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Communication

Molecular Cloning and Expression of the cDNAs Encoding Human and Yeast Mevalonate Pyrophosphate Decarboxylase*

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The importance of lowering serum cholesterol levels for the prevention of cardiovascular disease has been well documented. Because mevalonate pyrophosphate decarboxylase is a unique enzyme in the cholesterol biosynthetic pathway it is a potential therapeutic target for the treatment of hypercholesterolemia and other diseases. For this reason we cloned and expressed the cDNA for the human enzyme. We also cloned and expressed the yeast homolog using the human enzyme's similarity to a previously unidentified and incomplete genomic sequence. Northern blot analysis revealed a transcript of approximately 2 kilobases in a variety of human tissues. The recombinant human enzyme is a homodimer of 43-kDa subunits with a specific activity of 2.4 units/mg. Computer searches for similarity with known sequences showed that mevalonate pyrophosphate decarboxylase has little similarity to other proteins.

The mevalonate pyrophosphate decarboxylase (MPD)[1] enzyme of the cholesterol/ergosterol biosynthetic pathway converts the 6-carbon compound mevalonate pyrophosphate (MevPP) into isopentenyl pyrophosphate, the 5-carbon isoprene building block of a large family of biomolecules. Besides decarboxylation, this unusual enzyme dehydrates its substrate while hydrolyzing ATP. MPD, which performs the first committed step in the biosynthesis of isoprenes, has been purified from several animal and plant sources, but a comprehensive examination of the enzyme or its mechanism is lacking (1, 2).

The prodrug, 6-fluoromevalonate, has been shown to lower cholesterol biosynthesis (3, 4), block protein prenylation (5, 6), and inhibit the proliferation of various transformed cells (7, 8). Upon pyrophosphorylation with endogenous cellular enzymes this compound becomes a potent competitive inhibitor of MPD (3, 4). For these reasons MPD appears to be a potential pharmaceutical target for the treatment of hypercholesterolemia and unwanted cellular proliferation. In order to better study this enzyme we cloned and expressed the cDNA for MPD from human liver and, by sequence similarity, from yeast.

EXPERIMENTAL PROCEDURES

Isolation of Partial cDNA Clones—Purified rat liver MPD (9) was digested with trypsin, and the resulting peptides were isolated and sequenced (Harvard MicroChem). Using the amino acid sequences of the termini from one of these peptides we designed polymerase chain reaction (PCR) primers and amplified the corresponding cDNA fragment from a rat liver cDNA λ library (Stratagene 936513). This PCR reaction yielded a DNA fragment that when sequenced corresponded to the amino acid sequence of the peptide that was used as the template. A ^{32}P-labeled oligonucleotide encoding the unique DNA sequence between the PCR primers was then used to probe the same rat liver cDNA library. Three positive clones were isolated and sequenced. These clones were identical to each other, were incomplete because they only encoded the last half of the open reading frame (ORF), and contained a large 3′-untranslated region.[2]

A DNA fragment from the incomplete rat liver cDNA clone described above was then used to probe what was advertised as a human liver λ cDNA library (Stratagene 937220). We isolated and sequenced 11 positive clones. The largest clone was designated 18-1, and it encoded an estimated 90% of the ORF. This estimate was based on a molecular mass of 45 kDa for the rat protein (9) and on the absence of a methionine start codon.[2] Subsequent to our demonstration of MPD activity with human liver cDNA derived from another source (see below), we were informed by Stratagene that the advertised human liver cDNA library was actually made from a non-human tissue (possibly Chinese hamster ovary cells). This accident did not alter our final results because we had used human liver RNA to isolate the full-length cDNA sequence shown in Fig. 1 (see below).

5′- and 3′-RACE Experiments—5′-RACE experiments were performed on two independent sources of human liver RNA. Identical results were obtained with both preparations.

Primers for the 5′-RACE experiments were GSP1 (gene-specific primer 1, GGCCACATGCACCTTATAGC) and GSP2 (CTGTGAAGTCCTTGCTAATGG) and were purchased from Biosynthesis Inc. A 5′-RACE kit (18374–025) was purchased from Life Technologies, Inc. Total RNA was isolated from a 1-g sample of frozen human liver using a Life Technologies, Inc. total RNA isolation kit. A sample of human liver mRNA was also purchased from Clontech. Forty cycles of standard PCR conditions (94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min) were used to produce a 230-base pair (bp) band, which was subcloned into pCRII (Invitrogen) and transformed into *Escherichia coli*. Several independent subclones from the two different RNA sources were isolated and sequenced.

Primers for the 5′-RACE experiments were designed with the DNA sequence of the 18-1 clone (see above). At that time we did not know that 18-1 was derived from a non-human tissue. In spite of the coding differences due to the nonhuman sequence of the primers,[3] we obtained a 5′-end for the human liver MPD cDNA (nucleotides 1–170 of Fig. 1).

Using a unique DNA sequence obtained from the 5′-RACE experiments and a sample of human liver total RNA, we performed a 3′-RACE experiment and isolated the 3′-end of the cDNA (nucleotides 151-1812 of Fig. 1). The primers used for the 3′-RACE experiment were GSP1 (TGGTTCTGCCCATCAACTC) and GSP2 (ACTCTGCACCAGGACCAGTT). A 3′-RACE kit was purchased from Life Technologies, Inc. Total RNA prepared in the 5′-RACE experiment was utilized. PCR conditions were altered using the PCR Optimizer kit (Invitrogen). Conditions

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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) U49260 and U49261.

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[1] The abbreviations used are: MPD, mevalonate pyrophosphate decarboxylase; MEVPP, mevalonate pyrophosphate; bp, base pair(s); kbp, kilobase pair(s); ORF, open reading frame; PCR, polymerase chain reaction; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; GSP, gene-specific primer; EST, expression sequence tag; RACE, rapid amplification of cDNA ends.

[2] M. Toth and L. Huwyler, unpublished observations.

[3] In the 5′-RACE experiment primer GSP1 had 4 mismatches out of 20 bases while GSP2 had 3 mismatches out of 21 bases when compared with Fig. 1.

FIG. 1. **Nucleotide sequence of human liver mevalonate pyrophosphate decarboxylase cDNA and its deduced amino acid sequence.** ORF region is shown in *capital letters*.

were: 30 mM Tris-HCl, pH 9.0, 7.5 mM $(NH_4)SO_4$, 1.3 mM $MgCl_2$, 10% Me_2SO; 30 cycles of 94 °C for 1 min, 42 °C for 1 min, and 72 °C for 2 min. A 1.7-kbp band was isolated and subcloned, and several transformants were sequenced.

Full-length cDNA Amplification, Subcloning, and Mutagenesis of Human MPD—The primers for the full-length amplification of MPD cDNA were 5′ primer (GACCATGGCCTCGGAGAAG) and 3′ primer (CAGCAATGCTGGTTTATTC). We used the first strand synthesis material derived from a sample of human liver total RNA and the amplification conditions of the 3′-RACE experiment. A DNA fragment of 1.8 kbp was produced and subcloned into pCRII for sequencing.

The PCR primers used to modify the 1.8-kbp full-length cDNA fragment for subcloning into the expression vectors were 5′-BamNde (ACGGGATCCATATGGCCTCGGAG) and the 3′ primer of the full-length amplification. We used the 1.8-kbp DNA fragment from the full-length amplification and 20 standard PCR cycles. The resulting amplified DNA was first subcloned into pCRII and then into pET-14b (Novagen) using *Nde*I and *Xho*I or into pVL1392 (Invitrogen) using *Eco*RI.

The original subclone from the full-length PCR reaction contained five mutations compared with the consensus sequence of Fig. 1. Three of these mutations were coding changes ($A^{44}T$ gave $Thr^{13} \rightarrow Ser$, $G^{514}C$ gave $Glu^{169} \rightarrow Asp$, and $T^{1163}A$ gave $Cys^{386} \rightarrow Ser$) and two were noncoding ($T^{661}G$ gave AGT to AGG at Ser^{218}, and $a^{1460}g$ in the 3′-noncoding region). We used the Sculptor *in vitro* mutagenesis kit (Amersham Corp.) and three mutagenic oligonucleotides (at one time) to change the coding sequence of the original full-length subclone to the consensus sequence shown in Fig. 1. The activity of human MPD shown in Table I comes from this corrected subclone.

Growth, Extract Preparation, and Enzyme Activity Determination of Recombinant MPD—Strain BL21(DE3) was transformed with the pET14b (Novagen) construct for *E. coli* expression and induced with 0.4 mM isopropyl thiol-β-galactoside for 3 h. High Five cells (Invitrogen) were transfected with the pVL1392-derived subclone to give a high titer lysate. For baculovirus/insect cell expression, High Five cells were then infected with the high titer lysate and harvested after 72 h of growth.

Cell pellets from both expression systems were sonicated in homogenization buffer (20 mM Tris/HCl, pH 7.5, at 20 °C, 15 mM EDTA, 15 mM EGTA, 100 μM leupeptin, 0.75 mg/liter aprotinin, 0.1 mM phenylmethanesulfonyl fluoride) and assayed for enzyme activity and protein concentration. Activity was measured as the conversion of labeled MevPP into isopentenyl pyrophosphate as adapted from Cardemil and Jabalquinto (10). The assay solution contained 50 mM BisTris/HCl, pH 7.0, at 20 °C, 1 mM dithiothreitol, 10 mM $MgCl_2$, 5 mM ATP, and 3.8 μM [3-^{14}C]mevalonate pyrophosphate (DuPont NEN, 54 mCi/mmol). Assays were conducted at 37 °C for 1 h. To stop the reaction and to convert the substrates/products, 2 mg of alkaline phosphatase (Sigma P-3877) dissolved in 25 μl of 1 M Tris was introduced, and the sample was incubated for 30 more min. Two ml of Econofluor-2 (DuPont NEN) were then added, and the sample was capped, shaken, and counted on a scintillation counter. Because of the organic nature of the scintillation fluid, the dephosphorylated product (isopentenyl) is solubilized and counted whereas the dephosphorylated substrate (mevalonic acid) is not. A "no enzyme" sample was used to determine the background. Protein concentrations were determined using the dye-binding method (Bio-Rad).

Library Screening and Subcloning of Yeast MPD—A yeast cDNA library (Clontech YL1007b) was amplified and its DNA isolated using a λ DNA isolation kit (Promega). Using standard PCR conditions, the primers TTACACAGCATCCGTTACCG and GGAATCATGACCATCTTCAGC, and the isolated library DNA, we amplified a 530-bp fragment of the unidentified ORF next to *COQ2* (11). This DNA fragment was used to probe the same library with the ECL system of random primer labeling and detection (Amersham Corp.). Three overlapping positive

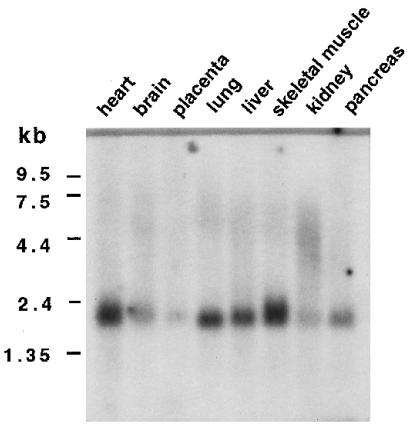

FIG. 2. **Northern blot of various human tissues probed with human MPD cDNA.** Size standards are along the *left margin*. See text for details.

TABLE I
Activity of recombinant mevalonate pyrophosphate decarboxylase in crude extracts

Expression systems used and enzymatic activity measured are as described under "Experimental Procedures."

System	Strain	Activity[a]
E. coli	Vector alone	<0.093
	Human MPD	52
	Yeast MPD	1700
Baculovirus (High Five cells)	No virus	37
	Human MPD	1800

[a] Expressed as nanomoles of product/mg of extract protein.

clones were identified. We modified one clone by PCR to introduce an ATG start codon and to allow subcloning into the same *E. coli* expression system as the human liver enzyme. The DNA sequence of the yeast clone expressing MPD activity has been submitted to the public data bank. Its deduced peptide sequence is displayed in Fig. 3.

Purification and Analysis of Recombinant Human MPD—250 ml of High Five cells (Invitrogen) were infected with the high titer lysate containing human MPD for 72 h and harvested by centrifugation. The cell pellet was sonicated, and enzyme activity was assayed as described above. The sonicate was centrifuged at 12,000 × *g* for 10 min at 4 °C, and a 40–70% ammonium sulfate fractionation of the supernatant was made. The precipitate from the ammonium sulfate fraction was resuspended in 50 mM potassium phosphate, pH 7.5, 1.3 M ammonium sulfate and applied to a 10-ml column of phenyl-Sepharose Fast Flow (Pharmacia Biotech Inc.) in the same buffer at 20 °C. A 2 × 50-ml linear gradient from 1.3 to 0 M ammonium sulfate over 4 h was used to elute activity. Active fractions were pooled, concentrated using a Centricon-30 concentrator (Amicon), and stored in assay buffer with 50% glycerol. We used a 10% SDS-polyacrylamide gel to estimate the purity of the sample. Gel filtration chromatography was performed at 20 °C using a Superdex 200 HR 10/30 column (Pharmacia) in 20 mM Tris/HCl, pH 8.0, 150 mM NaCl and a flow rate of 1 ml/min. Protein standards for size exclusion chromatography were purchased from Bio-Rad.

Northern Blot Analysis—A Northern blot of various human tissues, each lane containing 2 μg of mRNA, was purchased from Clontech (7760–1) and probed with a ^{32}P-labeled DNA fragment (Amersham Corp., Mega-prime kit RPN 1606) from the human MPD sequence (800-bp *Pst*I fragment, bp 246-1038 of Fig. 1). Exposure time was 3 days at −80 °C with an intensifying screen.

RESULTS AND DISCUSSION

The cDNA for human liver MPD was identified by using DNA sequence information originally obtained from a partial cDNA λ clone of rat liver MPD (see "Experimental Procedures") and from RACE experiments. We used the sequence information obtained in 5′- and 3′-RACE experiments to amplify a 1.8-kbp full-length cDNA directly from a sample of human liver total RNA. We sequenced three independent clones from two different full-length PCR reactions and obtained a consensus. Fig. 1 shows this consensus cDNA sequence with a representative poly(A) tail attached at the 3′-end. Large stretches of sequence identity with several expression sequence tag clones (EST) further substantiated the MPD cDNA sequence (see below). Inspection of Fig. 1 suggests that transcription starts at the methionine codon at bp 8 within a Kozak box sequence and continues for 400 amino acids producing a 43-kDa protein. A large 3′-untranslated region is also evident.

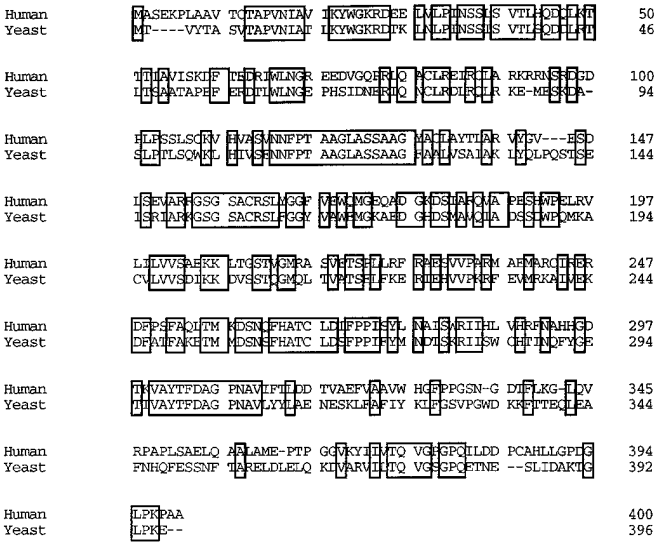

FIG. 3. **Comparison between the deduced human liver and yeast MPD protein sequences.** Identical regions are *boxed*.

Fig. 2 shows a Northern blot of various human tissues probed with the human MPD cDNA sequence. A transcript of approximately 2 kilobases in size was observed for liver, skeletal muscle, heart, brain, placenta, lung, kidney, and pancreatic tissues.

Because the clones from the full-length amplification had coding mutations compared with Fig. 1, one clone was modified and mutated so as to encode the consensus ORF (see "Experimental Procedures"). The resulting DNA fragment was subcloned into *E. coli* and baculovirus expression vectors. Shown in Table I are the MPD activity levels of extracts made from cells with these expression systems. In the *E. coli* system the cells containing human liver MPD cDNA showed substantial enzyme activity whereas the control cells had no detectable activity. Because *E. coli* does not inherently possess MPD activity (12) its presence indicates that we have cloned and expressed the human enzyme. The baculovirus/insect cell system showed a >50-fold increase in activity compared with the background level of uninfected cells.

To find if there was any similarity between MPD and any reported sequence we searched the publicly available data banks with the BLASTN, BLASTP, BLASTX, and TBLASTN programs (13). Only two classes of similarity emerged. One class included over 20 EST clones while the other class was an unidentified and incomplete ORF adjacent to the *COQ2* gene of *Saccharomyces cerevisiae* (11). From the level of similarity it appeared that the unidentified ORF was the yeast MPD gene. Based on the desirability of obtaining the yeast homolog of MPD, we decided to clone and to express this sequence. Consequently, we screened a yeast cDNA library with the unidentified ORF and obtained three overlapping clones. We subcloned the yeast cDNA sequence into the same *E. coli* expression system that was used for the human enzyme and found MPD activity as shown in Table I. In this expression system the yeast MPD clone yielded more enzyme activity in the crude extract than the human clone. The reason for this difference may be related to the presence of insoluble MPD

protein only with the human clone.[2]

Fig. 3 compares the predicted peptide sequences for both MPD enzymes, and it shows a 45% level of identity between the two homologs. Because the neighboring *COQ2* gene encodes *para*-hydroxybenzoate:polyprenyltransferase, it appears that this region of the yeast genome is involved with isoprene metabolism.

Significant DNA sequence identity to human liver MPD was also found with 20 EST clones derived from human tissues such as brain, spleen, and liver. We obtained and sequenced[2] one of the EST clones (ATCC 85596) that was derived from human fetal brain tissue and found it to be nearly identical to Fig. 1. Besides liver, brain tissue is another rich source of cholesterol biosynthesis. Apparently the human MPD cDNAs from infant brain and adult liver are identical.

We also found similarities with EST clones derived from *Arabidopsis thaliana*, *Oryza sativa*, and *Caenorhabditis elegans*. Though only partial sequence information is available, these EST clones would appear to be partial MPD cDNA sequences from those organisms.

We performed a scan for pattern recognition elements in the human MPD protein sequence using the PROSITE program from the EMBL-Heidelberg (14). No patterns were observed out of the 1011 tested. Combining the results of the BLAST and PROSITE searches, it appears that MPD is unique with little similarity to other protein sequences or to other non-MPD cDNA sequences.

We purified human MPD from the baculovirus/insect cell expression system to 50% purity as judged by SDS-polyacrylamide gel electrophoresis. We measured a specific activity of 2.4 units/mg and determined the apparent K_m values for MEVPP and ATP to be 2 and 600 μM, respectively. Further analysis using gel filtration chromatography revealed that the inclusion of 1 mM dithiothreitol was necessary to retain enzyme activity during elution. Omission of this reducing agent caused a loss of activity and a reduction in apparent molecular mass of the protein from 100 to 50 kDa. We interpret this to mean that active recombinant human MPD is a homodimer of 43-kDa subunits. These parameters are similar to those reported for MPD isolated from chicken liver (10), pig liver (15), and rat liver (9).

In summary, we have cloned and expressed the cDNAs for human liver and yeast MPD. Because of the relevance of MPD to cholesterol metabolism, studies are under way to observe if inhibitors of this enzyme will alter serum lipid levels. Because the prodrug 6-fluoromevalonate inhibits MPD activity after it becomes pyrophosphorylated and subsequently blocks the proliferation of Ras-transformed cells (7, 8), we also consider this enzyme to be a target for diseases such as cancer and restenosis. The production of recombinant MPD may allow a more complete picture of the mechanism and the structure of this remarkable enzyme. Furthermore, the cloning of yeast MPD should extend our understanding of sterol biosynthesis in this well studied eukaryote.

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