Human Pyridoxal Phosphatase

MOLECULAR CLONING, FUNCTIONAL EXPRESSION, AND TISSUE DISTRIBUTION*

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Pyridoxal phosphatase catalyzes the dephosphorylation of pyridoxal 5’-phosphate (PLP) and pyridoxine 5’-phosphate. A human brain cDNA clone was identified to the PLP phosphatase on the basis of peptide sequences obtained previously. The cDNA predicts a 296-amino acid protein with a calculated Mr of 31698. The open reading frame is encoded by two exons located on human chromosome 22q12.3, and the exon-intron junction contains the GT/AG consensus splice site. In addition, a full-length mouse PLP phosphatase cDNA of 1978 bp was also isolated. Mouse enzyme encodes a protein of 292 amino acids with Mr of 31512, and it is localized on chromosome 15.E1. Human and mouse PLP phosphatase share 93% identity in protein sequence. A BLAST search revealed the existence of putative proteins in organisms ranging from bacteria to mammals. Catalytically active human PLP phosphatase was expressed in Escherichia coli, and characteristics of the recombinant enzyme were similar to those of erythrocyte enzyme. The recombinant enzyme displayed Km and kcat values for pyridoxal of 2.5 μM and 1.52 s⁻¹, respectively. Human PLP phosphatase mRNA is differentially expressed in a tissue-specific manner. A single mRNA transcript of 2.1 kb was detected in all human tissues examined and was highly abundant in the brain. Obtaining the molecular properties for the human PLP phosphatase may provide new direction for investigating metabolic pathway involving vitamin B6.

Pyridoxal 5’-phosphate (PLP)† is the coenzymatically active form of vitamin B6 and plays an important role in maintaining the biochemical homeostasis of the body (1, 2). Thus, the other

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† The abbreviations used are: PLP, pyridoxal 5’-phosphate; PNP, pyridoxine 5’-phosphate; PMP, pyridoxamine 5’-phosphate; PLPP, PLP phosphatase; h, human; m, mouse; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends.
Human Recombinant Pyridoxal Phosphatase

EXPERIMENTAL PROCEDURES

Materials—We purchased a human brain cDNA library as well as a dot blot array containing poly(A)⁺ RNAs from human tissues from Clontech (Palo Alto, CA). Restriction endonuclease and other cloning reagents were purchased from New England Biolabs, Inc. (Beverly, MA) or Promega. Double-stranded DNA probes were radiolabeled with [α-³²P]dCTP (3000 Ci/mmol) from Amersham Biosciences using a commercial random priming kit (Amersham Biosciences). All other reagent-grade chemicals were obtained from standard suppliers.

Identification and Cloning of Human Brain PLP Phosphatase (hPLPP) cDNA—BLAST searches, conducted with PLP phosphatase peptides (AQGVLFDCDGVL and AVLVGYDEHFSFAK) as query sequences, revealed a full match with the predicted amino acid sequence of a CDNA (GenBank™/EBI accession number NM020315). This clone was used to design PCR primers for the cloning of human PLP phosphatase cDNA.

We employed 5'-rapid amplification of cDNA ends (RACE) using hPLPP-specific primer 1 (5'-CGATGCTGAAGTTCTCCGAGC-3'), AP1 anchor primer, and Marathon Ready cDNA (human whole brain, Clontech) as a template. PCR was carried out with betaine in a GeneAmp PCR system 2400 (PerkinElmer Life Sciences) for 30 cycles of denaturation (94° C, 30 s annealing; 60° C, 1 min; 72° C, 2 min extension). The resulting PCR product of 737 bp fragment revealed that the 5'-untranslated region is completely matched except 9 bp shorter than the cDNA from lung carcinoma. The full-length open reading frame (ORF) of 891 bp was amplified using the hPLPP-specific primers (sense, 5'-GATACCGCTGTCGGCCGTCGGA-3'; antisense, 5'-GATGC- CCGGTTGGCCTGTCGGCATG-3'). The PCR product was cloned into the plasmid pGEM-T vector (Promega) and sequenced (GenBank™ accession number AY125047).

A multiple protein sequence alignment of the human PLP phosphatase clone, along with the sequences most closely related to it, was performed using the ClustalW (version 1.74) program (23), and the resulting aligned sequence was shaded using Boxshade. Identification of the chromosomal localization of the human PLP phosphatase was performed by a BLASTN search of the human genome sequences of the National Center for Biotechnology Information (NCBI).

Cloning of Mouse Brain PLP Phosphatase (mPLPP) cDNA—We also identified a mouse expressed sequence tag (EST) clone (GenBank™ accession number AK043228) with high similarity to human PLP phosphatase. To obtain a full-length cDNA corresponding to this EST clone, 5'-RACE and 3'-RACE were performed using mouse brain cDNA (See-gene, Seoul, Korea) according to the manufacturer's protocol. Two consecutive PCR reactions using Ex Taq polymerase (Takara) were performed as follows: (1) 5'-RACE, 94° C for 3 min followed by 94° C for 1 min and 65° C for 40 s, followed by primer extension at 72° C for 1 min followed by 94° C annealing; (2) 3'-RACE primer (5'-TCCACGAGGCGATCTCTACT-3') and mPLPP-specific primer 1 (5'-ATCCACGCTCCCTCCTACT-3') and mPLPP-specific primer 2 (5'-CATGGAATGTCCGACGACTACGT-3') and mPLPP-specific primer 2 (5'-ATGGAAAAGGGGACTTCCAGG-3'). The PCR products were cloned and sequenced.

Expression in E. coli and Purification of Human PLP Phosphatase—The PLP phosphatase cDNA was cloned between the BamHI of the bacterial expression vector pQE30 (Qiagen) after PCR amplification. Transformants of E. coli M15/pPR4 were constructed with resulting pQE30-hPLPP construct were grown at 37° C in 200 ml of LB medium with 100 μg/ml ampicillin and 25 μg/ml kanamycin. The plasmid pREP4 constitutively expresses the Lac repressor protein encoded by the lacI gene to reduce the basal level of expression (Qiagen). When that culture had grown to an A₅₇₀ of 0.6, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After inducing the expression of the PLP phosphatase protein for 16 h at 25 °C cells were harvested, washed, and resuspended in 20 ml of 20 mM Tris-HCl buffer, pH 7.4, containing 200 mM NaCl and 20 mM imidazole.

The cell suspension was sonicated, and the lysate was cleared by centrifugation at 10,000 g × 4 for 30 min. The supernatant was then poured into the column loaded with the nickel-nitrioltriacetic acid agarose (Qiagen), washed with Tris buffer containing 40 mM imidazole, and eluted protein was eluted with 200 mM imidazole. The purity of the eluted protein was evaluated by 12% SDS-PAGE using Coomassie blue staining to visualize the protein.

Enzyme Assay and Kinetic Characterization—The enzymatic activity of PLP phosphatase was measured at pH 7.4 in 40 mM triethanolamine-HCl. The rate of production of pyridoxal from PLP was measured by following the decrease in absorbance at 390 nm for at least 3 min. The initial velocity data were fitted by a least-squares method to the Lineeweaver-Burk transformation of the following equation,

\[ v = \frac{V_{\max}}{K_m + [S]} \]

where \([S]\) represents the concentration of the varied substrate PLP, and \(K_m\) represents the Michaelis constant.

A molecular weight of 64,000 for PLP phosphatase with a specific activity of 1.4 using \(\mu\)mol⁻¹ min⁻¹ was used in the calculations of molecular enzyme concentrations. One unit of specific activity is defined as the amount of protein that catalyzes the formation of pyridoxal/min from PLP. PLP is known to have an extinction coefficient of 4900 cm⁻¹ M⁻¹ at pH 7. Protein concentrations were determined by the Bradford assay using the reagent and micro procedure from Pierce Chemical Co. Bovine serum albumin was used as a standard.

In addition, hydrolysis of all other substrates was measured in 40 mM triethanolamine-HCl, pH 7.4, containing 4 mM MgCl₂. The release of free phosphate from potential substrates was measured colorimetrically as the molybdate complex with malachite green (16, 24).

Multiple Tissue Expression Array—A Northern filter containing 8 human tissue-specific poly(A)⁺ RNAs and a dot blot array containing human poly(A)⁺ RNAs from various adult tissues, fetal tissues, and cancer cell lines were prehybridized at 65° C for 1 h in ExpressHyb™ hybridization solution (Clontech). The filters were then hybridized at 65° C for 16 h with ³²P-labeled cDNA probe containing the complete ORF. After washing as recommended by the manufacturer, blots were exposed to x-ray films at −70 °C with an intensifying screen for the appropriate time period. For scanning densitometry, the blot was scanned and BioLab Image software was used to quantify the signals. Blots were reprobed with β-actin as a loading control.

RESULTS

Identification and Cloning of the PLP Phosphatase cDNA from Human and Mouse—Using two tryptic peptide sequences from the erythrocytes PLP phosphatase previously reported by Gao and Fonda (21), we conducted NCBI BLAST searches of the human EST data base. A cDNA clone encoding both peptide sequences, designated a hypothetical protein dj37E 16.5 from lung carcinoma (GenBank™/EBI accession number NM020315) was identified. When the tryptic peptide sequences were aligned with the deduced amino acid sequence there was a perfect match, confirming that the cDNA in the EST GenBank™ data base codes for PLP phosphatase.

We used the sequence information for designing primers for PCR amplification utilizing a human brain cDNA library. Sequencing of this clone revealed that the brain cDNA is identical to the hypothetical protein from lung carcinoma. The ORF encodes a 296-amino acid protein with a molecular mass of 31698 Da. A computer calculation reveals that the isoelectric point for the protein is 6.12. ScanProsise software analysis by ExpASY showed that the deduced human protein has the following putative post-translational modification sites: 2 N-glycosylation sites, 7 phosphorylation sites, and 4 N-myristoylation sites.

In addition, we cloned a complete mouse homologue by 5'-RACE and 3'-RACE-PCR using an EST sequence (GenBank™/EBI accession number AK043228). As shown in Fig. 1, the longest cDNA contains 1978 bp consisting of a 879-bp ORF, a 13-bp 5'-untranslated region, and a 1086-bp 3'-noncoding region. The 3'-end of the sequence contains a poly(A) stretch, preceded by a putative polyadenylation signal AATAAA. The open reading frame encodes a 292-amino acid protein with a predicted molecular mass of 31512 Da and pl of 5.53. The deduced protein has a putative N-glycosylation site, 5 phosphorylation sites, and 4 amidation sites.

Using BLAST searches of the EST data bases, we found putative PLP phosphatases in various species. Multiple alignment of the protein sequence of PLP phosphatase with other homologous proteins or putative proteins was performed using the ClustalW program as shown in Fig. 2. All of the PLP...
phosphatases are highly homologous at the amino acid sequence level. The mouse PLP phosphatase sequence shows a high degree of similarity (94% identity and 98% similarity) with the human enzyme. The sequence identities of human PLP phosphatase to the hypothetical proteins from Danio rerio, Drosophila melanogaster, Caenorhabditis elegans, Arabidopsis thaliana, Saccharomyces cerevisiae, and E. Coli are 36, 28, 26, 25, 31, and 27%, respectively, and similarities share 77, 65, 68, 60, 70, and 66%, respectively. This result suggests that PLP phosphatase is highly homologous and widely expressed throughout evolution. The tryptic sequences from human erythrocytes enzyme that are perfectly matched with the deduced amino sequence are indicated by asterisks in Fig. 2.

Genomic Organization and Chromosomal Localization—Using PLPP cDNA as a query sequence, a BLAST analysis of the genomic sequence available through NCBI Web site mapped the PLP phosphatase gene to human chromosome 22q12.3. Mouse PLP phosphatase gene is located on chromosome 15.E1 with a very similar genomic organization to that observed in human. The ORF of PLP phosphatase is encoded by two exons...
as shown in Fig. 3. The intron/exon junctions followed the GT/AG rule (25) and were flanked by conserved sequences. The lariate branch-point in the consensus sequence is located at 23 nucleotides upstream of the 3′/H11032 splice site. The possible exon/intron junction site in mouse cDNA is also indicated by a slash in Fig. 1.

**Functional Expression and Catalytic Properties of the Human Recombinant Enzyme**—After *E. coli* M15/pQE30-hPLPP was induced with isopropyl-β-D-thiogalactopyranoside, the His6-tagged recombinant PLP phosphatase yielded a major band with an apparent molecular mass of 32 kDa on an SDS-PAGE gel corresponding to about 15% of the total applied protein (Fig. 4). Most of the expressed protein was found in the soluble extract after lysis of the cells. As shown in Table I, the crude extraction from 200 ml of cells contained 14.5 mg of total protein and 0.14 units/mg of PLP phosphatase activity. Purification by binding to Ni2+-nitrilotriacetic acid-agarose resin and elution with buffer containing 200 mM imidazole resulted in a near homogeneous preparation. The purified recombinant protein had a specific activity of 1.4 units/mg and appeared as a single band with a molecular mass of 32 kDa agreeing with the value calculated from the deduced sequence (Fig. 4, lane 6).

Activity of the human PLP phosphatase toward PLP, PNP, and PMP was determined (Table II). Recombinant human enzyme followed Michaelis-Menten kinetics with respect to PLP, and the Michaelis constant and $k_{cat}$ value for this reactant was $2.5 \mu M$ and $1.52 s^{-1}$, respectively. Km for PNP and PMP were 43 and $81 \mu M$, respectively, and $k_{cat}$ values were 1.25 and 0.45 s$^{-1}$, respectively, whereas activity with p-nitrophenyl phosphate was very low. Thus, the specificity constant of the recombinant PLP phosphatase was highest with PLP followed by PNP. The recombinant enzyme requires a divalent ion for activity, and Mg$^{2+}$ is the most effective in catalyzing the dephosphorylation of PLP.

To investigate the state of aggregation of recombinant human PLP phosphatase, the purified enzyme was examined by FPLC gel filtration at pH 7.4 in 50 mM triethanolamine-HCl buffer. The elution profile of the enzyme exhibits a symmetrical peak characterized by a molecular mass of about 60 kDa when the concentration of the recombinant protein in the fraction is around 10 μM (data not shown). These data clearly indicate
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**Fig. 3. Genomic organization of human and mouse PLP phosphatase genes.** Schematic representation of human (A) and mouse PLP phosphatase genes (B) arranged as two exons. Exon regions are denoted by boxes. Shadowed boxes represent ORF, and open boxes denote the 5'- and 3'-untranslated regions. Black lines indicate the introns. The consensus intron/exon junction sequences are underlined, and the intron/exon borders are marked by slashes.

**Fig. 4. Expression and purification of recombinant human PLP phosphatase.** Twelve percent of SDS-PAGE analysis of crude cell extracts of E. coli M15 containing the expression vector pQE30 without and with the coding sequence. Lane 1, low molecular mass standards (Bio-Rad Laboratory); lanes 2 and 3, crude extracts from cultured cells harboring pQE30 uninduced and induced with 1 mM isopropyl-1-thio-

that the recombinant enzyme is a dimer, agreeing with previous data for the erythrocyte enzyme.

**Tissue-specific Expression of Human PLP Phosphatase**—Dot blot array and Northern blot analyses were used to determine the expression pattern for PLP phosphatase in human tissues. As evident by multiple tissue expression (MTE™) array analysis, PLP phosphatase transcripts were detected in most tissues, indicating ubiquitous expression. However, the level of expression was markedly variable. PLP phosphatase was most highly represented in all the regions of central nerve system except the spinal cord (Fig. 5). The other major sites of expression were found in liver and testis. In fetus, expression levels of PLP phosphatase transcript showed a rather even distribution in all organs except brain. Like adult, fetal brain expressed PLP phosphatase transcript with a very high level. To determine the size of mRNA transcripts, Northern blots analyses were performed. A single 2.1-kb transcript was detected with variable intensity in all adult tissues examined: heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (data not shown).

**DISCUSSION**

In this report, we describe the first identification and characterization of cDNAs encoding a PLP phosphatase from human and mouse. A human cDNA clone of PLP phosphatase was identified by NCBI BLAST search on the basis of the amino acid sequences of two peptides from the purified human erythrocyte enzyme. Several lines of evidence confirm that the cDNA is indeed encoding the PLP phosphatase. First, the amino acid sequences of the two peptides of the purified protein are contained in the amino acid sequence deduced from the ORF (Fig. 2). Second, the molecular mass of the protein of 32 kDa calculated from the deduced cDNA sequence is identical to those of purified enzyme. The Kₚ values for the substrate PLP (2.5 μM) are practically indistinguishable from those of the erythrocyte (1.5 μM) (16).

Human PLP phosphatase cDNA encodes a 296-amino acid protein with a predicted molecular mass of 31698 Da. The hydropathy profile of PLP phosphatase indicated a rather even distribution of hydrophobic and hydrophilic regions throughout the molecule (data not shown). Therefore, no evidence for integral membrane domains in PLP phosphatase was found. The isolated cDNA contains a short 5'-untranslated segment similar to lung carcinoma cDNA. No larger product has been generated in repeated RACE experiments using different primer sets. Furthermore, the 2.1-kb message determined by Northern analysis agrees well with the size of the full-length cDNA, suggesting that the 5'-end of the cDNA is at or near the transcription start site. Therefore, the cloned human brain cDNA may be complete. By contrast, analysis the EST clone for mouse PLP phosphatase shows that it was incomplete. The mouse EST sequence (GenBank™/EMBL accession number AK043228) contains several unidentified nucleotides and a stop codon in ORF, but no polyadenylate tail. Thus, we cloned a full-length cDNA of mouse PLP phosphatase by 5'- and 3'-RACE-PCRs, and the sequence has been deposited in GenBank™/EMBL as accession number AY366300.

Comparison of the identified cDNA sequences with the genome sequences revealed the genomic structure and chromosomal localization. The PLP phosphatase gene is divided
into two exons, and the splice site sequences are remarkably well conserved as shown in Fig. 3. In mammals two ends of the intron are conserved by a donor-site consensus sequence AG/GURAGU and an acceptor-site consensus sequence (Y)nNCAG/G, where R, Y, and N indicate purine, pyrimidine, and any nucleotide, respectively (25, 26). A branch-point consensus sequence, YNCUGA, is usually about 15–40 nucleotides upstream of the 3’ splice site, and the adenosine in the sequence forms the 2’–5’ linkage in the lariat structure. In addition, the genomic sequences were examined for the pres-

<table>
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<th>Purification step</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>14.5</td>
<td>138</td>
<td>1</td>
<td>100%</td>
</tr>
<tr>
<td>Affinity chromatography: Ni-NTA agarose</td>
<td>1.4</td>
<td>1320</td>
<td>9.6</td>
<td>92.3%</td>
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### Table II

Kinetics of purified PLP-phosphatase with various substrates

<table>
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<tr>
<th>Compound</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (μmol/min/mg)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ μmol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP</td>
<td>2.5 ± 0.2</td>
<td>1.42 ± 0.04</td>
<td>1.52</td>
<td>6.08 × 10$^3$</td>
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<tr>
<td>PNP</td>
<td>43.4 ± 5.0</td>
<td>1.17 ± 0.17</td>
<td>1.25</td>
<td>2.70 × 10$^4$</td>
</tr>
<tr>
<td>PMP</td>
<td>80.6 ± 7.2</td>
<td>0.42 ± 0.06</td>
<td>0.45</td>
<td>5.58 × 10$^5$</td>
</tr>
<tr>
<td>p-NPP$^a$</td>
<td>0.04 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td></td>
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</tr>
</tbody>
</table>

$^a$Activity with 40 μM p-nitrophenylphosphate.

**Table I**

Purification of human recombinant PLP-phosphatase

**Fig. 5. Tissue-specific expression of the human PLP phosphatase mRNA.**

The human multiple tissue expression (MTE$^{TM}$) array was hybridized with a $^{32}$P-labeled human PLP phosphatase-specific cDNA probe. Tissue sources for the RNA are indicated below the blot.
ence of CpG islands using the CpG plot program in European Bioinformatics Institute (EBI), as defined by Gardiner-Garden and Frommer (27). The hPLPP gene contains a CpG island with a CG\text{obs}/CG\text{exp} ratio in excess of 0.8 and a GC content of 74% spanning a region from 343-bp upstream to 614-bp downstream of the start codon, whereas the mPLPP gene has a CpG island extending from \(-282\) bp to \(+643\) bp with a CG content of 71%.

We also reported here the functional expression of human PLP phosphatase. The expressed protein was purified by a one-step affinity chromatographic method, and consequently endogenous PLP phosphatase produced by E. coli was eliminated. Enzymological analysis of the recombinant human protein expressed from this cDNA clone showed a high specificity for the hydrolysis of PLP. Characteristics of the recombinant enzyme were similar to those from the native enzyme purified from human erythrocytes, except kinetic values for PNP (16). Noteworthy is the relatively high \(V_{\text{max}}\) and \(K_m\) with PNP as a substrate. As shown in Table II, the \(K_m\) values of recombinant enzyme for PNP and PLP were 43 and 2.5 \(\mu M\), respectively, whereas the \(V_{\text{max}}\) values for PNP were surprisingly high as 1.2 units/mg, which is almost same to the value for PLP (1.4 units/mg). Judging from the kinetic results, the catalytic efficiency (\(k_{\text{cat}}/K_m\)) for PNP was 20-fold lower than that of PLP. On the other hand, the kinetic values for the native enzyme were similar to those from the native enzyme purified from human erythrocytes, except kinetic values for PNP (16).

In conclusion, the results obtained in this study will provide valuable information on the action mechanism of human PLP phosphatase and the physiological role of this enzyme. The availability of the cDNAs encoding PLP phosphatase, and the recombinant enzyme will be helpful to expand our view of metabolic pathways that could involve PLP phosphatase and to evaluate its functional role in human cellular homeostasis.

REFERENCES