Primary Structure of Human Thromboxane Synthase Determined from the cDNA Sequence*

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Polymerase chain reaction techniques have been used to isolate a cDNA clone containing the entire protein coding region of thromboxane A₂ synthase (EC 5.3.99.5) from a human lung cDNA library. The cDNA clone hybridizes with a single 2.1-kilobase mRNA species in phorbol ester-induced human erythroleukemia and monocytic leukemia cell lines. A second cDNA, differing only by an insert of 163 base pairs near the 3'-end of the translated region, was also found to be present in the same library. The proteins predicted from both nucleic acid sequences include the three polypeptide sequences determined from amino acid sequencing of the purified human platelet enzyme, five potential sites for N-glycosylation, and a hydrophobic region that may serve to anchor the synthase in the endoplasmic reticulum membrane. The longer predicted protein, designated thromboxane synthase-I, contains 534 amino acids, with a Mₗ of 60,684, whereas the shorter protein, designated thromboxane synthase-II, contains 460 amino acids and has a Mₗ of 52,408. Although thromboxane synthase-II lacks the conserved cysteine that serves as the proximal heme ligand in the other cytochromes, significant sequence similarities exist among thromboxane synthase-I and -II and several P450s, particularly those in family 3. The overall amino acid identity is considerably less than 40%, making it likely that thromboxane synthase represents a previously undefined family of cytochrome P450.

Thromboxane A₂ (TXA) is a potent inducer of platelet aggregation and vasoconstrictor (1-3). It plays a pivotal role in thrombus formation, atherogenesis and traumatic or endotoxemic shock (4-6). Inhibition of its biosynthesis appears to have beneficial effects on reducing these catastrophic disorders (7). TXA is synthesized primarily in platelets and monocyte-macrophages and is also produced in lung, kidney, spleen, brain, and gastrointestinal tracts (8-11). Its biosynthesis involves arachidonate release, prostaglandin endoperoxide formation, and isomerization to TXA. The last step is catalyzed by TXA synthase. Besides forming thromboxane, purified TXA synthase also fragments prostaglandin H₂ into a hydroxyprostanoic acid and malondialdehyde; the ratio of products from the two pathways is about 1:1 but is somewhat variable (11, 12). The enzyme exhibits absorbance spectrum features characteristic of a cytochrome P450 but has no monoxygenase activity (11, 13). During catalysis, TXA synthase undergoes a "suicide" inactivation (14) that may be a physiological mechanism for limiting TXA formation. The level of TXA in cells can be increased by erythropoietin stimulation, diacylglycerols and 1,25-dihydroxy vitamin D₃ (15-17). Although the biosynthesis of TXA has been extensively studied, little is known regarding the chemistry of the enzyme due to the difficulty in obtaining a sufficient amount of active protein (11-13). We have recently reported the sequence of a cDNA encoding a part of TXA synthase (18). As a part of our investigation into the mechanisms of enzyme catalysis and into the control of expression of the gene, we report here the isolation of a full-length cDNA clone which contains the entire protein coding region of TXA synthase.

MATERIALS AND METHODS

Cloning and Sequencing of TXA Synthase cDNA—We recently obtained a ~700-bp TXA synthase cDNA using the "nested primer" PCR technique (18). This cDNA was used as a probe to screen a λgt11 human lung cDNA library (Clontech, Palo Alto, CA). The probe was ³²P-labeled by random primer and used to hybridize replicate filters containing about 2.5 × 10⁶ plaques under the following conditions: 6 × SSC (1 × SSC is 15 mM sodium citrate, pH 7.0, and 150 mM NaCl), 0.5% SDS, 2 × Denhardt's solution (50 × Denhardt's solution contains 1% each of Ficoll, bovine serum albumin, and polyvinylpyrrolidone), and 10 mM EDTA at 54 °C. The filters were washed with 2 × SSC and 0.5% SDS at 68 °C for 30 min, then with 0.5 × SSC and 0.5% SDS at 68 °C for 30 min. The EcoRI fragments of the inserts of the positive plaques were subcloned into the pGEM7Zf (Promega, Madison, WI) or M13 mp18 and sequenced using Sequenase (U. S. Biochemical Corp.) and the dideoxy chain termination method (19). Compression regions were sequenced with inosine mix substituted for guanosine mix.

RNA Blot Analysis—A human erythroleukemia cell line (HEL) and a monocytic leukemia cell line (THP-1) were obtained from American Type Culture Collection, Rockville, MD. Both cell lines were induced to differentiate by incubating with culture medium containing 50 nM PMA at 37 °C for 48 h. Total RNA was prepared as described (20). Poly(A⁺) RNA was prepared by using oligo-dT column chromatography. The RNA blot analysis was performed as follows. RNA preparations were size-fractionated by electrophoresis in a 1.0% agarose gel containing formaldehyde, transferred to Zetabind membrane (Bio-Rad), and then hybridized with the 1.9-kb cDNA which had been labeled with ³²P by random primers. Hybridization was performed at 54 °C in 1× Church buffer (0.5 mM NaOH, PO₄,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) M90646 and M90647.

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The abbreviations used are: TXA, thromboxane A₂; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; PMA, phorbol 12-myristate 13-acetate; bp, base pair(s); kb, kilobase(s).
Reverse Transcription and Polymerase Chain Reaction—Four oligonucleotides were synthesized for reverse transcription and PCR. P1 and P3 were complementary to the sense strand at positions 1802 and 1593, respectively. P2 and P4 corresponded to the sense strands at positions 61 and 1063, respectively. The sequences are: P1, 5'-GGGACATACGTCGATAT; P2, 5'-CTCTGACGTCGCTCGT-3'; P3, 5'-GGTGACATCTCGATATAGACCC; and P4, 5'-ACCTACCTAGCTGCGCCTAA. The added linker site is underlined.

Reverse transcription-PCR was performed according to a standard procedure (20), using 2 μg of poly(A)+ RNA from PMA-treated HEL cells as template and P1 (200 ng) as the primer in 20 μl of amplification buffer (10 mM Tris, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 0.01% gelatin) containing 20 units of Taq DNA polymerase. The entire cDNA sequences of the three clones were determined by dideoxy nucleotide sequencing using "13 universal primer, T7 primer, SP6 primer, and unique synthetic oligonucleotides as primers corresponding to the nucleotide sequences near the translational start and stop sites (see "Materials and Methods" section for details). A restriction enzyme digestion analysis of the resulting PCR product indicated that it was the same as the composite full-length lung cDNA except for the presence of an additional DNA fragment of about 160 bp between the HindIII and KpnI sites. A re-examination of the lung cDNA library by PCR, using primers corresponding to the sequences upstream of the HindIII site and downstream of the KpnI site (P3 and P4) gave roughly equal amounts of two PCR products, indicating that equal populations of two mRNA were present in the source of the lung library. One of these PCR products was about 370 bp long (as was expected from TXS 3), whereas the other was about 160 bp longer. The sequence of the longer fragment (530 bp) was determined, revealing a 163-bp segment (positions 1369-1531 in Fig. 1) in the lower panel. The putative transmembrane segment is indicated by the bar labeled M, the bars labeled E, F, I, J, and L indicate segments with sequence similarity to the E, F, I, J, and L helices in the P450cam structure (29).

While this manuscript was under review Yokoyama et al. (22) reported the isolation and sequencing of a TXA synthase clone from a human platelet library. Their sequence is almost exactly the same as that of the longer message we isolated from the human lung library. This confirms that the PCR product with the 163-bp insert is derived from the TXA synthase message itself rather than from a related cytochrome P450 message. It is not known whether the platelet library contains cDNA corresponding to that of the shorter message found in the lung library.

The total length of TXA synthase cDNA including the 163-bp insert but not the poly(A) tail is 2087 bp. The cDNA contained a 1602-bp open reading frame encoding 534 amino acids, flanked by 355 bp of 5′- and 169 bp of 3′-untranslated regions (Fig. 1). The cDNA lacking the 163-bp insert contained a 1380-bp open reading frame encoding 460 amino acids. The nucleotide sequences and deduced amino acid sequences are presented in Fig. 2; the longer protein is designated TXA synthase-I, the shorter one TXA synthase-II.

**RESULTS AND DISCUSSION**

Our strategy for the isolation of the full-length cDNA clone involved screening of the human lung Agt11 cDNA library with the 700-bp probe isolated previously (18). The location of this fragment relative to the full-length cDNA is indicated as PCR in Fig. 1 (panel A). Screening of approximately 5 × 10⁶ plaques from this library led to the identification of two positive clones, designated TXS 2 and TXS 4 (Fig. 1). TXS 4 was indeed from the same cDNA rather than from the presence of an additional DNA fragment of about 160 bp between the HindIII and KpnI sites. A re-examination of the
More than 90% of the sequence was determined on both DNA strands. The published partial amino acid sequences obtained from the NH₂ terminus and two tryptic peptides of the purified human platelet enzyme, including a total of 83 amino acids (12), are consistent with the deduced amino acid sequences of both TXA synthase-I and -II except for three residues: Asn → Gln at position 269, Asn → Gln at position 270, and Glu → Asp at position 278. These discrepancies may be due to genetic polymorphism or to difficulties in interpretation of amino acid sequencer data. The agreement between the peptide sequences and amino acid sequences deduced from the cDNA confirms the correct reading frame and is strong evidence for the authenticity of the cDNA clone. The amino acid compositions of TXA synthase-I and -II deduced from the cDNA sequences both agree reasonably well with that of the purified protein (11). Alternate splicing of the TXA synthase message might account for the presence of multiple forms of the synthase protein. A variable extent of glycosylation, synthase-I1 is 52,408.

The number of forms of TXA synthase, and their exact size, remains controversial. Two distinct species of TXA synthase, with molecular masses of 53 and 56 kDa, were detected in WI-38 human lung fibroblasts (11), and molecular mass values of 50 and 59 kDa have been reported for the human platelet protein (11,13). The published partial amino acid sequences obtained from the NH₂ terminus and two tryptic peptides of the purified human platelet enzyme, including a total of 83 amino acids (12), are consistent with the deduced amino acid sequences of both TXA synthase-I and -II except for three residues: Asn → Gln at position 269, Asn → Gln at position 270, and Glu → Asp at position 278. These discrepancies may be due to genetic polymorphism or to difficulties in interpretation of amino acid sequencer data. The agreement between the peptide sequences and amino acid sequences deduced from the cDNA confirms the correct reading frame and is strong evidence for the authenticity of the cDNA clone. The amino acid compositions of TXA synthase-I and -II deduced from the cDNA sequences both agree reasonably well with that of the purified protein (11). Alternate splicing of the TXA synthase message might account for the presence of multiple forms of the synthase protein. A variable extent of glycosylation, synthase-I1 is 52,408.

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The common NH₂-terminal portion of synthase-I and synthase-II contains a strongly hydrophobic segment whose secondary structure is predicted to be helical (indicated by M in Fig. 1 panel C). As shown in Fig. 4, the sequence of this segment is similar to those of the NH₂-terminal segments proposed to act as membrane anchor in eukaryotic P450s (31), and it may serve the same function for TXA synthase.

The heme prosthetic group and the residues that form the heme-binding pocket are important structural features in enzymes (31). The carboxyl-terminal region is where the synthase message in the HEL cells by both reverse transcription-PCR and RNA blotting (Fig. 3), indicates that other than TXA synthase are from GenBank. The accession numbers are as follows: II.A4, human P450IIIA4 (A29815; also known as 3A4 or nifedipine oxidase); HFLA, human P450 HFLA (JX0062; also known as 3A7 or HFL33); GLUC, human hepatic glucocorticoid-inducible P450 (A29410; also known as HLP or 3A3); and CAM, Pseudomonas putida camphor hydroxylase (A29660).

The sequence in the synthase (beginning at position 413) is REAQAQDCE, whereas that in the peroxidase (starting at position 908) is RAAADQDSE (33). The presence in the synthase of a cysteine in place of a serine makes this particular cysteine an interesting candidate for an alternative proximal heme ligand. The evidence for the presence of two distinct TXA synthase with selected family 3 cytochromes P450. The alignment was done according to Lawrence et al. (35). Hyphens indicate residues identical with those in both TXA synthase-I and II (Regions 1–3) or with residues in synthase-I alone (Region 4). The segments corresponding to helices E, F, I, J, and L, and the cysteine pocket, in P450cam, and the postulated transmembrane segment in the eukaryotic cytochromes P450, are indicated by horizontal arrows. Data on sequences other than TXA synthase are from GenBank. The accession numbers are as follows: II.A4, human P450IIIA4 (A29815; also known as 3A4 or nifedipine oxidase); HFLA, human P450 HFLA (JX0062; also known as 3A7 or HFL33); GLUC, human hepatic glucocorticoid-inducible P450 (A29410; also known as HLP or 3A3); and CAM, Pseudomonas putida camphor hydroxylase (A29660).

The carboxyl-terminal region of other P450s contains a conserved cysteine residue thought to be the proximal ligand for the heme iron, and also helix L, considered to form part of the helix-heme-helix sandwich structure in other P450 enzymes (31). The carboxyl-terminal region is where the sequence of TXA synthase-I diverges from that of TXA synthase-II, due to the 163-bp insert at position 1389 in the cDNA sequence. Both the cysteine pocket (with the crucial heme ligand at position 480) and the helix L regions are present in synthase-I; both are absent in synthase-II. TXA synthase-II thus might be expected to be unable to form heme ligands in the usual P450 fashion. However, one of the 11 cysteines in the deduced synthase sequences is in a short segment with considerable similarity to thyroid peroxidase.

The evidence for the presence of two distinct TXA synthase messages in the lung tissue raises the possibility of alternate splicing of the mRNA; the observation of only one species of synthase message in the HEL cells by both reverse transcription-PCR and RNA blotting (Fig. 3), indicates that any alternate splicing occurs in a tissue-specific manner. The differences between the two predicted sequences in strongly conserved segments near the carboxyl terminus suggests they might differ in catalytic activity. It is noteworthy that the ratio of the alternate products of catalysis by TXA synthase,
of synthase-I and -II remains an intriguing question. Whether this variation arises from different populations of synthase-I and -II remains an intriguing question.

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