Transcriptional Regulation of a Mesangium-Predominant Gene, Megsin

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Abstract. The cDNA of a new human mesangium-predominant gene, megsin, a novel member of the serpin superfamily, has recently been cloned. This study investigates the regulatory mechanisms of megsin gene expression. A genomic clone of the human megsin gene was obtained by screening bacterial artificial chromosome (BAC) library with the megsin cDNA. The analysis for exon-intron junctions of megsin genomic DNA demonstrated that the gene contained 8 exons and 7 introns, spanned 20 kbp, and that the genomic structure of the serpin superfamily was highly conserved. Fluorescence in situ hybridization (FISH) revealed that the megsin gene is localized in chromosome 18q21.3, close to the other serpin genes. The transcriptional start site, located by primer extension analysis, was 391 bp upstream from the start codon. The sequence and reporter analyses on 4021-bp-long 5'-flanking region of megsin gene demonstrated a consensus promoter segment within this region and a relatively strong promoter activity in human mesangial cells and A431, a human tumor cell line recently reported to express a novel serpin identical with megsin. Moreover, this study utilized site-directed and deletion mutagenesis analyses, and electrophoretic mobility shift assay identified one positive regulatory motif, an incomplete activator protein-1 (AP-1) binding motif (CTGATTAC) within the −120 to −112 region. This cis-acting element in the 5'-flanking region of megsin is involved in the activation of the megsin gene in mesangial cells.

Mesangial cells play an important role in maintaining the structure and function of the glomerulus and are thus involved in the pathogenesis of glomerular diseases (1,2). It is therefore important to identify and characterize the genes that are expressed predominantly by mesangial cells. Rapid large-scale DNA sequencing and computerized data processing allowed us to recently identify a new human mesangium predominant gene, megsin (3). This methodology has also been successful in the identification of tubules-specific genes (4,5). The full-length cDNA clone of megsin as well as its novel coded 380 amino acid protein exhibited a sequence similarity with those of the serine protease inhibitor (serpin) superfamily. This family includes plasminogen activator inhibitor type 2 (PAI-2), the squamous cell carcinoma antigen (SCCA), monocyte/macrophage elastase inhibitor, and maspin. It has been strongly conserved over the last 500 million years (6). Expression of megsin has also been shown to be upregulated in a variety of renal disorders with mesangial cell injury in humans (7,8) and in a mesangioproliferative glomerulonephritis model in rats (9). Of interest, overexpression of megsin led to progressive mesangial matrix expansion and an increase in the number of mesangial cells (10).

Several other genes, coding mainly for ion channels or transporters, are also expressed exclusively in renal tubular cells (11–16). The mechanisms responsible for their tissue specificity remain unknown at this stage. Elucidation of the transcriptional regulation of megsin should help us understand its tissue specificity and provide important insights into the mechanisms of cell type-dependent gene expression.

In the present study, we further characterize the structure, organization, and sequence of the mesangium dominant gene, megsin. We demonstrated that it is located in chromosome 18q21.3, close to other serpin genes. Finally, we identify, within the promoter region, a positive regulatory element involved in the activation of megsin gene transcription.

Materials and Methods

Isolation and Characterization of Human Megsin Gene

To isolate genomic DNA containing human megsin gene, bacterial artificial chromosome (BAC) library was screened with fragments of 5'- and 3'-untranslated regions of megsin. One positive clone (F581) was partially digested with BamHI, subcloned into the BamHI site of the pBluescript SK(−) (Stratagene, La Jolla, CA) for DNA sequencing analysis of 5'-flanking region of human megsin.

To analyze exon-intron junctions of megsin gene, the F581 clone and Genome Walker human genomic libraries (Clontech, Palo Alto, CA) were utilized.
**Cell Cultures**

Primary cultured human mesangial cells were purchased from Clontech. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml l-glutamine. A431 cells, human tumor cell line derived from epidermoid carcinoma, were purchased from Dai-Nihon Pharmacological Co. (Osaka, Japan). Primary cultured human dermal fibroblasts and umbilical vein endothelial cells (HUVEC) were purchased from Kurabo Co. (Tokyo, Japan). Human cell lines, including FL (from amnion), HeLa (from adenocarcinoma), and Chang liver (from liver) cells, were also purchased from ATCC (American Type Culture collection, Manassas, VA).

**Fluorescence In Situ Hybridization (FISH)**

DNA from bac BAC clone F581 encoding human megsin gene was labeled with digoxigenin dUTP by nick translation. Labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from phytohemagglutinin stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate, and 2 × SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated anti-digoxigenin antibodies followed by counterstaining with DAPI for one-color experiments. Probe detection for two color experiments was accomplished by incubating the slides in fluoresceinated anti-digoxigenin antibodies and Texas red avidin followed by counterstaining with DAPI.

**Primer Extension Analysis**

Ten micrograms of total RNA from cultured human mesangial cell were then mixed with 2.5 pmol of 5' 32P-labeled antisense primer corresponding to nucleotide +67 to +86 in the 5' untranslated region of megsin (5'-AGG CTG TCC AAA GGG GTA GCA GC-3'), denatured by heating at 65°C for 30 min, then annealed at room temperature for 90 min. Primer extension proceeded at 37°C for 60 min with 200 U/μl of SuperScript II RNase H– reverse transcriptase (Life Technologies BRL, Rockville, MD) in a buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.25 mM dNTPs). After ethanol precipitation, the products were analyzed on 8% acrylamide sequence gel. The subcloned 5' flanking region was sequenced using the same primer by the Tth polymerase Sequencing PRO DNA direct dideoxy nucleotide sequencing.

**Construction of Reporter Plasmids**

To assess human megsin gene promoter activity, enzyme restriction or PCR-amplified fragments of megsin gene were subcloned into pGL3-Basic vector (Promega, Madison, WI). Enzyme restriction fragments corresponding to nucleotides −4021, −2542, −1874, −1451, and −1052 to +130 in megsin transcript were generated by digestion of pBluescript SK(−) containing megsin gene from BAC clone, F581, with both BamHI, Stul, SacI, Pski, or KpnI and Xhol. A series of deletion mutants corresponding to nucleotides −1052 to +130 in megsin were prepared by PCR with a pair of primers spanning a region to be studied. These mutants contained 5'-flanking region of megsin from −834, −240, or −72 to +130. For the deleted fragment, which was deleted between −139 bp and −85 bp, the restriction fragments corresponding to nucleotides −240 to +130 in megsin was digested with EcoRV and HpaI and was then self-ligated.

For the site-directed mutations of AP-1 (from CTGATT to CAGAATTC) or Oct-1 (from CTGATTCATAC to CTGATTCGCACA) binding motifs in megsin gene, site-directed mutagenesis of the potential transcriptional regulatory domain was performed utilizing Quick Change site-directed mutagenesis kit (Stratagene). The deletion mutant of AP-1 binding motif was constructed by PCR-based technique. The mutations were verified by a direct dideoxy nucleotide sequencing.

**Transfection and Luciferase Assay**

Various kinds of human cells were transfected with 1 μg of megsin promoter-inserted pGL3-Basic vector containing firefly luciferase gene using LipofectAMINE PULS (Life Technologies-BRL) according to the manufacturer’s protocol. Transfection efficiencies were normalized by co-transfection with 5 ng of pRL CMV vector containing Renilla luciferase gene (Promega). At the completion of culture incubations, cell media were aspirated and the cells washed once in phosphate-buffered saline PBS then lysed in cell lysis buffer. The cell lysates were centrifuged, and the supernatant was analyzed for the promoter activities using the dual-luciferase reporter assay system (Promega) and a Lumat LB 9507 luminometer (EG & G Berthold, Bad Wildbad, Germany).

In some experiments, the transfected cells were subsequently treated with PDTC (pyrrolidine dithiocarbamate; Sigma, Dorset UK) for 24 h and then analyzed for the promoter activity as described above.

**Electrophoretic Mobility Shift Assays (EMSA)**

Nuclear extract from cultured human mesangial cells was obtained as described by Dignam et al. (17). Binding reactions were carried out on ice for 15 min in a mixture containing 10% glycerol, 5 mM MgCl2, 1 mM EDTA, 25 mM dithiothreitol, 50 mM KCl, 10 mM Hepes-KOH (pH 7.8), 2 μg of poly dl-dC, and 10 μg of nuclear extract, followed by further reaction with 80,000 cpm of 32P-end-labeled oligonucleotide probe at room temperature for 20 min. The complexes were subjected to electrophoresis in 4.5% polyacrylamide gels in running buffer containing 50 mM Tris-HCl, pH 8.5, 0.4 M glycine, and 2 mM EDTA. In this study, we used DNA fragment containing incomplete AP-1 binding motif of megsin (−127 bp to −108 bp; see Figure 5A) and AP-1 consensus oligonucleotide (Promega) as the probe. For competition studies, unlabeled competitor oligonucleotide was added by 100-fold molar excess to the reaction mixture. For super shift assay, anti-c-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used.

**Results**

**Isolation and Characterization of the Human Megsin Gene**

We have previously characterized a cDNA encoding human megsin (3). The 5'-flanking region and the exon-intron junctions of the human megsin gene were obtained by a PCR-based method with primers designed from megsin cDNA sequences. The structure and organization of the human megsin gene are shown in Figure 1. The megsin gene contains 8 exons and 7 introns and spans approximately 20 kbp. The exons range in size from 51 bp to 1141 bp, and exon-intron boundaries follow the GT/AG rule. Comparison of nucleotide sequences flanking exon-intron boundaries of the human megsin gene with those of another member of the serpin superfamily, PAI-2 (18), show that these sequences are well conserved, indicating a close phylogenetic relationship (Figure 1). The 4021-bp-long 5'-flanking region of the human megsin
gene has also been determined (GeneBank accession No. AF234618). Primer extension analysis located the transcriptional start site at \( 35 \text{bp downstream of a consensus TATA} \) and at \( 391 \text{bp upstream of the translational start site} \) (Figure 2). We demonstrate that the first intron is approximately 6.5 kbp long and is located between the transcriptional and translational start sites (Figure 1).

**Chromosomal Localization of Human Megsin Gene**

We performed fluorescence *in situ* hybridization utilizing as probe a megsin genomic DNA fragment, obtained from BAC clone F581 (Figure 3). The initial experiment resulted in the specific labeling of the long arm of a group E chromosome believed to be chromosome 18 on the basis of size, morphology, and banding pattern. In a second experiment, a biotin-labeled probe specific for the centromere of chromosome 18 was cohybridized with clone F581. This experiment resulted in the specific labeling of the centromere (red) and the long arm (green) of chromosome 18. Measurements of ten specifically labeled chromosomes 18 demonstrated that F581 is located at 68% of the distance from the centromere to the telomere of chromosome arm 18q, an area that corresponds to band 18q21.3. Out of total of 80 examined metaphase, 76 exhibited the specific labeling.

**Functional Analysis of Megsin Promoter**

The promoter activity of the isolated 5'-flanking region of human megsin was further investigated. We transfected the firefly luciferase reporter vector (pGL3-Basic) containing the 4151-bp-long 5'-flanking region (−4021bp to +130 bp), by lipofection into primary cultured cells, including human mesangial cells, dermal fibroblasts, and HUVEC. After normalization for *Renilla* luciferase activity, the firefly luciferase activity was statistically (mesangial cells *versus* dermal fibroblasts, \( P = 0.003 \); mesangial cells *versus* HUVEC, \( P = 0.006 \)) higher in cultured human mesangial cells than in other primary cultured cells (Figure 4A).

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**Table 1.**

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Data of human P4-5 are shown in parenthesis for comparison. \( \square \), untranslated region; \( \square \), open reading frame.

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**Figure 1.** Structure and organization of the human megsin gene.

**Figure 2.** Identification of the transcription start site of the human megsin gene. A primer extension analysis was performed with 10 μg of total RNA isolated from human cultured mesangial cells. The transcription start site (+1) and TATA box (−35) are indicated by arrows.

**Figure 3.** Localization of the human megsin gene to chromosome 18q21.3. The green dots indicated by arrows show the hybridization signals of the megsin gene probe to a metaphase chromosome spread. The red bands show binding of a specific probe for the centromere of chromosome 18.
Another group (19) has recently identified in a malignant A431 cell line a new serpin with megacaryocyte maturing activity. The amino acid sequence of the protein is identical with that of megsin. We thus transfected the firefly luciferase gene into the A431 cell line as well as in other human cell lines (FL, HeLa, Chang liver cells). Of interest, the firefly luciferase activity was also statistically increased (A431 versus FL, \( P < 0.001 \); A431 versus HeLa or Chang liver cells, \( P < 0.007 \)) in the A431 than in the others cells (Figure 4B). Altogether these data shows that the megsin promoter region is mainly active in cells expressing megsin.

To identify the regulatory element required for megsin expression, we generated various lengths of the megsin 5'-flanking region and subcloned them in a pGL3-basic vector. The slow growth of human cultured mesangial cells and their low yield in terms of the number of available cells led us to utilize A431 cells. The results of these experiments were subsequently confirmed in a limited number of human glomerular cell assays. As shown in Figure 5, the deletion from −4021 bp to −240 bp did not decrease the activity, whereas the deletion to −72 bp decreased the activity to approximately 5% of the initial promoter activity. The similar level of decrease in the promoter activity was observed in the deletion mutant, which was deleted between −139 bp and −85 bp (Figure 6B).

**Regulatory Element for Megsin Expression**

The motif search with the −139 to −85 region of human megsin revealed several potential transcription regulatory motifs, including AP-1 (Figure 6A), Oct-1, and TCF11 binding motifs. To identify the regulatory element within this region for transcriptional activation of megsin, we mutated the highly conserved transcription factor binding motifs, AP-1 and Oct-1, and assessed its promoter activity (see Materials and Methods).

When A431 cells were transfected with pGL3-Basic vector containing −240 to +130 region, both site-directed and deleted mutations for AP-1 led to the decrease in the transcriptional activation to approximately 50% of the initial promoter activity (Figure 6B). In contrast, Oct-1 mutation did not change the activity. These observations were confirmed in cultured human mesangial cells (Figure 6C).

Specific DNA-protein complexes were identified by oligo-
A nucleotide corresponding to the incomplete AP-1 site (Figure 7). A competitive inhibition assay relying on the massive addition of unlabeled AP-1 consensus oligonucleotide blocked the binding of the labeled probe. In EMSA using antibody to AP-1 binding transcriptional factor, c-fos, the specific DNA-protein complexes further shifted, demonstrating the binding of incomplete AP-1 site with AP-1 transcriptional factor.

Treatment with antioxidant PDTC, which dominantly activates AP-1, was performed to assess whether or not the AP-1 activator upregulates the activity of megsin promoter (−240 bp to +130 bp) containing the incomplete AP-1 site. Our luciferase assays showed that PDTC indeed upregulated the promoter activity in cultured human mesangial cells in a dose-dependent manner (Figure 7B).

Discussion

In the present study, we report the structure, organization, and sequence of the megsin gene. The megsin gene shows a similarity with other human chromosome 18 serpins (18) regarding the number of exon/intron and the positioning of exon-intron boundaries. The 18q21.3 locus has been previously shown to contain the genes encoding other serpins such as PAI-2, maspin, squamous cell carcinoma antigen 1 (SCCA1), and SCCA2 (20). We speculated that the megsin gene is located in the 18q21.3 locus because the megsin gene has a genetic organization similar to that of the other sepin genes and that the latter genes clusters in this region. The localization of the human megsin gene on chromosome 18q21.3 was indeed confirmed by our in situ hybridization experiments.

Megasin is expressed almost exclusively in mesangial cells. Still, two novel serpins with an identical cDNA/protein have also been identified either in malignant A431 cell lines (19) or in dendritic cells present in the human thymus (21). Still, megsin expression in two cell types tested in our laboratory is

Figure 6. Identification of the cis-acting element on megsin transcription. Comparison of the nucleotide sequences between the consensus activator protein-1 (AP-1) binding motif and the −120 to −112 region of megsin gene (A). We mutated the incomplete AP-1 and Oct-1 binding motifs in megsin gene and assessed its promoter activity in A431 cells (B) and human mesangial cells (C). pGL3-Basic, luciferase vector alone; −240bp-Basic, pGL3-Basic containing the 370-bp-long 5’-flanking region of megsin gene (−240 to +130 bp); −72bp-Basic, pGL3-Basic containing the 202-bp-long 5’-flanking region of megsin gene (−72 to +130 bp); −139 to −85 (Del)-Basic, −240bp-Basic with the deletion from −139 bp to −85 bp; AP-1(Mut)-Basic, −40bp-Basic with the site-directed mutation of AP-1; AP-1(Del)-Basic, −240bp-Basic with the deleted mutation of AP-1; Oct-1(Mut)-Basic, −240bp-Basic with the site-directed mutation of Oct-1.

Figure 7. Transcriptional activity of an incomplete AP-1 binding motif in megsin promoter. (A) Electrophoretic mobility shift assays (EMSA) was performed with nuclear extracts from human mesangial cells. Specific DNA-protein complexes (indicated by an arrow) were identified by the incomplete AP-1 site oligonucleotide of megsin promoter. The specific band was competed by unlabeled AP-1 consensus oligonucleotide and further shifted (indicated by an arrowhead) by antibody to AP-1 binding transcriptional factor, c-Fos. (B) AP-1 activator, PDTC, upregulates the transcriptional activity of megsin promoter. Human mesangial cells were transfected with megsin promoter (−240 bp to +130 bp) containing an incomplete AP-1 site, treated with various concentrations of PDTC for 24 h, and then assessed for its promoter activity by luciferase assay.
significantly lower than in mesangial cells (data not shown). The presence of megsin in A431 may reflect cancerous derepression. A431 cells provide us with alternative tools to study the regulatory mechanisms of megsin gene expression.

We further determined the sequence of the promoter region and discovered a significant positive regulatory element. In the 5′-flanking region of megsin gene (−4021 bp to +130 bp), we demonstrated the existence of a cis-acting element that activates the transcription of megsin gene in both A431 and mesangial cells. The use of the luciferase assay allowed us to demonstrate that the reporter construct with the 5′-flanking region of megsin gene is able to stimulate luciferase gene expression in both cell types. Although the promoter activity of megsin gene was stronger in A431 and mesangial cells, the reporter construct with the 4151-bp-long 5′-flanking region also induced a milder luciferase expression in other cell types. This observation suggests that the cell type-specific transcription regulation is not located solely in this promoter region.

Sequence analysis revealed various potential transcription factor binding sites, including an incomplete API binding site (22,23), a c-Myb binding site (24,25), an Oct-1 binding site (26), and an NF-κB binding motif (27,28) within the 5′-flanking region of megsin gene. A detailed analysis of the promoter region revealed a cis-acting element within the −72 to −72 region, which is able to activate the transcription of megsin; the element has highly conserved transcription factor binding motifs such as AP-1, Oct-1, and TCF11. Our site-directed and deletion mutagenesis analyses and EMSA identified one positive regulatory motif, an incomplete AP-1 binding motif (CTGATTCCAC) within −120 to −112 region. PDKT1, a dominant activator of AP-1, activates the megsin promoter (−240 bp to +130 bp), suggesting that AP-1 is a good candidate as a transcription factor of megsin among the molecules described above. In spite of these results, the 5′-flanking region of megsin gene tested in this study was not stimulated by the pathogenic mediators, e.g., TGF-β and PDGF. This observation indicates that the cell type-specific transcription of megsin is not regulated solely by this promoter region. Extensive investigation of more upstream of megsin promoter region as well as introns should be necessary for understanding of the inducible transcription regulation of megsin.

AP-1 is known to regulate the gene expression of various cytokines, chemotactic proteins, adhesion molecules, matrix proteins, and proteases involved in inflammation, immunologic responses, cell differentiation, and growth control. AP-1 DNA-binding activities in mesangial cells are regulated by the microenvironment (29), and Hernandez-Presa et al. (30) showed activated AP-1 in mesangial cells of immune complex glomerulonephritis rats utilizing Southwestern histochemical methods. Previous studies demonstrated that c-Jun/AP-1 activation is essential to the induction of matrix metalloproteinase-9 (MMP-9) by IL-1β or thrombin in cultured mesangial cells (31–33). We reported that megsin is upregulated in mesangioproliferative diseases in both humans and rats (3,7–9) and that overexpression of megsin induces progressive mesangial cell proliferation and expansion (10). These findings together with our results suggest that AP-1 may regulate matrix turnover by stimulating proteinases and protease inhibitors such as megsin in parallel.

Interestingly, the megsin promoter region includes one YB-1 binding site at −1588 bp to −1599 bp (GGG ATT GGT TAA). Mertens et al. (34) demonstrated that YB-1 is a major, cell type-specific transactivator of MMP-2 gene expression in glomerular mesangial cells. Still, our studies with deletion mutants of megsin promoter vectors have shown that the YB-1 binding site is not important for megsin gene transcription. They suggest that the different cell type–specific transcriptional regulation between megsin and MMP-2 relies on a different mechanism. Combinations of tissue-restricted and widely expressed factors sometimes mediate tissue-specific expression (35–38). However, the relatively low cell type specificity of this promoter region makes the tissue-specific regulation of megsin by these ubiquitous transcription factors unlikely. The cis-acting element demonstrated in this study might act in coordination with some tissue-specific enhancers and/or silencers in other regions such as introns.

In conclusion, we provide a detailed analysis of the genomic structure and the transcriptional regulation of a new human mesangium-predominant gene, megsin. The megsin gene is composed of eight exons and is localized to human chromosome 18q21.3, close to the other serpin genes. We have isolated the promoter region of human megsin gene and identified a positive cis-regulatory element. Our results demonstrate that the promoter region of −120 to −112 containing an incomplete AP-1 binding motif is important in the transcriptional activation of megsin. The actual sequence of the cis-elements identified in the megsin promoter by this study is a first step toward the elucidation of the molecular mechanism of mesangium-predominant gene regulation. Further studies such as cloning of a responsible transcriptional factor are required to completely understand the mesangium-predominant gene regulation of megsin.

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