

Cloning and Characterization of a Novel Subunit of Protein Serine/Threonine Phosphatase 4 from Mesangial Cells

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Abstract. Mesangial cells play an important role in maintaining glomeruli structure and function and in the pathogenesis of glomerular diseases. With a novel approach using a rapid large-scale DNA sequencing strategy and computerized data processing, a new human gene, PP4_{Rmeg} was cloned. The full-length cDNA clone of human PP4_{Rmeg} coded for a novel 950–amino acid protein, which was similar to a subunit of protein serine/threonine phosphatase 4 (PP4). Recombinant PP4_{Rmeg} produced in COS-7 cells bound to the catalytic subunit of PP4. PP4_{Rmeg} is therefore structurally and functionally related to the recently reported regulatory subunit of PP4, PP4_{R1}. Amino acid sequence analysis of rat PP4_{Rmeg} homologue revealed that the sequences were well conserved between human and rat (86.3% identity). Northern blot analyses of

human tissues and cultured cells demonstrated that the regulatory subunits were expressed abundantly in human cultured mesangial cells, although their expression was relatively ubiquitous. *In situ* hybridization studies in normal human renal tissues confirmed their expression in glomeruli *in vivo*. The expression was upregulated in glomeruli of anti-Thy1 glomerulonephritis rats before mesangial proliferation. These data demonstrate that PP4_{Rmeg} is a novel regulatory subunit of PP4, which is expressed ubiquitously but abundantly in mesangial cells. Its pathophysiologic role in mesangial cells and glomerulus remains unknown. As PP4 is an essential protein for nucleation, growth, and stabilization of microtubules at centrosomes/spindle pole bodies during cell division, PP4_{Rmeg} may play a role in regulation of mitosis in mesangial cells.

Mesangial cells play a central role in maintaining glomeruli structure and function. Numerous glomerular diseases, including chronic glomerulonephritis and diabetic nephropathy, two major causes of end-stage renal failure, are associated with proliferation of mesangial cells and/or accumulation of mesangial extracellular matrix, which eventually leads to the progression of glomerulosclerosis. To identify novel genes expressed and functioning in mesangial cells, we used a rapid large-scale DNA sequencing strategy and computerized data processing to compare the transcripts in cultured human mesangial cells with various cells and organs (1).

Using this novel methodology, we previously reported discovery of a new mesangium-predominant gene, megsin, which is a new member of the serine protease inhibitor (serpin) superfamily (2,3). Here, we cloned and characterized another novel gene, which was expressed ubiquitously but abundantly in mesangial cells.

Sequencing analysis of this gene demonstrated its structural homology with a regulatory subunit of protein serine/threonine phosphatase 4 (PP4_{R1}) (4). Protein serine/threonine phosphatases (PP) play an important role in the control of most biologic processes by catalyzing the dephosphorylating reactions and serving as an off switch of cell signaling (5). The four major PP families that have been identified are PP1, PP2A, PP2B, and PP2C. This classification is based on their substrate selectivity, inhibitor sensitivity, and requirement for divalent cations (6–10).

PP4 is classified as a PP2A family member. The catalytic subunit of PP4 (PP4c) is 65% identical to the catalytic subunit of PP2A (PP2Ac). However, PP4c does not associate with the regulatory subunits of PP2A (11). Instead, PP4c seems to associate with distinct regulatory subunits (4).

The biologic function of PP4 has not been understood well. However, several lines of evidence suggest its potential involvement at centrosomes/spindle pole bodies for nucleation, growth, and/or stabilization of microtubules (12), which play an important role in each of the mitotic processes by forming the mitotic spindle and functioning in many of the subcellular movements.

In the present study, we determined the structure of the novel gene and investigated its biologic function and localization. Using anti-Thy1 glomerulonephritis, a model of mesangioproliferative glomerulonephritis of rats, we also investi-

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gated the temporal profile of its expression in association with mesangial proliferation.

Materials and Methods

Cloning of Human $PP4_{Rmeg}$

Details of construction of the 3'-directed human mesangium cDNA library have been described elsewhere (1). Briefly, human glomeruli were isolated by differential sievings from a normal human kidney and were cultured in medium 199 (Life Technologies, Rockville, MD) containing 25 mM HEPES, 10% Nu-serum (Collaborative Biomedical Products, Bedford, MA), and antibiotics (50 μ g/ml penicillin, streptomycin, and Fungizone) in a 5% CO₂ incubator. PolyA⁺ RNA was isolated from human mesangial cells at passage 6 using the standard guanidine isothiocyanate method and oligo dT cellulose column (Amersham Pharmacia Biotech, Uppsala, Sweden). A vector primer is dam-methylated at the *Mbo*I sites (GATC) and has a 3' protrusion of homo dT stretch at one end. The double-stranded cDNA moiety was cleaved with *Mbo*I. The vector that was attached with the 3'-cDNA (from polyA to the nearest *Mbo*I site) was circularized by ligation and was transformed into *Escherichia coli*. The resulting transformants were selected randomly and were lysed individually by brief boiling. The lysate was used as a template for PCR. The resulting short double-stranded DNA was used for the cycle sequencing reaction and was analyzed using an autosequencer.

To identify the 5' end of $PP4_{Rmeg}$ cDNA, we used the 5'-Full RACE Core Set (Takara, Tokyo, Japan). The reverse transcriptase (RT) reaction was performed with 1.0 μ g of human mesangial cell polyA⁺ RNA with a gene-specific oligonucleotide. After degradation of the template mRNA with RNase H, the first-strand cDNA was circulated by T4 RNA ligase and an aliquot was used as a template for the first PCR amplification with gene-specific primers. The resulting PCR product was then used as a template for the second PCR amplification with nested gene-specific primers. The PCR products were cloned into the pCRII vector (Clontech, Palo Alto, CA), and DNA sequences were determined by dideoxy chain termination reaction using a DNA autosequencer.

Cloning of Rat $PP4_{Rmeg}$

To determine the sequence of rat $PP4_{Rmeg}$, we performed PCR with primers corresponding to highly homologous parts between human $PP4_{Rmeg}$ and mouse cDNA sequences that were obtained from the expression sequence tag database. Messenger RNA from cultured rat mesangial cells at passage of 10 to 20 with a Micro-FastTrack 2.0 mRNA Isolation Kit (Invitrogen, Groningen, the Netherlands) was reversed transcribed with Superscript II (Life Technologies) to generate template cDNA. The template was amplified in a DNA thermal cycler (Perkin Elmer Japan, Chiba, Japan) for 30 cycles using optimum conditions for each primer. To prevent misreading, we designed PCR primers so that each base could be determined in at least two different PCR products. We used a 5'RACE System, version 2.0 (Life Technologies) for the confirmation of the DNA sequence around the putative initiation codon. The PCR products were ligated into pGEM-T easy vector (Promega, Madison, WI), and DNA sequences of more than four clones were determined by dideoxy chain termination reaction with T7 and SP6 primers using a DNA autosequencer.

RT-PCR

Total RNA was isolated from cultured human mesangial cells (passage 10) and A431 cells (purchased from American Type Culture Collection, Manassas, VA) using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Approximately 2 μ g

of total RNA was reverse transcribed using an RNA-PCR kit (Takara). Ten microliters of cDNA was applied for PCR amplification.

PCR amplification was performed in a 25- μ l reaction containing 2.5 U of ExTaq (Takara), 2.5 mM each of the four deoxynucleoside triphosphates, and 50 pmol of the *Kpn*I- $PP4_{RS}$ (5'-CCG GTA CCG GGC GAC CAC AAG ATG GCG GAC CT-3') and *Sac*I- $PP4_{RS}$ (5'-TCG AGC TCC CGC TCT CAC AGT TGG TTC TGA ATC-3') primers. Specimens were amplified in a DNA Thermal Cycler. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s.

The PCR products were separated by electrophoresis in a 4% agarose gel and extracted using a QIAEX II kit (QIAGEN, Hilden, Germany). These products were ligated to pUC18 vector (Takara) after restriction enzyme digestion, and DNA sequences were confirmed by dideoxy chain termination reaction using a DNA autosequencer.

Production of $PP4_{Rmeg}$ Transfectants

Human $PP4_{Rmeg}$ cDNA was engineered with a c-Myc tag on the C-terminus as described below. The 5' terminal fragment of human $PP4_{Rmeg}$ (corresponding to 1 to 2210 bp) was obtained with digestion with *Eco*RI and *Hind*III. At the same time, the 3' terminal fragment was synthesized using a PCR-based method with the primers $PP4_{Rmeg}/2112$ (5'-CGA GCT TGC AGT TAT TCT TGG AGA TCA ATT GAC AGC TGC-3') and *Bam*HI- $PP4_{Rmeg}$ -AS (5'-CGG GAT CCA CGT AGG TTG AGG ACG CTG TGC TCA TGG C-3'). These two fragments were confirmed by sequence analysis, followed by simultaneous ligation into pcDNA 3.1/myc-His B vector (Invitrogen) with a Takara Ligation Kit Version 2.0.

The pcDNA 3.1/myc-His B vector containing $PP4_{Rmeg}$ was transfected into COS-7 cells cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum. Transfections were performed using 5 μ g of $PP4_{Rmeg}$ expression vector and 2.5 μ l of LIPOFECTAMINE Reagent (Life Technologies) according to the manufacturer's protocol. Sixty hours posttransfection, cells were dislodged by scraping in 1 ml of phosphate-buffered saline and stored at -80°C until use. Approximately 8×10^5 cells were lysed in 400 μ l of Buffer A (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.5% Triton X-100, 1 mM ethylenediaminetetraacetate, 1 mM ethyleneglycol-bis(β -aminoethyl ether)-*N,N'*-tetraacetic acid, 1 mM dithiothreitol, and a cocktail of protease inhibitors, which included leupeptin, pepstatin, aprotinin, benzamidine, and soybean trypsin inhibitors) by repetitive passage (8 to 10 times) through a 22-gauge needle. The cell lysates were clarified by centrifugation for 10 min at 4°C, and the supernatant was taken as cell lysates and solubilized in sodium dodecyl sulfate (SDS) sample buffer, which represented starting material.

Immunoprecipitation

The ability of recombinant $PP4_{Rmeg}$ (myc- $PP4_{Rmeg}$) to bind $PP4c$ was assessed by immunoprecipitation using specific antibodies. Cell lysates (350 μ g of protein) were incubated with 2 μ l of c-Myc antibody (Clontech) overnight while rotating at 4°C. Immune complexes were isolated by adding 40 μ l of a 50% slurry of Buffer A-washed GammaBind Plus Sepharose (Amersham Pharmacia Biotech) resin. The protein-antibody-beads complexes were pelleted by centrifugation, and the supernatant was taken, an aliquot of which was solubilized in SDS sample buffer, which represented unbound proteins. After the beads were washed six times with Buffer A, bound proteins were eluted with 30 μ l of 2 \times SDS sample buffer and then with 25 μ l of 1 \times SDS sample buffer, which represented immunopre-

precipitated proteins. Samples were analyzed by immunoblot analysis using an affinity-purified antibody raised against the amino acid residues 56 to 69 (ASENIFNRQMVARs) in PP4_{R1} (4) and an affinity-purified antibody directed against the C terminus of PP4c (4).

Immunoblot Analysis

Approximately 20 μ l of starting material, unbound protein, and immunoprecipitated protein were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Proteins were transferred to nylon-reinforced nitrocellulose in 10 mM 3-(cyclohexylamine)propanesulfonic acid buffer (pH 11.0) containing 10% methanol. The blots were incubated with affinity-purified antibody raised against the amino acid residues 56 to 69 (ASENIFNRQMVARs) in PP4_{R1} (4) and an affinity-purified antibody directed against the C terminus of PP4c (4), followed by incubation with goat anti-rabbit IgG-alkaline phosphatase conjugate. Immunocomplexes were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates for alkaline phosphatase.

Northern Blot Analysis

The expression of PP4 regulatory subunits (PP4_{RS}) mRNA was assessed in various human primary cultured cells and tissues, as well as in glomeruli isolated from anti-Thy1 glomerulonephritis rats. Cultured human mesangial cells, dermal fibroblasts, proximal tubule epithelial cells, umbilical vein endothelial cells, and aortic smooth muscle cells were purchased from Takara, and their mRNA were isolated at passage 5 to 15 using a Micro-FastTrack 2.0 Kit (Invitrogen). A human multiple tissue Northern blot containing polyA⁺ RNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas was purchased from Clontech. Total RNA was isolated from glomeruli of anti-Thy1 glomerulonephritis rats described below, using an ISOGEN kit (Nippon Gene).

PolyA⁺ RNA from cultured human cells (2 μ g) or total RNA from anti-Thy1 glomerulonephritis glomeruli (4 μ g) were electrophoretically separated in 1% agarose gels containing 2.2 mol/L formaldehyde, followed by capillary transfer to nitrocellulose membrane GeneScreenPlus (NEN, Boston, MA). The membrane was prehybridized in Quikhyb Hybridization solution (Stratagene, La Jolla, CA) at 68°C for 1 h, followed by hybridization in Quikhyb Hybridization solution containing labeled probe and salmon sperm DNA at 68°C for 2 h. After hybridization, membranes were washed with 0.2 \times SSC/0.1% SDS at 60°C.

As a probe for human PP4 regulatory subunits (PP4_{RS}), we used a 625-bp-long cDNA fragment cut with *PvuII* (corresponding to 2734 to 3359 bp). As a probe for rat PP4_{RS}, we used a 1141-bp-long cDNA fragment (corresponding to 894 to 2034 bp). These DNA fragments were radiolabeled using Random Primer DNA Labeling Kit (Takara) and purified with ProbeQuant G-50 Micro columns (Amersham Pharmacia Biotech).

Quantification of the PP4_R mRNA signals was obtained by densitometry using National Institutes of Health image software (version 1.62; Bethesda, MD). The intensities of the signals were normalized using β -actin mRNA or 28S ribosomal RNA as loading controls.

In Situ Hybridization

In situ hybridization was performed as described previously (13). Briefly, fresh kidney biopsy tissues were embedded in OCT compound, frozen quickly, and stored at -80°C until use. Sections of tissues were cut 4 μ m thick and fixed in 4% paraformaldehyde in phosphate-buffered saline deproteinized by HCl and digested with proteinase K (Sigma Chemical, St. Louis, MO). The specimens were

incubated in a prehybridization buffer, drained, and hybridized overnight with digoxigenin (DIG)-labeled oligonucleotide probe in the prehybridization buffer. As a probe, we used 34-bp-long synthesized oligonucleotide corresponding to nucleotides 2879 to 2912. After hybridization, the DIG-labeled probe was visualized by immunohistochemical staining using a mouse monoclonal anti-DIG antibody (Boehringer Mannheim, Mannheim, Germany), horseradish peroxidase-conjugated rabbit anti-mouse antibody (DAKO, Kyoto, Japan), and horseradish peroxidase-conjugated swine anti-rabbit antibody (DAKO). Color was developed with diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl (pH 7.6) and 0.03% H₂O₂. Sections were briefly counterstained with hematoxylin, rinsed, dehydrated, cleared in xylene, and mounted.

Anti-Thy1 Glomerulonephritis Rat Experiment

Anti-Thy1 glomerulonephritis was induced in 21 male Wistar rats (Nippon Clea, Tokyo, Japan) that weighed approximately 200 g using a hybridoma line producing anti-rat Thy1.1 antibody from European Collection of Animal Cell Culture (Sulisbury, UK); purification of anti-Thy1.1 monoclonal antibody was performed as reported (14). To induce anti-Thy1 glomerulonephritis, purified anti-rat Thy1.1 antibody (2.4 mg/kg body wt) was injected intravenously. Three rats were killed at each time point (days 2, 4, 7, 14, and 28) along with three control rats. Rat kidneys were removed, followed by glomerular isolation by standard sieving method (15) and preparation of total RNA for Northern blot analysis. A portion of tissue was fixed in methyl-Carnoy's solution, embedded in paraffin, sectioned 4 μ m thick, and stained with the periodic acid-Schiff reagent and counterstained with hematoxylin. Immunohistochemical staining with mouse antiproliferating cell nuclear antigen (PCNA) monoclonal antibody PC10 (DAKO) was performed as described previously (16). The number of intraglomerular cells and PCNA-positive glomerular cells on 20 randomly selected glomeruli was determined. Glomerular cross-sections containing only a minor portion of the glomerular tuft (<20 discrete capillary segments per cross-section) were not used.

Results

Cloning of PP4_{Rmeg}

A large-scale sequencing of a 3'-directed cDNA library of cultured human mesangial cells was performed to determine partial sequences of 1836 randomly picked clones (1,2). The sequences of the clones was compared with the DNA database GenBank using the FASTA program (17). Several clones, which have not been recorded in any other databases, were found to be abundantly expressed in human mesangial cells. We obtained a clone, which was abundant among those detected in our human mesangial cell cDNA library. This clone consisted of 0.25% of all of the mRNA population.

Using PCR techniques, we isolated a full-length cDNA. Nucleotide sequencing analysis of this clone revealed an open reading frame of 2850 nucleotides coding for a predicted protein of 950 amino acids (Figure 1). The sequence around the initiation codon (AAGATGG) was consistent with Kozak's consensus sequence (A/GCCATGG). Stop codons in all three reading frames were identified upstream of the initiation codon.

Nucleic acid homology searches of the database using the BLASTN program revealed that this gene was highly homologous to a recently identified regulatory subunit of protein

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human 1 : MADLSLLQED LQEDADQFGV DDYSSSESDVI IIPSA18LDFVS QDEMLTPLGR LDKYAAASENI
***** * * * * *
rat 1 : MADLSLLQED LPEDADQLGV DDYSSSESDVI IIPSA18LDFVS QDEMLTPLGR LDKYAAASENV

human 61 : FNRQMVARSL LDTLREVCDD ERDCIAVLER ISRLADDSEP TVRAELMEQV PHIALFCQEN
*****
rat 61 : FNRQMVARSL LDTLREVCGE ERDCIAVLER ISRLADDSEP TVRAELMEQV PHIALFCQEN

human 121 : RPSIPYAFSK FLLPIVVRYL ADQNNQVRKT SQAALLALE QELIERFDVE TKVCPVLIEL
*****
rat 121 : RPSIPYAFSK YLLPIVVRYL ADQNNQVRKT SQAALLALE QELIERLDVE TKVCPVLIDL

human 181 : TAPDSNDDVK TEAVAIMCKM APMVGKDITE RLILPRFCEM CCDCRMFHVH KVCAANPGDI
*****
rat 181 : TAPDSNDDVK TEAVAIMCKM APMVGKDITE RLILPRFCEM CCDCRMFHVH KVCAANPGDI

human 241 : CSVVGQQATE EMLLPRFFQL CSDNVWVGRK ACAECFMAVS CATCQEIRRT KLSALFINLI
*****
rat 241 : CSVVGQQATE EMLLPRFFQL CSDNVWVGRK ACAECFMAVS CATCQEIRRT KLSALFINLI

human 301 : SDPSRWVROA AFQSLGPFIS TFANPSSSGQ YFKEESKSSE EMSVENKNRT RDQEAPEDVQ
***** * * * *
rat 301 : SDPSRWVROA AFQSLGPFIS TFANPSSSGQ CFKDESKSS- ---EDKDRI RDDGVVQEEQ

human 361 : VRPEDTPSDL SVSNSSVILE NTMEDHAAEA SGKPLGEISV PLDSSLCTL SSSHQEAAS
* * * * *
rat 356 : SRPEDAPSDL SAPHSSARLD GTLEGCAEET FGDSAGDMRV PADSSLCTL SSESPEEAAS

human 421 : -NENDKK-PG NYKSMLRPEV GTTSQDSALL DQELYNSFHF WRTPLPEIDL DIELEQNSGG
* * * * *
rat 416 : DAESGKKHDN NSKSASRPDV GTSSPEPTPL DQEMFNSFHF WRTPLPQIDL DKELQQDPGE

human 479 : KPSPEGPEEE SEGVPVSSPN ITMATRKELE EMIENLEPHI DDPDKVAQVE VLSAALRASS
**** * * * *
rat 476 : RPSPERTGDA PAAPVPGSPS ITMATRKELE EMIENLEPHM DDPDKVAQVE VLSAALARAST

human 539 : LDAHEETISI EKSDLQD-- ELDINELPNC KINQEDSVPL ISDAVENMDS TLHYIH--ND
**** * * * *
rat 536 : LDAHDEAGGA EQRSELQDDA VGAGGELPNC SISEDTESEPL VIAAENMEA TPDYIHGGAD

human 595 : SDLSNNSFS PDEERRTKVQ DVVPQALLDQ YLSMTDPSRA QTVDTETIAKH CAYSLPGVAL
** * * * *
rat 596 : VGPGGGGFS PDEERRPKVQ DVVPQALLDQ YLSMTDPSRA QTVDTETIAKH CAYSLPGVAL

human 655 : TLGRQNWCHL RETYETLASD MQWKVRRTLA FSIHELAVIL GDQLTAADLV PIFNGFLKDL
*****
rat 656 : TLGRQNWCHL RETYETLASD MQWKVRRTLA FSIHELAVIL GDQLTAADLV PIFNGFLKDL

human 715 : DEVRIQVLKH LHDFLKLHI DKRREYLYQL QEFVTDNSR NWRFRAELAE QLLILLELYS
*****
rat 716 : DEVRIQVLKH LHDFLKLHI DKRREYLYQL QEFVTDNSR NWRFRAELAE QLLILLELYS

human 775 : PRDVYDYLRP IALNLCADKV SSVRWISYKL VSEMVKKLHA ATPPTFGVDL INELVENFGR
*****
rat 776 : PRDVYDYLRP IALNLCADKV SSVRWISYKL VSEMVKKLHM ATPPTFGVEL INELVENFGR

human 835 : CPKWSGRQAF VFVCQTVIED DCLPMDQFAV HLMPHLLTLA NDRVFNVRVL LAKTLRQTL
*****
rat 836 : CPKWSGRQAF VFVCQTVIED DCLPMDQFAV HLMPHLLTLA NDRVFNVRVL LAKTLRQTL

human 895 : EKDYFLASAS CHQEAVEQTI MALQMDRSD VKYFASIHPA STKISEDAMS TASSTY
* * * * *
rat 896 : EKEYFLASAS CHQEAVEQTI MALQMDRSD VKYFASIHPS STKLSSEDAMS TASSTY

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Figure 1. Amino acid sequences of human and rat PP4_{Rmeg}. The amino acid sequences were well conserved between human and rat PP4_{Rmeg} (86.3% homology). Thirteen regions homologous to the heat repeats found in the A subunit of PP2A (18) are underlined. The heat repeat sequences were highly conserved between human and rat. The portion enclosed by a box indicates the 18 amino acids substituting for 18-Ser in PP4_{R1}. The accession numbers for the nucleotide sequence of human and rat PP4_{Rmeg} are AF200478 and AF332004, respectively (DNA Data Bank of Japan, European Molecular Biology Laboratory, and GenBank).

phosphatase 4 (PP4_{R1}) (4). The deduced amino acid sequence of PP4_{Rmeg} was completely identical to PP4_{R1}, except for the replacement of serine-18 in PP4_{R1} by 18 amino acids (FGVD-DYSSSESDVIIIIPSA) in PP4_{Rmeg}. PP4_{Rmeg} has 13 regions homologous to the “heat” repeats found in the A subunit of PP2A (18).

We confirmed by RT-PCR that cultured human mesangial cells and the A431 cell line contained both PP4_{Rmeg} and PP4_{R1} transcripts (Figure 2). Nucleotide sequence analyses of the 275- and 326-bp PCR products established that they were PP4_{R1} and PP4_{Rmeg}, respectively.

The size of the myc-tagged PP4_{Rmeg} protein produced by the *in vitro* translation system using the rabbit reticulocyte lysates with canine pancreatic microsomal membranes was 135 kD, which was much higher than the calculated molecular weight of the deduced amino acid sequence of the tagged PP4_{Rmeg} (107 kD; data not shown). This finding suggests the existence of some posttranslational modification(s). The size of the myc-tagged protein was almost identical when the *in vitro* translation was performed in the absence of canine microsomal membranes, excluding the possibility of *N*-glycosylation of PP4_{Rmeg}.

To investigate whether PP4_{Rmeg} was conserved in other species, we cloned a partial cDNA of the rat homologue of PP4_{Rmeg} from cultured rat mesangial cells. The determined DNA sequences covered the putative open reading frame, and the 18 amino acids that we found in the *N*-terminus of human PP4_{Rmeg} also existed in rat PP4_{Rmeg}. The DNA sequence of this region was almost identical with that of human PP4_{Rmeg}. At the amino acid level, rat PP4_{Rmeg} was 86.3% identical to human PP4_{Rmeg} (Figure 1). The regions of the heat repeat sequences were highly conserved in rat PP4_{Rmeg}.

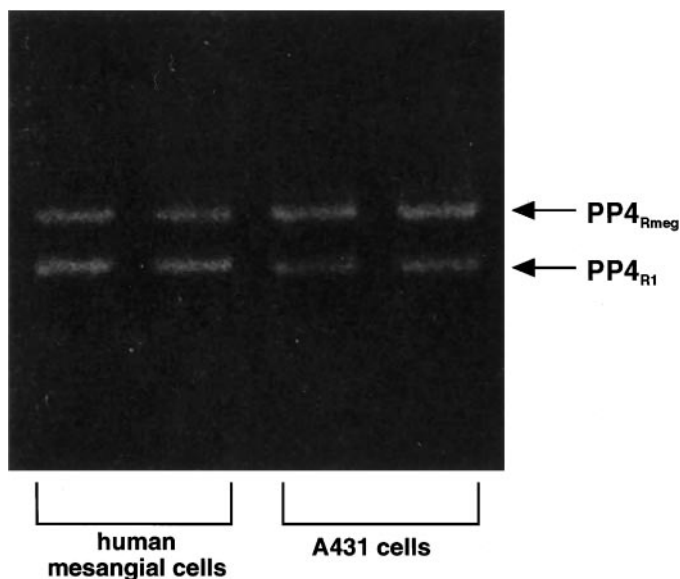


Figure 2. Detection of PP4_{Rmeg} and PP4_{R1} transcripts by reverse transcriptase-PCR in cultured human mesangial cells and A431 cells. Total RNA from two independently cultured mesangial and A431 cells were reverse transcribed, and the fragments containing 18-Ser in PP4_{R1} (275 bp) or the substitutive 18 amino acids in PP4_{Rmeg} (326 bp) were amplified by PCR.

Binding of PP4_{Rmeg} to PP4c

The high homology of PP4_{Rmeg} with the recently reported regulatory subunit of PP4, PP4_{R1}, suggested that PP4_{Rmeg} might bind to PP4c. To test this, we transfected COS-7 cells with Myc-PP4_{Rmeg}, and recombinant Myc-PP4_{Rmeg} (M.W.: 135 kD, Figure 3A) was immunoprecipitated from the cell lysates using anti-Myc antibody. The immune complexes were analyzed for PP4c by immunoblotting. As shown in Figure 3B, PP4c co-precipitated with recombinant Myc-PP4_{Rmeg} from cell lysates of Myc-PP4_{Rmeg} transfectants. No PP4c was detected in the anti-Myc immune complexes from lysates of mock-transfected cells. These results indicate that recombinant PP4_{Rmeg} bound to endogenous PP4c, suggesting that PP4_{Rmeg} functions as a regulatory subunit of PP4.

PP4_{Rmeg} Is Abundantly Expressed in Human Mesangial Cells

The high homology made it difficult to discriminate between PP4_{Rmeg} and PP4_{R1}. Consequently, we performed Northern blot analysis using a probe that recognizes a common portion of the PP4 regulatory subunits (PP4_{RS}).

A single transcript of 4.5 kb was detected in cultured mesangial cells (Figure 4A). PP4_{RS} were also detected in other human cells, *e.g.*, dermal fibroblasts, tubular epithelial cells, umbilical vein endothelial cells, and aortic smooth muscle cells, but were most highly expressed in mesangial cells. The PP4_R mRNA were ubiquitously expressed in the various organs and most highly expressed in heart and placenta (Figure 4B).

The expression of PP4_R mRNAs *in vivo* was examined in

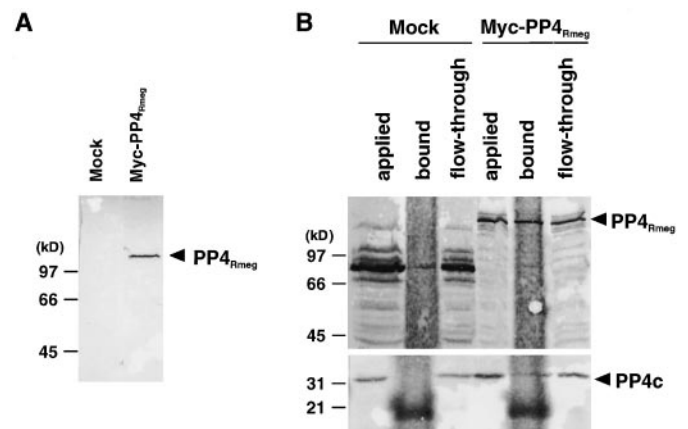


Figure 3. Association of PP4_{Rmeg} with PP4c. (A) The cell lysates obtained from mock- or Myc-PP4_{Rmeg}-transfected COS-7 cells were analyzed by immunoblotting with anti-Myc antibody to detect recombinant Myc-PP4_{Rmeg}. (B) The mock- or Myc-PP4_{Rmeg}-transfectant lysates were reacted with anti-Myc antibody and the thus-precipitated immune complexes were analyzed by immunoblotting with anti-PP4_{R1} (anti-PP4_{Rmeg}) antibody (top) or anti-PP4c antibody (bottom). The “applied” fraction represents starting material, which is cell lysate solubilized in sodium dodecyl sulfate sample buffer. The “bound” fraction represents immunoprecipitated proteins. The “flow-through” fraction represents unbound proteins present in the supernatant after centrifugation of the protein-antibody-beads complex.

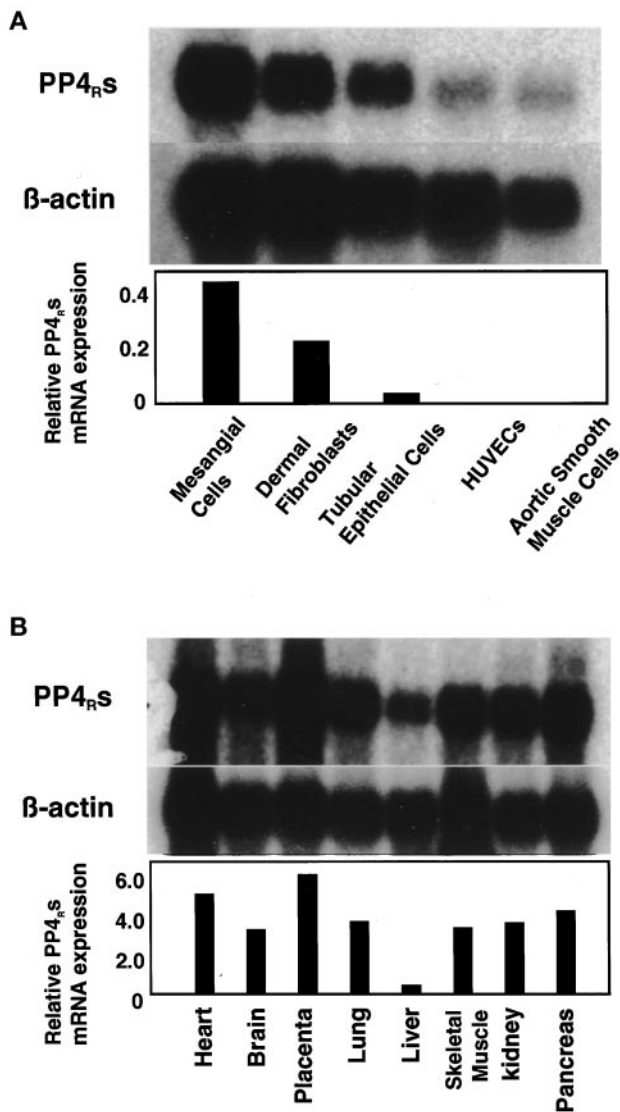


Figure 4. Detection of PP4_{R,s} (PP4_{R,meq} and PP4_{R,1}) transcripts in tissues and cells. Expression of PP4_{R,s} in primary culture cells (A) and various human tissues (B) was examined by Northern blotting. Top, PP4_{R,s} (PP4_{R,meq} and PP4_{R,1}). Middle, β -actin. Bottom, the ratios of intensities of PP4_{R,s} to β -actin transcripts calculated by densitometry using NIH image software. HUVECs, human umbilical vein endothelial cells.

normal human renal tissues by *in situ* hybridization. The strongest signals were detected mainly within the glomerulus (Figure 5). By contrast, the signals in the tubular and interstitial regions were less intense.

Upregulation of the PP4 Regulatory Subunits Expression in Anti-Thy1 Glomerulonephritis Rats

To investigate whether the PP4_{R,s} expression is altered *in vivo* in glomerular disease, we used anti-Thy1 glomerulonephritis rats. This is a well-established model of mesangial proliferative glomerulonephritis (19). Periodic acid-Schiff staining of the renal tissues demonstrated characteristic features of anti-Thy1 glomerulonephritis, including mesangial

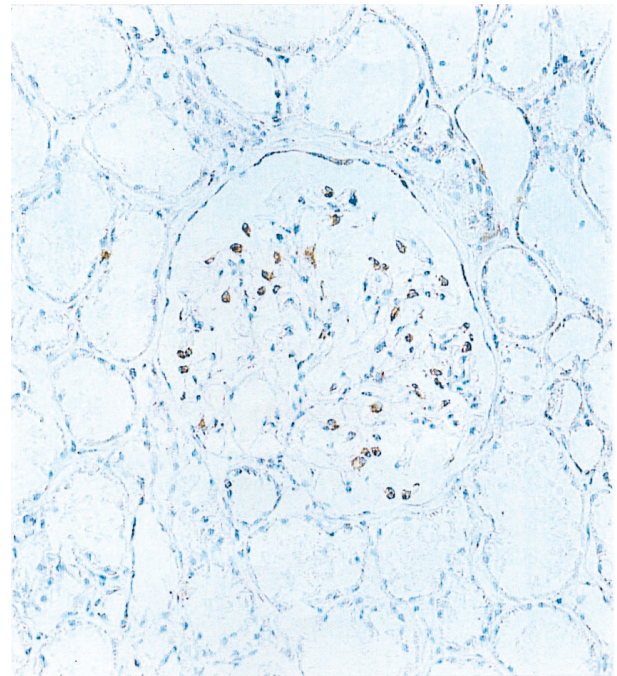


Figure 5. *In situ* hybridization detection of PP4_{R,s} transcripts in human renal tissues. The signals for PP4 regulatory subunits PP4_{R,meq} and PP4_{R,1} are observed in a glomerulus of normal subjects. Magnification, $\times 40$.

hypercellularity, matrix accumulation, and microaneurysm formation (19). Northern blot analysis showed that PP4_{R,s} mRNA expression reached peak on day 2 and returned to the control levels thereafter (Figure 6A). Total cell number in glomeruli decreased on day 2, did not recover on day 4, and demonstrated only a slight increase on day 7 (Table 1). Immunohistochemical staining showed that the number of PCNA-positive cells in glomeruli reached its peak on day 7 (Table 1). Whereas the number of PCNA-positive cells on day 1 was approximately half of that on day 2, the PP4_{R,s} expression level was almost identical to the peak value on day 2, suggesting that expression of PP4_{R,s} precedes mesangial proliferation (Figure 6B, Table 1).

Discussion

Here we report the cloning and characterization of a novel subunit of PP4. PP dephosphorylate many kinds of proteins and play biologically important roles. The result that the amino acid sequences of PP4_{R,meq} were well conserved between human and rat reflects that PP4 has biologically basic functions. The sequences were especially well conserved in the regions of the heat repeat, which may be a potential candidate as a binding site to other subunits. It is likely that PP4c binds to the C terminus of PP4_{R,meq}, because this is where the A subunit of PP2A and PP4_{R,meq} share the greatest homology; however, the precise binding site remains to be determined.

Structural and functional analyses of PP4_{R,meq} revealed that it was closely related to the recently reported novel regulatory subunit of PP4, PP4_{R,1} (4). The amino acid sequences of

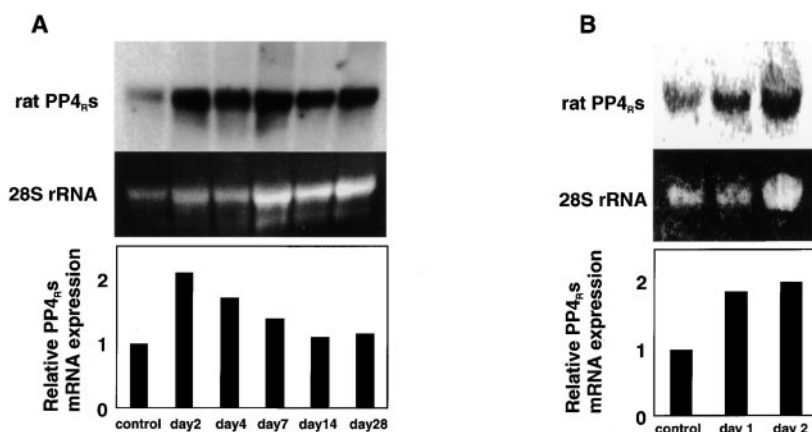


Figure 6. Kinetics of PP4_RS expression in glomeruli of anti-Thy1 glomerulonephritis rats at the time points from day 2 to day 28 (A) and of day 1 and day 2 (B). The expression of PP4_RS transcripts in glomeruli of anti-Thy1 glomerulonephritis rats was examined by Northern blotting (top). The bottom panel demonstrated the ratios of intensities of PP4_RS transcripts to ethidium bromide staining of 28S rRNA (middle) calculated by densitometry using NIH image software.

PP4_{R_{meg}} and PP4_{R₁} were almost identical except that serine-18 in PP4_{R₁} was replaced by 18 amino acids (FGVDDYSSSESD-VIIIPSA) in PP4_{R_{meg}}. Despite the additional amino acid insertion at the N-terminus, PP4_{R_{meg}} seems to be biologically similar to PP4_{R₁}. Our immunoprecipitation studies demonstrated that recombinant PP4_{R_{meg}} can associate with endogenous PP4c in cell lysates, supporting the idea that PP4_{R_{meg}} functions as a regulatory subunit of PP4. In good agreement with this, Kloeker and Wadzinski (4) previously demonstrated that PP4_{R₁} migrated as a doublet on SDS-PAGE and exhibited a fairly broad peak (approximately 105 kD) by mass spectrometry, suggesting the possibility of the isoform(s) of PP4_{R₁}.

According to the genomic information in the GenBank database, PP4_{R_{meg}} seems to be localized on chromosome 18. The PP4_{R_{meg}}-specific sequence, which is 51 bp long, is located on the 5' part of the third exon consisting of 136-bp nucleotides. The sequences of the 3' end of intron 2 and the PP4_{R_{meg}}-

Table 1. The number of intraglomerular cells and PCNA-positive cells in the renal tissues of anti-Thy1 glomerulonephritis rats at the time points from day 2 to day 28 and of day 1 and day 2

Time Points	Total Cells	PCNA-Positive Cells
Days 2 to 28		
control	44.9 ± 11.2	1.6 ± 1.9
day 2	35.4 ± 7.0 ^a	3.5 ± 2.9 ^b
day 4	37.5 ± 9.9 ^b	4.2 ± 3.1 ^a
day 7	51.8 ± 11.4 ^c	11.0 ± 5.0 ^a
day 14	46.9 ± 11.0	1.0 ± 1.5
day 28	46.9 ± 10.0	0.3 ± 0.6
Days 1 and 2		
control	54.2 ± 7.9	1.1 ± 0.8
day 1	43.2 ± 5.6 ^a	3.3 ± 1.5 ^a
day 2	38.0 ± 6.0 ^a	4.8 ± 1.5 ^a

^a P < 0.001, ^b P < 0.01, ^c P < 0.05 versus control.

specific part are consistent with consensus sequences for donor sites of alternative splicing. PP4_{R_{meg}} and PP4_{R₁} therefore may be splicing variants of a common gene. Both PP4_{R_{meg}} and PP4_{R₁} are expressed in human mesangial cells and A431 cells. However, the difference of function and enzymatic activity between these two molecules remains to be determined.

In the present study, we showed ubiquitous expression of PP4 regulatory subunits in various human organs. This result is in accordance with the fact that PP4 is the protein with biologically basic functions. Although our Northern blot analysis demonstrated that PP4_RS were expressed to some degree in most, if not all, cultured cells examined thus far, we observed higher expression of PP4_RS mRNA in cultured mesangial cells. The result of the present *in situ* hybridization studies also supported high expression of PP4_RS in glomerular cells *in vivo*.

Our immunoprecipitation studies demonstrating the association of PP4_{R_{meg}} with PP4c suggest that a functional role for PP4_{R_{meg}} is similar to PP4_{R₁}. This may give us a clue to its biologic functions. PP4 has been localized at centrosomes/spindle pole bodies in human and *Drosophila* cells (11). The *Drosophila melanogaster* strain containing a mutated PP4c gene exhibits a semilethal phenotype with abnormal mitotic spindle microtubules (12). Most cell-cycle-dependent regulation of microtubule assembly occurs through changes in microtubule-associated protein phosphorylation state (reviewed in reference 20). Thus, PP4 may regulate cell mitosis by locally dephosphorylating centrosomal or microtubule-associated proteins, thereby regulating microtubule growth initiation or stability. We speculate that PP4_{R_{meg}} controls cell mitosis by regulating the activities of key phosphoproteins. Although some proteins, including γ -tubulin and CP60, have been considered to be potential candidates (12), the *in vivo* substrates have not identified. To uncover the mechanism of cytokinesis, it will be important to identify the substrates and to determine the precise function of PP4 in cell mitosis.

In this context, of particular interest is the potential involve-

ment of PP4_{Rmeg} in the regulation of mesangial cell division and proliferation. This stimulated us to investigate expression profiles of PP4_{Rmeg} in mesangial injury and repair. Our present results using anti-Thy1 glomerulonephritis rats suggest a link between the upregulation of PP4_{Rmeg} expression and mesangial proliferation. After intravenous administration of anti-Thy1 antibody, the number of endogenous glomerular cells decreases as a result of mesangiolysis. This phase is transient and followed by the recovery phase with mesangial proliferation (19). Our studies demonstrate that the upregulation of PP4_{Rmeg} mRNA expression preceded mesangial proliferation. These results are consistent with our hypothesis that PP4_{Rmeg} serves as a regulator of cell division in glomeruli. However, functional studies are required to conclude that PP4_{Rmeg} plays a role in this process.

Acknowledgments

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