

Mouse Inter-Alpha-Trypsin Inhibitor Family Heavy Chain-Related Protein is an Acute Phase Protein Induced by Inflammation

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The mouse counterpart of the human inter-alpha-trypsin inhibitor family heavy chain-related protein (IHRP), inter-alpha-trypsin inhibitor family heavy chain-related protein, was partially purified from mouse serum through Western blot analysis using anti-human IHRP antibody. The amino-terminal amino acid sequence was then determined, and the cDNA clone encoding mouse IHRP was isolated and characterized. The mouse IHRP amino-terminal amino acid sequence matched the predicted sequence from the cDNA, and has high homology compared with human and pig IHRP. Furthermore, the whole mouse IHRP amino acid sequence predicted from the cDNA nucleotide sequence showed reasonable homology to that of both human and pig IHRP. Mouse IHRP expression was induced by treatment with typical inflammation-inducing compounds, such as turpentine and lipopolysaccharide.

Key words — acute phase, inter-alpha-trypsin inhibitor, inflammation, lipopolysaccharide, turpentine

INTRODUCTION

Inter-alpha-trypsin inhibitor (ITI) family heavy chain-related protein (IHRP) is a novel human glycoprotein that shows significant homology in amino acid sequencing to the heavy chains of the ITI family from human plasma.^{1,2} ITI family (ITI, pre-alpha-trypsin inhibitor and H2/bikunin) are composed of bikunin and one or two heavy chains, such as HC1, HC2 or HC3. ITI family heavy chains reportedly stabilize the cumulus extracellular matrix due to hyaluronan binding.^{3,4}

We identified IHRP as a single chain protein without bikunin, since IHRP does not have a consistent amino acid sequence, such as DPHFII, to complex with bikunin *via* chondroitin sulfate. Although IHRP is known as inter-alpha-trypsin inhibitor heavy chain 4, it differs from the ITI family heavy chains, HC1, HC2 and HC3. Therefore, IHRP is likely to have a distinct function. We have recently reported that IHRP inhibited actin polymerization

and phagocytosis of polymorphonuclear cells.⁵

Human IHRP cDNA cloning has revealed that IHRP is identical to PK-120, consistent with Nishimura *et al.*⁶ The partial N-terminal amino acid sequence of pig-MAP, which is reportedly a major acute phase protein, is also identical to that of pig IHRP, as we previously reported, with the exception of Ser22 and Lys23.⁷ Pig IHRP seems to be an acute phase reactant protein due to its inductive role in the acute phase.

We purified human IHRP homologue from mouse serum by using Western blot analysis with anti-human IHRP polyclonal antibody and cloned its cDNA, because the molecular mass (120 kDa) of the protein, which observes in mouse serum by Western blot analysis with anti-human IHRP polyclonal antibody, is about same it of pre-alpha trypsin inhibitor belongs to inter-alpha-trypsin inhibitor family.

To elucidate the role of IHRP during inflammation, the process of expression was investigated using by both Northern and Western blot analyses.

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MATERIALS AND METHODS

Materials — Polyethylene glycol (PEG) 4000 and turpentine oil were purchased from Wako Pure Chemicals (Japan) and endotoxin lipopolysaccharide (LPS), from *Escherichia coli* (*E. coli*) O55 was obtained from Difco Laboratories (MI, U.S.A.). A HiTrap Q, HiPrep 16/60 Sephacryl S-200 HR column and ³²P-labeled nucleotide were obtained from Amersham Bioscience (NJ, U.S.A.), while the UK-50 membrane was obtained from Toyo (Japan). Restriction enzymes were purchased from Nippon Gene Co., Ltd. (Toyama, Japan), Toyobo Co., Ltd. (Osaka, Japan), and Boehringer Mannheim Co. (IN, U.S.A.), and used according to the respective manufacturer's instructions. Recombinant Taq DNA polymerase was obtained from Takara Shuzo (Japan). The nitrocellulose filter was purchased from Schleicher and Schuell, while the agarose type 2 was obtained from Sigma Chemical Co. (MO, U.S.A.) and the low-melting-point agarose (NuSieve) from FMC Co. (ME, U.S.A.). A random-primer labeling kit was purchased from Du pont-New England. The male mice (Balb/c, 4 weeks old) were obtained from Sankyo Laboratory.

Polyclonal anti-human IHRP antibody, which was cross-reactive with mouse IHRP, was obtained by rabbit immunized with human IHRP.¹⁾

Partial Purification of Mouse IHRP — Mouse serum (30 ml) was mixed in 10 mM Tris-HCl (pH 7.0) containing 10 mM lysine-HCl (20 ml). The dissolved sample was applied to a HiTrap Q column (three HiTrap Q 5 ml columns were connected tandem) equilibrated with 10 mM Tris-HCl (pH 7.0) containing 10 mM lysine-HCl. The column was washed thoroughly with the same buffer to remove the unbound proteins, and the bound proteins were then eluted by a linear gradient of 0–0.5 M NaCl in the same buffer. The fractions containing IHRP were detected by Western blot analysis with anti-human IHRP antibody. The fractions containing IHRP were concentrated to 5 ml by ultrafiltration with a Centricon 100 concentrator. The concentrated sample was applied to HiPrep 16/60 Sephacryl S-200 HR (1.6 × 60 cm) equilibrated with 10 mM Tris-HCl (pH 7.0) containing 10 mM lysine-HCl and 0.15 M NaCl at a flow rate of 0.8 ml/min.

The experimental protocol was approved by The Institutional Animal Care and Use Committee of Showa University.

Analysis of N-Terminal Amino Acid Sequence of Partially Purified Mouse IHRP — The par-

tially purified IHRP was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. The separated proteins were transferred onto a PolyVinylDene Fluoride (PVDF) membrane, stained with 0.1% Coomassie brilliant blue R-250 for 1 min, and then destained with 50% methanol. The protein bands were cut off separately and subjected to N-terminal sequence analysis with a protein sequencer 473A (Applied Biosystems, CA, U.S.A.).

Electrophoresis and Western Blot Analysis — Protein samples were analyzed by 7.5% SDS-PAGE and then the separated proteins were transferred onto a nitrocellulose membrane. After blotting, the membrane was blocked with 10 mM Tris-based saline, 0.1% tween 20 and 5% fat-free dry milk for 1 hr and incubated with anti-human IHRP antibody (1000-fold dilution) for 1 hr. The membrane was washed three times for 5 min with 10 mM Tris-based saline, 0.1% tween 20 (TBS-T), and then incubated with peroxidase-conjugated anti-rabbit IgG in TBS-T (1000-fold dilution). The membrane was washed as above, developed using the ECL-PLUS detection system (Amersham Bioscience), and then exposed to Fuji X-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Cloning of Mouse IHRP cDNA — The human IHRP cDNA labeled with ³²P was used as a screening probe. The mouse liver cDNA library constructed in λZAP phage vector was screened by cross hybridization. Hybridization was carried out in a solution containing 5 × saline citrate (SSC), 5 × Denhardt's, 50 mM Tris-HCl (pH 7.5), 100 mg/ml of denatured salmon sperm DNA, 0.1% SDS, 10% dextran sulfate and 50% formamide at 42°C, followed by sequential washings of the membranes with 2 × SSC containing 0.1% SDS at 37°C and with 0.1 × SSC containing 0.1% SDS at 37°C. Several rounds of screening were carried out in order to obtain positive clones.

Characterization of the Isolated Clones — Three clones (15, 133 and 134) were isolated by cross hybridization. After retrieving the insert DNAs in Bluescript vector, the nucleotide sequences were determined by the dideoxynucleotide chain termination method⁸⁾ with a DNA sequencer model DSQ-1000 (Shimadzu, Kyoto, Japan). The three cDNA clones, which had an overall length of 2300 bp, did not contain full-length cDNA. To obtain the full-length cDNA encoding mouse IHRP, the same cDNA library was rescreened using the cloned cDNA as a probe. Eight clones were isolated by rescreening.

The clones containing the longest insert cDNA were further analyzed by sequencing.

Treatment of Mice with Inflammation Inducers

— The mice were injected with turpentine oil (TUR, 0.5 ml/100 g body weight, sc.) or endotoxin lipopolysaccharide (LPS, 0.5 mg/kg body weight, i.p.) dissolved in physiological saline to induce inflammation. Prior to the induction of inflammation, some mice were pre-treated with dexamethasone (DEX, 25 mg/kg of body weight, sc.) dissolved in 5% acacia (Arabic gum, GUM) solution to prevent inflammation. After inflammation was induced the animals were sacrificed by exsanguination under ether anesthesia. The animal sera and livers were then collected.

Isolation of RNA and Northern Blot Analysis

— Total RNA was isolated from livers with ISOGEN-LS (Nippon Gene). The RNAs (50 µg, denatured with formaldehyde for 15 min at 65°C) were separated on 1% agarose gel containing 2.2 M formaldehyde. Isolated RNA was then transferred onto a nylon membrane (Amersham Bioscience, Hybound-N+). Next, the RNA was fixed by UV cross-linking and hybridized using a ³²P-labeled mouse IHRP cDNA probe. The membrane was washed with 2 × SSC containing 0.1% SDS at 55°C for 30 min. The membrane was then exposed to X-ray film (Fuji film) at -70°C. The radioactive band on that membrane was evaluated using an imaging analyzer (Fuji Bio Imaging Analyzer model BAS 2000). GAPDH mRNA was determined with its cDNA probe and then used as an internal control. The equalization of the RNA samples was confirmed by the ethidium bromide patterns of the ribosomal RNA bands.

RESULT

By screening approximately 8×10^5 recombinant clones from a mouse liver cDNA library using the ³²P-labeled human IHRP cDNA fragment as a probe, three positive clones were obtained. The nucleotide sequences of these clones overlapped each other, indicating the possibility that the three clones resulted from a single mRNA; however all of the samples had a truncated nucleotide sequence at the 5' end. To obtain the full-length cDNA from mouse IHRP, the same library was rescreened using the cloned mouse IHRP cDNA fragment as a probe, and eight positive clones were isolated. After these clones were analyzed as described above, clone 110,

which had the longest cDNA insert, was sequenced and ascertained to possess the full-length nucleotide sequence for mouse IHRP. This clone contained an insert of 3000 bp and poly (A) tail of 31 bases, and the nucleotide sequence was well-matched to that reported by Tao Cai *et al.*⁹⁾ The open reading frame begins 5 bases downstream from the 5'-end and extends for 940 amino acids to a TAG stop codon. Although the nucleotide sequence surrounds the first-frame initiation codon at nucleotides 6–8, GGCAGATGA, it does not match well with the consensus sequence, CCACCATGG, as described by Kozak.^{10,11)} This codon appears to be the most likely candidate for the translation start site because the methionine was followed by a 27 amino acid hydrophobic residue, which may function as a signal peptide sequence. The length of the hydrophobic amino acid stretch is suitable for a signal peptide. The N-terminal of a mature protein comprised of 912 amino acid residues consists of 15 residues, as confirmed by the protein sequencer. Figure 1 illustrates that the first methionine residue within the mouse IHRP is conserved in human and pig IHRP, while the second methionine residue is substituted by threonine in the human sequence and by glycine in the pig sequence. Thus, the first methionine residue in the mouse sequence is likely to be the first amino acid of the putative polypeptide and is consequently assigned as 1. In the 3' non-coding region, a consensus polyadenylation signal, AATAAA,¹²⁾ and a 3' poly (A) tail are positioned 20 nucleotides upstream of the first A of the poly (A) tract and 3' end, respectively. The open reading frame encodes the putative signal sequence of 28 amino acid residues.

The IHRP protein was partially purified from mouse serum through analysis with SDS-PAGE and Western blot analysis using anti-human IHRP antibody (Fig. 2). The molecular mass of the mouse IHRP protein estimated by SDS-PAGE was approximately 110 kDa. The N-terminal amino acid sequence of partially purified mouse IHRP protein was identical to that of human IHRP and completely matched to the amino acid sequence predicted from the nucleotide sequence of the cloned mouse IHRP cDNA. This indicates that the cDNA encodes mouse IHRP and the anti-human IHRP antibody specifically recognizes mouse IHRP.

IHRP gene expression in the mouse liver was remarkably induced by turpentine treatment. After turpentine administration, IHRP mRNA expression reached a maximum at 12 hr and gradually declined thereafter (Fig. 3). The IHRP gene expression was



Fig. 1. Comparison of the Amino Acid Sequence of Mouse IHRP with those of Human, Pig and rat IHRP

Sequences were aligned using the Genetyx program (SDC, Tokyo, Japan). Amino acid residues identical in two or more species among the four species investigated (human, pig, rat and mouse), are shown by black boxes. The arrowhead indicates the N-terminal amino acid of purified IHRP from human, pig and mouse.

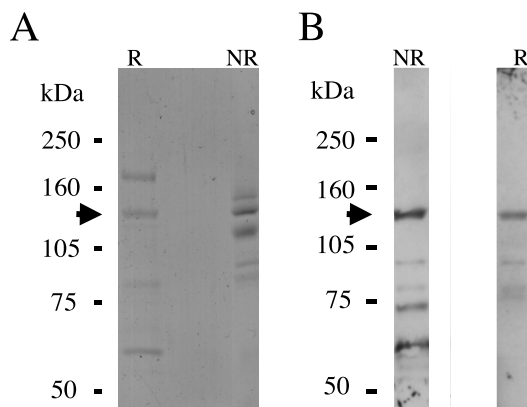


Fig. 2. SDS-PAGE and Western Blot Analysis of Partially Purified Mouse IHRP

Partially purified mouse IHRP and serum were analyzed by SDS-PAGE (7.5% gel) under non-reducing (NR) and reducing (R) conditions. After electrophoresis the proteins in the gel were stained with Coomassie brilliant blue R-250 (A) and followed by Western blot analysis using anti-human IHRP antibody (B). The arrow indicates mouse IHRP band, which was subjected to amino acid sequence analysis.

also induced by LPS administration (Fig. 4). Next we examined the effect of dexamethasone on the induction of IHRP gene expression by these stimulators (Fig. 5). The mice were pre-treated with dexamethasone, and injected with turpentine oil. The induction of IHRP gene expression by turpentine oil was effectively inhibited by the dexamethasone pre-treatment while the induction. To observe the RNA band of IHRP at time 0 Figs. 4 and 5, the membrane was exposed to X-ray film at long period. Therefore the RNA band in the vicinity of 18S (Fig. 4) or IHRP (Fig. 5) was detected. But those bands were unclear. The level of serum IHRP was examined by Western blot analysis (Fig. 6). In the case of turpentine oil, the level of IHRP in the serum from the mouse one day after treatment was more than ten-fold that at day 0 and gradually decreased, reaching control levels on day 3. In the case of LPS, the change of IHRP level was similar to that of the turpentine (data not shown).

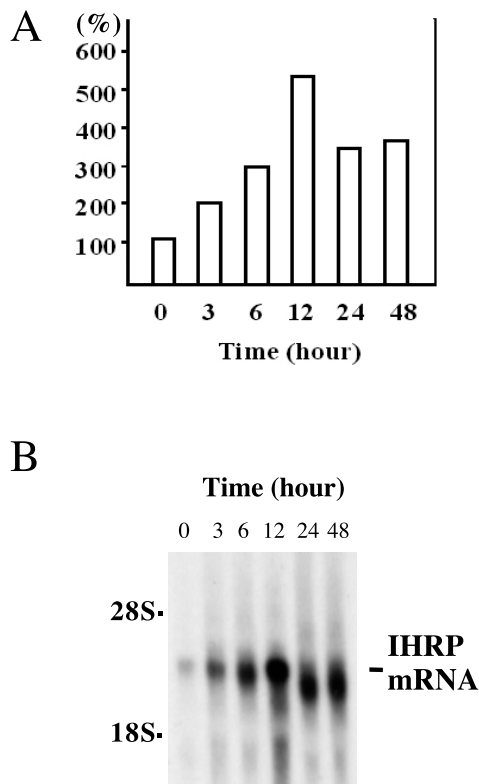


Fig. 3. Time Course of IHRP mRNA Induced by Turpentine Oil

The mice were injected with turpentine oil (0.5 ml/100 g of body weight). The total RNA was prepared from the liver at each time interval, analyzed by Northern blot (A), and quantified by BAS2000 (B).

DISCUSSION

In order to elucidate the physiological function of IHRP, purification of mouse IHRP was carried out and cDNA was isolated, while examining the role of this protein in an inflammatory reaction. The cDNA of mouse IHRP was cloned from the mouse liver cDNA library using human IHRP cDNA as a probe. On the other hand, using the cross reactivity of an anti-human IHRP antibody to mouse IHRP, partial purification of the mouse IHRP was carried out, and the partial amino acid sequence was determined as the protein chemistry target. The obtained partial amino acid sequence of mouse IHRP concurs with that expected from cloned mouse IHRP cDNA, which has significant homology to those of human, pig and rat IHRP (Fig. 1). The molecular weight of mouse IHRP, which was calculated as 101546.95 from the amino acid sequence predicted from its cDNA, was slightly smaller than the 110 kDa estimated by SDS-PAGE. This difference may be due to N-glycosylation because there are 6

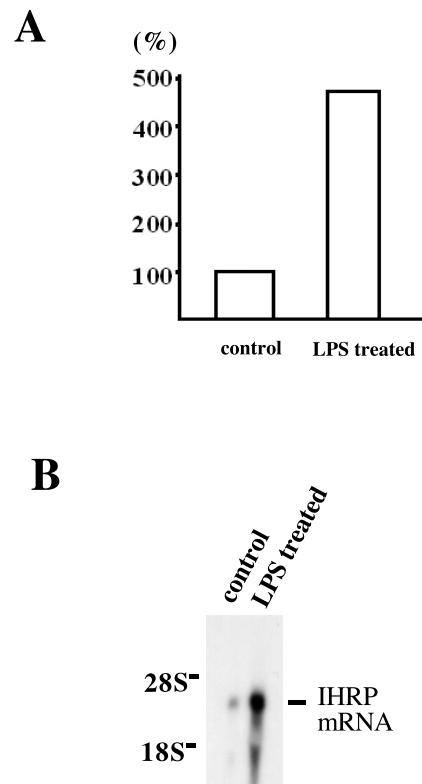


Fig. 4. The Effect of LPS on the Expression of IHRP mRNA

The mice were treated with LPS dissolved in PBS (0.5 mg/kg body weight). After 24 hr, the total RNA was prepared from mouse liver and analyzed by Northern blot analysis (A), and quantified by BAS2000 (B).

potential N-glycosylation sites in mouse IHRP. The three sites are well conserved in human, pig and mouse IHRP at the same positions, Asn 81, Asn 207 and Asn 517 in mouse IHRP.

The nucleotide sequence of cloned mouse IHRP cDNA coincides with Tao Cai *et al.* These authors reported that the mouse IHRP/*itih-4* protein is necessary in the development of early embryonic mouse liver.¹³ Pig MAP is considered to be the IHRP counterpart of an acute phase protein, based on Northern blot analysis of the mouse IHRP expression during the inflammatory reaction induced by turpentine or LPS. Mouse IHRP mRNA expression peaked and then gradually fell to control levels 12 hr after the administration of turpentine or LPS (Figs. 3 and 4). Western blot analysis also showed the same pattern (data not shown). From these results, it is thought that mouse IHRP is also an acute phase protein.

Interestingly, when an inflammatory reaction occurred after turpentine treatment, the increase in mouse IHRP was remarkably reduced by pre-treatment with dexamethasone, taking into consideration

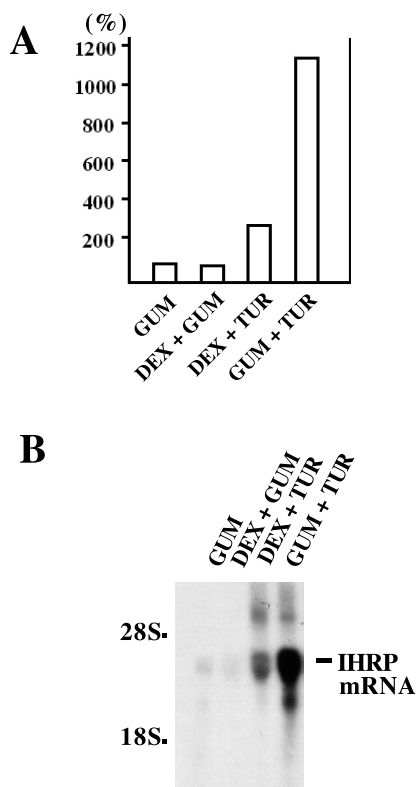


Fig. 5. The Effect of Dexamethasone on IHRP mRNA Induction by Turpentine Oil and LPS

Before injection of turpentine oil or LPS, the mice were pretreated with dexamethasone dissolved in 5% acacia. The control mice were either injected with 5% acacia or dexamethasone dissolved in 5% acacia. After 12 hr total RNA in mouse liver was analyzed by Northern blot (A) and quantified by BAS2000 (B). DEX; dexamethasone, TUR; turpentine oil, LPS; endotoxin lipopolysaccharide.

rat and mouse revealed high homology throughout their entire amino acid sequences, with the exception of approximately one hundred residues from Val 605 to Pro 716 in human IHRP.¹⁴ In this region IHRP has a kallikrein cleavage site and mouse IHRP contains the putative kallikrein cleavage site (Phe 626-Arg 627). This region is coded by small exons (exons 14, 15, 16, 17, 18 and 19) in the human IHRP gene.¹⁵ It can be speculated that this region divides the protein into two functional domains, the N-terminal domain similar to ITI heavy chains and the unique C-terminal region. In other words, this region may serve as a target site for protease to produce two functionally different polypeptides.

Recently, we found the IHRP binding protein (IBP) in human leukocytes.⁵ IBP has been identified as actin, also known as the cytoskeletal fibril protein, where IHRP inhibits the actin polymerization and phagocytosis of polymorphonuclear cells.⁵

These results suggest that IHRP induced an inflammatory reaction is an acute phase protein, and might use as an inflammation's marker in mice.

The cDNA cloned in this study and the anti-human IHRP antibody, which is cross reactive to mouse IHRP, are useful to elucidate the function of IHRP. Thus, IHRP cDNA and antibody prove to be useful investigative tools.

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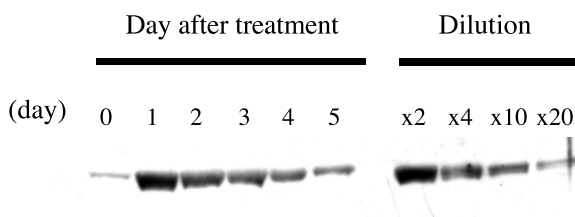


Fig. 6. Induction of Mouse IHRP Expression in the Sera of Mice Treated with Turpentine Oil

The mice were injected with turpentine oil (0.5 ml/100 g of body weight). After 0, 1, 2, 3, 4 and 5 days, serum was taken from mice and then analyzed by Western blot against anti-human IHRP antibody. Day 1 serum was serially diluted, $\times 2$, $\times 4$, $\times 10$ and $\times 20$, and compared to Day 0.

the suppression of IHRP induction by turpentine was a result of dexamethasone's control over the inflammatory reaction instead of its direct suppression of IHRP.

As previously reported, IHRP from human, pig,

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