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Molecular cloning and functional expression of rat leukotriene A_4 hydrolase using the polymerase chain reaction

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We isolated a cDNA encoding rat leukotriene A_4 (LTA_4) hydrolase from mesangial cells by the polymerase chain reaction according to the human amino acid sequence. The deduced amino acid sequence shows that rat LTA_4 hydrolase is a 609 amino acid protein with an M_r 69 kDa. Comparison of human LTA_4 hydrolase revealed 93% homology, and include zinc-binding motifs of aminopeptidases. COS-7 cells transfected with the cDNA revealed substantial LTA_4 hydrolase activity, and their activities were abolished by preincubation with captopril, representing the first reported cDNA expression of recombinant enzyme in mammalian cells. RNA blot analysis indicated that LTA_4 hydrolase was expressed in glomerular endothelial, epithelial and mesangial cells.

Leukotriene A_4 hydrolase; PCR (polymerase chain reaction); cDNA cloning; Glomerular cell; Captopril

1. INTRODUCTION

Leukotriene A_4 (LTA_4) hydrolase is a key enzyme which hydrolyzes an unstable epoxide moiety LTA_4 to yield dihydroxy fatty acid leukotriene B_4 (LTB_4) which is regarded as a potent pro-inflammatory mediator [1]. Glomerular LTB_4 synthesis is enhanced early during immune injury, but suppressed below control level by 24 h, suggesting that regulation of LTA_4 hydrolase activity in indigenous glomerular cells may be a principal determinant of glomerular LTB_4 synthesis [2]. In order to determine the cellular origin of LTB_4 and the regulation of its biosynthesis during inflamation, we needed to determine, at first, the cellular localization of LTA_4 hydrolase expression. Moreover, molecular-biological techniques used for the precise quantification of mRNA expression require strict homology between the cDNA and the target mRNA. LTA_4 hydrolase has been purified as a soluble protein [3], and the cDNA was recently cloned and sequenced [4–6], but that of rat is not available at present. It was, therefore, necessary to obtain a specific cDNA probe for rat which is one of the established experimental animals for inflammation.

We report here the isolation, characterization and sequence of cDNA encoding the rat LTA_4 hydrolase using the polymerase chain reaction (PCR), and its functional expression in mammalian cells. We also report the distribution of its expression in indigenous glomeruiar cells.

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2. MATERIALS AND METHODS

2.1. *Cell culture and PCR cloning*

Rat mesangial, glomerular endothelial, and epithelial cells were cultured as described previously [7]. Total RNA was extracted from rat mesangial cells [8], reverse-transcribed, and amplified by PCR using degenerate oligonucleotides A1 (5′-TAGCGAATTCAARAC-STGGGATCACTTYTGG-3′) and A2 (5′-TAGCGTCGACTTGA-AGT TGTACACCTCYTGCAT-3′) according to the human LTA_4 hydrolase cDNA sequence [4]. PCR schedule was three cycles of 45 s at 94°C, 60 s at 44°C, 90 s at 72°C followed by 30 cycles with a 55°C annealing temperature. The 5′ end of the cDNA pLT2 was amplified by PCR using 5′ primer B1 (5′-CATGCCCGAGRTMG MGGAT-ACYTGYTC-3′) and 3′ primer B2 (5′-GGTATGTCCTTCGTTT AA CCA-3′); three cycles of 45 s at 94°C, 1 min at 37°C, 2 min at 72°C followed by 30 cycles with a 50°C annealing temperature. Extension of the cDNA 3′ end (pLT3) was accomplished by the method described by Frohman et al. [9].

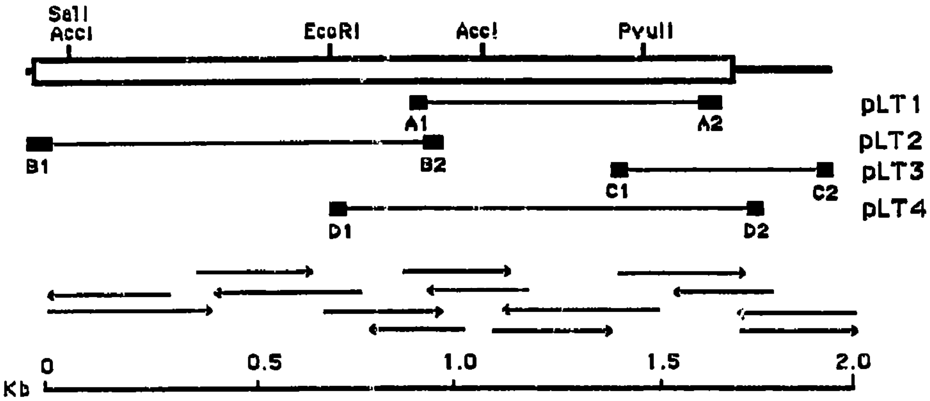
2.2. *Northern blotting and DNA sequencing*

Total RNA from glomerular cells were size fractionated in 1% agarose gels containing formaldehyde, transferred to nylon membranes. Hybridization and washing were carried out according to standard procedures [10]. PCR fragment pLT2 was ^{32}P-labeled with random hexamer. PCR-amplified cDNA fragments were gel-purified and sequenced by the dideoxy chain-termination method (Sequenase Kit, USB)

2.3. *Expression of cloned rat LTA_4 hydrolase*

The expression plasmid encoding rat LTA_4 hydrolase was constructed as follows. (i) PCR amplification was performed using primers D1 (5′-TATGAATTCTCTGAGACTGAATC-3′) and D2 (5′-TTCCTTAATCC ACTTTCAGATC-3′) to obtain a clone encoding the C-terminal region (pLT4). The PCR cycle consisted 25 cycles of 45 s at 94°C, 1 min at 50°C, and 2 min at 72°C. The PCR product pLT4 was subcloned into pCR 1000 (Invitrogen), and digested with *Not*I and *Eco*RI, and subsequently cloned into pcDNA I (Invitrogen) to give pCDNA-3′LT. (ii) DNA fragment pLT2 was subcloned into pCR 1000 and cleaved with *Eco*RI to give 1.1 kbp fragment. (iii) pCDNA-3′LT was digested with *Eco*RI, treated with calf alkaline

A



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ATG CCC CAG GTC GAG GAT ACT TGT TCC TTG GCT TCT CCA GCC TCG GTC TGC CGA ACC CAG CAC CTC CAT CTT CGC TGC AGC GTC GAC TTC     90
Met Pro Glu Val Glu Asp Thr Cys Ser Leu Ala Ser Pro Ala Ser Val Cys Arg Thr Gln His Leu His Leu Arg Cys Ser Val Asp Phe     29

GCT CGC CGG GCA CTG ACC GGG ACC GCC GCG CTC ACG GTC CAG TCA CAG GAG GAT AAT CTG CGC ACG CTG ACT TTG GAT ACA AAA GAC CTT    180
Ala Arg Arg Ala Leu Thr Gly Thr Ala Ala Leu Thr Val Gln Ser Gln Glu Asp Asn Leu Arg Thr Leu Thr Leu Asp Thr Lys Asp Leu     59

ACG ATA GAA AAA GTG GTG ATC AAC GGA CAA GAA GTC AAA TAC ACT CTT GGA GAA AGC CAG GGT TAC AAA GGA TCG CCG ATG GAA ATC TCC    270
Thr Ile Glu Lys Val Val Ile Asn Gly Gln Glu Val Lys Tyr Thr Leu Gly Glu Ser Gln Gly Tyr Lys Gly Ser Pro Met Glu Ile Ser     89

CTT CCC ATC GCT CTG AGC AAA AAT CAA GAG GTC GTT ATA GAG ATT TCC TTC GAG ACC TCT CCC AAG TCC TCG GCC CTT CAG TGG CTC ACT    360
Leu Pro Ile Ala Leu Ser Lys Asn Gln Glu Val Val Ile Glu Ile Ser Phe Glu Thr Ser Pro Lys Ser Ser Ala Leu Gln Trp Leu Thr    119

CCC GAG CAG ACT TCA GGA AAG CAG CAT CCA TAC CTC TTC AGT CAG TGG GAG GCC ATC CAC TGC AGG GCA ATC CTC CCT TGC CAA GAC ACT    450
Pro Glu Gln Thr Ser Gly Lys Gln His Pro Tyr Leu Phe Ser Gln Trp Glu Ala Ile His Cys Arg Ala Ile Leu Pro Cys Gln Asp Thr    149

TCT GTG AAG TTA ACC TAC ACC GCA GAG GTA TCT GTC CCC AAA GAA CTG GTG GCT CTC ATG AGT GCC ATC CGT GAT GGA GAA GCC CCT GAC    540
Ser Val Lys Leu Thr Tyr Thr Ala Glu Val Ser Val Pro Lys Glu Leu Val Ala Leu Met Ser Ala Ile Arg Asp Gly Glu Ala Pro Asp    179

CCA GAA GAC CCC AGC AGG AAG ATA TAC AGA TTT AAC CAG AGG GTT CCC ATC CCG TGC TAC CTG ATT GCT TTA GTC GTT GGC GCT TTA GAA    630
Pro Glu Asp Pro Ser Arg Lys Ile Tyr Arg Phe Asn Gln Arg Val Pro Ile Pro Cys Tyr Leu Ile Ala Leu Val Val Gly Ala Leu Glu    209

AGC AGG CAA ATT GGC CCA AGA ACT CTG GTG TGG TCT GAG AAA GAG CAG GTG GAG AAG TCT GCT TAT GAA TTC TCT GAG ACT GAA TCC ATG    720
Ser Arg Gln Ile Gly Pro Arg Thr Leu Val Trp Ser Glu Lys Glu Gln Val Glu Lys Ser Ala Tyr Glu Phe Ser Glu Thr Glu Ser Met    239

CTT AAA ATT GCA GAA GAT CTG GGG GGA CCC TAT GTT TGG GGA CAG TAC GAC CTG TTG GTC CTG CCC CCA TCC TTC CCG TAC GGA GGC ATG    810
Leu Lys Ile Ala Glu Asp Leu Gly Gly Pro Tyr Val Trp Gly Gln Tyr Asp Leu Leu Val Leu Pro Pro Ser Phe Pro Tyr Gly Gly Met    269

GAG AAT CCT TGT CTC ACA TTC GTA ACG CCT ACT CTA TTG GCA GGT GAC AAG TCA CTC TCT AAT GTT ATT GCA CAC GAA ATA TCG CAT AGC    900
Glu Asn Pro Cys Leu Thr Phe Val Thr Pro Thr Leu Leu Ala Gly Asp Lys Ser Leu Ser Asn Val Ile Ala His Glu Ile Ser His Ser    299

TGG ACA GGG AAC CTA GTG ACT AAC AAG ACT TGG GAT CAC TTC TGG TTA AAC GAA GGA CAT ACC GTC TAT TTA GAG CGC CAC ATT TGT GGC    990
Trp Thr Gly Asn Leu Val Thr Asn Lys Thr Trp Asp His Phe Trp Leu Asn Glu Gly His Thr Val Tyr Leu Glu Arg His Ile Cys Gly    329

CGG TTG TTT GGG GAA AAA TTC AGA CAT TTC CAC GCT TTA GGA GGA TGG GGA GAG CTA CAG AAC ACG ATA AAC ACT TTC GGG GAG TCC CAT   1080
Arg Leu Phe Gly Glu Lys Phe Arg His Phe His Ala Leu Gly Gly Trp Gly Glu Leu Gln Asn Thr Ile Lys Thr Phe Gly Glu Ser His    359

CCC TTC ACC AAG CTC GTG GTC GAT CTG AAG GAC GTA GAC CCC GAT GTG GCC TAC TCC TCC ATT CCC TAC GAG AAG GGC TTC GCC TTG CTC   1170
Pro Phe Thr Lys Leu Val Val Asp Leu Lys Asp Val Asp Pro Asp Val Ala Tyr Ser Ser Ile Pro Tyr Glu Lys Gly Phe Ala Leu Leu    389

TTC TAC CTG GAA CAA CTT CTT GGC GGA CCG GAG GTG TTC CTA GGA TTC TTA AAG GCT TAT GTG GAG AAG TTT TCC TAC CAG AGC GTA ACC   1260
Phe Tyr Leu Glu Gln Leu Leu Gly Gly Pro Glu Val Phe Leu Gly Phe Leu Lys Ala Tyr Val Glu Lys Phe Ser Tyr Gln Ser Val Thr    419

ACT GAT GAC TGG AAG AGT TTC CTG TAT GCT CAC TTT AAA GAT AAG GTG GAT CTT CTC AAT CAA GTT GAT TGG AAT GCC TGG CTC TAT GCT   1350
Thr Asp Asp Trp Lys Ser Phe Leu Tyr Ala His Phe Lys Asp Lys Val Asp Leu Leu Asn Gln Val Asp Trp Asn Ala Trp Leu Tyr Ala    449

CCT GGC CTG CCT CCT GTC AAA CCC AAT TAC GAT GTG ACT CTC ACG AAT GCC TGC ATC GCC TTA AGT CAA AGA TGG GTC ACT GCC AAA GAG   1440
Pro Gly Leu Pro Pro Val Lys Pro Asn Tyr Asp Val Thr Leu Thr Asn Ala Cys Ile Ala Leu Ser Gln Arg Trp Val Thr Ala Lys Glu    479

GAA GAT TTA AAT TCA TTC AGC ATC GAA GAC CTG AAG GAC CTC TCG TCC CAT CAG CTG AAT GAG TTC CTG GCA CAG GTG CTT CAG AGG GCA   1530
Glu Asp Leu Asn Ser Phe Ser Ile Glu Asp Leu Lys Asp Leu Ser Ser His Gln Leu Asn Glu Phe Leu Ala Gln Val Leu Gln Arg Ala    509

CCT CTT CCC TTG GGG CAC ATA AAG CGA ATG CAA GAG GTG TAC AAC TTC AAT GCC ATT AAC AAT TCT GAA ATA CGA TTC AGA TGG TTA CGG   1620
Pro Leu Pro Leu Gly His Ile Lys Arg Met Gln Glu Val Tyr Asn Phe Asn Ala Ile Asn Asn Ser Glu Ile Arg Phe Arg Trp Leu Arg    539

CTC TGC ATT CAA TCG AAG TGG GAA CAA GCA ATT CCT TTG GCC CTA AAG ATG GCA ACT GAA CAA GGA AGG ATG AAA TTT ACA CGA CCT CTA   1710
Leu Cys Ile Gln Ser Lys Trp Glu Glu Ala Ile Pro Leu Ala Leu Lys Met Ala Thr Glu Gln Gly Arg Met Lys Phe Thr Arg Pro Leu    569

TTC AAG GAC CTC GCT GCC TTC GAC AAA TCC CAC GAT CAA GCT GTC CGC ACC TAC CAG GAA CAT AAA GCC TGT ATG CAT CCC GTG ACC GCC   1800
Phe Lys Asp Leu Ala Ala Phe Asp Lys Ser His Asp Gln Ala Val Arg Thr Tyr Gln Glu His Lys Ala Cys Met His Pro Val Thr Ala    599

ATG CTG GTG GGG AAA GAT CTG AAA GTG GAT TAA GGAACTGAGTGTTGCTGATTTCAGAAAATTCCTTTTTTTAAGAAAAAAAATCGAACCTATTAAGAAATACAAAAT   1909
Met Leu Val Gly Lys Asp Leu Lys Val Asp ***                                                                                 609

TTTAGCTCTCGTTCT(A)                                                                                                         1941
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rat     MPEVEDTCSLASPASVCRTQHLHLRCSVDFARRALTGTAALTVQSQEDNLRTLTLDTKDLTIEKVVINGQEVKYTLGESQGYKGSPMEIS   89
human      IV              K          T  T                 S V                    A   R S
mouse       A                            T             E   S

rat     LPIALSKNQEVVIEISFETSPKSSALQWLTPEQTSGKQHPYLFSQWEAIHCRAILPCQDT-SVKLTYTAEVSVPKELVALMSAIRDGEAP  179
human             I                          E       CQ             P                           T
mouse             I                                  CQ             P

rat     DPEDPSRKIYRFNQRVPIPCYLIALVVGALESRQIGPRTLVWSEKEQVEKSAYEFSETESMLKIAEDLGGPYVWGQYDLLVLPPSFPYGG  269
human             K I K
mouse                                                       N

rat     MENPCLTFVTPTLLAGDKSLSNVIAHEISHSWTGNLVTNKTWDHFWLNEGHTVYLERHICGRLFGEKFRHFHALGGWGELQNTIKTFGES  359
human                                                                          N          SV     T
mouse

rat     HPFTKLVVDLKDVDPDVAYSSIPYEKGFALLFYLEQLLGGPEVFLGFLKAYVEKFSYQSVTTDDWKSFLYAHFKDKVDLLNQVDWNAWLY  449
human             T I        V                    I              K I      D   SY      V
mouse                                                       K

rat     APGLPPVKPNYDVTLTNACIALSQRWVTAKEEDLNSFSIEDLKDLSSHQLNEFLAQVLQRAPLPLGHIKRMQEVYNFNAINNSEIRFRWL  539
human   S     I     M             I    D     NAT                T
mouse                                     S    A                   K

rat     RLCIQSKWEEAIPLALKMATEQGRMKFTRPLFKDLAAFDKSHDQAVRTYQEHKACMHPVTAMLVGKDLKVD                    610
human            D                                            S
mouse                                                 H       S          R
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Fig. 2. Homology of LTA_4 hydrolase amino acid sequences among rat, human, and mouse. Amino acids of human LTA_4 hydrolase are numbered from the N-terminal proline residue. Amino acid residues of human and mouse that are different from that of rat are shown under the sequence of rat LTA_4 hydrolase, and identical residues are written as blanks. One amino acid gap (Pro-150) of rat LTA_4 hydrolase is indicate as a hyphen. Underlined sequences indicate the zinc ion binding motif.

phosphatase, and ligated with 1.1 kbp *Eco*RI fragment of pLT2 to yield pcDNA1-LT. The orientation of *Eco*RI fragment was checked by *Sal*I/*Pvu*II digestion. COS-7 cells were transfected with 12 μg of pcDNA1-LT plasmid or vector-only DNA by calcium phosphate method [10]. Sixty hours after transfection, cells were washed with phosphate-buffered saline and sonicated. After centrifugation, the supernatant was used for LTA_4 hydrolase assay as described previously [11]. In some experiments, the supernatant was preincubated with 70 μM of captopril at 37°C for 2 min.

3. RESULTS AND DISCUSSION

We cloned a cDNA encoding rat LTA_4 hydrolase from mesangial cells using PCR. Based on the disclosed amino acid sequence [4], we found two regions flanking a 600 bp cDNA and providing relatively low degeneracies, which are corresponding to primers A1 and A2. Additional cDNAs encompassing toward the 5′ and 3′ ends were obtained by using conventional reverse-transcription PCR (RT-PCR) or RACE-PCR, respectively [9]. The cleavage sites that have been verified by restriction endonuclease digestion are shown (Fig. 1A). There is an open reading frame encoding 609 amino acids following a start codon ATG in the sequence, and the molecular weight is estimated as 69,001 (Fig. 1B). The cloned sequence contains 0.1 kbp of 3′-untranslated sequence including a poly A stretch, but polyadenylation signals were not found as in human or mouse cDNA sequences [4–6]. Amino acid sequence comparison to the human LTA_4 hydrolase reveals a high degree of homology (93%), with one amino acid gap at Pro-150 (Fig. 2).

In a series of recent studies [12–15], it has been established that LTA_4 hydrolase possesses intrinsic aminopeptidase activity and bears strong functional resemblance to a superfamily of zinc-metallohydrolases. In particular, three residues involved in zinc binding and one residue of the active sites of aminopeptidases N are conserved in LTA_4 hydrolase [12–15]. In the present studies, we document that the sequences of the zinc ion binding motif and peptidase active site; VIAHEISHSW (293–302) and LNEGH (317–321) are identical to their human and mouse counterparts (Fig. 2).

The 1.8 kb rat cDNA clone (nucleotide from −1 to 1837) was subcloned into a mammalian expression vector pcDNAI (designated as pcDNAI-LT) was transfected to COS-7 cells under the control of the cytomegalovirus promoter. Northern blot showed a substantially

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Fig. 1. (A) Partial restriction map and the strategies for PCR and sequencing of cloned cDNAs encoding rat LTA_4 hydrolase. The location of oligonucleotide primers are indicated by solid boxes, and resulting cDNA fragments are shown by solid bars and designated on the right. Direction and extent of sequencing determinations are indicated by arrows. The open reading frame is indicated as an open box. (B) Nucleotide and deduced amino acid sequence of rat LTA_4 hydrolase. Nucleotides are numbered beginning with the first residue of the ATG initiator codon. Amino acids are numbered from the N-terminal proline residue. Underlined sequences indicate the zinc ion binding motif, and (A) stands for poly A stretch.

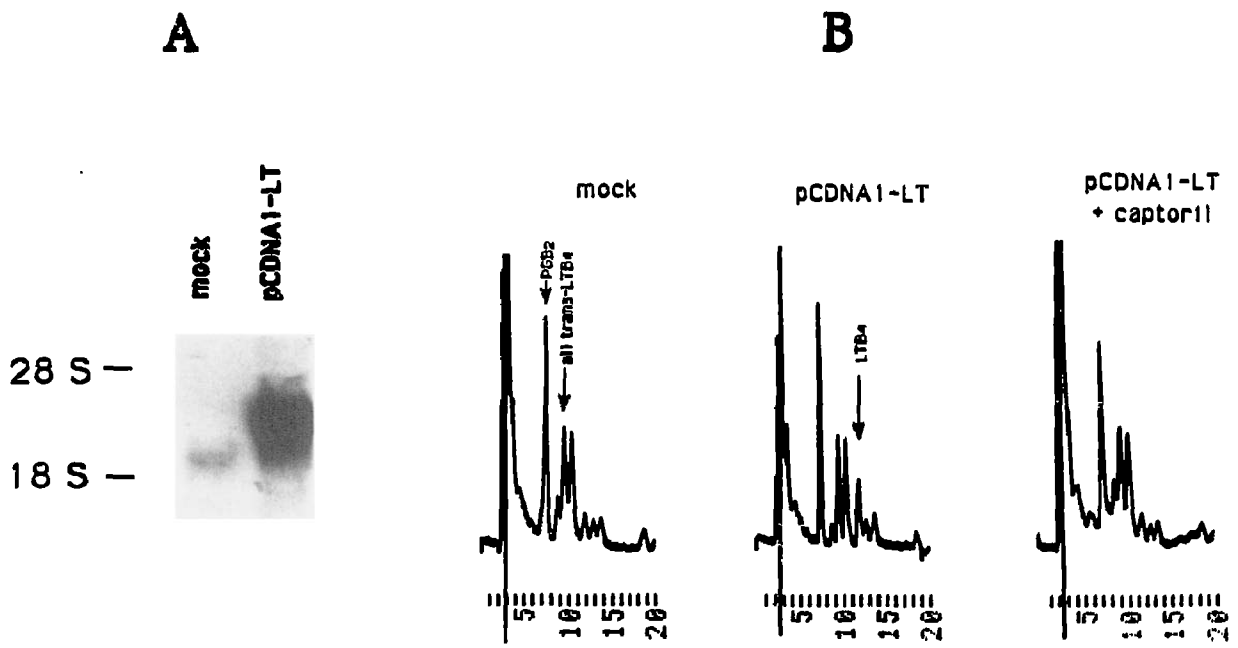

Fig. 3. (A) Autoradiogram of Northern analysis. Twenty micrograms of total RNA from mock-transfected COS cells (mock) and pcDNA1-LT-transfected cells (pcDNA1-LT) were electrophoresed on a 1% agarose gel containing formaldehyde, blotted to nylon membranes, and hybridized to ^{32}P-labeled pLT2. Positions of 28 S and 18 S ribosomal RNA are indicated on the left. (B) Reverse-phase HPLC chromatograms of LTA_4 hydrolase products formed from mock-transfected COS cells (left), pcDNA1-LT-transfected COS cells (middle), and pcDNA1-LT-transfected COS cells preincubated with captopril (right). UV-monitoring was carried out at 270 nm. The peaks of LTB_4 and PGB_2 were determined by the retention times of authentic standards.

high expression of LTA_4 hydrolase mRNA in pcDNA1-LT-transfected COS cells although a small amount of native expression was detected in mock-transfected cells

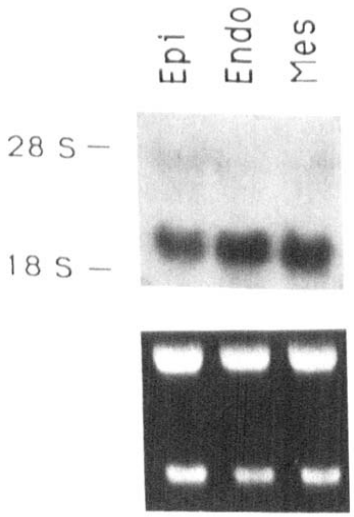

Fig. 4. Autoradiogram of Northern analysis (upper panel) and ethidium bromide staining of agarose gel (lower panel). Twenty micrograms of total RNA from rat glomerular epithelial cells (Epi), glomerular endothelial cells (Endo), and mesangial cells (Mes) were analyzed as in Fig. 3B. The equivalent amount of total RNA per lane was monitored by 28 S (upper bands) and 18 S (lower bands) ribosomal RNA. Filters were washed three times with 0.1 × SSC, 0.1% SDS at 60°C for 15 min, and were autoradiographed for 12 h at −70°C using an intensifying screen.

(Fig. 3A). The difference of mRNA size between them is presumably due to the additional non-coding sequence between the *Not*I site and polyadenylation signal in the pcDNAI vector.

An increase in LTB_4 production of pcDNA1-LT-transfected cells was observed, whereas mock-transfected cells displayed a low level of LTB_4 production, presumably due to endogenous LTA_4 hydrolase (Fig. 3B). The amount of produced LTB_4 calculated from the peak area ratio LTB_4/PGB_2 revealed that LTA_4 hydrolase expression was 4.5 times higher in pCDNA1- LT-transfected cells than in mock-transfected cells. In addition, preincubation with captopril abolished LTB_4 synthesis in transfected COS cells (Fig. 3C), presumably due to the binding capacity of its sulfhydril group to the zinc-binding domain of LTA_4 hydrolase. [15,16].

Evaluation of LTA_4 hydrolase gene expression revealed that a single mRNA of 2.2 kb was detected all types of cultured glomerular cells, and the expression in epithelial cells was slightly lower than in mesangial cells and endothelial cells. (Fig. 4).

The availability of this rat-specific probe should allow for the quantitative and histopathological evaluation of LTA_4 hydrolase gene expression throughout the time course of experimental glomerular immune injury, and hopefully provide insight into its pathophysiology.

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