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MOLECULAR CLONING AND POSTTRANSCRIPTIONAL REGULATION OF MACROPHAGE INFLAMMATORY PROTEIN-1α IN ALVEOLAR MACROPHAGES*

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SUMMARY: Macrophage inflammatory protein-1α (MIP-1α) belongs to the "chemokine" superfamily of chemoattractant pro-inflammatory cytokines. MIP-1α is chemotactic for monocytes and neutrophils and thus, plays an important role in initiation and control of inflammation. We have isolated and sequenced a cDNA clone encoding rat MIP-1α. This 0.75 kb cDNA includes a single open reading frame of 92 amino acids. Expression of MIP-1α mRNA was characterized in NR8383, a rat alveolar macrophage cell line (RAM). In resting RAM cells, MIP-1α mRNA decayed rapidly, with a half life of less than 2 hours. Lipopolysaccharide (LPS) treatment of RAM cells resulted in a dose-dependent increase in MIP-1α steady state mRNA expression. The induction of MIP-1α mRNA by LPS was partially the result of mRNA stabilization, as half life increased to over 6 hours.

Macrophage inflammatory protein-1 (MIP-1) belongs to the newly-recognized "chemokine" supergene family of cytokines with monocyte/macrophage and neutrophil chemotactic properties. Native MIP-1 was originally reported as a low molecular weight, heparin-binding protein composed of two subunits, MIP-1α and MIP-1β (1). MIP-1α is a member of the CC family of chemokines, named according to the position of the first two cysteine residues at the amino terminus (2). It has been reported that MIP-1α is primarily

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ABBREVIATIONS:

bp, base pair; EDTA, ethylenediamine tetraacetic acid; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; PCR, polymerase chain reaction; RAM, rat alveolar macrophage; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate; TE, Tris-EDTA; UTR, untranslated region.

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chemotactic for mononuclear phagocytes, but may also be chemotactic for neutrophils and lymphocytes (1,3,4,5). It also plays an important role in the control of haemopoietic stem cell proliferation (6,7).

Rats are widely-used animal models for studying acute and chronic inflammation in the lungs (8,9). A recent study has suggested that MIP-1α is one of the primary mediators inducing pulmonary inflammation in the rat (10). Additional characterization of this rat chemokine will be necessary for our continuing studies of gene structure, expression and regulation in models of pulmonary inflammation. We have cloned a cDNA for rat MIP-1α and provide the nucleotide sequence for this clone. The cDNA was used as a probe to investigate MIP-1α mRNA expression in a rat alveolar macrophage cell line in response to an inflammatory stimulus and to investigate postranscriptional regulation of this mRNA.

MATERIALS AND METHODS

Cell culture: Tissue culture supplies and related materials were purchased from Sigma (St. Louis, MO) unless otherwise stated. The rat alveolar macrophage (RAM) cell line, NR8383, was generously provided by Dr. R. Helmke (11). Cells were cultured in RPMI 1640 supplemented with 5% equine serum, 2 mM L-glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in a humidified incubator at 37°C with 5% CO_2. When used, lipopolysaccharide (LPS, *Escherichia coli*) was added to a final concentration of 1 or 10 μg/ml for 4 h.

RNA extraction: Total cellular RNA was isolated from RAM cells using a modified guanidinum method (12). Cells were lysed in 4 M guanidine thiocyanate/25 mM Na citrate (pH 7.0)/0.5% N-lauroyl-sarcosine/0.1 M 2-mercaptoethanol. The mixture was layered on 5.7 M CsCl/0.1 M Na_2ethylenediamine tetraacetic acid (EDTA), and centrifuged at 47,000 rpm (268,000 x g) for 4 h in a Beckman SW55 Ti rotor. Pelleted RNA was resuspended in diethyl pyrocarbonate-treated TE buffer (10 mM Tris/1 mM EDTA, pH 7.4).

Synthesis of a rat MIP-1α probe: A 180 bp partial rat MIP-1α cDNA was synthesized by the reverse transcription-polymerase chain reaction (RT-PCR). Oligonucleotides were synthesized according to the sequence of mouse MIP-1α (1). The 5' primer was 5'-TATGGAGCTGACACCCCGAC-3' (bases 146-165) and the 3' primer was 5'-GATGTATTCTTGGACCCAGGT-3' (bases 305-325). First-strand cDNA was reverse-transcribed from 1 μg of total RNA, isolated from LPS-stimulated RAM cells, using Ready-To-Go T-Primed First Strand Kit (Pharmacia, Piscataway, NJ). Subsequently, 15 pmole of the 5'- and 3'-primers and 2.5 U of Taq DNA polymerase were added and subjected to PCR amplification: 94°C 1 min, 42°C 2 min, 72°C 3 min for 9 cycles; 94°C 1 min, 50°C 2 min, 72°C 3 min for 19 cycles; 55°C 3 min, 72°C 7 min for 1 cycle. The PCR product was resolved in 1% agarose, visualized by ethidium bromide staining, extracted from the gel and labeled with α[^{32}P]-dATP (6,000 Ci/mmol, DuPont-NEN, Boston, MA) by random primer labeling (Life Technologies, Inc., Gaithersburg, MD).

Library screening and sequencing: A rat alveolar macrophage cDNA library, constructed from poly(A)$^+$ RNA extracted from LPS-stimulated macrophages as previously described (8), was screened by plaque hybridization. Approximately 5,000 recombinant phages were plated on each of ten 15-mm petri dishes, and then blotted onto nitrocellulose filters (MSI Inc.,

Westboro, MA). The filters were prehybridized for 3 h in Church buffer (0.5 M $NaPO_4$/1 mM EDTA/7% sodium dodecyl sulfate (SDS)/150 µg/ml tRNA) (13) at 65°C and then hybridized with the [^{32}P]-labeled MIP-1α cDNA for 16 h. Filters were washed once in 0.1 X SSC (20 X = 3 M NaCl, 0.3 M Na_3citrate)/0.1% SDS at room temperature and twice at 52°C before autoradiography. After secondary screening, positive phages were resuspended in SM buffer (85 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris, pH 7.5, 0.01% gelatin). An aliquot of this buffer from 10 independent colonies was boiled for 5 min and subjected to PCR with primers corresponding to T3 and T7 promoter sequences on pBluescript. The PCR product having the largest insert size (800 bp) was subcloned into the vector pCRII and transformed into host cells INVαF' (Invitrogen, San Diego, CA). A single clone containing the MIP-1α insert was sequenced on both strands using the dideoxy procedure with universal primers or a series of synthesized 17-mer oligonucleotide primers (14).

Northern analysis: Total RNA (10 µg/lane) was denatured in 50% formamide/7% formaldehyde, resolved in a 1% agarose/7% formaldehyde gel and transferred and UV cross-linked to nylon membranes (MSI, Inc.). Equal loading in each lane was confirmed by either ethidium bromide staining of ribosomal RNA or by probing blots with radiolabeled mouse β-actin (15). Prehybridization (3 h) and hybridization with [^{32}P]-labeled MIP-1α or β-actin cDNA (overnight) were carried out in Church buffer at 65°C. Blots were washed once in 0.1 X SSC/0.1% SDS at room temperature and twice at 52°C before autoradiography.

mRNA half-life determination: Following treatment of RAM cells with 10 µg/ml LPS or media alone for 4 h as above, actinomycin D was added to the media to a final concentration of 5µg/ml. Cells were sampled at selected times through 6 h and levels of mRNA quantified by Northern analysis. The integrated band values, as determined by densitometry, were normalized to β-actin gene expression.

RESULTS AND DISCUSSION

Eight positive plaques were identified from 50,000 screened using the RT-PCR generated rat MIP-1α probe. One insert was subcloned into the pCRII vector and both strands sequenced (Fig. 1A). The MIP-1α cDNA is 755 base pairs long with 69 bp in the 5' untranslated region (UTR), a single open reading frame of 276 bp (92 amino acids), and 410 bp in the 3' untranslated region (Fig. 2). Relative to the first ATG, nucleotides at positions -3 to -1 are ACC, consistent with the consensus sequence for translation initiation (16). The deduced protein has 92 amino acids (MW = 10 kDa) with 4 conserved cysteines at positions characteristic of the CC chemokine family (2). The deduced rat MIP-1α protein shares 89% and 75% homology to its mouse and human counterparts, respectively (Fig. 3). The deduced peptide also contains a hydrophobic signal sequence at the N-terminus (Fig. 1B). Based on the sequence pattern of amino acids near the signal cleavage site (17), we assigned the first 23 amino acids as the signal peptide and localized the signal peptide cleavage site between Ser (amino acid 23) and Ala (amino acid 24). The predicted mature secreted MIP-1α peptide contains 69 amino acids with a calculated molecular weight of 8 kDa.

Sequence analysis of this clone revealed that it includes the complete 3'-UTR, which terminated in a poly(A)$^+$ sequence at nucleotide 755 and had a consensus polyadenylation

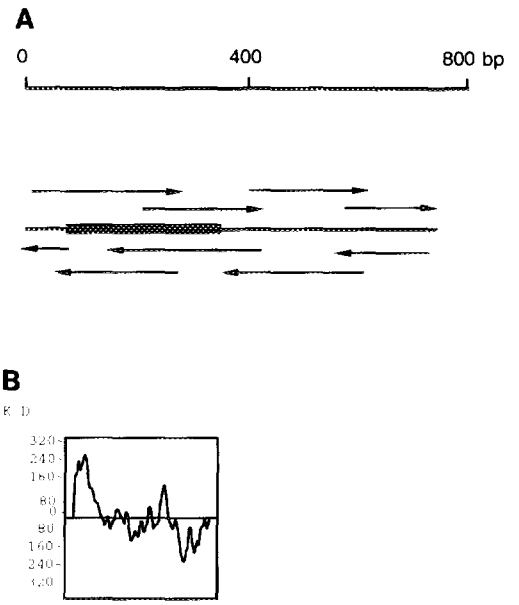

Figure 1. A) Schematic representation and sequencing strategy for rat MIP-1α cDNA. Arrows indicate direction and extent of individual sequencing reactions using synthetic oligonucleotide primers. The open reading frame is indicated by a bold line. B) A Kyte-Doolittle (K-D) hydropathy plot was derived from the predicted amino acid sequence.

signal (AATAAA) 17 bases upstream of the poly $(A)^+$ tail. The 3'-UTR sequence of the rat MIP-1α gene was particularly AT-rich. There were six copies of the ATTTA sequence, which has been identified in many cytokine, lymphokine, oncogene, and inducible growth factor mRNAs (18, 19). The presence of multiple copies of this AUUUA motif in the 3'-UTR of cytokine mRNAs is known to accelerate cytoplasmic decay of mRNAs (18, 20). Identification of these sequences suggests that MIP-1α gene expression is regulated, in part, at the level of mRNA stability.

The rat MIP-1α cDNA sequence provided us with a valuable tool to further dissect the mechanisms by which MIP-1α gene expression is controlled. A cDNA probe prepared from the cloned MIP-1α was used to study the expression of MIP-1α mRNA in RAM cells in response to an acute inflammatory stimulus, LPS. Our probe hybridized with a single band at approximately 0.9 kb (Fig. 4). Untreated RAM cells, similar to primary alveolar macrophages, demonstrated a low, but detectable steady-state level of MIP-1α mRNA. This expression was markedly up-regulated after 4 h exposure to 1 or 10 μg/ml LPS in a dose-dependent manner (Fig. 4). Increased MIP-1α mRNA levels in response to LPS are most likely the result of increased gene transcription, a decrease in mRNA turnover, or both. As discussed above, we suspected that the six AUUUA elements in the 3'-UTR of rat MIP-1α

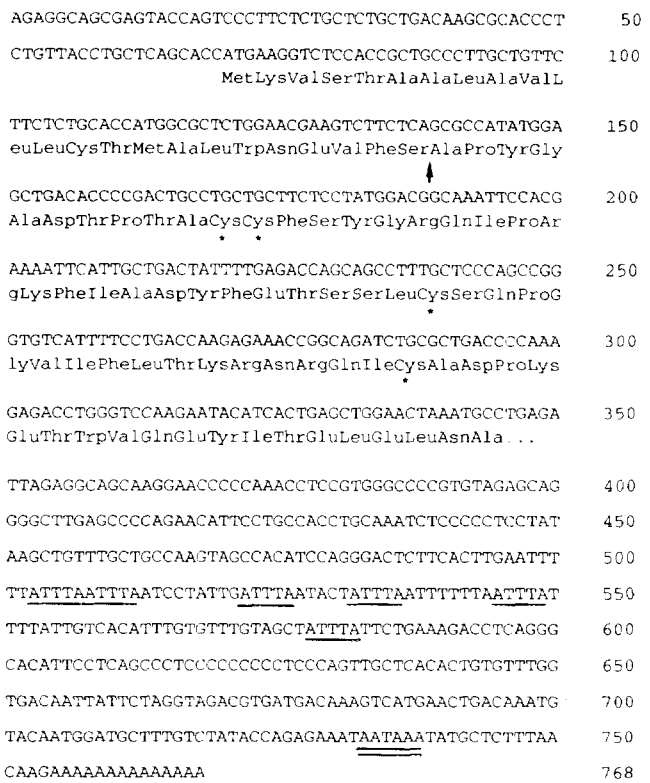

Figure 2. Nucleotide and deduced amino acid sequence of rat MIP-1α. The signal peptide cleavage site is indicated by a vertical arrow. * identifies the four cysteine residues conserved in the CC subfamily of chemokines. The characteristic cytokine mRNA destabilization sequences are underlined and the putative polyadenylation signal AATAAA is double underlined.

mRNA modulate mRNA stability, an important mechanism of post-transcriptional control (21, 22). The contribution of changes in MIP-1α mRNA stability to its increased levels in response to LPS was evaluated by measuring MIP-1α mRNA half life ($t_{1/2}$). In the presence of actinomycin D, MIP-1α mRNA from untreated macrophages decayed quickly with a calculated half-life of 1.8 h (Fig. 5). MIP-1α mRNA is not constitutively expressed in other

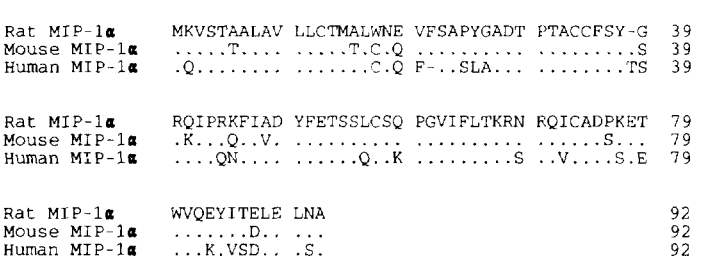

Figure 3. Comparison of rat, mouse and human MIP-1α peptide sequences. Amino acids identical to the rat counterpart are represented by dots. Dash (-) represents assigned spacing of amino acids.

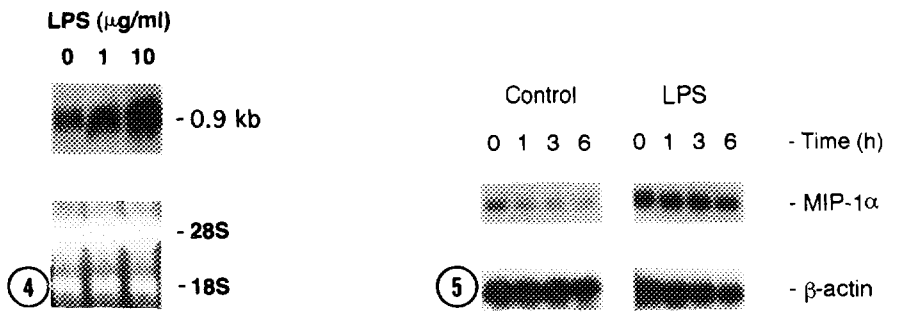

Figure 4. Induction of MIP-1α mRNA levels by lipopolysaccharide (LPS) in a rat alveolar macrophage cell line (NR8383). Cells were incubated with 0, 1, or 10 μg/ml LPS for 4 h. Total cellular RNA was extracted and 10 μg of each sample was resolved in 1% agarose under denaturing conditions, transferred to nylon membrane, and hybridized with a MIP-1α cDNA probe. Upper panel, autoradiogram of Northern blot hybridized with radiolabeled MIP-1α cDNA. Lower panel, ethidium bromide-stained, formaldehyde-agarose gel prior to transfer to nylon membrane, indicating similar loading among lanes.

Figure 5. LPS-induced stabilization of MIP-1α mRNA in a rat alveolar macrophage cell line NR8383. Cells were stimulated with 10 μg/ml LPS for 4 h. Actinomycin D was then added to a final concentration of 5 μg/ml, and at times indicated, total RNA was isolated and Northern analysis was performed as described in Figure 4. Upper panel, autoradiogram of Northern blot hybridized with radiolabeled MIP-1α cDNA. Lower panel, the same blot probed with radiolabeled β-actin cDNA as a control. The MIP-1α mRNA half life from untreated cells was approximately 1.8 h, whereas LPS treatment increased the half life to greater than 6 h.

inflammatory cell types (e.g. polymorphonuclear leukocytes , PMNs) and, therefore, other estimates of $t_{1/2}$ for this mRNA are unavailable (23). The half-life of IL-8, a CXC chemokine, has been reported as less than 1 h in human transitional and renal cell carcinomas (24). LPS treatment significantly increased the $t_{1/2}$ of MIP-1α mRNA, with essentially no change in mRNA levels through the 6 hours examined after actinomycin D addition (Fig. 5). This $t_{1/2}$ is substantially longer than the half-life of 40 min, reported for human MIP-1α mRNA from PMNs also treated 4 h with LPS (23). We suspect increases in MIP-1α mRNA levels in PMNs, in response to LPS, is primarily transcriptionally regulated, whereas, posttranscriptional regulation has a more dominant role in increasing MIP-1α mRNA levels in macrophages. These results demonstrate that posttranscriptional control plays an important role in the LPS-induced up-regulation of MIP-1α mRNA expression. We suspect this mechanism will also be important for regulating expression of other chemokines involved in pulmonary inflammation.

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