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ISOLATION OF PARTIAL COMPLEMENTARY DNA ENCODING HUMAN THROMBOXANE SYNTHASE

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SUMMARY Thromboxane synthase catalyzes the biosynthesis of thromboxane A_2 which plays a key role in the proaggregatory and vasoconstrictive processes. In this communication, we reported the successful cloning of thromboxane synthase cDNA from a human lung cDNA library. Oligonucleotides were synthesized according to the direct amino acid sequence of 2 peptides derived from purified human thromboxane synthase. Polymerase chain reaction was carried out using these oligonucleotides as primers to isolate a complementary DNA from human lung cDNA library. The longest cDNA thus obtained was 687 base pairs in length. Amino acid sequences deduced from the cDNA contained all three peptide sequences reported, confirming the authenticity of the cDNA clone. © 1991 Academic Press, Inc.

Thromboxane A_2 (TXA_2) is a potent inducer of platelet aggregation and a constrictor of vascular and respiratory smooth muscles (1-3). It has been implicated as a mediator of several important disease processes, such as thrombosis, atherosclerosis and asthma (4,5). TXA_2 is produced by these tissues upon injury or activation. Its biosynthesis is catalyzed by a series of enzymes including phospholipase A_2, prostaglandin H synthase and thromboxane synthase (3). Thromboxane synthase catalyzes the final step of conversion of prostaglandin H_2 to TXA_2. However, this enzyme is thought also to catalyze the conversion of prostaglandin H_2 to 5, 8, 10-heptadeca- trienoic acid (HHT) and malondialdlyde (MDA). Although this remains controversial as prostaglandin H_2 may

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be converted to HHT and MDA by nonenzymatic hydrolysis (6). Recent studies indicate that like PGH synthase, this enzyme undergoes suicidal autoinactivation (7). The mechanism by which this occurs remains unclear. Lack of understanding of the enzymatic mechanisms and the enzyme regulation stems from the difficulty in obtaining sufficient quantities of purified enzymes because of its low abundance in cells. Recently, thromboxane synthase was purified from porcine lung (8) and human platelets (9) by affinity chromatography. Unfortunately, the purified enzyme loses most of its activity due to low pH elution. An alternative way to obtain a fully active enzyme in large quantity is to use molecular biological techniques to express the cDNA in cells. Up to date, cDNA of thromboxane synthase has not been cloned. As an initial step to achieve this goal, we attempted to clone the cDNA based on the partial peptide sequences reported by Nusing et al (9). In this communication, we report the successful cloning of thromboxane synthase using the polymerase chain reaction technique. Partial sequence of the cDNA is reported here. The deduced amino acid sequence from this clone is in agreement with the partial amino acid sequence derived from purified enzyme.

MATERIALS AND METHODS

Polymerase Chain Reaction (PCR). Based on the direct amino acid sequence of the N-terminal peptide and the tryptic Peptide 2 reported by Nusing et al (9), the oligonucleotide primers were synthesized according to human codon usage frequency (10). The nucleotide sequences of primers are shown in Fig. 1. A human lung cDNA library constructed in λgt 11 was purchased from Clontech. About 10^5 phages from cDNA library were grown per plate and phages from ten plates were harvested and combined. Phage DNAs used as template for PCR were prepared by standard procedures (11). An aliquot of cDNA (0.5 μg) was used for PCR amplification in 50 μl of reaction mixture containing 50 mM KCl, 10 mM Tris, pH 8.3 at 23°C, 1.5 mM $MgCl_2$, 0.01% gelatin, 1 μM of each primer, 200 μM of dNTP and 2.5 units of Taq DNA polymerase (Perkin Elmer-Cetus). Thirty five cycles were carried out in a GTC-1 thermal cycler (Precision Scientific) equipped with an external cooling system (50% methanol + 50% water at -10°C).

In the case that less than 100-bp PCR product was expected, amplification was carried out as follows, 1st cycle, 3 min at 94°C, 1 min at 42°C and then, 1 min at 72°C; 2nd-34th cycles, 40 sec at 94°C, 30 sec at 42°C, 20 sec at 72°C; final cycle, 40 sec at 94°C,

30 sec at 42°C, 5 min at 72°C. If more than 100-bp PCR product was expected, PCR conditions are: first cycle, 5 min at 94°C, 2 min at 42°C, 3 min at 72°C; 2nd-34th cycles: 1 min at 94°C, 1 min at 42°C, 3 min at 72°; final cycle: 1 min at 94°C, 1 min at 42°C, 5 min at 72°C. The PCR products were resolved on a 5% polyacrylamide gel, the DNA fragment of the anticipated size was excised and extracted with 50 µl of 10 mM Tris, pH 7.5 and 0.1 mM EDTA. Subsequently, the extracted DNA was re-amplified using kinased primers before blunt-ended ligation into SmaI site of pGEM-7Zf (Promega) for nucleotide sequencing.

DNA Sequencing. Nucleotide sequences were determined by dideoxy chain termination method (12) using sequenase (United States Biochemicals). Alkali-denatured plasmid DNA was sequenced in conjunction with custom-synthesized 20-mer oligonucleotide primers (complementary to the cDNA sequence) and primers to the sequences flanking the cloning site. Overlapping sequences were obtained for both strands of the cDNA.

RESULTS AND DISCUSSION

PCR has been used to facilitate a number of techniques in molecular biology, including cloning, sequencing and modifying specific nucleotide sequences. Recently, Nusing et al (9) purified the thromboxane synthase from human platelets and reported the amino acid sequence of 3 peptides (N-terminal peptide and peptides 1 and 2) which provide useful information for PCR cloning. Since successful PCR does not require a perfect complementarity of a primer to its target sequence, we designed several nucleotide sequences as primers based on human frequent condon usage. Each primer has 20-24 bases in length and 16-256 redundancies. These primers span the N-terminal and Peptide 2 regions reported by Nusing et al, as shown in Fig. 1. We then amplified the human lung cDNA library (about 10^6 complexities) with a combination of these primers. In one case, the cDNA library was first amplified with primers W25 and W26, which correspond to amino acid residue number 1 to 7 of the N-terminal peptide and number 15 to 22 of Peptide 2, respectively (Fig. 1). After amplification, the PCR products were diluted 3000-fold with 10 mM Tris, 0.1 mM EDTA and 1 µl of the diluted sample was re-amplified with primers W27 and W26. We designated such combination of amplification as 25-27/26-26. A 68-bp DNA fragment was expected as predicted from the direct amino

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N-terminus:

Met-Glu-Ala-Leu-Gly-Phe-Leu-Lys-Leu-Glu-Val-Asn-Gly
    G   C   G   G   T       A       G   G
ATG GGA GCT CTC GGC TTC CT> AAG CTG GAG GTC AAC GGC
         W25                              W21

-Pro-Met-Val-Thr-Val-Ala-Leu-?-Val-Ala-Leu-Leu-Ala
   A
 CCT ATG>

Peptide 2:

Val-Pro-Leu-Ala-Arg-Ile-Leu-Pro-Asn-Lys-Asn-Arg-Asp-Glu
  G   A   C   C C C   T   C           A   C C C
GTC CCT CTG GCT AGT ATC CTG CC> AAC AAG AAT AGT GAC GAG
         W27                                W24

-Leu-Asn-Gly-Phe-Phe-Asn-Lys-Leu-Ile-Arg-Asn-Val-Ile-Ala-Leu
 GTG AA>
          C   A   A       T               C   A   G   G
     <TTG CCG AAG AAG TTG TTC GAC     <TTG CAG TAG CGA GAC
          W26                                W28

-Arg-Asp-Asn-
 G G   A
 TCA CTG TT

Peptide 1

Ile-Lys-Gln-Val-Leu-Val-Glu-Asn-Phe-Ser-Asn-Phe-Thr-Asn-Arg
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Fig. 1. Sequences of amino acid and corresponding customer-synthesized oligonucleotides. Direct amino acid sequencing was from published results (9). The names and 5'→3' direction of oligonucleotides are indicated.

acid sequence. This DNA fragment was extracted from the polyacrylamide gel, amplified with W27 and W26, subcloned into pGEM-7Zf and finally sequenced with T7 primer. The nucleotide sequence along with the deduced amino acid sequence between W27 and W26 was shown in Fig. 2A. The deduced amino acid sequence is in agreement with the reported amino acid sequence. Similarly, the PCR products of 27-24/28-28 gave a DNA fragment of 71-bp in length. The amino acid sequence predicted from the sequence between W24 and W28, again, agrees well with the direct amino acid sequencing (Fig. 2A).

In an attempt to isolate a longer stretch of thromboxane synthase cDNA, we first synthesized two oligonucleotides which were the non-coding strands of the PCR products of 25-27/26-26 and 27-24/28-28, designated as W30 and W29, respectively. Then we amplified the cDNA library with a combination of primers, i.e. 25-

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A. Amplification          Sequence
   25-27/26-26          C AAT AAG AAC CGA GAC GAA CTG
                          Asn Lys Asn Arg Asp Glu Leu

   27-24/28-28          T GGC TTT TTT AAC AAA CTC ATT AGG
                          Gly Phe Phe Asn Lys Leu Ile Arg

B. GTG ACG GTG GCC CTG TCA GTG GCT CTC TTG GCC CTC CTG AAA TGG TAC
   Val Thr Val Ala Leu Ser Val Ala Leu Leu Ala His Leu Lys Trp Tyr

   TCC ACA TCA GCA TTC TCA AGA CTG GAG AAG TTA GGC CTC AGA CAT CCC
   Ser Thr Ser Ala Phe Ser Arg Leu Glu Lys Leu Gly Leu Arg His Pro

   AAG CCT TCT CCT TTC ATT GGA AAC TTG ACA TTT TTC CGC CAG GGT TTT
   Lys Pro Ser Pro Phe Ile Gly Asn Leu Thr Phe Phe Arg Gln Gly Phe

   TGG GAA AGC CAA ATG GAG CTC AGA AAG CTG TAT GGA CCT CTG TGT GGG
   Trp Glu Ser Gln Met Glu Leu Arg Lys Leu Tyr Gly Pro Leu Cys Gly

   TAC TAT CTT GGT CGT CGG ATG TTT ATT GTT ATT TCT GAG CCA GAC ATG
   Tyr Tyr Leu Gly Arg Arg Met Phe Ile Val Ile Ser Glu Pro Asp Met

   ATC AAG CAG GTG TTG GTT CAG AAC TTC AGT AAC TTT ACC AAC AGA ATG
   Ile Lys Gln Val Leu Val Glu Asn Phe Ser Asn Phe Thr Asn Arg Met

   GCG TCG GGT TTG GAG TTC AAG TCG GTA GCC GAC AGC GTT CTG TTT TTA
   Ala Ser Gly Leu Glu Phe Lys Ser Val Ala Asp Ser Val Leu Phe Leu

   CGT GAC AAA AGA TGG GAA GAG GTC AGA GGT GCC CTG ATG TCT GCT TTC
   Arg Asp Lys Arg Trp Glu Glu Val Arg Gly Ala Leu Met Ser Ala Phe

   AGT CCT GAA AAG CTG AAC GAG ATG GTT CCC CTC ATC AGC CAA GCC TGC
   Ser Pro Glu Lys Leu Asn Glu Met Val Pro Leu Ile Ser Gln Ala Cys

   GAC CTT CTC CTG GCT CAT TTA AAA CGC TAT GCG GAA TCT GGG GAC GCA
   Asp Leu Leu Leu Ala His Leu Lys Arg Tyr Ala Glu Ser Gly Asp Ala

   TTT GAC ATC CAG AGG TGC TAC TGC AAT TAC ACC ACA GAT GTG GTT GCC
   Phe Asp Ile Gln Arg Cys Tyr Cys Asn Tyr Thr Thr Asp Val Val Ala

   AGC GTC CCG TTT GGC ACC CCG GTG GAC TCC TGG CAG GCC CCT GAG GAT
   Ser Val Pro Phe Gly Thr Pro Val Asp Ser Trp Gln Ala Pro Glu Asp

   CCC TTT GTG AAA CAC TGC AAG CGT TTC TTC GAA TTC TGC ATC CCC AGA
   Pro Phe Val Lys His Cys Lys Arg Phe Phe Glu Phe Cys Ile Pro Arg

   CCT ATC CTG GTT TTA CTC TTA TCA TTT CCA TCC ATA ATG GTC CCA CTG
   Pro Ile Leu Val Leu Leu Leu Ser Phe Pro Ser Ile Met Val Pro Leu

   GCC CGG ATT TTG CCC
   Ala Arg Ile Leu Pro
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Fig. 2. Partial nucleotide sequence of thromboxane synthase obtained from PCR amplification. (A) Nucleotide sequences within the Peptide 2. "Amplification" indicates the primers used for PCR. The deduced amino acid is shown under each codon. (B) Nucleotide sequence and the deduced amino acid sequence of the human thromboxane synthase cDNA between N-terminus and the Peptide 2. The amino acid sequences underlined indicate the three peptides obtained from direct amino acid sequencing of the thromboxane synthase.

21/26-28, 25-21/30-29, 25-21-21/30-29-28. The PCR products of all the combinations revealed a common band of nearly 700 bp in size. This band was extracted, amplified with W21 and W30 and subsequently cloned into pGEM-7Zf. Fig. 2B shows the nucleotide sequence between W21 and W30. The deduced amino acid sequences of

both N-terminus and Peptide 2 are consistent with the reported sequences. In addition, it also contained the third reported amino acid sequence, i.e. Peptide 1 described by Nusing et al (9) (Fig. 1). These results provided strong evidence that this cDNA clone was indeed the authentic cDNA encoding the thromboxane synthase.

We estimate that we have obtained about half of thromboxane synthase cDNA predicted from the presumed molecular weight of human thromboxane synthase of 58 kDa by using PCR alone. This 700-bp cDNA is an important tool for isolating the full length cDNA and genomic DNA of thromboxane synthase. It will also be valuable for determining gene expression by Northern blot analysis. Availability of this cDNA will advance the understanding of mechanism of enzyme catalysis and regulation of thromboxane synthase gene expression.

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