RESOLUTION OF THE TWO COMPONENTS OF MACROPHAGE INFLAMMATORY PROTEIN 1, AND CLONING AND CHARACTERIZATION OF ONE OF THOSE COMPONENTS, MACROPHAGE INFLAMMATORY PROTEIN 1β

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The host inflammatory response to infection and tissue injury is characterized by increased blood flow, changes in vascular permeability, and margination and emigration of leukocytes. A number of well-characterized macrophage-derived mediators, including cachectin/TNF and IL-1, have been implicated in these processes (1, 2). Other, newly-isolated monokines are suspected to play an important role as well. Recently, we reported the purification of one such monokine, a heparin-binding protein secreted by macrophages in response to stimulation by LPS (3). This novel monokine, referred to as macrophage inflammatory protein 1 (MIP-1),[1] causes a local inflammatory response when injected into the footpads of C3H/HeJ mice, and induces superoxide production in human neutrophils in vitro (3). In vitro, it is also mildly chemokinetic for human neutrophils. Since one hallmark of inflammation is the mobilization and activation of neutrophils, we conjecture that MIP-1 may be an important endogenous mediator of acute or chronic inflammation.

On SDS-PAGE, purified native murine MIP-1 migrates as a doublet composed of peptides with nearly identical apparent mol wt of ~8,000 (3). NH_2-terminal amino acid sequence analysis of native MIP-1 reveals one dominant sequence with minor amino acids at several positions. A synthetic oligonucleotide probe pool corresponding to the dominant NH_2-terminal amino acid sequence was used to isolate a cDNA clone containing the coding sequence for murine MIP-1 (4). This cDNA clone has now been sequenced (4) and expressed in yeast (unpublished work). From this initial work, however, it had not been possible to distinguish whether the two peptide bands of purified "doublet" represented two allelic forms of the same gene product, two alternative splicings of a unique genetic locus, or differing proteolytic digests of a single mature protein.

To clarify the nature of the MIP-1 doublet, we chromatographically separated and partially sequenced the two component peptides. This resolution allowed us to at-

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[1] *Abbreviations used in this paper:* DTT, dithiothreitol; MIP-1, macrophage inflammatory protein 1.

tribute our previous MIP-1 cDNA clone to one peptide (MIP-1α), and to clone a second cDNA corresponding to the second peptide (MIP-1β) of the originally purified MIP-1 doublet. The MIP-1α and MIP-1β cDNA clones are clearly distinct but highly homologous, share several common features in noncoding flanking regions, code for homologous proteins whose predicted hydrophilicity profiles are strikingly similar, and help to define a growing family of closely related proteins produced by various "activated" cell types.

Materials and Methods

Cell Culture. The mouse macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Rockville, MD). Cells were maintained as described previously (3). Conditioned medium from LPS-stimulated cells was generated as follows: RAW 264.7 cells were grown in 150-mm tissue culture dishes (Falcon Labware, Oxnard, CA) in RPMI 1640 culture medium supplemented with 10% FCS until they reached confluency. Confluent monolayers were washed five times in HBSS and covered with serum-free RPMI 1640 containing 1.0 μg/ml LPS (LPS W, *E. coli* 0127:B8; Difco Laboratories, Inc., Detroit, MI). After incubation at 37°C for 16 h, conditioned medium was harvested, filtered through a 0.22-μm filter, and stored frozen at −20°C until use.

Purification of MIP-1β. MIP-1 was purified as described previously (3). In brief, conditioned medium from LPS-stimulated RAW 264.7 cells was concentrated 20-fold and diafiltrated against 0.02 M Tris/HCl buffer, pH 8.0, using a hollow fiber concentration system equipped with a 10,000-mol wt cutoff (DC 2; Amicon Corp., Danvers, MA). Octyl glucoside was added to a final concentration of 1% (wt/vol), and the MIP-1-containing concentrate was fractionated over a Mono Q 10:10 anion exchange column (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.02 M Tris/HCl buffer, pH 8.0. A linear gradient from 0 to 1.0 M NaCl was used to elute adsorbed proteins. MIP-1 was monitored throughout by its pattern of migration on SDS-PAGE. MIP-1-containing fractions were pooled, concentrated, and subjected to high performance gel filtration chromatography over a Superose 12 column (Pharmacia Fine Chemicals) equilibrated in 0.1 M ammonium acetate buffer, pH 7.4. As described previously, MIP-1 eluted in the void volume (3). Fractions containing MIP-1 were pooled, concentrated using a PM-10 membrane in a stirred cell (Amicon Corp.), and diafiltrated against 0.01 M sodium phosphate buffer, pH 6.4. The two components of MIP-1 were then resolved by SDS-hydroxylapatite chromatography according to the procedure of Moss and Rosenblum (5). In brief, a 500-μg aliquot of MIP-1 was equilibrated in 0.01 M sodium phosphate buffer, pH 6.4, containing 1% SDS and 1% mercaptoethanol and placed in a boiling water bath for 2 min. The sample was immediately diluted 10-fold with 0.01 M sodium phosphate buffer (pH 6.4) containing 0.1% SDS and 1.0 mM dithiothreitol (DTT) (buffer A), applied directly to a hydroxylapatite column (Bio Gel HPHT; Bio-Rad Laboratories, Richmond, CA) pre-equilibrated in buffer A. A 25-ml linear gradient from 0.01 to 0.35 M sodium phosphate buffer (pH 6.4) containing 0.1% SDS and 1.0 mM DTT was used for elution.

Protein Determination. Protein concentration was measured by Bradford assay (6) with BSA as standard (Bio-Rad Laboratories).

Protein Sequencing. NH_2-terminal amino acid sequence analysis of MIP-1α and MIP-1β were performed on a gas phase sequenator (Applied Biosystems, Inc., Foster City, CA). The Dayhoff protein sequence bank was searched for homologous amino acid sequences using the computer program FASTP.

Hydrophilicity Plots. The hydrophilicity plots of MIP-1α and MIP-1β were calculated by a modification of the algorithm of Hopp and Woods (7).

Construction of cDNA Library. A cDNA library from LPS-stimulated RAW 264.7 cells was obtained as described previously (4). In brief, confluent monolayers of RAW 264.7 cells were washed five times in HBSS and covered with serum-free RPMI 1640 culture medium containing 1.0 μg/ml LPS. After incubation at 37°C for 2 h, total RNA was extracted into 6 M guanidinium thiocyanate (8). Poly(A)$^+$ RNA was then isolated by two cycles of oligodT-cellulose chromatography according to the procedure of Maniatis et al. (9). dscDNA

was prepared from the poly(A)$^+$ RNA according to the procedure of Gubler and Hoffman (10). Internal Eco R1 sites were methylated, Eco R1 linkers added, and the cDNA was inserted into the Eco R1 sites of bacteriophage λ gt10 (11).

Construction of the Probe Pools. An oligonucleotide probe pool corresponding to amino acids 2–8 of a partial NH_2-terminal sequence of MIP-1β were synthesized as described by Warner et al. (12). This particular sequence was chosen because it was the region of least apparent homology between the dominant (MIP-1α) and minor (MIP-1β) sequences.

Screening of the Library. Duplicate nitrocellulose filter lifts of the plated library (4×10^5 plaques) were hybridized overnight at 42°C in 4× SSC, 2X Denhardt's solution, 40 mM sodium phosphate buffer, pH 7.0, 0.3 mg/ml sonicated salmon sperm DNA, 0.1% SDS, and $2.5–5 \times 10^4$ cpm/ml per degeneracy of the ^{32}P-ATP 5′ end-labeled synthetic oligonucleotide probe pool. After hybridization, the filters were washed; the final washes were done under conditions of moderate stringency: 2X SSC, 0.1% SDS at 50°C. Plaques that were positive on duplicate filters were subjected to a second round of low density plating and screening. In this way two independent positive phage clones were isolated from which DNA was prepared for further analysis.

DNA Sequence Analysis. The cDNA inserts to be analyzed were subcloned into M13 phage vectors and DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (13).

Results and Discussion

As described previously, native murine MIP-1 is isolated as a doublet with a subunit mol wt of ~8,000 (3). Although the two components are separated to some extent by SDS-PAGE, their electrophoretic mobilities are so similar that preparative SDS-PAGE appeared an impractical means of purification. We subjected native MIP-1 to SDS-hydroxylapatite chromatography, a technique that has been used successfully to separate protein subunits of similar electrophoretic mobility (5). As can be seen in Fig. 1, two distinct protein peaks are observed after fractionation of native MIP-1 on a hydroxylapatite column in the presence of SDS. The first peak elutes at 0.24 M sodium phosphate and the second at 0.27 M sodium phosphate. SDS-PAGE analysis of the column fractions revealed that the first peak corresponded to the lower mol wt band of MIP-1, while the second peak corresponded to the higher mol wt band. Using this technique, each component was obtained in pure form (>95% as judged by SDS-PAGE) for NH_2-terminal amino acid sequence analysis.

The NH_2-terminal amino acid sequence of the lower mol wt component of MIP-1 (now referred to as MIP-1α) is shown in Fig. 2 *A*. The NH_2-terminal amino acid sequence reported here is identical to the dominant sequence obtained when native MIP-1 (doublet) was sequenced previously (3). The NH_2-terminal sequence anal-

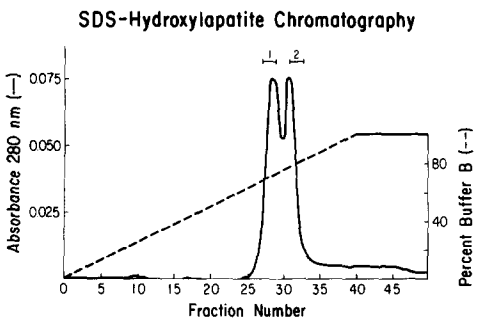

FIGURE 1. Fractionation of MIP-1 into component peptides by SDS-hydroxylapatite chromatography. MIP-1 (500 μg) was applied to SDS-hydroxylapatite and fractionated as described in Materials and Methods. 0.5-ml fractions were collected, analyzed by SDS-PAGE, and pooled as indicated by the brackets (*1*, MIP-1α; *2*, MIP-1β).

A

ala-pro-tyr-gly-ala-asp-thr-pro-thr-ala- X - X -phe-ser-tyr-ser-arg-lys-ile-

pro-arg-gln-phe-ile-val-asp-tyr-phe-glu-thr-ser-

B

ala-pro-met-gly-ser-asp-pro-pro-thr-ser- X - X -phe- X -tyr-

C

(met) (pro) (thr)

ala-pro-tyr-gly-ala-asp-thr-pro-thr-ala- X - X -phe-ser-tyr-ser-arg-lys-ile-

pro-arg-gln-phe-ile-val-asp-tyr-phe-glu-thr-ser-

FIGURE 2. (*A*) NH_2-terminal amino acid sequence of purified MIP-1α; (*B*) NH_2-terminal amino acid sequence of MIP-1β. The residues underscored (2–8) were those used to construct an oligonucleotide probe pool for MIP-1β; (*C*) Original NH_2-terminal amino acid consensus sequence reported for purified native MIP-1 (doublet). Residues corresponding to minor amino acids at variance with the dominant sequence are given in parenthesis. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00801.

ysis of the higher mol wt component of MIP-1 (now referred to as MIP-1β) is shown in Fig. 2 *B*. The minor amino acids observed at positions 3 and 7 in the original NH_2-terminal sequence correspond to differences in amino acid residues between MIP-1α and MIP-1β. In Fig. 2 *C*, the original NH_2-terminal amino acid consensus sequence for native MIP-1 is given for comparison (3). It is clear from the nucleotide sequence reported by Davatelis et al. (4) that the MIP-1 cDNA clone corresponds to MIP-1α, ruling out proteolytic digestion as a source of the two bands of MIP-1.

A cDNA clone containing the coding sequence for MIP-1β was isolated and characterized. To accomplish this, a cDNA library from LPS-stimulated RAW 264.7 cells was prepared according to the procedure described in Materials and Methods. The oligonucleotide probe pool used to screen the cDNA library was generated against the sequence underscored in Fig. 2 *B*. Three of these seven MIP-1β amino acids differ from the corresponding sequence of MIP-1α. The probe pool used to screen the library was 5′-CXATGGGX$^{TC}_{AG}$CGACCCXCC-3′. The third position choices were made based on the codon usage reported for cloned murine genes and the codon usage of MIP-1α (13).

Utilizing the labeled probe pool, the library was screened under conditions of moderate stringency. Two independent clones were isolated out of 4×10^5 recombinant phage plaques screened. This was a considerably lower frequency than expected. Upon DNA sequencing of the isolated clones, it was found that the choice of C for the third position of the Ser5 codon was incorrect; it was actually a T, thereby resulting in no perfect match of any probe pool sequence to the actual MIP-1β sequence. Thus, MIP-1β sequence representation in the library is likely to be underestimated based on hybridization with this probe pool. Subsequent screening of the library with unique oligonucleotide probes specific for comparable regions of MIP-1α and MIP-1β indicate that MIP-1β-specific sequences are fivefold less abundant than MIP-1α sequences.

After plaque purification, DNA inserts (~700 bp) from the two positive recombinant phage were shown to crosshybridize to the full MIP-1α cDNA probe. Insert cDNA was isolated from each recombinant phage population, cloned into m13, and the complete nucleotide sequence was determined. The two nucleotide sequences

differed only in the length of 5′ untranslated sequence present; the longer nucleotide sequence is presented in Fig. 3.

The MIP-1β cDNA is 650 bp long; which by primer extension analysis is no more than 11–12 nucleotides short of a complete cDNA sequence. The nucleotide sequence of MIP-1β contains a single open reading frame beginning with the first ATG codon encountered at the 5′ end of the sequence (nucleotides +62 to +64) after an in-frame stop codon. The methionine specified by this codon defines the start of a signal sequence (residues −23 and −1) with characteristic features including α helix and a centrally located hydrophobic region (14). The sequence surrounding this ATG codon conforms to the consensus sequence shared by many mRNAs of higher eukaryotes (15).

The TGA termination triplet is located 91 codons downstream of the initiating codon. The 3′ untranslated region of MIP-1β is composed of 315 nucleotides and contains the hexanucleotide AATAAA (nucleotides +283 to +288), which precedes the site of polyadenylation in many eukaryotic mRNAs (16).

There is a single sequence at nucleotides +174 to +181 that precisely matches the cytokine 3′ untranslated consensus sequence (TTATTTAT) defined by Caput et al. (17) that is characteristic of many immunomodulatory proteins. In addition, there are two additional sequences present that have a single mismatch to this consensus sequence (nucleotides +165 to +172 and nucleotides +185 to +192).

The mature peptide sequence, starting at position 1, is 69 amino acids in length.

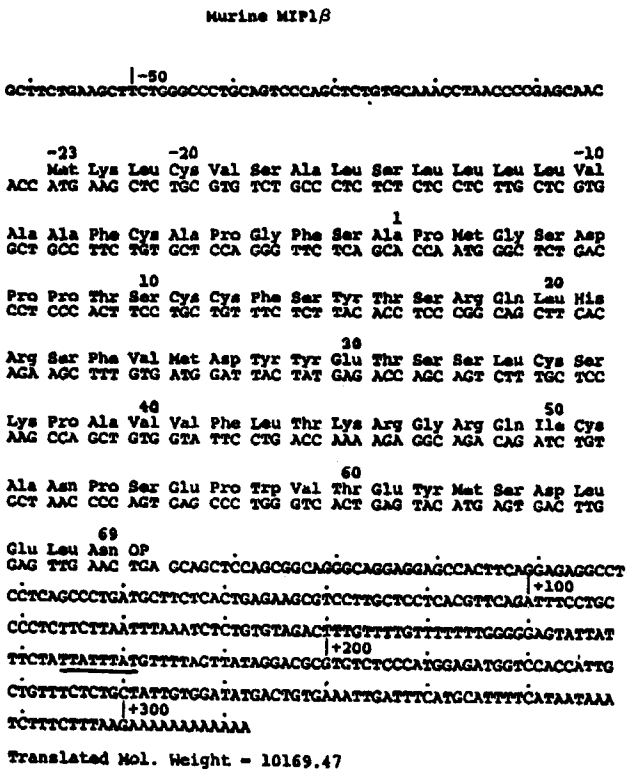

FIGURE 3. The complete nucleotide sequence of the cDNA clone for MIP-1β is shown. The predicted translated mol wt is 10,169. The mature protein sequence, starting at position one, is 69 amino acids in length and has a predicted mol wt of 7,832. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00801.

The mature protein, as determined from its cDNA clone, has a mol wt of 7,832, which agrees with the mol wt predicted by SDS-PAGE. The amino acid sequence predicted from the cloned cDNA sequence is consistent with the first 13 amino acids obtained from NH_2-terminal amino acid sequencing of purified native MIP-1β.

MIP-1β has a single *N*-linked glycosylation site present in the mature protein at amino acids 53–55 (Asn-Pro-Ser). Whether native MIP-1 is actually glycosylated at this position has not yet been determined, but it is well recognized that proline at position X in the Asn-X-Ser, Thr signal for *N*-linked glycosylation results in impaired or no glycosylation at that site (18). Hydrophilicity plots for MIP-1α and MIP-1β, which indicate the predicted polarity profiles of the two proteins, are shown in Fig. 4. It is clear from Fig. 4 that the local polarity distribution within MIP-1α and MIP-1β are strikingly similar.

The entire nucleotide sequence of murine MIP-1β cDNA was compared with sequences in the GenBank nucleotide database (rodent, primate, other mammals, and other vertebrate libraries) as well as to the MIP-1α cDNA sequence (4). The MIP-1β and MIP-1α cDNAs are 56.7% identical; the only significant homology found in GenBank was to cDNA clone pLD78 isolated from a human T cell lymphocyte cDNA library on the basis of its mRNA induction by either a tumor promoter or PHA (19). Murine MIP-1β cDNA showed 57.1% identity in a 592-nucleotide overlap to human LD78 cDNA. Murine MIP-1α cDNA showed even higher homology, 69% identity in a 746 nucleotide overlap.

When the predicted amino acid sequence of MIP-1β was tested for homology to sequences in the Dayhoff data base using the FASTP computer homology algorithm, no strikingly high homology was found. However, comparison of the predicted protein sequences of murine MIP-1β, murine MIP-1α, human LD78, as well as the recently reported predicted sequences for the PDGF, IL-1, and dsRNA-inducible murine cytokine JE (20), and a murine T cell activation protein, TCA3 (21), indicates that these proteins are clearly homologous (Fig. 5). The deduced amino acid sequence of MIP-1β shows 59.8% identity to that of MIP-1α, and 58.7%, 38.9%, and 31.9% identity to the predicted amino acid sequences of LD78, JE, and TCA3, respectively. Common to all these sequences is the presence of four conserved cysteines in each of the mature protein sequences. Interestingly, the predicted amino acid sequence of murine MIP-1α is more homologous to that of human LD78 (75.3%

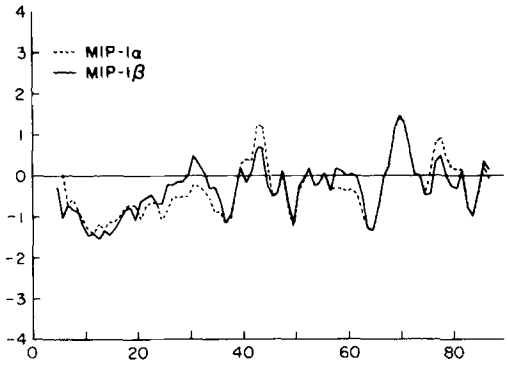

FIGURE 4. Hydrophilicity plots of MIP-1α (-----) and MIP-1β (———) indicating the calculated polarity profiles of the two peptides. The *y*-axis represents hydrophilicity values derived by a modification of the Hopp and Woods algorithm (7). The *x*-axis shows position along the amino acid sequence.

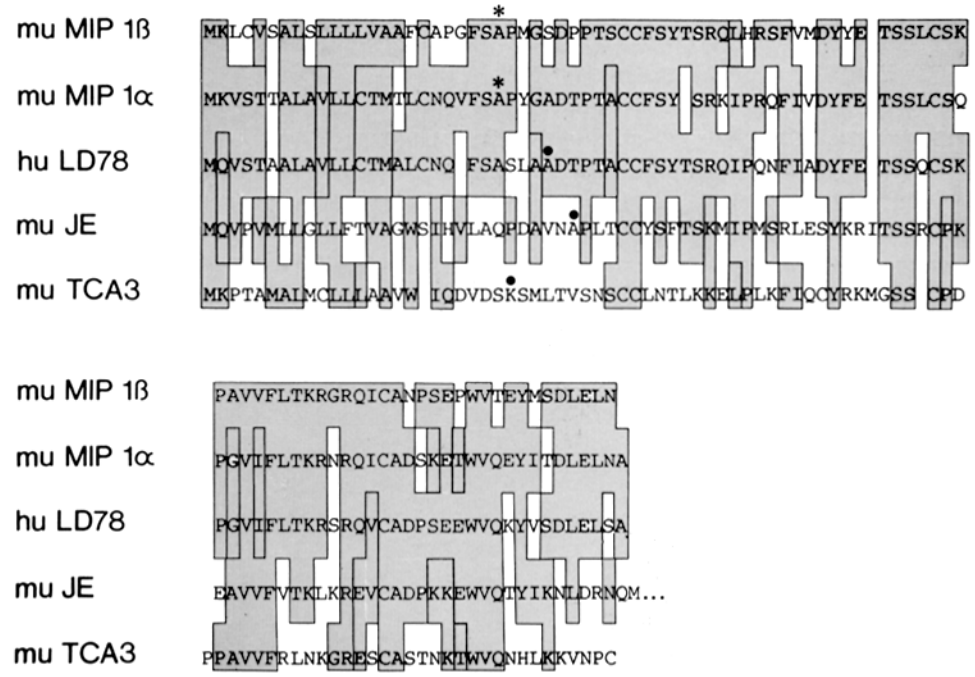

FIGURE 5. Homology of MIP-1β and related proteins. Sequences (cDNA) were obtained from the literature and the predicted protein sequences were aligned using the FASTP program algorithm. Amino acids that are identical between at least two sequences are enclosed in shaded boxes. (*) The NH_2-terminal of the putative mature protein as determined by NH_2-terminal sequence analysis of purified secreted protein. (●) The NH_2-terminal amino acid predicted to be the start of the mature protein. Small case prefixes refer to species: mu, murine; hu, human. References for sequences are as follows: MIP-1β (this work); MIP-1α (4); LD78 (19); JE (21); and TCA3 (20).

identity) than it is to the predicted amino acid sequence of MIP-1β (59.8% identity). Thus, these proteins share similarities in sequence that appear to define a family of peptides that may be involved in inflammatory and/or immune responses.

The quaternary structure of native MIP-1 has not yet been determined. The two component peptides, MIP-1α and MIP-1β, appear to be produced in equal amounts by macrophages in response to LPS. Further work should elucidate any relationship that exists between these two proteins. In particular it is important to determine if a combination of both components is required for some or all of the bioactivities exhibited by purified native MIP-1. This work is currently in progress. The availability of recombinant MIP-1α and MIP-1β will help to reveal other activities associated with this monokine as well.

Summary

A number of macrophage-derived mediators have been implicated in the vascular changes of inflammation. We recently reported the isolation of a novel monokine, macrophage inflammatory protein 1 (MIP-1), which causes local inflammatory responses in vivo, and induces superoxide production by neutrophils in vitro. Purified

native MIP-1 comprises two peptides with very similar physical characteristics. We report here the resolution of MIP-1 into component peptides by SDS-hydroxylapatite chromatography, and compare the NH_2-terminal sequences of the two peptides, now referred to as MIP-1α and MIP-1β. A synthetic oligonucleotide probe pool corresponding to the NH_2-terminal amino acid sequence of MIP-1β was used to isolate a cDNA clone containing its coding sequence. The sequence codes for a 109 amino acid-long polypeptide, of which 69 amino acids correspond to the mature product. Comparison of this MIP-1β cDNA with our previously cloned MIP-1α sequence reveals that the MIP-1 peptides, members of a growing family of potential inflammatory mediators, are distinct but highly homologous (58.9% sequence identity) products of different genes.

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