Purification and Structural Analysis of a Murine Chemotactic Cytokine (CP-10) with Sequence Homology to S100 Proteins*

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In delayed-type hypersensitivity reactions, cytokine-mediated cell migration leads to localized accumulation of neutrophils and mononuclear cells over 4–48 h. In contrast to transient (2–6 h) responses elicited by other chemotactic factors, earlier studies indicated that a chemotactic activity previously described in our laboratory elicited skin test responses over 24 h, identical to those induced by injection of antigen into a sensitized test subject. We have isolated this factor, a 10.3-kDa chemotactic protein designated CP-10, from supernatants of activated murine spleen cells. Purification to homogeneity was achieved using affinity chromatography on iminodiacetic acid-immobilized copper and cation-exchange, mixed mode (cation exchange/metal affinity), reversed-phase, and size-exclusion high performance liquid chromatography. CP-10 had maximal chemotactic activity for neutrophils at 10^{-12} M. The 76-amino acid sequence, obtained by automated N-terminal microsequence analysis of native CP-10, and fragments derived from trypsin digestion and cyanogen bromide cleavage indicated no sequence identity with any known cytokine or chemotactic factor but revealed up to 55% sequence homology with S100, Ca^{+2}-binding proteins. CP-10 appears to be the first protein of this family with a well defined function affecting cell migration, and its biological potency suggests an important role for this cytokine in cellular immune reactions.

The delayed-type hypersensitivity (DTH)1 reaction is a widely studied example of cellular immunity and is characterized by the accumulation of leukocytes, over 4–48 h, at the site of intradermally injected antigen into a sensitized subject. Polymorphonuclear leukocytes and mononuclear cells are dominant in the reaction, and the composition of the inflammatory infiltrate depends on the temporal stage of the lesion (for review see Ref. 1). Chemotactic factors play an important role in the recruitment and accumulation of these cells (2–4). We previously reported properties of a cytokine produced by concanavalin A (ConA)-activated murine spleen cells which induced skin responses with histopathological changes similar to antigen-elicited DTH reactions when injected intradermally, in a highly enriched form, into test animals, (5, 6). The histopathological changes which occurred were reminiscent of those described for skin reactive factor, a poorly characterized cytokine distinct from macrophage migration inhibition factor (MIF) (7) which provokes DTH-like responses when injected into normal skin (8). In vitro experiments confirmed that this protein fraction was immunologically distinct from complement-derived C5a and was chemotactic for neutrophils and macrophages. We describe here the purification of this chemotactic protein (CP), called "CP-10" according to its relative molecular mass of 10.3 kDa. Structural analysis yielded 86% of the total sequence which was later confirmed by sequence analysis of the complementary DNA. The data indicated that CP-10 was different from other known cytokines or chemotactic factors but was highly homologous with Ca^{+2}-binding proteins, in particular to an S100 protein recently identified in cells of myeloid origin and referred to as MIF-related protein (MRP-8), cystic fibrosis antigen, or calgranulin A (9–11).

MATERIALS AND METHODS

Preparation of Cytokine-containing Supernatants and Chemotaxis Assay—Spleen cells from QS mice were cultured for 24 h with Sepharose-ConA (Pharmacia, North Ryde, Australia) and the supernatant harvested as described earlier (12). Prior to storage at −70 °C, 0.1 mg/ml DNase, 0.1 mg/ml RNase (Boehringer Mannheim, North Ryde, Australia), 0.01% Tween 20 (Sigma), and 0.25 mM phenylmethylsulfonyl fluoride (Sigma) were added to supernatants. Aliquots of aqueous chromatography fractions were diluted directly with RPMI 1640 (GIBCO) before assay. Aliquots (5–50 μl) of organic solvent-containing fractions were freeze-dried in the presence of a total of 2 μg of ovalbumin (Calbiochem; 5 × crystalline) and reconstituted to the original volume with RPMI 1640. A minimum of two dilutions of each individual fraction was tested for chemotactic activity. Samples stored before testing were kept at −80 °C, preferably in the solvent used for elution.

Chemotaxis of mouse polymorphonuclear leukocytes was measured in a 48-well microchemotaxis chamber (Neuro Probe Inc., Bethesda, MD) as described (6). A sample of endotoxin-activated mouse serum (EAMS) containing mainly complement component C5a and prepared as detailed earlier (6) was used in every assay as a positive control. The chamber was incubated for 1.5–2 h at 37 °C in an

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§This abbreviation is used for: DTH, delayed-type hypersensitivity; acetanilide; CH3CN; ConA, concanavalin A; CP, chemotactic protein; EAMS, endotoxin-activated mouse serum; HPLC, high performance liquid chromatography; LC, liquid chromatographic; IDAA, iminodiacetic acid; MIF, migration inhibition factor; MRP-8, MIF-related protein; PAGE, polyacrylamide gel electrophoresis; RP, reversed phase; SDS, sodium dodecyl sulfate; SE, size exclusion.


The abbreviations used are: DTH, delayed-type hypersensitivity; acetanilide, CH3CN; ConA, concanavalin A; CP, chemotactic protein; EAMS, endotoxin-activated mouse serum; HPLC, high performance liquid chromatography; LC, liquid chromatographic; IDAA, iminodiacetic acid; MIF, migration inhibition factor; MRP-8, MIF-related protein; PAGE, polyacrylamide gel electrophoresis; RP, reversed phase; SDS, sodium dodecyl sulfate; SE, size exclusion.
atmosphere of 5% CO₂ in air after which the filter was removed, air-dried, and stained. Migrating cells were quantitated over five microscope fields (X 10 objective) using planimetry measurements obtained from Image Analysis (Wild-Leitz, Lane Cove, Australia).

**Results**

Nonmetallic Waters HPLC Instrumentation—Nonmetallic Waters HPLC systems were equipped with model M490 and M484 absorbance detectors were used for all liquid chromatographic (LC) separations except for most of the reverse phase HPLC which was performed on a Hewlett Packard model 1090 liquid chromatographic, equipped with a model 1040 diode-array detector. For preparative reversed-phase HPLC on the Waters preparative solvent line was flushed with equilibration buffer until the absorbance reached the baseline level of the solvent in use.

**Preparative Copper Affinity and Cation-exchange HPLC—** Batches of 5-5 liters of supernatant were concentrated to 150-200 ml and dialyzed against 20 mM Tris/HC1, 0.01% Tween 20, pH 8.5 (buffer A) containing 1.0 M NaCl and 0.25 mM phenylmethylsulfonyl fluoride, using a spiral ultrafiltration cartridge (molecular mass cut-off: 10 kDa, Amicon Scientific, Australia), followed by centrifugation at 10,000 × g at 4 °C for 30 min. The clear supernatant was sequentially absorbed at 3 ml/min onto TSK-HW50S gel-bound chelates (imino-diacetic acid, IDAA, Pierce Chemical Co.) of Zn²⁺ and Cu²⁺ (15), which were eluted into individual columns (100 × 20 mm; Advanced Purification-1 glass columns, AP-1, Millipore/Waters, Lane Cove, Australia), starting with absorption onto the Zn²⁺ column. After washing with equilibration buffer (see above), the columns were disassembled, and bound material eluted separately with a gradient of 0–30 mM imidazole in buffer A containing 40 mM NaCl at a flow rate of 5 ml/min over 30 min. Chemotactically active fractions eluting from the Cu²⁺ affinity column were loaded at 3 ml/min directly onto a column (10 × 200 mm; AP-1 column, Millipore/Waters) of Poly Cat A (polysarcatic acid) silica (Poly LC, Columbia, MD), equilibrated with 20 mM sodium acetate, 20 mM NaCl, 0.01% Tween, pH 5.5. The Cu²⁺ proteins were eluted at the above flow rate using a linear gradient of 0.02–1.0 M NaCl in this buffer over 65 min. Aliquots from pools of successive 1-min fractions were collected, and appropriate dilutions tested for chemotactic activity.

**Mixed Mode (Cu²⁺ Affinity/Cation-exchange) HPLC—** A 10 × 100-mm AP-1 glass column (Millipore/Waters), packed under constant flow with a 1:1 mixture of IDAA-Cu²⁺ TSK 65 F and Poly Cat A silica and equilibrated at 2.25 ml/min with 1.0 M NaCl in buffer A was loaded at this flow rate with pooled fractions from preparative cation-exchange HPLC adjusted to 1.0 M NaCl. Following sample application (see above) the NaCl concentration was reduced to 40 mM using a 30 ml/min flow rate. Fractions with peak activity were collected, and peak fractions were pooled into a ternary gradient of 0–30 mM imidazole and 0.04–1.0 M NaCl in buffer A. One-min fractions were collected and assayed for chemotactic activity in pools of representative fractions. Fractions of interest were combined for further purification.

**Recoversed-phase (RP) HPLC—** Material from the previous purification step was adjusted to 0.1% trifluoroacetic acid and loaded onto a Bakerbond C₄-wide pore HPLC column (4.6 × 250 mm, J. T. Baker Chemical Co.), equilibrated in 0.1% aqueous trifluoroacetic acid (solvent B). Proteins were eluted with a three-step gradient of acetonitrile (CH₃CN) in solvent B (as indicated in Fig. 2) ranging from 55% to 98% CH₃CN in 55 min. Appropriate dilutions of aliquots (5%) of individual 1-min fractions were assayed for chemotactic activity. Fractions with peak activity were combined with dilutions of CH₃CN/CH₃CN and rechromatographed on C₄ RP-HPLC (Aquapore RP300 Cartridge, 4.6 × 100 mm, Applied Biosystems, Foster City, CA). The cartridge, equilibrated in solvent B, was loaded with the sample by multiple 1-ml injections, and proteins were eluted with a 1%/min CH₃CN gradient in solvent B over 60 min. Eluting material was collected manually according to the absorbance at 214 nm, 5% aliquots were assayed for chemotactic activity, and pool peaks were analyzed by SDS-PAGE (14) and silverstaining (15).

**Size-exclusion HPLC—** The volume of the manually collected eluate from C₄ RP-HPLC with maximal chemotactic activity (~1 ml) was reduced to 50 μl on a Speed Vac concentrator (Savant), adjusted to 40% CH₃CN, 0.1% aqueous trifluoroacetic acid in a total volume of 100 μl, and injected onto a TSK G 3000 SW high performance gel filtration column (7.5 × 300 mm, LKB) equilibrated with 40% CH₃CN in solvent B at 0.5 ml/min as described (16). Fractions were collected manually according to the absorbance profile of the eluate at 214 nm and dilutions of 5% aliquots of individual fractions tested for chemotactic activity. The chemotactic protein obtained from this purification step was concentrated on C₅ RP-HPLC as described above, and relative molecular mass and homogeneity of the reduced protein were determined in 55% CH₃CN in solvent B for elution. Purification of carboxymethylated CP-10 (CM-CP-10) and peptide mixtures derived from trypsin digestion and cyanoargent bromide (CnBr) cleavage were fractionated as described (17, 18).

**Structure Analysis—** Carboxymethylated CM-CP-10, trypsin digestion of CM-CP-10, and CnBr cleavage were carried out as described (18, 19). Automated N-terminal amino acid sequence analysis of native CP-10, tryptic peptides, and CnBr cleavage fragments was performed on Applied Biosystems sequencers (models 477A and 470 A) (18) which had been modified to allow for injection of the total phenylthiohydantoin amino acid derivative (20).

Amino acid sequence comparisons were made by searching the following data bases: EMBL/GenBank and the NBRF Protein and Nucleotide Data Base, release 24.

**Protein Assay—** The protein concentration of samples was measured using the BCA dye-protein assay (Pierce Chemical Co.) with lysozyme (Boehringer Mannheim) as standard. Protein concentrations of RP-HPLC eluates were estimated using the absorbance at 214 nm and calibration of the peak area with lysozyme (Maxima Peak Integration Software, Dynamic Solutions, Millipore/Waters).

**RESULTS**

**Purification of CP-10—** Initial (6) and further characterization of preparations containing the chemotactic factor indicated the formation of urea-resistant aggregates of heterogeneous molecular weights, a basic isoelectric point (or close association with basic proteins), and high affinity to immobilized Cu²⁺, but not to Zn²⁺. Accordingly, the following purification protocol was established: the concentrated (40-fold) supernatant from 1-2 × 10¹² cells was sequentially loaded onto Zn²⁺, then Cu²⁺ affinity columns (13). The chemotactic factor was displaced from immobilized Cu²⁺ with a gradient of imidazole (0–30 mM) and applied directly to preparative cation-exchange HPLC equilibrated at pH 5.25. Proteins with chemotactic activity eluted in a complex peak with 0.34–0.48 M NaCl (Fig. 1). This material was pumped onto a column containing 1:1 mixture of IDAA-chelated Cu²⁺ and weak cation-exchange (aspartic acid) resin equilibrated with 1.0 M NaCl at pH 6.5. Retention of proteins under these conditions (85% of loaded material) was predominantly due to affinity for Cu²⁺. The column was eluted with gradients of imidazole and NaCl, and active fractions comprising 50% of the retained material were pooled for further purification. Combined fractions, adjusted to 0.1% trifluoroacetic acid, were fractionated on C₄ (Fig. 2) and C₅ RP-HPLC (Fig. 3, inset) and chemotactic activity eluted at CH₃CN concentrations of 45.5 and 64.5%, respectively. The manually collected fraction from C₅ RP-HPLC revealed a number of protein bands upon SDS-PAGE (not shown) and was subjected to size-exclusion HPLC in 40% acetonitrile, 0.1% trifluoroacetic acid, 59.9% H₂O. Chemotactic protein separated (retention time, 21.5–24.9 min, Fig. 4) after a major absorbance peak of contaminating material and eluted with an apparent mass of 10.3 kDa (Fig. 3). This preparation (Fig. 3, indicated by a horizontal bar) yielded a symmetrical absorbance peak on C₅ RP-HPLC with maximal chemotactic activity for neutrophils
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Earlier characterized a heparin-binding fraction from supernatants of ConA-stimulated murine spleen cells that mimicked DTH skin test responses when injected into naïve animals. The chemotactic activity was detected in a fraction containing macrophage procoagulant-inducing factor, and preliminary characterization indicated size heterogeneity of the proteins involved. Pilot experiments suggested a tendency of the chemotactic factor to form 40 (6 M)-resistant aggregates after concentration in aqueous buffers, extreme susceptibility to adherence to surfaces during buffer changes or lyophilization, and instability to freeze-thawing. These properties prompted the design of a purification scheme whereby concentration, buffer change, and purification were achieved almost exclusively by specific adsorption of proteins to column supports. Earlier studies had suggested that the chemotactic agent contained in the heparin-binding fraction of the cytokine supernatant had a basic pl (6) and strong affinity to Cu²⁺ but did not bind to immobilized Zn²⁺ (data not shown). This was exploited in the initial concentration and purification step by passing the concentrated supernatant through immobilized Zn²⁺ and Cu²⁺ columns, depleting the preparation of Zn²⁺-binding components according to a method developed by Porath et al. (13, 23).

Chemotactic activity with affinity to immobilized Cu²⁺ bound effectively to a cation exchange resin (Fig. 1) even at near-neutral pH (6.8) (data not shown). In the following step, sequential adsorption at neutral pH to Cu²⁺ affinity and cation-exchange stationary phases on a mixed mode LC support removed contaminating proteins during the loading phase (high NaCl concentration suppressed initial interaction of proteins with the cation exchanger) and separated chemotactically active fractions comprising 50% of retained protein during subsequent elution with imidazole/NaCl gradients.

The elution pattern of CP-10 on reversed phase HPLC indicated that CP-10 was strongly hydrophobic (Figs. 2 and 4). This confirmed the notion that aggregation of CP-10 in concentrated protein solution, and its nonspecific adsorption to surfaces, was caused by nonpolar interactions. Consequently, we used a CH₃CN/trifluoroacetic acid buffer system during fractionation of CP-10 by size exclusion (Fig. 3). In addition, this step was carried out at a final stage in the

**Fig. 1.** Preparative cation exchange HPLC of Cu²⁺ affinity LC-derived chemotactic fractions. Pooled material with affinity to Cu²⁺ was fractionated on weak cation exchange silica (see "Materials and Methods") using a gradient of NaCl (---) and absorbance of the eluate monitored at 280 nm (---). Chemotactic activity in aliquots (0.1 ml) of three successive 1-min fractions was assayed at 1% (□) and 0.5% (□). Chemotactic activity of 5% EAMS is shown for comparison (□). Fractions of interest (black bar) were combined for further purification.

**Fig. 2.** RP-HPLC on a C₃ BakerbondWP column of CP-10-containing fractions from mixed mode HPLC. Proteins absorbed to the RP-HPLC column were eluted with CH₃CN (---). Eluting material was monitored at 214 nm (---); aliquots (50 µl) of 1-ml fractions were freeze-dried in the presence of carrier protein and tested for chemotactic activity at 5% (□) and 1.25% (□) of the original volume; black bar, fractions pooled for further purification.

**Fig. 3.** SE-HPLC of CP-10-containing material eluted from CuCl₂ RP-HPLC. Active fractions eluting from CuCl₂ RP-HPLC were combined, fractionated on CuCl₂ RP-HPLC (inset), and the absorbance recorded at 214 nm (---). Individual peaks were assayed for chemotactic activity. The fraction of interest (inset, black bar) was applied to a SE-HPLC column (see "Materials and Methods") pre-calibrated with molecular weight standard proteins: ovalbumin (Mr = 44,000), soybean trypsin inhibitor (Mr = 20,100), cytochrome c (Mr = 12,400), and aprotinin (Mr = 6,000). The eluate was collected manually at intervals indicated by the bars; 5% aliquots were tested for chemotactic activity at 1:40 (□) and 1:80 (□) dilutions, and fractions (black bar) were pooled for final purification on RP-HPLC.

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at 10⁻¹² M (Fig. 4a). Analysis of this material on SDS-PAGE revealed a homogeneous protein band at 10 kDa (Fig. 4b). Total recovery of chemotactic protein of this purity (98% as indicated after silver staining) was 10⁻¹¹ µg from 5-6 liters of spleen cell conditioned medium.

**Amino Acid Sequence Analysis of CP-10—Automated N-terminal microsequence analysis of 1.5 to 2.0 pg (-200 pmol) of terminal microsequence analysis of 1.5-2.0 pg (-200 pmol) of terminal microsequence analysis of 1.5-2.0 pg (-200 pmol) of terminal microsequence analysis of 1.5-2.0 pg (-200 pmol) of terminal microsequence analysis of 1.5-2.0 µg (~200 pmol) of homogeneous protein yielded 38 residues (Table I) which bore a striking sequence identity to a putative Ca²⁺-binding site together with flanking α-helices (Fig. 6, residues 11-38), characteristic of S100 proteins (21, 22). This sequence was confirmed and extended to amino acid residue 76 by analysis of microbore C₃ RP-HPLC-purified tryptic and CNBr fragments (Fig. 5, T1, T2 and CN-1, CN-2), derived from trypsin digestion of ~25 µg of CP-10, respectively. These sequences revealed a second, C-terminal Ca²⁺-binding site typical of S100 proteins. Comparisons of the summary sequence suggested 55% sequence identity of CP-10 with MRP-8 (Fig. 6) but no structural homologies with other chemotactic factors or cytokines.

**DISCUSSION**

Migration of phagocytic cells into inflammatory lesions is an important feature of cell-mediated immune reactions. We
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Retention Time (min)

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Automated Edman degradation of CP10

Defined cytokines with chemotactic activity which may have copurified with CP-10 during initial purification steps (Figs. 1 and 2) include tumor necrosis factor α (pI 4.7) (24), which is chemotactic for monocytes and neutrophils (25), and macrophage inflammatory protein 1 (pI 4.6), a macrophage-derived cytokine of the interleukin-8/thromboglobulin family of proteins with affinity for heparin, which induces chemokinesis of neutrophils (26). Other members of this superfamily with chemotactic activity for neutrophils and/or macrophages, but with neutral or basic pI, include platelet factor 4 (27), monocyte-derived neutrophil chemotactic factor (28, 29), and neutrophil activating proteins 1 (30) and 3 (31). Proteins of the interleukin-8/thromboglobulin family are thought to have functionally important roles in inflammation immune regulation (for a review see Ref. 32).

No attempts were made to calculate the yield of chemotactic activity from starting material because unfractionated starting material, e.g. ConA-stimulated spleen cell supernatant, contains low levels of total chemotactic activity which become obvious only after partial purification (6). In addition, the method of production of supernatants is likely to yield some of the cytokines listed above which would contribute to the

FIG. 4. Cs RP-HPLC and SDS-PAGE of CP-10; titration of chemotactic activity. a, chemotactic protein eluting from SE-HPLC was applied to a Cs Aquapore HPLC cartridge and eluted with a gradient of CH₃CN (---). Homogenous CP-10 was collected manually according to the absorbance at 214 nm (---) and diluted serially for chemotaxis assays. Mean values of a representative experiment are shown (inset to the figure). Chemotactic activity of 5% EAMS (○) is given for comparison. b, an aliquot of the protein tested for chemotactic activity was reduced with 0.5% dithiothreitol and analyzed by SDS-Tricine PAGE and silver staining. Tracks A and C, protein standards; M, values shown on either side; B, RP-HPLC-purified CP-10.

FIG. 5. Separation of tryptic and CnBr fragments on microbore HPLC. Homogeneous CP-10 derived from Cs RP-HPLC was either treated with CnBr (lower panel) or S-carboxymethylated and digested with trypsin (upper panel) as outlined under "Materials and Methods." Diluted reaction mixtures were applied to microbore RP-HPLC, and peptides eluting with a gradient of CH₃CN (---) were trapped manually, according to the absorbance at 214 nm (---), for automatic sequence analysis.
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Sequence Position
CP-10 N-terminal
CP-10 Tryptic (T)
CP-10 Cnl-cle (CN)
CP-10 Consensus
MRP-8 Consensus
Ca2+-binding site

Sequence Position
CP-10 Tryptic (T)
CP-10 Cnl-cle (CN)
CP-10 Consensus
MRP-8 Consensus
Ca2+-binding site

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Fig. 6. Partial amino acid sequence, structural characteristics, and homology of CP-10 with MRP-8. Fragments of the consensus sequence (bold letters) obtained from N-terminal sequence analysis (sequence position 1–38) and sequencing of tryptic peptides (boxed) T1 (residue 7–30), T2 (residue 36–55), T3 (residue 56–76), and of CNBr peptides (boxed) CN1 (residue 1–22), CN2 (residue 37–57) are shown. Areas of sequence identity between the consensus sequences of CP-10 and MRP-8 (bold) are indicated by shaded boxes. Structural characteristics of S100 proteins, i.e., N- and C-terminal Ca2+-binding sites are denoted by a (α-helix-forming residue) and X, Y, Z, –X, –Y, –Z (Ca2+-coordinating residues), as originally proposed by Kretsinger (21) and by “−” denoting “hinge” region residues (37).

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total chemotactic activity but which cannot be distinguished from CP-10 because of the lack of specificity of chemotaxis assays for individual chemoattractants.

The biological potency of CP-10 for neutrophils was up to 1000-fold greater than that reported for other chemotactic agents including complement-derived C5a, leukotriene B4, bacterial cell wall-derived peptide (Met-Leu-Phe), and interleukin 8 (neutrophil-activating protein 1) (28, 30, 31, 33–35) and makes CP-10 one of the most potent chemoattractants described to date. It is unlikely that contaminating material (see Fig. 4b; major contaminant of M, 14,000; approximately 2% of total protein) contributed to the bioactivity at a CP-10 concentration of 10−12 M.

The M, 8,888 calculated from the 76-amino acid sequence of CP-10 (Fig. 6) and an estimated apparent molecular mass of 10.3 kDa suggested either additional C-terminal residues or glycosylation. No indication for N-linked glycosylation was obtained by amino acid sequence analysis. Details of the structure presented in Fig. 6 have facilitated the isolation of a cDNA clone encoding CP-10. Characterization of this clone has confirmed and extended the amino acid sequence data and predicts a molecular weight for CP-10 (88 residues) of M, 10,163, in good agreement with the experimentally determined relative molecular mass (Figs. 3 and 4b). Partial purification of the chemotactic activity found in supernatants of ConA-activated spleen cells by heparin affinity chromatography and isoelectric focusing suggested a basic pI of the associated protein(s) (6) in contrast to a calculated pI of 5.5 deduced from the amino acid composition of the CP-10 sequence (theoretical pI from partial sequence, 4.8). This discrepancy could be due to association of CP-10 with basic protein(s) which may explain the broad elution profile of CP-10 on cation exchange LC (Fig. 1). Functional expression of the CP-10 cDNA in mammalian cells and purification of a recombinant protein with chemotactic activity have confirmed the identity of the sequenced protein and chemotactic cytokine described here.

A putative N-terminal and “typical” C-terminal Ca2+-binding domain as defined by Kretsinger (21, 36) are evident at amino acid positions 20–32 and 58–68, respectively (excluding adjacent α-helices) (Fig. 6). Sequence and location of these Ca2+-binding sites indicate strong homology of CP-10 with the highly conserved family of S100 proteins (for review see Ref. 22), a structurally well defined group of the “EF-hand family” of Ca2+-binding proteins (21). Comparison of the CP-10 sequence with protein sequences stored in available data bases revealed maximal identity (55%) with MRP-8 (also referred to as cystic fibrosis antigen, L-1 antigen, or calgranulin A (10, 11)). In common with other S100 proteins a main region of sequence divergence between CP-10 and MRP-8 is found in the region between the two Ca2+-binding sites (Fig. 6 (~)), a domain thought to confer physiological specificity on S100 proteins (37). The first residue of this “hinge” region (position 41) is occupied by a single cysteine, which, by analogy with MRP-8 and other S100 proteins may be involved in formation of homodimers and/or heterodimers (10, 11, 38–44). This may provide another explanation for the size heterogeneity observed during earlier attempts to purify CP-10 (6). Furthermore, the dimerized form of recombinant S100β, but not a mutant form with a single cysteine to valine change (unable to form dimers), stimulates glial cell proliferation at nanomolar concentrations (42), supporting earlier suggestions that biological activity is associated with the disulfide form of S100β (41).

In contrast to their well characterized physicochemical properties, reports detailing the functions of the majority of S100 proteins are scarce (37, 45). S100 proteins are highly conserved between species (37), and up to 96% homology is found between rat and bovine S100β (46). This suggests that functions other than Ca2+ binding may have restricted mutation of these proteins since Ca2+-binding domains (and adjacent α-helices) cover only 66–77% of their structure (45). A limited number of biochemical activities and biological functions related to Ca2+-dependent processes has been suggested and includes promotion of microtubule dissociation and inhibition of microtubule assembly (47), inhibition of protein kinase substrate phosphorylation (48), involvement in the differentiation of glial cells (49), and stimulation of neurite outgrowth of chicken cerebral cortex neurons (41). MRP-8 is found in elevated levels in the plasma of cystic fibrosis patients, in the cytoplasm of normal neutrophils, epithelial cells, and chronically activated macrophages and keratinocytes (10, 11, 50, 51) suggesting a role for this protein in chronic inflammation and myeloid cell differentiation (10, 50). MRP-8, associated with another S100 protein, MRP-14, was purified as a complex from supernatants of activated human mononuclear cells using a monoclonal antibody to the human cytokine, macrophage MIF. However, neither recombinant MRP-8 nor -14, either alone or in combination, has MIF...
activity (10), and to date no functional bioassay with the levels of sensitivity obtained with CP-10 has been reported for either of the individual proteins.

Our finding that CP-10 is one of the most potent chemotactic factors described to date and is structurally related to the S100 protein family supports recent suggestions for extracellular roles of these proteins (41, 42). The implication that CP-10 participates in the first line of host defense against the invasion of foreign organisms (6) and the finding that it is a member of the evolutionary highly conserved family of S100 proteins support the proposal for a fundamental biological function of this cytokine.

REFERENCES