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cDNA CLONING OF RAT LRP, A RECEPTOR LIKE PROTEIN TYROSINE PHOSPHATASE, AND EVIDENCE FOR ITS GENE REGULATION IN CULTURED RAT MESANGIAL CELLS

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SUMMARY: Protein tyrosine phosphatases (PTPases) are a family of enzymes that play a crucial role in the regulation of signal transduction mediated by reversible protein tyrosine phosphorylation. To understand the significance of PTPases in physiological and pathophysiological processes in the kidney, we isolated three cDNA segments encoding PTPases (LAR, LRP and a novel PTPase) from rat kidney by polymerase chain reaction (PCR). Using PCR product as a probe, we isolated a full-length cDNA of rat LRP. LRP cDNA encoded a single membrane spanning protein consisted of 796 amino acids, with two tandemly located intracellular PTPase domains. By Northern analysis, a ubiquitous pattern of LRP gene expression in rat tissues was demonstrated. In cultured rat mesangial cells, LRP mRNA was detected and the mRNA level was suppressed by either interleukin-1 or interleukin-6 treatment.

Protein tyrosine phosphorylation, which is regulated by the activities of both protein tyrosine kinases and protein tyrosine phosphatases (PTPases; E.C.3.1.3.48), is crucial for diverse cellular processes such as proliferation and differentiation. Protein tyrosine kinases have been paid more attention than PTPases partly because of the better molecular characterization of protein tyrosine kinases (1) compared to PTPases. Since the molecular characterization of PTPase 1B by protein sequence and consequent cDNA cloning (2,3), diversity and structures of PTPases have been demonstrated by cDNA cloning, and the number of PTPase gene family is still growing (4). Currently, two types of PTPases, cytosolic and transmembrane types are known. Cytosolic type PTPases possesses one catalytic domain, while most of the transmembrane type PTPases have two tandem repeat of catalytic domains and diverse extracellular domains.

Mesangial cell abnormalities including proliferation and extracellular matrix expansion are hallmarks of chronic glomerular diseases (5). Cultured rat mesangial cells are known to respond to cytokines; interleukin-1 (IL-1) and interleukin-6 (IL-6) have been demonstrated to promote mesangial cell proliferation (6,7).

The abbreviations used are: PTPase, protein tyrosine phosphatase; LAR, leukocyte common antigen related protein; LRP, leukocyte common antigen related phosphatase; PCR, polymerase chain reaction; IL, Interleukin.

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Tyrosine kinase activation has been reported to be a component of the response of cells to IL-1 (8) and IL-6 (9). However, there has been little study which focuses the physiological and pathophysiological roles of PTPases. To elucidate the involvement of PTPases in disease states of kidney, we undertook cDNA cloning of PTPases from rat kidney mRNA using PCR and identified three cDNA segments of PTPases including LRP. We cloned a full-length cDNA of LRP, and further we studied the effect of IL-1 and IL-6 on the gene expression of LRP in cultured rat mesangial cells to test the potential role of LRP in the mesangial cell proliferation process.

MATERIALS AND METHODS

Materials and general methods: Restriction endonucleases and modifying enzymes were purchased from Takara Shuzo, Life Science, Toyobo. Recombinant Thermus aquatics (Taq) polymerase was obtained from Perkin-Elmer Cetus. Sequenase was purchased from United States Biochemicals. Oligonucleotide primers were synthesized on Applied Biosystems 380A DNA synthesizer. Standard DNA manipulations and cloning procedures were done according to Sambrook et al. (10). Total RNA was isolated by acid guanidium thiocyanate-phenol-chloroform extraction as described (11). [α-32P] dATP (3000 Ci/mmol) and Colony Plaque Screen were purchased from Du Pont-New England Nuclear. hrIL-1β was purchased from Genzyme. hrIL-6 was kindly provided by Prof. Hirano (Osaka Univ. Med. Sch.).

Polymerase chain reaction and subcloning of PTPase domains: Total RNA from rat kidney was converted to single stranded cDNA by random hexamer priming and MMLV reverse transcriptase. This cDNA was used as a template for in vitro amplification with Taq DNA polymerase and degenerate sense and antisense primers (Fig. 1). The PCR was carried out as follows. The first and second cycles were for 1 min at 94°C (denaturation), 1 min at 37°C (annealing) and 1 min at 72°C (extension). The following 28 cycles were carried out under the same conditions except for the annealing temperature (3rd and 4th cycles at 45°C and 5th to last cycles at 55°C). Fragments of expected size (about 350 bp) were isolated from polyacrylamide gel, subcloned into pUC 119 and sequenced.

Isolation of LRP cDNA clones: A rat λgt10 kidney cDNA library (Clontech) was screened with the purified insert of p34-7 clone as a probe.

5'-End isolation of LRP cDNA: 5'-End of the LRP cDNA was isolated by PCR as already described (12) with modifications. Five μg of total RNA was reverse transcribed using random hexamer as primers. After purification of first strand cDNA from the excess nucleotides, the cDNA was polyadenylated by terminal deoxynucleotidyl transferase. Amplification of 5'-end of the cDNA was performed directly on 1 μl aliquot of the polyadenylayted cDNA pool essentially as described (12).

Culture of rat mesangial cells: Cultured mesangial cells were prepared as previously described (13). Briefly, glomeruli were isolated from male Sprague-Dawly rats by consecutive sieving with three different meshes (120, 280, and 53 μm). The isolated glomeruli were seeded in plastic culture dishes and were cultured in RPMI 1640 medium supplemented with 20% fetal calf serum. The cultures were kept at 37°C in a 95% air and 5% CO_2 atmosphere. The first subculture was done on day 21 of the primary culture, when most of the cells grown were of mesangial origin. Cells of passages 4 and 5 were used in this study.

RESULTS AND DISCUSSION

Isolation of PTPase homologs from rat kidney RNA

We used the following PCR-based protocol to isolate members of PTPase gene family from rat kidney RNA. Degenerate oligonucleotides were designed corresponding to conserved regions in the catalytic domain of PTPase (N/GDFWRM and VHCSAG) (14) (Fig. 1). PCR was carried out for 30 cycles on the first strand cDNA synthesized from rat kidney RNA. PCR products of expected size (350bp) were subcloned into pUC 119 and sequenced. We found three putative PTPase cDNA clones. Amino acid

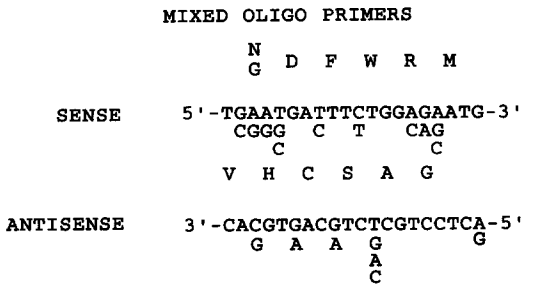

Figure 1. Primers used for polymerase chain reaction. The degenerate primer set was derived from the amino acid sequence indicated by the single letter code. Below the amino acid sequence, derived nucleotide sequences are shown.

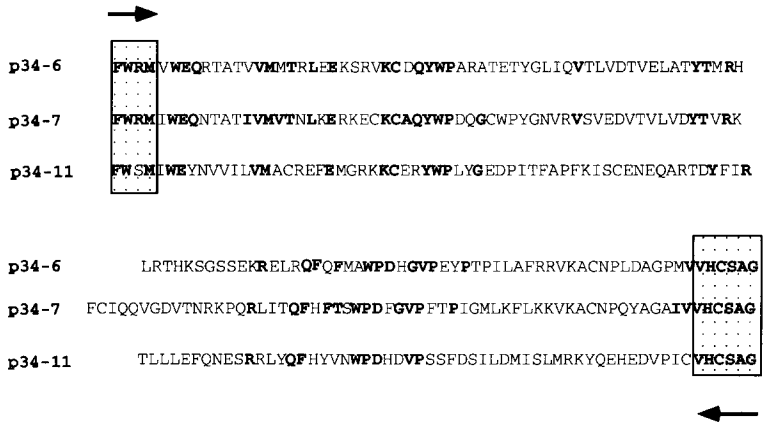

Figure 2. Deduced amino acid sequences of PCR products for PTPases from rat kidney RNA. Amino acid sequences derived from PCR primers are boxed and these sequences may not represent actual ones. Arrows show primers used for PCR.

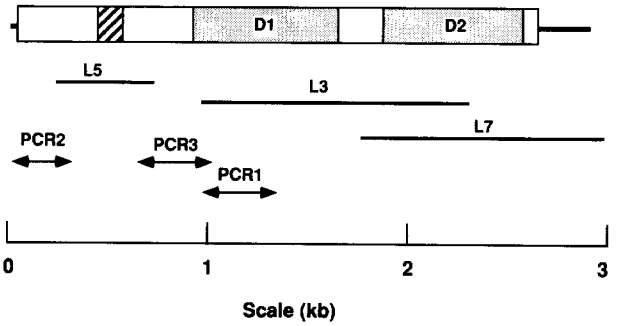

Figure 3. Schematic diagram of the rat LRP cDNA, and the relationship of the isolated cDNA clones. The open reading frame is indicated by box. Striped box represents a transmembrane portion and dotted boxes represent catalytic domains D1 and D2. The solid bars show the isolated cDNA clones, and the lines with arrows represent the cDNA clones obtained by PCR. p34-7 is designated as PCR-1 in this figure.

sequences derived from the nucleotide sequences of these cDNA clones are shown in Fig. 2. By comparison of the sequences with the Genebank nucleotide sequence data bank, two were highly homologous or identical to known PTPases, while the other one was only distantly related to known sequences. p34-6 was identical to LAR (leukocyte common antigen related protein) (15, 16), while p34-7 (as PCR-1 in Fig. 3) showed 94.6% homology to mouse LRP (14) and appeared to represent its rat counterpart. p34-11 has conserved amino acids characteristics for PTPase catalytic domain. However it has relatively low DNA

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CTAGGTCAGCATGGATTCCTGGTTCATTCTTATCCTGTTTGGCAGTGGTCTAATACACATTAGTGCCAACAACACTACTACAGTTTCACCTTCTTTAGGA      100
        MetAspSerTrpPheIleLeuIleLeuPheGlySerGlyLeuIleHisIleSerAlaAsnAsnThrThrThrValSerProSerLeuGly

ACTACAAGATTAATTAAAACATCAACAACAGAATCGGCTAAGGAAGAGAATAAAACTTCAAATTCAACCTCTTCAGTAATTTCTCTTTCTGTGACACCAA      200
ThrThrArgLeuIleLysThrSerThrThrGluSerAlaLysGluGluAsnLysThrSerAsnSerThrSerSerValIleSerLeuSerValThrProThr

CATTCAGCCCAAATCTAACTCTGGAACCCACCTATGTGACTACTGTTAATTCTTCACACTCTGACAATGGGACCAAGAGAGCAGCCAGCACGGAATCTGG      300
 PheSerProAsnLeuThrLeuGluProThrTyrValThrThrValAsnSerSerHisSerAspAsnGlyThrLysArgAlaAlaSerThrGluSerGly

AGGCACTACCATTTCACCAAACGAACATACGGAACATGGCTTACTGATAACCAGTTCACGGATGCCAGAACAGAACCCTGGGAGGGGAATTCCAGCAGT      400
 GlyThrThrIleSerProAsnGlyThrTyrGlyThrTrpLeuThrAspAsnGlnPheThrAspAlaArgThrGluProTrpGluGlyAsnSerSerSer

GCAGCAACCACTCCAGAAACCTTCCCCCCTGCAGATGAGACACCAATCATTGCGGTGATGGTGGCCCTGTCCTCTCTGCTAGTAATTCTCTTTATTATTA      500
AlaAlaThrThrProGluThrPheProProAlaAspGluThrProIleIleAlaValMetValAlaLeuSerSerLeuLeuValIleLeuPheIleIleIle

TAGTTCTGTACATGTTAAGGTTTAAGAAATACAAGGAAGCTGGGAGCCATTCCAATTCTTTCCGCCTGTCAAATGGCCGGACAGAGGATGTGGAGCCCCA      600
 ValLeuTyrMetLeuArgPheLysLysTyrLysGlnAlaGlySerHisSerAsnSerPheArgLeuSerAsnGlyArgThrGluAspValGluProGln

AAGTGTACCACTTCTGGCCAGGTCCCCGAGCACCAACAGAAAGTACCCACCACTGCCTGTGGACAAGCTGGAAGAGGAGATTAACCGGAGAATGGCTGAT      700
 SerValProLeuLeuAlaArgSerProSerThrAsnArgLysTyrProProLeuProValAspLysLeuGluGluGluIleAsnArgArgMetAlaAsp

GACAATAAGCTCTTCAGAGAGGAATTCAACGCCCTCCCTGCCTGTCCAATCCAGGCCACCTGTGAGGCTGCCTCCAAGGAAGAAAACAAGGAAAAAAACC      800
AspAsnLysLeuPheArgGluGluPheAsnAlaLeuProAlaCysProIleGlnAlaThrCysGluAlaAlaSerLysGluGluAsnLysGluLysAsnArg

GATATGTAAACCTCCTGCCTTATGACCACTCCAGAGTGCACCTGACACCTGTTGAAGGGGTTCCAGATTCTGATTACATCAACGCCTCATTCATTAATGG      900
  TyrValAsnLeuLeuProTyrAspHisSerArgValHisLeuThrProValGluGlyValProAspSerAspTyrIleAsnAlaSerPheIleAsnGly

CTACCAGGAAAAGAACAAATTCATTGCTGCACAAGGACCAAAAGAAGAAACGGTGAATGATTTCTGGAGAATGATATGGGAACAGAACACAGCCACCATT     1000
 TyrGlnGluLysAsnLysPheIleAlaAlaGlnGlyProLysGluGluThrValAsnAspPheTrpArgMetIleTrpGluGlnAsnThrAlaThrIle

GTCATGGTGACCAACCTGAAGGAGAGAAAGGAGTGTAAATGTGCCCAGTATTGGCCAGACCAAGGCTGCTGGCCATATGGGAATGTCCGTGTGTCTGTCG     1100
ValMetValThrAsnLeuLysGluArgLysGluCysLysCysAlaGlnTyrTrpProAspGlnGlyCysTrpProTyrGlyAsnValArgValSerValGlu

AGGATGTGACTGTTCTGGTGGACTACACAGTACGGAAATTCTGCATCCAGCAGGTGGGCGACGTCACCAACAGGAAGCCACAGCGCCTCATCACTCAGTT     1200
  AspValThrValLeuValAspTyrThrValArgLysPheCysIleGlnGlnValGlyAspValThrAsnArgLysProGlnArgLeuIleThrGlnPhe

CCATTTCACCAGCTGGCCAGACTTTGGGGTGCCTTTTACCCCAATTGGCATGCTCAAGTTCCTCAAGAAGGTGAAAGCCTGTAACCCTCAGTATGCAGGG     1300
 HisPheThrSerTrpProAspPheGlyValProPheThrProIleGlyMetLeuLysPheLeuLysLysValLysAlaCysAsnProGlnTyrAlaGly

GCTATCGTGGTCCACTGCAGTGCAGGTGTAGGGCGCACTGGCACCTTTGTTGTCATCGATGCCATGCTGGACATGATGCATTCGGAGCGCAAAGTGGATG     1400
AlaIleValValHisCysSerAlaGlyValGlyArgThrGlyThrPheValValIleAspAlaMetLeuAspMetMetHisSerGluArgLysValAspVal

TATATGGGTTTGTGAGCCGGATCCGGGCCCAGCGCTGCCAGATGGTCCAGACAGACATGCAGTACGTCTTCATATACCAGGCCCTTCTGGAGCATTATCT     1500
  TyrGlyPheValSerArgIleArgAlaGlnArgCysGlnMetValGlnThrAspMetGlnTyrValPheIleTyrGlnAlaLeuLeuGluHisTyrLeu

ATATGGCGACACAGAGCTGGAAGTGACTTCCCTAGACACCCACCTGCAAAAAATTTATAACAAGATCCCAGGAACTAGCAACAATGGACTAGAGGAGGAA     1600
  TyrGlyAspThrGluLeuGluValThrSerLeuAspThrHisLeuGlnLysIleTyrAsnLysIleProGlyThrSerAsnAsnGlyLeuGluGluGlu

TTTAAGAAATTAACTTCAATCAAAATCCAAAATGACAAGATGCGTACTGGTTACCTTCCAGCCAACATGAAGAAGAACCGAGTTTTACAGATCATTCCAT     1700
PheLysLysLeuThrSerIleLysIleGlnAsnAspLysMetArgThrGlyTyrLeuProAlaAsnMetLysLysAsnArgValLeuGlnIleIleProTyr

ATGAATTTAACAGAGTGATCATTCCAGTCAAACGGGGCGAAGAGAACACAGACTATGTGAACGCATCCTTCATTGATCCGTACCGGCAGAAAGATTCCTA     1800
  GluPheAsnArgValIleIleProValLysArgGlyGluGluAsnThrAspTyrValAsnAlaSerPheIleAspProTyrArgGlnLysAspSerTyr

ATCGCCAGCCAGGGCCCTCTTCTCCACACAACTGAGGACTTCTGGCGAATGATCTGGGAATGGAAGTCCTGCTCCATTGTAATGCTAACAGAACTGGAA     1900
 IleAlaSerGlnGlyProLeuLeuHisThrThrGluAspPheTrpArgMetIleTrpGluTrpLysSerCysSerIleValMetLeuThrGluLeuGlu

GAAAGAGGCCAGGAGAAGTGTGCCCAGTACTGGCCATCTGATGGCCTGGTGTCCTATGGAGACATCACAGTTGAGCTGAAGAAGGAGGAGGAATGTGAGA     2000
GluArgGlyGlnGluLysCysAlaGlnTyrTrpProSerAspGlyLeuValSerTyrGlyAspIleThrValGluLeuLysLysGluGluGluCysGluSer

GCTACACTGTCCGAGACCTCCTGGTCACCAACACCAGGGAGAACAAGAGTCGGCAGATCCGGCAGTTCCACTTCCACGGCTGGCCTGAGGTGGGCATCCC     2100
  TyrThrValArgAspLeuLeuValThrAsnThrArgGluAsnLysSerArgGlnIleArgGlnPheHisPheHisGlyTrpProGluValGlyIlePro

CAGCGATGGGAAGGGTATGATCAACATCATCGCAGCAGTCGCAGAAGCAGCAGCAGTCAGGGAACCATCCCATCACTGTGCACTGCAGTGCCCGGGCA     2200
 SerAspGlyLysGlyMetIleAsnIleIleAlaAlaValGlnLysGlnGlnGlnSerGlyAsnHisProIleThrValHisCysSerAlaGlyAla

GGACGGACAGGGACCTTTTGTGCCTTGAGCACAGTCCTGGAGTGCGTGAAAGCAGAAGGAATTTTCGATGTCTTCCAAACTGTCAAGACGCTGCCGCTGC     2300
GlyArgThrGlyThrPheCysAlaLeuSerThrValLeuGluCysValLysAlaGluGlyIleLeuAspValPheGlnThrValLysThrLeuArgLeuGln

AGAGGCCACACATGGTCCAGATACTGGAACAGTATGAATTCTGCTACAAGGTGGTACAGGAGTACATCGATTCCTTCTCAGATTATGCCAACTTGAAGTG     2400
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GAGTCATTCCAGGTAACTCGGTGCCAACTAAAACAAAGCACAAAGTTCTCGGAGCTCTTGAGGAAATGGTTGTCTCACGTCCCCAGGCCGGCCTCTTCCC     2800
TTCCCTGTCCCTCTCCAGTGCACCCTCATCTCCCACCAATATCAGCCACCTCAGGAGTGGGGAATAATGAGACCAGAGCAGCATCCCTGGCACCACACTA     2900
GGAATCATCAGGTAATAAACACTCTTGATTCCCGGAAAAAAAAAAAAAAAAAAA
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Figure 4. Nucleic acid sequence and translated amino acid sequence of the rat LRP cDNA. Nine nucleotide insertion is boxed. The polyadenylation site is underlined.

homology to reported PTPases with 40% homology to mouse LRP as highest one. Thus, we assume that p34-11 represents a new member of PTPase gene family.

Isolation and analysis of LRP cDNA clones from rat kidney

Since the nucleotide sequence of the rat LRP has not been reported, we screened rat kidney cDNA library in λgt10 using p34-7 as a probe and we isolated λL3. The library was screened again using λL3 cDNA as a probe and λL7 was isolated (Fig. 3). λL7 clone contained polyadenylation signal of AATAAA and poly(A) tail. Since no clone containing upstream sequences of the λL3 clone was isolated, we cloned some part of the cDNA(PCR-3) by PCR using primers derived from mouse LRP sequences. Using PCR-3 cDNA as a probe, λL5 was obtained from the library. 5'-End of the cDNA (PCR-2) was obtained by PCR as described in the MATERIALS AND METHODS. The composite cDNA sequence contained one long open reading frame spanning from nucleotide 11 to 2398 (Fig.4). Thus, rat LRP is consisted of 796 amino acid residues. We did not find 108 nucleotide insertion of alternative spliced exon reported in the mouse LRP (14). To note, there was 9 nucleotides insertion at position 324-332 which is not present in the mouse LRP sequence. These sequences are also present in the LRP genomic DNA (data not shown). Except this insertion, there was 95.1 % homology between rat and mouse LRP coding regions.

Gene expression of LRP in rat tissues

Northern blot analysis revealed a ubiquitous pattern of expression of LRP mRNA. A 3 kb mRNA was present in all tissues examined, with brain showing the highest levels of expression followed by heart, kidney and lung (Fig.5). This tissue distribution pattern of LRP mRNA in rat is similar to that in mouse as already described (14). Since the mRNA level of LRP in the kidney is relatively high, LRP may have some physiological significance in various processes in the kidney.

Regulation of LRP mRNA expression in cultured rat mesangial cells by interleukins

To study the potential role of LRP in mesangial cell abnormalities, we tested if LRP mRNA is expressed and is regulated by external stimuli in cultured rat mesangial cells (Fig. 6). In control cells, LRP mRNA (3kb) was observed, and 12 hour-treatment with IL-1(100U/ml) or IL-6 (100U/ml) suppressed the LRP mRNA level compared to control. 10% fetal calf serum had little effect on the LRP gene expression. Since IL-1 and IL-6 are growth promoters of mesangial cells, these results raise the possibility that IL-1 and IL-6 stimulate mesangial cell proliferation via the suppression of LPR activity in mesangial cells. Further study is required to uncover the relevance of LRP to mesangial cell growth regulation. This study will enable us to gain some insight into whether LRP has physiological role in normal mesangial cell function and whether perturbation of the PTPase activity may lead to the pathological state of the glomeruli.

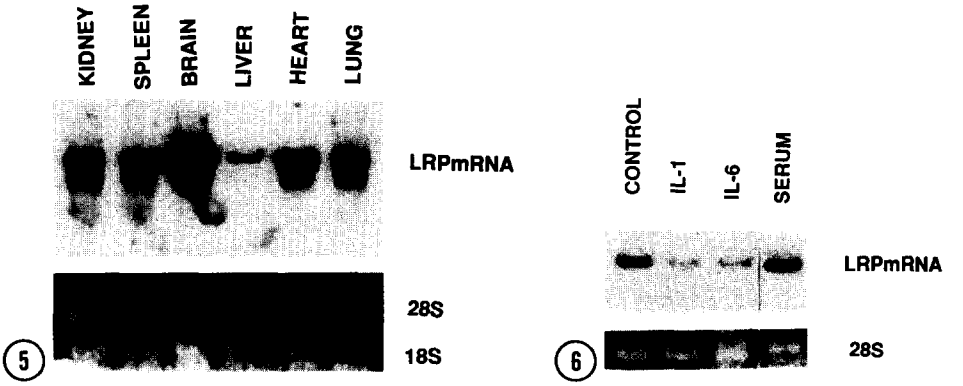

Figure 5. Expression of LRP in rat tissues. Total RNA was isolated from the indicated tissues and was separated on a 0.8% agarose formaldehyde gel, transferred to nitrocellulose. The blots were probed with PCR product, p34-7. All lanes were loaded with 5 μg of total RNA. Ethidium bromide stain of 18S and 28S ribosomal RNAs was shown as a control in the lower panel.

Figure 6. Effect of IL-1 and IL-6 on the LRP mRNA level in cultured rat mesangial cells. Subconfluent monolayers of mesangial cells were washed with phosphate-buffered saline and incubated in serum-free RPMI 1640 media for 12 hours prior to the addition of ligands. Then cells were incubated in serum-free RPMI 1640 plus ligands (no addition; control, IL-1 (100U/ml), IL-6 (100U/ml), 10% fetal calf serum) for 12 hours, and total RNA was isolated. 10 μg of total RNA was separated on a 0.8 % agarose formaldehyde gel, transferred to nitrocellulose. The insert of p34-7 was used as a probe. Ethidium bromide stain of 28S ribosomal RNA was shown as a control in the lower panel.

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