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Cloning and expression of a novel form of leukotriene B_4 ω-hydroxylase from human liver

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Abstract

We have isolated and sequenced a cDNA for human liver LTB_4 ω-hydroxylase. The cDNA encoded a protein of 520 amino acids with a molecular weight of 59,853 Da. The cDNA-deduced amino acid sequence showed 87.3% homology to that of human polymorphonuclear leukocytes (PMN) LTB_4 ω-hydroxylase (CYP4F3). Northern blot analysis revealed that the mRNA hybridized to the specific cDNA fragment is expressed in human liver, but not in human PMN. The microsomes from yeast cells transfected with the cDNA catalyzed the ω-hydroxylation of LTB_4 with a K_m of 44.8 μM. These results clearly show that a new form of the CYP4F LTB_4 ω-hydroxylase exists in human liver.

Key words: Cytochrome P-450; Leukotriene B_4; ω-Hydroxylase; cDNA cloning; Human liver

1. Introduction

Leukotriene (LT) B_4 is a potent chemotactic agent and plays a role as a mediator of inflammation [1]. Human polymorphonuclear leukocytes (PMN) not only generate LTB_4 from arachidonic acid by the 5-lipoxygenase pathway, but also convert LTB_4 into biologically less active products by the ω-oxidation pathway, the first step of which is catalyzed by LTB_4 ω-hydroxylase [2–6]. Recent studies in our laboratories revealed that LTB_4 ω-hydroxylase is a P-450 [7], which shows about 40% amino acid sequence homology to several ω-hydroxylases toward fatty acids and prostaglandins belonging to the CYP4A subfamily [8]. This new P-450 was named CYP4F3 [7] according to the uniform nomenclature system of the P-450 superfamily [9]. On the other hand, the liver is the principal organ for metabolic inactivation of LTB_4 as well as cysteinyl leukotrienes. Rat liver microsomes or hepatocyte suspensions catalyze the ω-hydroxylation of LTB_4 [10–13]. Sumimoto et al. [11] provided evidence for the participation of a P-450 in the ω-hydroxylation of LTB_4. However, nothing is known about the enzyme system for the metabolism of LTB_4 in human liver. In the present study, we have found the capability of human liver microsomes to ω-hydroxylate LTB_4. Furthermore, we have cloned human liver LTB_4 ω-hydroxylase. This enzyme shows 87.3% amino acid sequence homology to the human PMN LTB_4 ω-hydroxylase (CYP4F3), indicating that it is a new member of the CYP4F subfamily. Nevertheless, the human liver LTB_4 ω-hydroxylase differs from the PMN enzyme in its K_m value and tissue localization.

2. Materials and methods

2.1. Materials

Restriction enzymes, T4 DNA ligase, and a large fragment of *Escherichia coli* DNA polymerase I (Klenow fragment) were purchased from Takara Shuzo (Kyoto); λgt11 cDNA library of human liver (HL1115b #25596) was from Clontech Laboratories (Palo Alto); [α-^{32}P]dCTP (111 TBq/mmol) was from Radiochemical Center (Amersham). *Saccharomyces cerevisiae* strain AH22 (a leu2 his4 can1 cir$^+$) was donated by Dr. T. Hatano (Fukuyama University), and the yeast expression vector pAAH5 carrying the ADH1 promoter and terminator was from Dr. B. D. Hall (University of Washington). NADPH-P-450 reductase was prepared from rabbit liver microsomes by the method of Taniguchi et al. [14].

2.2. Cloning procedure

cDNA clones for human liver LTB_4 ω-hydroxylase were isolated from 4.0×10^5 recombinant phages with cDNA under high stringency conditions using the cDNA for human PMN LTB_4 ω-hydroxylase (CYP4F3) [7] as a probe. Plaque hybridization was carried out with the ^{32}P-labeled cDNA (specific activity, 2.5×10^8 cpm/μg) at 65°C in 50 mM Tris buffer (pH 7.5) containing 1 M NaCl, 10 mM EDTA, 0.1% sodium *N*-lauroylsarcosinate, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin, and 100 μg/ml salmon sperm DNA. The filters were washed twice at 65°C for 30 min in 1 × SSC (0.15 M NaCl and 0.015 M sodium citrate) and 0.1% *N*-lauroylsarcosinate. Positive clones were subjected to four rounds of plaque purification, and inserted cDNAs were subcloned into pUC18 plasmid vector at the *Eco*RI site.

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Abbreviations: P-450, cytochrome P-450; LTB_4, leukotriene B_4; PMN, polymorphonuclear leukocytes; bp, base pair(s). CYP refers to a cytochrome P-450 gene(s) and cDNA(s), which is the nomenclature recommended by Nelson et al. [9].

The nucleotide sequence reported in this paper has been submitted to DDBJ, EMBL, and GenBank with accession number D26480.

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2.3. DNA sequence analysis

The plasmid-containing cDNA insert of human liver LTB_4 ω-hydroxylase was digested with several restriction enzymes to identify fragments convenient for sequence analysis. Restriction fragments were inserted into appropriate vectors, M13 mp18 and M13 mp19, at the polylinker site, and then sequenced by the dideoxy method [15]. All sequencings were performed in both directions.

2.4. Northern blot analysis of mRNA from human liver

Four cDNA fragments including a 1749-bp CYP4F3 cDNA [7] insert (specific activity, 2.1×10^8 cpm/mg) and its 142-bp *Hin*fI–*Pvu*II restriction fragment, 236–377 (specific activity, 1.2×10^8 cpm/mg), and a 2227-bp LB3 cDNA insert (specific activity, 2.3×10^8 cpm/mg) and its 106-bp *Pvu*II–*Pvu*II fragment, 279–384 (specific activity, 1.5×10^8 cpm/mg) were used as probes (Fig. 1). Poly(A) RNA (1 μg) from the human liver, kidney, and PMN were denatured, electrophoresed on a 0.8% agarose gel containing 2.2 M foramide, then transferred onto a nitrocellulose filter. The filters were hybridized with each ^{32}P-labeled probe and washed twice at 50°C for 30 min in 0.1× SSC and 0.2% sodium *N*-lauroylsarcosinate as described [7].

2.5. Expression of liver LTB_4 ω-hydroxylase in yeast cells

Yeast expression vector, pAAH5, and LB3 cDNA were used to express liver LTB_4 ω-hydroxylase. *Hin*dIII linkers (CCCAAGCTTA-AAAAA) were constructed just before the initiation codon, ATG, and at *Eco*RV restriction site in position 1714 of LB3 cDNA by the polymerase chain reaction. This modified cDNA fragment was digested with *Hin*dIII, and inserted into expression vector, pAAH5 at the *Hin*dIII site. Its orientation was determined by restriction enzyme mapping. Transfection and cultivation of yeast cells were carried out as described [7]. Yeast microsomes were suspended in 0.1 M potassium phosphate buffer (pH 7.5) containing 20% glycerol, 1 mM DTT, and 1 mM EDTA to give a concentration of 20 mg of protein per ml.

2.6. Activity assay

LTB_4 ω-hydroxylase activity was determined as described [7]. The reaction mixture containing 340 mM sucrose, 20 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM DTT, 1 mM NADPH, 70 μM LTB_4, and human liver microsomes (0.2 mg of protein) or yeast microsomes (0.1–0.5 mg of protein), in a total volume of 0.2 ml, was incubated at 37°C for 10 min. The reaction product was extracted and determined as described [7]. Microsomal activities of fatty acid and prostaglandin (PG) A_1 hydroxylation were determined as described [16]. The concentration of P-450 was determined by the method of Omura and Sato [17]. Microsomes from yeast harboring pAAH5 without the cDNA insert were used for control experiments.

3. Results and discussion

3.1. LTB_4 ω-hydroxylation by human liver microsomes

We have examined P-450 contents and LTB_4 ω-hydroxylase activities of microsomes prepared from the normal portions of 14 human liver specimens resected for hepatocellular carcinoma (Table 1). Incubation of the individual microsomes with LTB_4 in the presence of NADPH for 10 min resulted in the formation of ω-hydroxy LTB_4 (20-OH-LTB_4). No (ω-1)-hydroxy LTB_4 (19-OH-LTB_4) was detected. The LTB_4 ω-hydroxylase activity was strongly inhibited by carbon monoxide, 1 mM SKF 525-A, and 1 mM metyrapone. No activity was observed in the absence of NADPH. These results suggest the presence of a P-450(s) responsible for the ω-hydroxylation of LTB_4 in human liver microsomes.

3.2. Isolation of cDNA clone for human liver LTB_4 ω-hydroxylase

We observed that the mRNA hybridized to the whole CYP4F3 cDNA [7] is expressed in the human liver and kidney as well as human PMN (Fig. 1A). In contrast, a significant signal was observed only in human PMN, but not in human liver and kidney, when the 142-bp *Hin*fI–*Pvu*II restriction fragment at position 236–377 of CYP4F3 cDNA was used as a probe (Fig. 1B). These findings prompted us to isolate a cDNA specific to human liver LTB_4 ω-hydroxylase. Approximately 4.0×10^5 plaques from a human liver cDNA library were screened with the CYP4F3 cDNA [7] as a probe. Sixteen positive plaques were obtained and subjected to four rounds of plaque purification. Seven clones of longer than 1.5-kbp were sequenced to about 200-bp of both ends. One clone, designated LB3, had an initiation codon, ATG, in its 5′-end. The other 6 clones carried the same, but shorter cDNA insert. LB3 cDNA contained an insert of about 2.2-kbp in size.

Fig. 2 shows the nucleotide and deduced amino acid sequences for LB3 cDNA together with those for PMN LTB_4 ω-hydroxylase (CYP4F3) for comparison. LB3 cDNA contained 2,227 nucleotides and lacked the poly(A) tail. An open reading frame at position 42–1601 was followed by a termination codon at position 1602–1604 and a 621-bp 3′-untranslated sequence. The open reading frame encoded a protein of 520 amino acids with a molecular weight of 59,853 Da. An invariant cysteine

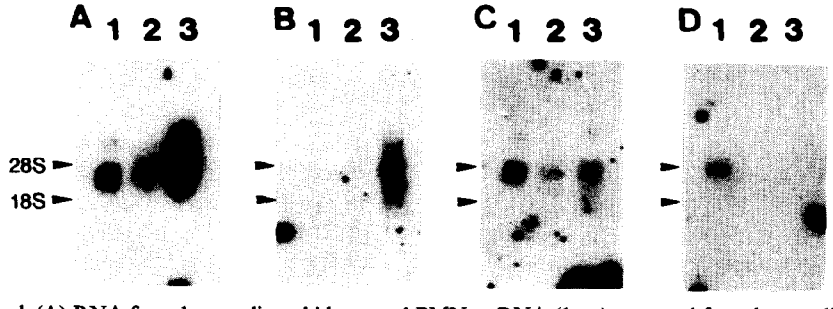

Fig. 1. Blot analysis of the poly(A) RNA from human liver, kidney, and PMN. mRNA (1 μg) prepared from human liver, kidney, and PMN were separated on an 0.8% denaturing agarose gel, transferred to the nitrocellulose filter, and hybridized with four ^{32}P-labeled cDNA fragments. (A) 1749-bp human PMN LTB_4 ω-hydroxylase (CYP4F3) cDNA insert [7]; (B) 142-bp *Hin*fI–*Pvu*II restriction fragment (236–377) of CYP4F3 [7]; (C) 2227-bp LB3 cDNA insert; (D) 106-bp *Pvu*II–*Pvu*II fragment (279–384) of LB3 cDNA insert. Lane 1, liver; lane 2, kidney; lane 3, PMN.

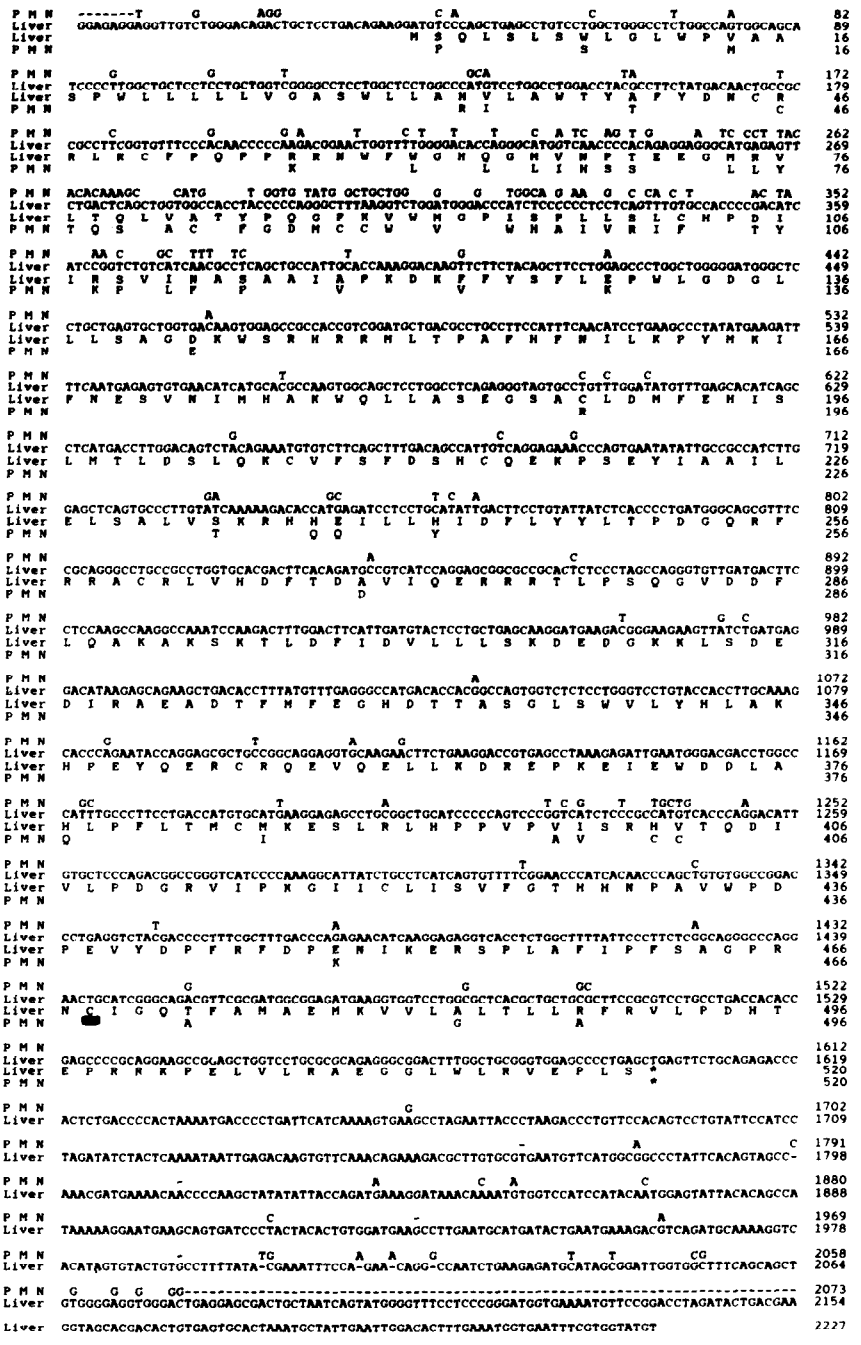

Fig. 2. Nucleotide sequences of human liver and PMN LTB_4 ω-hydroxylase cDNAs and their deduced amino acid sequences. The whole nucleotide and amino acid sequences of liver LTB_4 ω-hydroxylase cDNA are shown ('liver'). Only the nucleotides and amino acids of PMN LTB_4 ω-hydroxylase cDNA (CYP4F3) [7] that are not identical with those of the liver enzyme cDNA are presented ('PMN'). A putative heme-binding cysteine residue is underlined.

residue was found to be located at position 468 in the conserved region among all P-450s examined [18,19]. The cDNA-deduced amino acid sequence showed 87.3% homology with that of CYP4F3 [7]. However, the regions with low homology between the two P-450s were found to be present at position 61–114 of the amino acid sequence (29.6%) and at position 223–381 (LB3) of the nucleotide sequence (49.7%), although other sequences showed high homology (91.5%) with each other. The primary structure of the protein encoded by LB3 cDNA had 31–45% homology with those of members of the CYP4A [20–22], CYP4B [23] and CYP4C [24] subfamilies, and showed 78.5% homology with that of CYP4F1, the mRNA of which was detected in rat hepatic tumors [25]. These results indicate that the P-450 encoded by LB3 cDNA is a new member of the CYP4F subfamily.

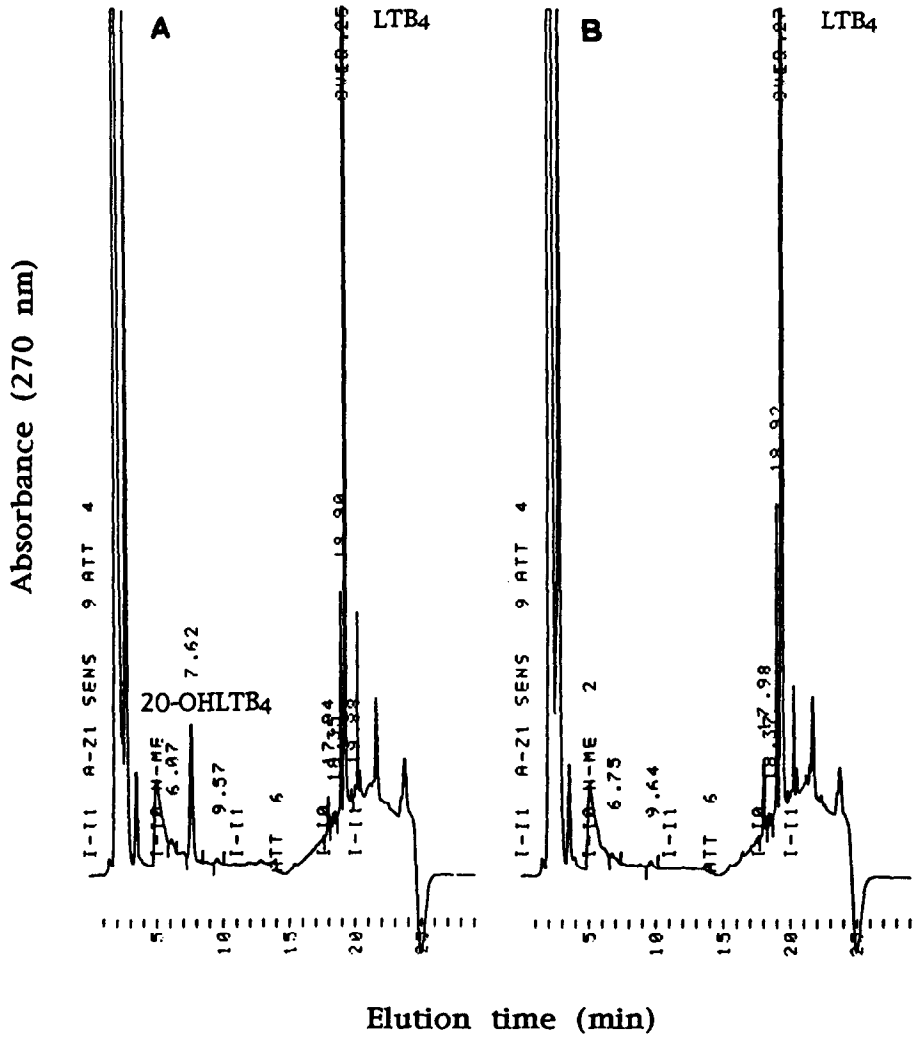

Fig. 3. HPLC of 20-OH-LTB$_4$ produced from LTB$_4$ by yeast microsomes transfected with LB3 cDNA. Reaction conditions are described in the text. (A) Incubation for 5 min; (B) zero time.

Table 1
Microsomal LTB$_4$ ω-hydroxylase activity in human liver

Case	Specific content of P-450 (nmol P-450/mg protein)	Activity (pmol product/min/mg protein)
1	0.12	41
2	0.07	18
3	0.21	18
4	0.13	46
5	0.38	78
6	0.07	38
7	0.07	49
8	0.10	20
9	0.08	22
10	0.05	11
11	0.22	32
12	0.08	42
13	0.07	39
14	0.15	81

The reaction conditions are given in the text.

3.3. Expression in yeast

Microsomal fractions obtained from yeast cells transfected with the expression vector pAAH5 carrying recombinant LB3 cDNA showed approximately 0.06 nmol of P-450/mg of protein, whereas no P-450 was detected in control yeast cells. The transfected yeast microsomes catalyzed the hydroxylation of LTB$_4$ solely at the ω-position with a turnover rate of 2.6 nmol/min/nmol of P-450 (Fig. 3). No activity was detected with laurate, arachidonate, or PGA$_1$. The apparent K_m for LTB$_4$ was 44.8 μM. This value is 63-fold higher than that for PMN LTB$_4$ ω-hydroxylase (CYP4F3)[7]. Addition of rabbit liver NADPH-P-450 reductase had little effect. In contrast, no activity was observed in the absence of NADPH. These results demonstrate that LB3 cDNA encodes human liver LTB$_4$ ω-hydroxylase.

3.4. Northern blot analysis

Northern blot analysis showed that the whole LB3

cDNA hybridized strongly with mRNA in human liver and PMN, with a weaker hybridization signal being observed in human kidney (Fig. 1C). However, when the cDNA fragment at position 279–384 (*Pvu*II–*Pvu*II) of LB3 cDNA was used as a probe to minimize cross-hybridization, a single signal was detected only in human liver (Fig. 1D). These results resemble those of Northern blot analysis with the whole CYP4F3 cDNA and its specific cDNA fragment as probes (Fig. 1A and B). These findings indicate that human liver and PMN LTB_4 ω-hydroxylases are specifically expressed in the corresponding tissues.

In humans, LTB_4 is metabolized by two different pathways, one of which is ω-oxidation in PMN [2–6], the other being the 10, 11-reductase pathway in various human cells such as lung macrophage [26], glomerular mesangial cells [27] and keratinocytes [28], and lung tissue [29]. However, little information is available on the metabolism of LTB_4 in human liver. In this paper, we showed that LTB_4 is hydroxylated at the ω-position by microsomes from all of the 14 human liver specimens examined. Furthermore, we have isolated and sequenced the cDNA encoding human liver LTB_4 ω-hydroxylase. This enzyme exhibited 87.3% homology with human PMN LTB_4 ω-hydroxylase (CYP4F3)[7] in its amino acid sequence, indicating that it is a member of the CYP4F subfamily. However, the K_m of the liver enzyme is 63-fold higher than that of the PMN enzyme. These two enzyme are separately expressed in the liver and PMN. Preliminary studies on the purification indicate that the liver enzyme is much more unstable compared to the PMN enzyme. The liver is the major site for the uptake and degradation of leukotrienes. We suggest that the human liver LTB_4 ω-hydroxylase is of great importance for the inactivation of LTB_4 in human liver.

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