestingly, three ESTs, D82589, T27336, and T27433, had a 3-base pair deletion that causes deletion of the NH2-terminal residue of the mature protein (see “Discussion”). By searching the dbEST with the full-length LARC sequence as a query, we identified one more EST, D17012, which contains the sequence of the 3’-noncoding region (Fig. 1).

The LARC cDNA is approximately 0.8 kb long and contains the longest open reading frame of a 96-amino acid protein that starts at the first methionine codon (Fig. 2A). The nucleotide sequence around this methionine codon conforms to the consensus sequence shared by many mRNAs of higher eukaryotes (30). The NH2-terminal end of the deduced amino acid sequence is highly hydrophobic and is consistent with a typical signal peptide sequence (31). The cleavage site is predicted to be between Ala-26 and Ala-27. The primary sequence of LARC contains no putative N-glycosylation site. The 3’-noncoding region does not contain the typical AATAAA polyadenylation signal but contains two copies of the AATAAG sequence that is also used as a polyadenylation signal in the human α-globin gene (32). The 3’-noncoding region also contains three copies of the mRNA destabilization signal (ATTATA) that is often present in the 3’-noncoding regions of many cytokine mRNAs (33).

Amino Acid Sequence Homology With Other CC Chemo- kines—LARC shows relatively low sequence similarity with other human CC chemokines, having the highest similarity to MIP-1β (28% identity) (Fig. 2B). The alignment data in B, evolutionary distances between the human CC chemokines were estimated using the GeneWorks (IntelliGenetics, Mountain View, CA). The location of the branch points is not to scale.
Fig. 3. Northern blot analysis of poly(A)+ RNAs from human cell lines and tissues. A, leukemic cell lines, Jurkat, K562, and U937, and Bowes melanoma cell lines. Poly(A)+ RNAs were prepared from the cells that had been stimulated with PMA for 6 h (+) or left unstimulated (−) and transferred onto a nylon membrane. B, normal tissues. The filters were obtained from Clontech. These filters were hybridized with 32P-labeled LARC 3′-RACE fragment (Fig. 1). After autoradiography, the filters were rehybridized with the GAPDH cDNA probe. PBL, peripheral blood leukocyte.

...due to this YAC contig (20). Surprisingly, however, the result was inconsistent with our radiation hybrid mapping data. The GeneBridge 4 Radiation Hybrid panel consisting of 91 hybrid DNAs (Research Genetics, Whitehead Institute Center for Genomic Research order) was used. The result of radiation hybrid screening was 1000000000 0000000011 1110100100 0100000010 0100110001 0000000000 0111000001 0010000010 0000000000 0 0 and 1 represent negative and positive PCR assays, respectively. C, YAC identification. Twenty YACs containing a STS D2S159 were analyzed by PCR with LARC primers.

Fig. 4. Chromosomal localization of the LARC gene and identification of YAC clones containing the LARC gene. A, PCR analysis of somatic cell hybrid DNAs containing a single human chromosome. Hybrid DNAs from the NIGMS human × rodent somatic cell mapping panel No. 2 version 2 were analyzed by PCR with LARC primers. Lanes are labeled 1–22, X, and Y to indicate the human chromosome retained in each hybrid. Human (H), Chinese hamster (C), and mouse (M) genomic DNA controls were used to identify bands generated by the parental cells. N, negative control (PCR reaction without DNA). S, size marker. B, localization of the LARC gene by radiation hybrid mapping. The GeneBridge 4 Radiation Hybrid panel consisting of 91 hybrid DNAs (Research Genetics, Whitehead Institute Center for Genomic Research order) was used. The result of radiation hybrid screening was 1000000000 0000000011 1110100100 0100000010 0100110001 0000000000 0111000001 0010000010 0000000000 0 0 and 1 represent negative and positive PCR assays, respectively. C, YAC identification. Twenty YACs containing a STS D2S159 were analyzed by PCR with LARC primers.

...ues that are conserved in other CC chemokines are replaced in LARC (Tyr → Phe-49, Thr → Ala-54; indicated by filled circles in Fig. 2B). One of these residues, Tyr, has been shown to be important for monocyte chemotaxis (45, 46). A phylogenetic tree based on the similarity scores highlights the low relatedness of LARC to other CC chemokine family members (Fig. 2C).

Induced and Constitutive Expression of LARC mRNA—We examined the expression of LARC mRNA in various human cell lines and tissues. Poly(A)+ RNAs were extracted from Jurkat, K562, U937, and Bowes melanoma with and without PMA stimulation for 6 h and analyzed by Northern hybridization. While there was no detectable LARC mRNA in unstimulated cells, LARC mRNA was strongly induced in U937 and Bowes melanoma after stimulation with PMA (Fig. 3A). No such induction was, however, observed in Jurkat and K562. The detected LARC mRNA was approximately 0.9 kb long, which corresponded to the size of the LARC cDNA plus a poly(A) tail.

We then examined expression of LARC mRNA in various human tissues. LARC mRNA was found to be constitutively expressed at high levels in liver and, to a lesser extent, in lung (Fig. 3B). When another batch of the commercial Northern filters was examined, expression of LARC mRNA in lung was quite low compared with that in liver (data not shown). Very low levels of expression were also seen in thymus, prostate, testis, small intestine, and colon. In testis, two transcripts, approximately 1.0 and 1.3 kb long, were observed instead of the 0.9-kb one. Transcripts were not detected in spleen and peripheral blood leukocytes. Although three LARC ESTs (D25839, T27336, and T27433) were derived from the pancreatic islet cDNA library, no constitutive expression was detected in pancreas even using different batches of filters.

Chromosomal Localization of the LARC Gene—We tested whether the LARC gene was present in the YAC contig of several mega bases corresponding to human chromosome 17q11.2. All CC chemokines reported so far and four newly identified CC chemokine genes (NCC-1→4) have been mapped to this YAC contig (20). Surprisingly, however, the result was negative (data not shown). We therefore examined the chromosomal location of the LARC gene by using the DNA panel of the somatic cell hybrids containing human monochromosomes. The LARC gene was localized to chromosome 2 (Fig. 4A). To map the location of the LARC gene more precisely, radiation hybrid mapping was performed using the GeneBridge 4 panel (27) (Fig. 4B). The resulting PCR data were analyzed at the Radiation Hybrid mapping server at the Whitehead Institute/MIT Center for Genome Research. The results showed that the LARC gene is located 0.0 centi-Ray (3.7 centi-Ray is approximately 1 mega base pairs) apart from a STS marker D2S159 that is mapped between the bands q33 and q37 of chromosome 2 (47). We therefore analyzed 20 YAC clones containing D2S159 for the LARC gene by PCR. Two clones, 770_f_5 (1540 kb) and 933_c_7 (1690 kb), were positive for LARC (Fig. 4C). One of the clones, 933_c_7, has already been used for fluorescence in situ hybridization analysis and has been mapped to a locus 93–95% from the top of chromosome 2 (48). This fluorescence in situ hybridization result is consistent with our radiation hybrid mapping data.

Expression of LARC Protein—To obtain LARC protein, recombinant baculovirus was prepared. Insect cells BTI-TN-5B1-4 were infected with the virus, and recombinant LARC was purified from the culture supernatants by cation-exchange chromatography and reverse-phase high performance liquid chromatography. LARC was eluted from the reverse-phase column as a single major peak (Fig. 5A). The yield of purified protein was ~0.4 μg per ml of starting culture supernatant.
When analyzed by SDS-polyacrylamide gel electrophoresis and silver staining, the purified protein migrated as a single band of 8 kDa (Fig. 5B). Amino acid sequence analysis demonstrated that the NH$_2$ terminus of recombinant LARC started at Ala-27 of the predicted sequence. These results agreed with the predicted signal cleavage site and molecular weight of the mature protein.

Chemotaxis—LARC was not significantly chemotactic for THP-1 cells (Fig. 6A) ($n = 4$, maximal stimulation index of 2.2 ± 0.7 at 1 μg/ml) or monocytes (Fig. 6B) ($n = 4$, maximal index of 1.0 ± 0.4 at 1 μg/ml), whereas MCP-3 induced strong chemotaxis in THP-1 and monocytes at 1 and 10 ng/ml, respectively. However, LARC was found to be chemotactic for lymphocytes (Fig. 6C) from 100 ng/ml ($n = 8$, maximal index of 3.3 ± 0.5) onwards and at 1 μg/ml the chemotactic response ($n = 8$, maximal index of 8.2 ± 2.1) was almost as strong as with MCP-3 at 100 ng/ml. LARC was also slightly chemotactic for neutrophils (Fig. 6D) from 100 ng/ml ($n = 6$, maximal index of 4.0 ± 0.7) onward at 1 μg/ml. The chemotactic index for neutrophils, however, remained much below that observed with IL-8.

Characterization of LARC Receptor on Lymphocytes—We next investigated the specific binding of LARC to human blood leukocytes. An expression vector was constructed to produce LARC fused with a SEAP-(His)$_6$ tag. The LARC-SEAP(His)$_6$ protein was secreted as a single major protein with an expected apparent molecular mass of 74 kDa (data not shown). This fusion protein has an AP activity for quantitative tracing and a (His)$_6$ tag in its COOH terminus for easy purification. Amino acid sequence analysis of the purified fusion protein demonstrated that the NH$_2$ terminus of LARC-SEAP(His)$_6$ started at Ala-27. As LARC has chemotactic activity for lymphocytes, we characterized the receptor for LARC on these cells. When binding was performed with increasing concentrations of LARC-SEAP(His)$_6$ (Fig. 7A), a single class of receptor with a $K_d$ of 0.4 nM and 2100 sites/cell was observed (Fig. 7B). Competition experiments showed that LARC fully inhibited binding of LARC-SEAP(His)$_6$ to lymphocytes with an IC$_{50}$ of 3.2 nM (Fig. 7C). On the other hand, similar competition experiments showed monocytes to have only low affinity binding sites for LARC with an IC$_{50}$ of about 200 nM (data not shown). Other CC and CXC chemokines, TARC, MCP-1, RANTES, MIP-1α/β/LD78α, and IL-8, had virtually no inhibitory effect on LARC-SEAP(His)$_6$ binding to lymphocytes (Fig. 7D), indicating that the LARC receptor is highly specific for LARC.

**FIG. 5.** Purification of recombinant LARC expressed in the baculovirus system. A, reverse-phase high performance liquid chromatogram of the pooled HiTrap-S fractions. The culture supernatant of BTI-TN-5B1–4 cells infected with the LARC recombinant virus was loaded on a HiTrap-S column and eluted with a salt gradient. The pooled fractions were then loaded on a Cosmocil SC4–300 column and eluted with a gradient of acetonitrile. B, silver stain of purified recombinant LARC. Proteins were separated on a 15% SDS-polyacrylamide gel electrophoresis gel and detected by silver staining. Positions of size markers (kDa) are shown on the right.

**FIG. 6.** Chemotaxis assay. Homogeneous recombinant LARC was tested for a dose-dependent chemotactic activity (microchamber assay) on THP-1 cells (A), freshly isolated peripheral blood monocytes (B), lymphocytes (C), and granulocytes (D). Chemotactic responses are expressed as stimulation indexes (significant chemotaxis corresponds to an index $>2$). For each cell type, a positive control chemokine was included for comparison (MCP-3 and IL-8).
A Novel CC Chemokine LARC

In the present report, we have described a novel human CC chemokine designated LARC, which was first identified from the EST data base. LARC is only distantly related to other CC chemokine members so far identified, although it has certain characteristics of the CC chemokines (Fig. 2). For example, LARC retains three out of five amino acid residues found in most CC chemokines in addition to the conserved four cysteine residues. However, the well-conserved tyrosine and threonine residues present between the second and third cysteine residues in other CC chemokines are replaced in LARC with phenylalanine and alanine, respectively. The well-conserved tyrosine residue has been shown to be critical for monocyte chemotaxis (45, 46). The recently cloned CC chemokine TARC (18), which is chemotactic for T cells but not for monocytes, does not contain the tyrosine residue either. The presence of the tyrosine residue, however, may not be sufficient for monocyte chemotaxis since eotaxin contains the tyrosine residue but does not attract monocytes (15–17).

Among the five ESTs containing the coding region of LARC, three encode proteins lacking the NH₂-terminal alanine residue that is present in the other two ESTs and the isolated cDNAs. If the signal sequence cleavage site of this LARC variant (LARCvar) is the same as that of LARC, the NH₂-terminal residue of its mature protein is serine (Fig. 2). The NH₂-terminal sequences of chemokines have been shown to markedly affect their activities and binding to receptors (49–52). In the case of MCP-1, deletion of one NH₂-terminal residue has been shown to change the target cell specificity (53). Therefore, it may be interesting to see whether LARC and LARCvar have the same activities and cell specificities.

The LARC gene has been localized to a marker D2S159 that has been mapped to the bands close to q33 and q37 on chromosome 2 (Fig. 4). This was unexpected since all the other CC chemokine genes and four newly identified putative CC chemokine genes (NCCE-1—4) have been mapped to chromosome 17q11.2 (20). Another example is the CXC chemokine SDF-1/PBSF gene that has been localized on human chromosome 10 instead of chromosome 4 (13). The CC and CXC chemokines on human chromosomes 17 and 4, respectively, have been presumably generated from successive gene duplication events. Since LARC is only distantly related to other CC chemokines, LARC may have been generated before amplification of the CC chemokines on chromosome 17. This also suggests that there might be LARC-related chemokines on chromosome 2.

We have identified two YAC clones from chromosome 2 containing the LARC gene. These two YAC clones and the clones from the CC chemokine cluster on chromosome 17 (20) may be useful tools for localizing new CC chemokine genes. Since ESTs are only partial sequences, it may be sometimes helpful to know whether or not a particular EST is mapped to one of these YAC clones by PCR. An EST mapped to one of these YAC clones is more likely to be a CC chemokine cDNA. Furthermore, such YAC clones may be useful for cloning new CC chemokine genes by, for example, exon trapping.

Because of its unorthodox chromosomal location, the question remained whether LARC was a true chemokine. Recombinant LARC was prepared and tested on various cell types. Whereas monocytes and THP-1 cells did not respond to LARC, lymphocytes reacted positively to LARC with a typical bell-shaped dose-response curve (Fig. 6). Granulocytes were also weakly attracted at a concentration of 1 μg/ml. These chemotaxis results were confirmed by binding experiments using a LARC-alkaline phosphatase fusion protein. This method was previously employed for identification of a specific receptor for a CXC chemokine IP-10 (54). Lymphocytes showed a single class of high-affinity receptor for LARC with a Kd of 0.4 nM that was not shared by other CC and CXC chemokines (Fig. 7). On the other hand, monocytes possessed only a substantial level of low-affinity binding sites for LARC (data not shown). We extended the above analysis and found that LARC did not bind to the CC chemokine receptors CCR1, 2B, 3, 4, and 5 and also to the chemokine receptor-like orphan receptors EB11 and BLR1 (data not shown).

Compared with the concentration required for LARC binding to the receptor (Kd = 0.4 nM, Fig. 7B), considerably higher concentrations (~100 ng/ml that corresponds to approximately 12 nM, Fig. 6) were required for the significant chemotactic responses of lymphocytes. Since chemokines are now known as pleiotropic cytokines, this discrepancy may indicate that LARC has physiological functions other than chemotactic activity. For example, CC chemokine I-309 has been shown to be chemotactic for monocytic cell line THP-1 at ~50 ng/ml but has an anti-apoptotic activity at 0.1–2 ng/ml (55). Cloning of the LARC receptor will demonstrate its distribution among various types of cells and will help to elucidate the biological functions of LARC.

In conclusion, LARC is a novel inducible CC chemokine that is mainly expressed in liver and mainly attracts lymphocytes through a single class of high-affinity receptors unique to LARC. The LARC gene is on human chromosome 2. The biological roles of LARC in inflammatory and immunological responses as well as its physiological functions in the liver remain to be established.

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