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Regulated Production and Molecular Diversity of Human Liver and Activation-Regulated Chemokine/ Macrophage Inflammatory Protein-3α from Normal and Transformed Cells[1]

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Liver and activation-regulated chemokine (LARC), also designated macrophage inflammatory protein-3α (MIP-3α), Exodus, or CCL20, is a C-C chemokine that attracts immature dendritic cells and memory T lymphocytes, both expressing CCR6. Depending on the cell type, this chemokine was found to be inducible by cytokines (IL-1β) and by bacterial, viral, or plant products (including LPS, dsRNA, and PMA) as measured by a specific ELISA. Although coinduced with monocyte chemotactic protein-1 (MCP-1) and IL-8 by dsRNA, measles virus, and IL-1β in diploid fibroblasts, leukocytes produced LARC/MIP-3α only in response to LPS. However, in myelomonocytic THP-1 cells LARC/MIP-3α was better induced by phorbol ester, whereas in HEp-2 epidermal carcinoma cells IL-1β was the superior inducer. The production levels of LARC/MIP-3α (1–10 ng/ml) were, on the average, 10- to 100-fold lower than those of IL-8 and MCP-1, but were comparable to those of other less abundantly secreted chemokines. Natural LARC/MIP-3α protein isolated from stimulated leukocytes or tumor cell lines showed molecular diversity, in that NH_2- and COOH-terminally truncated forms were purified and identified by amino acid sequence analysis and mass spectrometry. In contrast to other chemokines, including MCP-1 and IL-8, the natural processing did not affect the calcium-mobilizing capacity of LARC/MIP-3α through its receptor CCR6. Furthermore, truncated natural LARC/MIP-3α isoforms were equally chemotactic for lymphocytes as intact rLARC/MIP-3α. It is concluded that in addition to its role in homeostatic trafficking of leukocytes, LARC/MIP-3α can function as an inflammatory chemokine during host defense. *The Journal of Immunology*, **2000, 165: 4470–4477.**

Chemokines are a large family of chemotactic cytokines characterized by the conservation of Cys residues. Depending on the presence and positioning (adjacent or not) of the first two Cys residues, chemokines are classified in C, C-X_3-C, C-X-C, and C-C subfamilies (1). Many chemokines have been identified based on their capacity to attract (in vitro or in vivo) specific leukocyte subsets. In addition to their roles in inflammation and infection, chemokines affect other biological processes, such as hemopoiesis and angiogenesis. More recently, a number of cDNA sequences, structurally related to chemokines, have been discovered by screening databases of expressed sequence tags (ESTs)[3] (2). The corresponding chemokines (recombinant or synthetic) have been identified biochemically, but their potential role as chemoattractant for leukocytes is only partially understood. Some of these chemokines, such as the recently described C-C chemokine liver and activation-regulated chemokine (LARC) (3), also called macrophage inflammatory protein-3α (MIP-3α) (4) or Exodus (5), are expected to play a crucial role in trafficking and homing of lymphocytes and dendritic cells into secondary lymphoid organs and in the maturation of leukocytes (6–8). The three different acronyms for this single gene product are the consequence of the independent identification of LARC/MIP-3α through bioinformatics on ESTs. In contrast to most human C-C chemokine genes, which are mapped on chromosome 17, the LARC/MIP-3α gene is localized on chromosome 2 (3). Based on its gene *SCYA20* (9), LARC/MIP-3α protein is also designated C-C chemokine ligand 20 (CCL20) following a new classification system (10).

The spectrum of target cells that chemotactically respond to LARC/MIP-3α is restricted to memory T lymphocytes (3, 11, 12) and immature dendritic cells (11, 13–16). In addition, this chemokine has been reported to inhibit the proliferation of hemopoietic progenitors in vitro (5, 17). LARC/MIP-3α is exerting its activity through binding to CCR6 (11, 12, 18–20), which is not shared by any other known chemokine, but is nevertheless binding a member of the structurally unrelated β-defensins (21). Concordant with the target cells for LARC/MIP-3α, CCR6 is found to be expressed on immature dendritic cells and memory T lymphocytes as well as on B lymphocytes, in various lymphoid organs, and in pancreas (11–16, 18, 19, 22–24). Up-regulation of CCR6 expression on human neutrophils by cytokines can explain the slight chemotactic response of these cells to LARC/MIP-3α (3, 25).

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[3] Abbreviations used in this paper: ESTs, expressed sequence tags; $[Ca^{2+}]_i$, intracellular calcium concentration; CPG, controlled pore glass; EMEM, Eagle's MEM with Earle's salts; ENA-78, epithelial cell-derived neutrophil attractant-78; HEK, human embryonic kidney; LARC, liver and activation-regulated chemokine; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; poly(rI:rC), polyriboinosinic:polyribocytidylic acid; RP, reverse phase.

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LARC/MIP-3α mRNA is reported to be expressed at mucosal sites; in lymphoid tissues such as liver, lung, peripheral lymph nodes, thymus, tonsils, and appendix; and in PBMC (3–5, 11, 13, 26). However, little is known about the regulation of the LARC/MIP-3α protein production in different cell types. Additionally, many chemokines are post-translationally modified due to NH_2- and/or COOH-terminal processing. Selective NH_2-terminal cleavage of most C-C chemokines by enzymes (e.g., CD26/dipeptidyl peptidase IV) results in impaired receptor recognition and signaling properties and hence loss of chemotactic activity (27). In contrast, NH_2- or COOH-terminal processing of some inflammatory C-X-C chemokines causes an increase in chemotactic potency (28). To better understand the role of natural LARC/MIP-3α in physiologic and pathologic conditions, we have studied the gene regulation and molecular diversity of LARC/MIP-3α at the protein level. Here we report its inducible production in various normal tissue cells and tumor cell types. In addition, natural LARC/MIP-3α isoforms, isolated from normal and transformed cell cultures, were identified and compared biochemically and biologically.

Materials and Methods

Induction of chemokines in PBMC and cultured cells

Human PBMC were isolated from single blood donations (Blood Transfusion Center, Antwerp, Belgium). Erythrocytes were removed by sedimentation (30 min) with hydroxyethyl starch (Plasmasteril, Fresenius Hemotechnology, Bad Homburg, Germany). Mononuclear cells and granulocytes were separated by density gradient centrifugation (400 × *g*, 30 min) on Ficoll-sodium diatrizoate (Lymphoprep, Life Technologies, Paisley, UK). For induction experiments, mononuclear cells were seeded at 2×10^6 cells/ml in serum-free Eagle's MEM with Earle's salts (EMEM; Life Technologies) in 25-cm^2 flasks (5 ml) or in multiwell dishes (1 ml/well, 24 × 1.9 cm^2; Life Technologies). Different concentrations of the following inducers were added: pure natural human IL-1β (29), human rIFN-γ (Bioferon, Laupheim, Germany), the dsRNA polyriboinosinic: polyribocytidylic acid (poly(rI:rC); P-L Biochemicals, Milwaukee, WI), measles virus (Attenuvax strain, $10^{6.5}$ 50% tissue culture infectious doses/ml) ($TCID_{50}$/ml), PMA (Sigma, St. Louis, MO), Con A (Calbiochem, La Jolla, CA), or LPS from *Escherichia coli* (0111:B4, Difco Laboratories, Detroit, MI). Conditioned medium was collected after 48 h and was stored at −20°C until assay.

Human diploid fibroblasts (E_6SM, a strain of embryonic skin and muscle cells), the human osteosarcoma cell line MG-63, and the human epidermal larynxcarcinoma cell line HEp-2 were grown in EMEM supplemented with 10% FCS (Life Technologies). For induction experiments, confluent monolayers (grown in 25-cm^2 flasks) were induced in 5 ml of EMEM containing 0.5% FCS by stimulation with different concentrations of the inducers mentioned above. The human myelomonocytic cell line THP-1 was grown in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) supplemented with 10% FCS. After centrifugation and resuspension of the THP-1 cells in EMEM containing 2% FCS, cells were seeded at 2×10^6 cells/ml in 25-cm^2 flasks, and the above-mentioned inducer substances were added. After 48- to 72-h stimulation at 37°C, cell supernatants were harvested and kept at −20°C until assay.

Chemokine immunoassays

For detection of LARC/MIP-3α protein, a classical sandwich ELISA was developed. Plates coated with a mouse mAb raised against human rLARC/MIP-3α (mAb3B7) were provided by Dr. J.-M. Jaspar (BioSource Europe, Nivelles, Belgium). Recombinant LARC/MIP-3α (PeproTech, Rocky Hill, NJ) was used as a standard. Samples diluted in blocking buffer (PBS containing 0.05% Tween-20 and 0.1% casein) were added, as was the polyclonal rabbit anti-human LARC/MIP-3α Ab (dilution, 1/3000; PeproTech) used for capturing. After 2 h of incubation, detection was performed with peroxidase-conjugated $F(ab')_2$ donkey anti-rabbit Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) and 3,3′,5,5′-tetramethylbenzidine dihydrochloride hydrate (Aldrich, Milwaukee, WI). The detection limit of the LARC/MIP-3α ELISA was about 0.2 ng/ml, and its specificity was demonstrated by the lack of cross-reactivity with other chemokines (including granulocyte chemotactic protein-2, epithelial cell-derived neutrophil attractant-78 (ENA-78), monocyte chemotactic protein-1 (MCP-1), MCP-2, and IL-8 at a concentration of 100 ng/ml) and other potentially cross-reactive agents (e.g., IL-1β, IFN-γ, poly(rI:rC), PMA, Con A, and LPS, used as inducers).

Recombinant MCP-1 was a gift from Dr. J. J. Oppenheim (National Cancer Institute, Frederick, MD); natural IL-8 was purified from osteosarcoma cell-conditioned medium (30). MCP-1 and IL-8 were quantified with a classical sandwich ELISA as previously described (28, 31). Polyclonal rabbit anti-human MCP-1 and polyclonal goat anti-human IL-8 (both purified by protein A affinity chromatography) were used for coating in the MCP-1 and IL-8 ELISA, respectively. Monoclonal mouse anti-human MCP-1 and anti-human IL-8 (both from R&D Systems, Abingdon, U.K.) were used as capture Abs. Peroxidase-labeled goat anti-mouse polyclonal Ab (Jackson ImmunoResearch Laboratories) and 3,3′,5,5′-tetramethylbenzidine dihydrochloride hydrate were applied for detection. The detection limits for MCP-1 and IL-8 were 0.1 and 0.02 ng/ml, respectively.

Production and purification of natural LARC/MIP-3α

Human LARC/MIP-3α protein was purified from conditioned medium of stimulated MG-63 osteosarcoma cells, myelomonocytic THP-1 cells, and PBMC. MG-63 cell monolayers (126 flasks, 175 cm^2; Life Technologies) were grown to confluence in EMEM containing 10% FCS and were stimulated in EMEM plus 2% FCS with a semipurified cytokine mixture (derived from mitogen-stimulated mononuclear cells). THP-1 cells were grown in suspension in RPMI containing 10% FCS and were induced with PMA (10 ng/ml) in EMEM containing 2% FCS (42 flasks, 175 cm^2, 1.8×10^6 cells/ml). PBMC (50×10^9 cells, isolated from 116 blood donations; see above) were suspended in Spinner flasks (5×10^6 cells/ml) in EMEM plus 1% FCS supplemented with Con A (2 μg/ml) and LPS (2 μg/ml). After 48 h (for MG-63 cells and PBMC) or 72 h (for THP-1 cells) of incubation at 37°C, conditioned medium was collected and stored at −20°C until processing.

Natural LARC/MIP-3α was isolated through a four-step concentration and purification procedure, as previously described (32). Briefly, the stimulated conditioned medium from THP-1 and MG-63 cells was concentrated and partially purified by adsorption to controlled pore glass (CPG) beads (CPG 10-350, Serva, Heidelberg, Germany) and elution with 0.3 M glycine/HCl, pH 2.0. The acid CPG eluate was neutralized with NaOH and further purified by Ab affinity chromatography, using a polyclonal Ab against CPG-purified fibroblast-derived cytokines. The Ig fraction from 20 ml of the antiserum was coupled to 6 g of cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). LARC/MIP-3α immunoreactivity was bound to the column (1.5 × 15 cm, 20 ml/h) at neutral pH and was recovered in 0.5 M NaCl and 0.1 M citrate/HCl, pH 2.0. Alternatively, the PBMC-derived conditioned medium was concentrated by adsorption to silicic acid (Matrex, Amicon, Beverly, MA) and elution with 50% ethylene glycol in 1.4 M NaCl/PBS, pH 7.4. After dialysis against 50 mM NaCl and 50 mM Tris-HCl (pH 7.4), the silicic acid eluate was further purified by heparin affinity chromatography. The eluate was loaded onto a heparin-Sepharose column (Pharmacia), and the proteins were eluted in a NaCl gradient (0.05–2 M NaCl) in 50 mM Tris-HCl, pH 7.4.

Fractions recovered by Ab or heparin affinity chromatography containing the LARC/MIP-3α immunoreactivity were prepared for Mono S cation exchange chromatography (Pharmacia) by dialysis against equilibration/loading buffer (50 mM formate, pH 4.0). A linear NaCl gradient (0–1 M) was used to elute (1 ml/min) proteins in 50 mM formate, pH 4.0. Absorbance was monitored at 280 nm as a measure for protein concentration. Finally, LARC/MIP-3α was purified to homogeneity by reverse phase HPLC (RP-HPLC). Samples were injected on a 220 × 2.1-mm C_8 Aquapore RP-300 column (PE Biosystems, Foster City, CA), equilibrated with 0.1% trifluoroacetic acid in water (pH 2.0), and the proteins were eluted (0.4 ml/min) with an acetonitrile gradient (0–80%) in 0.1% trifluoroacetic acid/H_2O, pH 2.0. The column effluent was monitored by a spectrophotometer at 220 nm.

SDS-PAGE, immunoblotting, amino acid sequence analysis, and mass spectrometry

Natural LARC/MIP-3α was analyzed for M_r and purity by SDS-PAGE under reducing conditions on Tris/tricine gels (33). Proteins were stained with silver, and the molecular mass markers used were carbonic anhydrase (M_r, 29,000), β-lactoglobulin (M_r, 18,400), lysozyme (M_r, 14,400), and bovine trypsin inhibitor (M_r, 6,200) (Life Technologies).

Alternatively, the proteins were transferred to a Problot membrane (PE Biosystems) after SDS-PAGE to enable immunoblotting. The M_r markers used (Bio-Rad, Hercules, CA) were the prestained SDS-PAGE standards lysozyme (M_r, 21,300) and aprotinin (M_r, 7,600). The Problot membrane was incubated overnight with specific rabbit polyclonal anti-human LARC/MIP-3α antiserum (dilution, 1/1000; PeproTech), which was also used in

the LARC/MIP-3α ELISA, and subsequently for 1 h with alkaline phosphatase-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Immunoreactive proteins were stained by incubating the membrane in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color solution (Sigma).

The identity and the NH_2-terminal sequence of purified chemokines were determined by Edman degradation on a pulsed liquid phase protein sequencer (477A/120A, PE Biosystems) with *N*-methylpiperidine as a coupling base and on-line detection of phenylthiohydantoin amino acids. The presence of Cys residues was obvious from the absence of any detectable signal (32).

The molecular mass of the purified LARC/MIP-3α forms was determined on an electrospray ion trap mass spectrometer (Drs. A. Schneider and A. Ingendoh, Esquire, Bruker Daltonik, Bremen, Germany). C_8 RP-HPLC purified proteins were diluted 5-fold (final LARC/MIP-3α concentration, 0.2–1 μg/ml) in methanol/water (1/1) including 0.1% acetic acid and applied to the mass spectrometer by direct infusion at a flow rate of 2 μl/min. Average relative molecular masses were calculated from the summation of 100 spectra, with an accuracy of ±1.0.

Chemotaxis and intracellular calcium mobilization

Blood lymphocytes were purified by incubating PBMC (see above) for 15 min at 4°C with paramagnetic microbeads conjugated with mAb against CD3. The cell suspension was passed over a column placed in a magnetic field (VarioMACS, Miltenyi Biotec, Bergisch Gladbach, Germany). After this positive magnetic cell sorting, a cell purity (analyzed by FACS) of >80% was reached for lymphocytes ($CD3^+$).

LARC/MIP-3α was tested for its chemotactic potency on lymphocytes in the Boyden microchamber (48-well chemotaxis microchamber, Neuro Probe, Gaithersburg, MD). The lower compartments of the microchamber were filled with test samples, dilutions of synthetic MCP-3 (34) as a positive control, and the dilution buffer HBSS (Life Technologies) supplemented with pyrogen-free human serum albumin (1 mg/ml; Red Cross, Leuven, Belgium) as a negative control (27 μl). The upper compartments were filled with a lymphocyte suspension (50 μl) at 10×10^6 cells/ml in HBSS with human serum albumin. The lower compartments were separated from the upper compartments by a 5-μm pore size, polyvinyl pyrrolidone-free polycarbonate membrane (Nuclepore, Acton, MA) that was coated with 20 μg/ml fibronectin (Life Technologies) for 24 h at 4°C. After 4-h incubation at 37°C, the cells that migrated through the membrane were fixed, stained with Hemacolor solutions (Merck, Darmstadt, Germany), and counted microscopically in 10 oil immersion fields at ×500 magnification. The chemotactic activity of a sample (triplicates in each chamber) was expressed as a chemotactic index; this was the number of cells that migrated in response to the test sample divided by the number of cells that migrated in response to the negative control.

The intracellular calcium concentration ($[Ca^{2+}]_i$) was measured using the fluorescent indicator fura-2 (fura-2/acetoxymethylester, Molecular Probes Europe, Leiden, The Netherlands). Human CCR6 transfectants were generated using human embryonic kidney cells (HEK 293) (22) and were provided by Dr. G. Márquez (Department of Immunology and Oncology, National Center of Biotechnology, Madrid, Spain). Transfected cells were grown in DMEM with 4.5 g/L glucose and L-Gln (BioWhittaker Europe) supplemented with 10% FCS in the presence of geneticin at 450 μg/ml (G418, Life Technologies). For detection of the intracellular calcium concentration, cells (10^7 cells/ml) were incubated for 30 min at 37°C in the growth medium containing 2.5 μM fura-2 and 0.01% Pluronic F-127 (Sigma). Cells were washed twice and resuspended at a concentration of 10^6 cells/ml in HBSS (1 mM Ca^{2+}) supplemented with 0.1% FCS and buffered at pH 7.4 with 10 mM HEPES/NaOH. Cells were allowed to equilibrate at 37°C for 10 min before fura-2 fluorescence was measured in an LS50B luminescence spectrophotometer (PerkinElmer, Norwalk, CT). Upon excitation of the cell suspension at 340 and 380 nm, emission was measured at 510 nm. The $[Ca^{2+}]_i$ was calculated according to the Grynkiewicz equation (35). To determine the R_{max}, cells were lysed by addition of 50 μM digitonin. The R_{min} was determined by the addition of 10 mM EGTA to the lysed cells after adjusting the pH to 8.5 with 20 mM Tris. The K_d used for calibration was 224 nM.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney *U* test. The significance levels of differences with controls are indicated in the figures.

Results

Regulated production of LARC/MIP-3α in human tumor cells

Human osteosarcoma (MG-63) cells are a good source of chemokines such as IL-8 (30) and MCP-1 (36) when stimulated with cytokines or after viral infection. Indeed, about 200 ng/ml of IL-8 (Fig. 1*A*) and MCP-1 (36) was induced by 100 U/ml of IL-1β or by infection with $10^{5.2}$ 50% tissue culture infectious doses/ml of measles virus. However, induction of MG-63 cells for 48 h with IL-1β yielded 500-fold lower (0.4 ng/ml) LARC/MIP-3α production as measured by a specific ELISA. A higher production of LARC/MIP-3α (~2 ng/ml) by MG-63 sarcoma cells was obtained after infection with measles virus. Although the tumor-promoting agent PMA did not induce detectable LARC/MIP-3α or MCP-1 (data not shown) at 100 ng/ml, it clearly stimulated IL-8 production (Fig. 1*A*). It can be concluded that in MG-63 sarcoma cells, LARC/MIP-3α induction quantitatively and qualitatively differed from that of other chemokines, such as IL-8 and MCP-1.

In contrast to MG-63 cells, in human epidermal carcinoma (HEp-2) cells the conditions for LARC/MIP-3α induction were more similar to those for IL-8 (Fig. 1*B*). IL-1β, measles virus, and PMA were capable of inducing both chemokines, whereas IFN-γ, which induced MCP-1 in HEp-2 cells (data not shown), failed to stimulate LARC/MIP-3α and IL-8 production. It should be noted that the maximal amount of LARC/MIP-3α produced by HEp-2 cells (10 ng/ml) is about 5-fold higher than that produced by MG-63 cells. As a result, HEp-2 cells produced only 10-fold less LARC/MIP-3α compared with IL-8 (Fig. 1*B*). These data show that LARC/MIP-3α production is differently regulated in these sarcoma and carcinoma cells. Indeed, LARC/MIP-3α was best produced by measles virus in MG-63 cells, whereas in HEp-2 cells IL-1β was superior.

Further, myelomonocytic THP-1 cells were found to be good producers of both LARC/MIP-3α and IL-8 after stimulation with PMA or LPS (Fig. 2). In contrast, MCP-1 was dose-dependently induced in THP-1 cells by both LPS and IFN-γ, whereas PMA failed to do so. Measles virus and IL-1β, which induced sarcoma cells and carcinoma cells to produce chemokines, did not stimulate

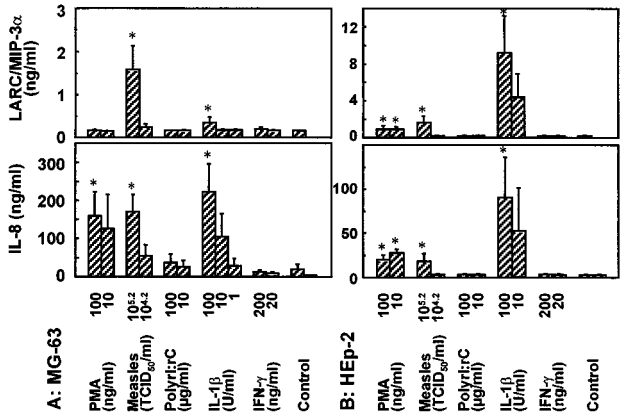

FIGURE 1. Induction of human LARC/MIP-3α in sarcoma and carcinoma cells by cytokines and cytokine inducers. Confluent monolayers of MG-63 osteosarcoma (*A*) and HEp-2 epidermal carcinoma (*B*) cells were stimulated for 48 h with different concentrations of various chemokine inducers (as indicated in *Materials and Methods*) or were left untreated (control). The production of LARC/MIP-3α and IL-8 was measured by specific ELISAs. The results represent the mean ± SEM of three independent experiments. Statistically significant increases in chemokine production above the control value, determined by the Mann-Whitney *U* test, are indicated (*, $p < 0.05$).

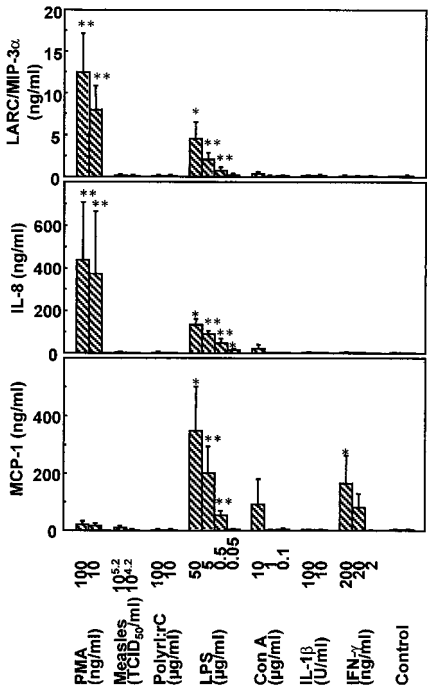

FIGURE 2. Regulated production of LARC/MIP-3α in stimulated monocytic cells. THP-1 monocytic cells (2×10^6 cells/ml) were induced for 48 h with various cytokines (IL-1β and IFN-γ) and cytokine inducers (PMA, measles virus, poly(rI:rC), LPS, and Con A). The production of LARC/MIP-3α, IL-8, and MCP-1 was measured by ELISA. Values represent the mean ± SEM of four independent experiments. The statistical significance of difference from control (unstimulated) cells is indicated (*, $p < 0.05$; **, $p < 0.01$).

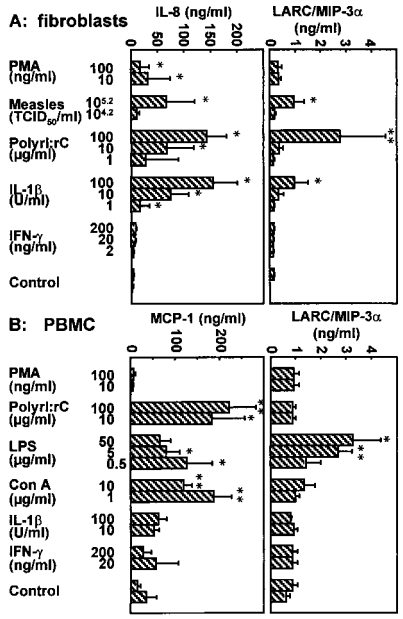

FIGURE 3. Induction of LARC/MIP-3α in human diploid fibroblasts and PBMC. Monolayers of cultured skin fibroblasts (*A*) and suspension cultures of freshly isolated PBMC (*B*) at 2×10^6 cells/ml were stimulated with various chemokine inducers or were left untreated (control). Chemokine concentrations were determined by specific ELISA, and the mean ± SEM were calculated from four (fibroblasts; *A*) and six (PBMC; *B*) independent experiments, respectively. Significant differences from controls are indicated (*, $p < 0.05$; **, $p < 0.01$).

the leukemic cell line to produce LARC/MIP-3α, IL-8, or MCP-1. Finally, these three chemokines were only marginally induced in THP-1 cells by the mitogen Con A. Collectively, these results indicate that the production of LARC/MIP-3α varied depending on the tumor cell line and the inducer used.

Induction of human LARC/MIP-3α in diploid fibroblasts and PBMC

To evaluate LARC/MIP-3α gene regulation and protein secretion by normal cells, similar induction experiments were performed on cultured diploid skin-muscle fibroblasts and freshly isolated PBMC. Fig. 3*A* shows that in diploid fibroblasts the dsRNA poly(rI:rC), measles virus, and IL-1β were the predominant inducers of LARC/MIP-3α. This dose-dependent induction pattern is again similar to that of IL-8 (Fig. 3*A*). The finding that normal connective tissue cells responded best to dsRNA to produce LARC/MIP-3α is in contrast with the fact that the tumor cell lines (see above) failed to secrete this chemokine after induction with poly(rI:rC). On a quantitative basis, LARC/MIP-3α production by normal fibroblasts and sarcoma cells was low compared with that by carcinoma and myelomonocytic cells, indicating that connective tissue is not the primary source of LARC/MIP-3α.

In PBMC, LPS induced a significant level of LARC/MIP-3α (Fig. 3*B*). Measles virus also induced LARC/MIP-3α in mononuclear cells (data not shown), but not in THP-1 cells (Fig. 2). In contrast, this myelomonocytic cell line was most responsive to PMA, which failed to induce LARC/MIP-3α in PBMC (Fig. 3*B*). Although PBMC did not respond to the other stimuli tested, the capacity of the mononuclear cell population to respond to other inducers was confirmed by the significant induction of MCP-1 by poly(rI:rC) and Con A (Fig. 3*B*). Taken together, the induction experiments demonstrate that in normal leukocytes and fibroblasts LARC/MIP-3α induction is differently regulated, and compared with corresponding leukemic and sarcoma cell lines and to other chemokines, there is only limited parallelism of inducibility.

Isolation and characterization of natural LARC/MIP-3α isoforms

In view of the existing heterogeneity of chemokines due to post-translational modification and the subsequent alterations in biological activities, natural LARC/MIP-3α forms were isolated from different cellular sources. To that goal, conditioned media from cytokine-stimulated MG-63 osteosarcoma cells, phorbol ester-induced monocytic THP-1 cells, and PBMC stimulated by endotoxin and mitogen were produced on a large scale (3–10 liters). These conditioned media were processed through a standard procedure for chemokine purification, including adsorption (to CPG or to silicic acid), affinity chromatography (Ab or heparin), cation exchange chromatography and RP-HPLC (32). The LARC/MIP-3α concentration was quantified by the same ELISA used to measure LARC/MIP-3α in crude induction samples (see above). After cation exchange chromatography, LARC/MIP-3α from MG-63 cells eluted in the NaCl gradient as a single sharp peak of immunoreactivity (data not shown), separate from the other chemokines present in the crude conditioned medium (IL-8, granulocyte chemotactic protein-2, GRO, IFN-γ-inducible protein-10, MCP-1, MCP-2, MCP-3, and RANTES). This confirms the specificity of the LARC/MIP-3α ELISA. Further purification of this LARC/MIP-3α peak by RP-HPLC showed that the chemokine again eluted in a single peak of immunoreactivity corresponding to a 6.0-kDa protein (data not shown). Although only a minute amount of purified LARC/MIP-3α protein was recovered, NH_2-terminal sequence analysis of this 6.0-kDa protein allowed us to identify it as LARC/MIP-3α. The natural product showed heterogeneity, in

that NH_2-terminally intact LARC/MIP-3α and an isoform missing the NH_2-terminal Ala were detected. Thus, on the basis of the ELISA only a single chemokine, i.e., LARC/MIP-3α, was purified from a crude chemokine mixture.

Because THP-1 cells stimulated with PMA were quantitatively a better source of LARC/MIP-3α than MG-63 cells (Figs. 1*A* and 2), an identical isolation procedure was applied for THP-1 cell-conditioned medium. LARC/MIP-3α from THP-1 cells eluted at a similar position from the cation exchange column and yielded again a single 6.0-kDa protein peak on RP-HPLC (data not shown). NH_2-terminal sequence analysis and mass spectrometry revealed the presence of the two isoforms, intact LARC/MIP-$3\alpha_{1-70}$ and truncated LARC/MIP-$3\alpha_{2-70}$, that differ in length by only one residue at the NH_2 terminus. However, both isoforms were COOH-terminally intact. The amounts of purified LARC/MIP-3α protein were insufficient to continue further fractionation.

To determine whether LARC/MIP-3α heterogeneity also occurred in normal cells, LARC/MIP-3α present in the conditioned medium from stimulated PBMC was purified to homogeneity (Fig. 4). All LARC/MIP-3α immunoreactivity specifically bound to the heparin-Sepharose column, but was not separated from other chemokines present, such as MIP-1α, MIP-1β, ENA-78, MCP-1, and IL-8 (data not shown). After subsequent cation exchange chromatography, LARC/MIP-3α eluted over a broad range (0.55–0.90 M NaCl), indicating additional heterogeneity of leukocyte-derived LARC/MIP-3α (Fig. 4*A*). The LARC/MIP-3α immunoreactivity was not due to cross-reactivity with other abundantly present chemokines such as MCP-1, as can be seen by its distinct elution pattern (Fig. 4*A*). Final purification of leukocyte-derived LARC/MIP-3α by RP-HPLC revealed two separate peaks of LARC/MIP-3α protein (Fig. 4*B*). The first peak (fraction 70) eluting at 34% acetonitrile corresponded to a 6.0-kDa protein upon SDS-PAGE (Fig. 5*A*). Its LARC/MIP-3α authenticity was confirmed by immunoblotting in parallel with rLARC/MIP-3α (Fig. 5*B*). However, this LARC/MIP-3α form appeared to be underestimated in the ELISA, indicating a biochemical difference from the rLARC/MIP-3α used as a standard. The second, major LARC/MIP-3α peak (fractions 73 and 74) eluting on RP-HPLC at 35% acetonitrile (Fig. 4*B*) clearly contained a 5- to 5.5-kDa protein doublet (Fig. 5*A*), which was confirmed to be LARC/MIP-3α by immunoblotting (Fig. 5*B*). Sufficient LARC/MIP-3α protein was available from this second LARC/MIP-3α peak to demonstrate again by amino acid sequence analysis the presence of NH_2-terminal heterogeneity identical with that for LARC/MIP-3α forms recovered from THP-1 cells. However, this minor difference (one amino acid) in length at the NH_2 terminus could not explain the LARC/MIP-3α protein doublets (5 and 5.5 kDa, fractions 73–74) isolated from PBMC.

To further decipher the complex molecular heterogeneity of natural LARC/MIP-3α, RP-HPLC fractions were subjected to ion trap mass spectrometry. It can be seen from the deconvoluted spectra (Fig. 6*A*) that 6.0-kDa LARC/MIP-3α (fraction 70) from leukocytes is composed of proteins for which the average relative molecular mass corresponds to intact LARC/MIP-$3\alpha_{1-70}$ and the truncated LARC/MIP-$3\alpha_{2-70}$ and LARC/MIP-$3\alpha_{1-69}$ forms, missing one residue at the NH_2 and COOH terminus, respectively. Indeed, the average relative molecular mass (7895.1 ± 1.0) of LARC/MIP-$3\alpha_{1-69}$ fully corresponded to the theoretical value (M_r, 7894.3) of LARC/MIP-3α missing the COOH-terminal Met. Because the experimentally obtained average relative molecular masses for LARC/MIP-$3\alpha_{1-70}$ and LARC/MIP-$3\alpha_{2-70}$ were about 16 ± 1 Da higher than the theoretical masses, in all probability the COOH-terminal Met residue is oxidized. The composition of the 5- to 5.5-kDa LARC/MIP-3α doublet (fraction 73) was found to be even more complex in COOH-terminal processing (Fig. 6*B*). LARC/MIP-$3\alpha_{1-67}$ and LARC/MIP-$3\alpha_{1-64}$, missing three and six COOH-terminal residues, respectively, were the predominant LARC/MIP-3α forms in this fraction. In addition, their corresponding forms lacking the NH_2-terminal Ala residue, i.e., LARC/MIP-$3\alpha_{2-67}$ and LARC/MIP-$3\alpha_{2-64}$ were present. Finally, two additional LARC/MIP-3α forms were discovered, of which the obtained average relative molecular masses (7713.8 ± 1.0 and 7360.2 ± 1.0, respectively) did not correspond to the theoretical value of any intact or truncated LARC/MIP-3α. These minor forms showed, respectively, 132.2 ± 1.0 and 134.7 ± 1.0 Da of difference in molecular mass from LARC/MIP-$3\alpha_{2-67}$ and LARC/

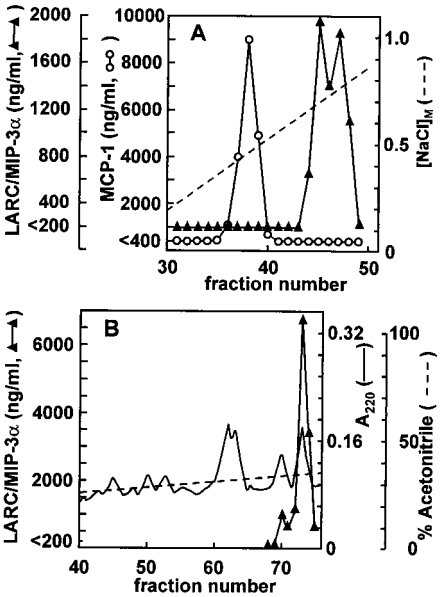

FIGURE 4. Purification of natural LARC/MIP-3α protein produced by stimulated PBMC. Conditioned medium from PBMC induced by LPS and Con A was prepurified by heparin-Sepharose chromatography, followed by cation exchange chromatography (*A*). Chemokines were eluted from the cation exchange column in a linear NaCl gradient (dashed line). Final LARC/MIP-3α purification occurred on a C_8 RP-HPLC column (*B*). Proteins were recovered from the C_8 column in an acetonitrile gradient (dashed line), and absorbance was monitored at 220 nm (solid line). LARC/MIP-3α (▲) and MCP-1 (○) immunoreactivities were measured by ELISA.

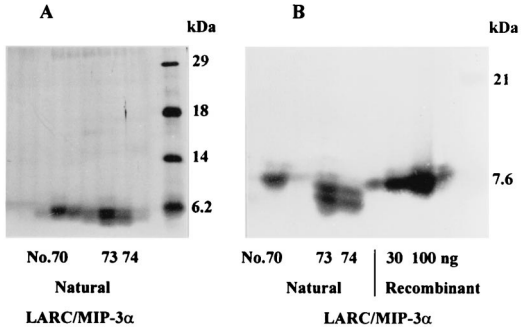

FIGURE 5. Biochemical analysis of natural LARC/MIP-3α isoforms isolated from PBMC. Leukocyte-derived LARC/MIP-3α, purified to homogeneity by RP-HPLC (see Fig. 4*B*), was subjected to SDS-PAGE (*A*; 20 μl/lane of RP-HPLC fractions 69–75) and immunoblotting with polyclonal Ab against LARC/MIP-3α (*B*; 15 μl/lane of RP-HPLC fraction 70, 10 μl/lane of fractions 73 and 74 vs 30 and 100 ng rLARC/MIP-3α as control). The m.w. markers are described in *Materials and Methods*.

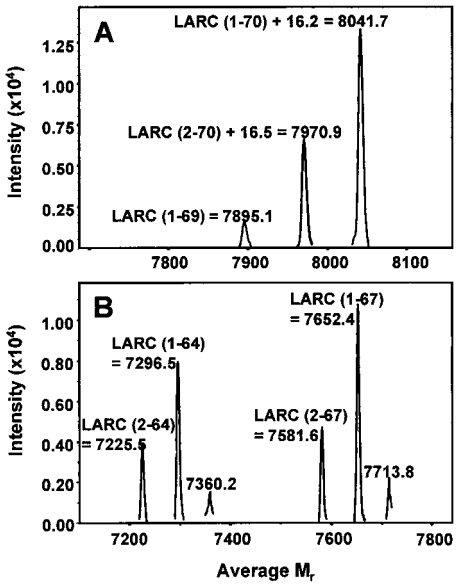

FIGURE 6. Mass spectrometric analysis of pure natural LARC/MIP-3α from PBMC. The molecular mass of RP-HPLC purified LARC/MIP-3α from PBMC (see Fig. 5) was determined on an electrospray ion trap mass spectrometer. Average relative molecular masses (with an accuracy of $\pm$1.0), calculated as the sum of 100 spectra, are given for RP-HPLC fractions 70 (*A*) and 73 (*B*).

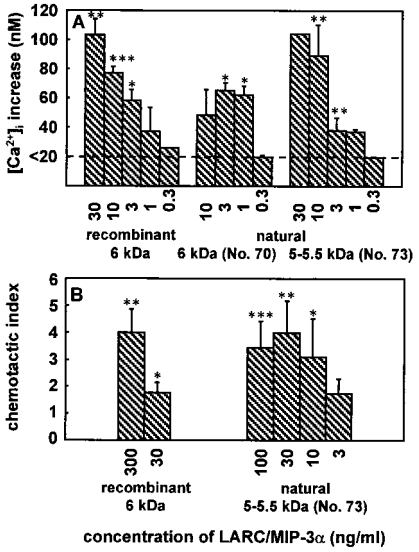

FIGURE 7. Biological activities of natural LARC/MIP-3α isoforms. Pure PBMC-derived LARC/MIP-3α (RP-HPLC fractions 70 and 73; see Fig. 5) was compared at various concentrations with rLARC/MIP-3α for calcium-mobilizing capacity (mean increase in $[Ca^{2+}]_i \pm$ SEM from two to four independent experiments) in HEK 293 cells transfected with CCR6 (*A*) and for chemotactic potency (mean chemotactic index $\pm$ SEM from four independent experiments) on freshly isolated blood $CD3^+$ lymphocytes (*B*). The dotted line in *A* indicates the detection limit (20 nM) for increases in $[Ca^{2+}]_i$. Statistically significant calcium increases and chemotactic responses are indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

MIP-3α_{2-64}, which may be explained by *O*-glycosylation, e.g., with a single ribose. It should be noted that intact LARC/MIP-3α_{1-70}, without any minor modification such as an oxidized COOH-terminal Met and with a molecular mass corresponding to the theoretical one, was not recovered from any of the PBMC- or THP-1 cell-derived preparations tested. In view of their physical separation (RP-HPLC elution pattern) and their different compositions (molecular mass), the first and second RP-HPLC peaks of leukocyte-derived LARC/MIP-3α were further compared at the biological level.

Chemotactic potency and receptor signaling capacity of natural LARC/MIP-3α isoforms

Natural LARC/MIP-3α was tested for its capacity to mobilize intracellular calcium in HEK 293 cells transfected with CCR6, the single receptor for LARC/MIP-3α. Fig. 7*A* indicates that both natural 6-kDa LARC/MIP-3α (RP-HPLC fraction 70) and 5- to 5.5-kDa LARC/MIP-3α (RP-HPLC fraction 73) could dose-dependently induce increases in $[Ca^{2+}]_i$; 1–3 ng/ml was the minimal effective concentration. As a control, rLARC/MIP-3α was already active at 3–10 ng/ml. It must therefore be concluded that the natural LARC/MIP-3α isoforms that differ in COOH-terminal truncation (fraction 70 vs fraction 73) were equally potent in exerting calcium mobilization via CCR6.

Because rLARC/MIP-3α has been reported to chemoattract lymphocytes, natural LARC/MIP-3α was tested in the microchamber migration assay using $CD3^+$ lymphocytes freshly purified from peripheral blood. Fig. 7*B* shows that the 5- to 5.5-kDa LARC/MIP-3α isoforms were already chemotactic for lymphocytes at 10 ng/ml, whereas rLARC/MIP-3α was still active at 30 ng/ml. In view of the fact that the natural LARC/MIP-3α preparations are still mixtures of various isoforms, minor differences in chemotactic potency cannot be excluded.

Discussion

Chemokines have recently been classified according to their constitutive or inducible production (37). Many chemokines, such as IL-8 and MCP-1, are produced by various cell types in response to cytokines (such as IL-1 and IFN-γ) and to bacterial and viral products (1, 37). These inducible chemokines are mainly responsible for the recruitment of phagocytes to sites of inflammation or infection. Other chemokines, such as stromal cell-derived factor-1, are secreted constitutively and are probably more implicated in trafficking of leukocytes under physiologic conditions. However, many chemokines can be classified in both categories (37). LARC/MIP-3α, also designated Exodus and CCL20 (following a new classification system), is such a recently discovered C-C chemokine (3–5, 10) that stimulates migration of monocyte-derived and $CD34^+$-derived immature dendritic cells as well as of memory T cells through its receptor CCR6 (3, 11–16). LARC/MIP-3α mRNA is expressed in various tissues, including lymphoid organs (3–5, 11), and occurs in different transcripts (3, 4). However, the regulated production of LARC/MIP-3α protein in various cell types as well as the biochemical and biological characteristics of natural LARC/MIP-3α protein isoforms have not yet been evaluated.

In this study we report the induction of LARC/MIP-3α protein in normal and transformed cell types as measured by a specific and sensitive ELISA. It was found that the induction of LARC/MIP-3α is differently regulated in skin fibroblasts and PBMC, which responded best to dsRNA and LPS, respectively. In fibroblasts, LARC/MIP-3α was coinduced with the proinflammatory chemokines IL-8 and MCP-1 (36) by dsRNA, IL-1β, and measles virus. In leukocytes, MCP-1 was best induced by plant (Con A) and viral (dsRNA) products, whereas LARC/MIP-3α was predominantly induced by bacterial endotoxin. This corresponds with the reported detection of LARC/MIP-3α mRNA in PBMC after induction with LPS (5). The production levels of LARC/MIP-3α protein in normal fibroblasts and leukocytes were about 100-fold lower than those of IL-8 or MCP-1. However a 3- to 10-fold increase in

LARC/MIP-3α protein expression was readily reached after appropriate induction of these cells.

In IL-1β-stimulated HEp-2 epidermal carcinoma cells and PMA-treated THP-1 monocytic cells, 3-fold higher levels of LARC/MIP-3α were obtained than in normal cells, indicative of a potential role for LARC/MIP-3α in tumor biology. In this respect, monocytic (U937 and THP-1) cells and Bowes melanoma cells were reported to contain increased mRNA levels of LARC/MIP-3α after PMA treatment (3, 5). The finding that epithelial cells are good producers of LARC/MIP-3α in response to inflammatory stimuli such as IL-1β fits with the reported detection of LARC/MIP-3α mRNA in epithelial cells of the appendix and in pancreatic adenocarcinoma cells (24, 26). In addition, in situ hybridization demonstrated the presence of LARC/MIP-3α mRNA in epithelial crypts of inflamed tonsils (13). This indicates that immature dendritic cells are possibly chemoattracted by LARC/MIP-3α produced by the epithelium to infiltrate the site of inflammation for Ag uptake. Indeed, immature dendritic cells derived from $CD34^+$ hemopoietic progenitor cells or from monocytes, but not mature dendritic cells and monocytes, are chemotactically responsive to LARC/MIP-3α (11, 13–16). Expression of LARC/MIP-3α mRNA has also been observed in lymphoid tissues such as peripheral lymph nodes and thymus, in addition to liver, lung, PBMC, fetal lung, and fetal liver (3–5, 11). Finally, LARC/MIP-3α mRNA expression can be down-regulated in activated monocytes by the anti-inflammatory cytokine IL-10 (4).

Until the present, little has been known about the molecular heterogeneity of LARC/MIP-3α protein. Although messages of different sizes have been demonstrated for LARC/MIP-3α (3, 4), it is not clear how this is reflected at the protein level. This study demonstrates that the bulk of LARC/MIP-3α, derived from either normal leukocytes or tumor cells, is secreted as two protein isoforms that differ in one additional amino acid at the NH_2 terminus. This NH_2-terminal truncation at the protein level is in agreement with published data showing that some of the ESTs containing the coding region for human or mouse LARC/MIP-3α lack the codon for the Ala^{27} residue (3, 5, 11, 26, 38). Inspection of the genomic sequence of murine LARC/MIP-3α revealed two potential splice acceptor sites in the boundary of the first intron and the second exon. As the resulting two species of transcripts corresponded exactly to those for human LARC/MIP-3α with and without the codon for the Ala^{27} residue, and both transcripts appeared to be expressed in various mouse tissues, it was concluded that such LARC/MIP-3α heterogeneity at the transcriptional level is due to alternative splicing in both humans and mice (26). In addition, post-translational modification of LARC/MIP-3α protein is implicated. Indeed, mass spectrometry on LARC/MIP-3α from stimulated PBMC and THP-1 cells has revealed the existence of COOH-terminal processing, generating LARC/MIP-3α with an oxidized Met or truncated isoforms missing one, three, or six residues. Although it was impossible to further fractionate these isoforms chromatographically and hence to test these as individual molecules, it can indirectly be deduced that neither NH_2-terminal nor COOH-terminal processing has significant consequences for CCR6 receptor recognition. Indeed, it was demonstrated that separated PBMC-derived LARC/MIP-3α isoforms that differ in COOH-terminal truncation (RP-HPLC fraction 70 vs fraction 73) were equally potent in mobilizing intracellular calcium (Fig. 7*A*). In addition, intact recombinant LARC/MIP-3α has been shown to be only 2-fold more active than the truncated isoform missing the NH_2-terminal Ala in chemoattracting T cells (11). Furthermore, in chemotaxis assays using mononuclear cells it was demonstrated that the two synthetic NH_2-terminal isoforms of LARC/MIP-3α were nearly equipotent (5). This is in contrast with most chemokines, which are rather sensitive to NH_2-terminal truncation, e.g., by CD26/dipeptidyl peptidase IV, resulting in partial or complete loss of receptor binding, signaling, and chemotactic capacity (27). Because LARC/MIP-3α has no Pro or Ala at the penultimate position, it cannot be a substrate for CD26. Nevertheless, CD26 has been reported to be coexpressed with CCR6 on memory T lymphocytes (12). Because the ELISA for LARC/MIP-3α, like other chemokine ELISAs, does not discriminate between intact and NH_2- or COOH-terminally truncated forms, it is difficult to say whether a particular isoform is produced in response to a particular stimulus or under specific pathologic conditions. Such evidence can only be provided by production and purification of chemokines for each induction condition tested, followed by identification of the purified isoforms through mass spectrometry and sequencing. Complete purification of LARC/MIP-3α isoforms derived from monocytic THP-1 cells (stimulated with PMA) and from PBMC (induced by LPS and Con A) demonstrated the presence of equal amounts of the two NH_2-terminally processed forms of LARC/MIP-3α. Although based on rather limited experience, it was also noticed that the COOH-terminally truncated forms were only recovered from PBMC and not from THP-1 cells. It is not clear, however, whether this reflects a difference between normal vs tumor cells in producing certain isoforms, because PBMC contain a mixture of cell types and possibly a higher content of proteolytic activity. Similarly, relatively more truncated isoforms of the chemokines GRO and ENA-78 were recovered when isolated from PBMC compared with tissue or tumor cells (28).

It can be concluded that LARC/MIP-3α not only acts as a constitutively expressed chemokine involved in the trafficking of dendritic cells and lymphocytes. Indeed, this study clearly indicates that the production of LARC/MIP-3α is positively regulated by inflammatory signals such as cytokines and micro-organisms. Furthermore, neutrophils can be induced by cytokines to express CCR6 and to migrate in response to LARC/MIP-3α (25). This latter is in agreement with the initial observation that LARC/MIP-3α was weakly active on human neutrophils (3). Like many inflammatory chemokines (e.g., MCP-1, IL-8), LARC/MIP-3α was inducible in most cell types, including epithelial cells, fibroblasts, leukocytes, and tumor cells, indicative of its possible role in various disease states. However, only a limited parallelism in inducibility with other chemokines was obtained at several levels. Epithelial cells were the best cell sources for LARC/MIP-3α, whereas fibroblasts and leukocytes were superior for MCP-1 and IL-8. Secondly, the amount of LARC/MIP-3α produced was much lower than that of IL-8 and MCP-1, but was comparable to those of MCP-2 (31) and MCP-3 (39). Finally, the optimal inducers for LARC/MIP-3α production were different from those of other chemokines. This further illustrates that the chemokine network is only apparently redundant and that LARC/MIP-3α has at least in part a specific role in the immune response. This study confirms that natural LARC/MIP-3α exerts chemotactic activity on lymphocytes and mobilizes intracellular calcium via CCR6. Additional regulation of activity by post-translational processing, as described for many inflammatory chemokines, seems not to apply for LARC/MIP-3α, because the various natural molecular forms of this chemokine behave similarly in signaling through their receptor CCR6. However, this does not exclude minor biological differences at the level of cell migration, as post-translational modifications can differently alter the chemotactic and calcium-mobilizing capacities of chemokines. The finding that β-defensins can compete for CCR6 binding sites and exert chemotactic activity on the same target cells as LARC/MIP-3α (21) indicates an additional but unique mechanism of intervention via CCR6 during host defense against microbial infection.

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