Molecular Cloning and Expression of a Mouse Thiamin Pyrophosphokinase cDNA*

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Thiamin pyrophosphokinase (EC 2.7.6.2) catalyzes the pyrophosphorylation of thiamin with adenosine 5'-triphosphate to form thiamin pyrophosphate. A mouse thiamin pyrophosphokinase cDNA clone (mTPK1) was isolated using a combination of mouse expressed sequence tag database analysis, a two-step polymerase chain reaction procedure, and functional complementation screening with a Saccharomyces cerevisiae thiamin pyrophosphokinase-deficient mutant (thi80). The predicted protein contained 243 amino acid residues with a calculated molecular weight of 27,068. When the intact mTPK1 open reading frame was expressed as a glutathione S-transferase fusion protein in Escherichia coli lacking thiamin pyrophosphokinase, marked enzyme activity was detected in the bacterial cells. The corresponding 2.5-kilobase pair mRNA was expressed in a tissue-dependent manner and was found at relatively high levels in the kidney and liver, indicating that the mode of expression of mTPK1 genes differs with cell type. The expression of mTPK1 genes in cultured mouse neuroblastoma and normal liver cells was unaffected by the thiamin concentration in the medium (10 μM versus 3.0 μM). This is the first report on identification of the primary sequence for mammalian thiamin pyrophosphokinase.

Vitamin B1 is a water-soluble vitamin found in four different forms: thiamin, thiamin monophosphate, thiamin pyrophosphate (TPD),1 and thiamin triphosphate (1, 2). Thiamin is the main form of the vitamin in blood plasma of animals and can be taken into cells across the membrane via carrier-mediated processes (3–5). Inside the cells, thiamin is phosphorylated to TDP, a dominant form in almost all cells. TDP functions as a precursor for thiamin triphosphate that is suspected to have a specific role in neuronal activity (8, 9).

An important aspect of thiamin metabolism in mammals is the regulation of the intracellular levels of the four thiamin forms in a narrow range. The main factors involved in this regulation are membrane-associated thiamin transport systems and the cellular enzyme, thiamin pyrophosphokinase (TPK; EC 2.7.6.2). A number of biochemical analyses suggest that several types of thiamin transport systems exist in mammals, and in several cells the driving force for thiamin uptake appears to be its phosphorylation to TDP (3–5). Recently, the mammalian thiamin transport protein, THTR-1, was identified as the gene mutated in thiamin-responsive megaloblastic anemia (Online Mendelian Inheritance in Man number: 249270) (10–12). However, although the enzyme has been purified from several mammalian sources (13–15), the nucleotide sequences of TPK have been determined only for the microorganisms (16, 17) Saccharomyces cerevisiae (THI80), Schizosaccharomyces pombe (tnr3), and Paracoccis denitrificans (pTPKI). The isolation of the cDNA or genomic DNA for TPK from mammalian cells is indispensable for the elucidation of thiamin metabolism and its regulation in higher animals. In this study, we isolated a new mouse cDNA, whose translation product is homologous to the TPKs of the microorganisms, by the combination of expressed sequence tag (EST) database analysis, a two-step polymerase chain reaction (PCR) procedure (18), and functional complementation screening using the yeast TPK-deficient mutant (thi80) (19). The expression experiment in Escherichia coli showed that the cloned cDNA, termed mTPK1, produced the TPK activity. We also investigated the steady-state mRNA levels in various mouse tissues and two cultured cells grown under thiamin-sufficient or -deficient conditions.

EXPERIMENTAL PROCEDURES

Organisms and Cultures—The E. coli strains DH5a and BL21 were used to amplify plasmids and express the recombinant protein, respectively. The S. cerevisiae thi80 mutant strain used in this study was T48-2D (α thi80–1 ura3–52 his3 A200 leu2–1 trp1–Δ65) (16). The media and the growth conditions for the yeast and bacterial cells were as described previously (16). Glucose in the yeast minimal medium was replaced with 2% galactose for inducing the transcription from the GAL1 promoter in the yeast expression vector pYES2 (Invitrogen). The mouse neuroblastoma cell line, Neuro 2a (ATCC CCL-131), and the
mouse normal liver cell line, NMuLi (ATCC CRL-1638), were purchased from Dainippon Pharmaceutical Co. Ltd.; Suita, Japan. The mouse cells were grown in the special Dulbecco’s modified Eagle’s medium without thiamin (Life Technologies, Inc.) and supplemented with glucose (6 mg/ml), 5% fetal bovine serum (Equitech-Bio; Ingram, TX), and 2 mM glutamine at 37 °C in 5% CO2. Under this condition the only source of thiamin was the fetal bovine serum, and the final thiamin concentration was 3.0 nM, which was determined using the method previously described (20); this medium will be referred to as thiamin-deficient medium.

For amplification of the 5'-cDNA. obtained following the 5'-gated with yeast expression vector and employed for functional complementation screening with the S. cerevisiae TPK-deficient mutant. The full-length cDNA was obtained following the 5'- and 3'-RACE methods using R4 and F3 primers, respectively.

Enzyme Assays—Thiamin-repressible acid phosphatase activity of the thi80 mutant on plates was detected using a staining method based on the corresponding thiochrome (24). The protein was determined using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

The deduced amino acid sequence of the S. pombe TPK gene, tnr3, was used to search a mouse EST database, and the entry
Fig. 3. Expression of the GST-mTPK1 fusion protein in E. coli.

A, SDS-polyacrylamide gel electrophoresis. Crude extracts (10 μg of protein) of bacterial cells expressing GST alone (lane 2) and GST-mTPK1 (lane 3) were analyzed using 7.5% SDS-polyacrylamide gel electrophoresis as directed by Laemmli (33) and followed by Coomassie Blue staining. The position of the predicted GST-mTPK1 fusion protein is indicated by the arrow. Lane 1 shows molecular standards proteins (Bio-Rad): phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000). MW, molecular weight.

B, chromatogram of thiochrome from TDP on high-performance liquid chromatography. Crude extracts (10 μg of protein) of bacterial cells expressing GST alone (panel 2) or GST-mTPK1 (panel 3) were incubated for 30 min at 37 °C in the TPK assay mixture, and 100 μl of the solution, after conversion to the thiochrome, were injected onto the column. Panel 1 indicates the chromatogram from 1 nmol of authentic thiochrome. The position of the predicted GST-mTPK1 fusion protein is indicated by the arrow. Lane 1 indicates the chromatogram from 1 nmol of authentic thiochrome. Blue staining. The position of the predicted GST-mTPK1 fusion protein is indicated by the arrow. lane 1 shows molecular standards proteins (Bio-Rad): phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000). MW, molecular weight. B, chromatogram of thiochrome from TDP on high-performance liquid chromatography. Crude extracts (10 μg of protein) of bacterial cells expressing GST alone (panel 2) or GST-mTPK1 (panel 3) were incubated for 30 min at 37 °C in the TPK assay mixture, and 100 μl of the solution, after conversion to the thiochrome, were injected onto the column. Panel 1 indicates the chromatogram from 1 nmol of authentic thiochrome. Blue staining. The position of the predicted GST-mTPK1 fusion protein is indicated by the arrow. lane 1 shows molecular standards proteins (Bio-Rad): phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000). MW, molecular weight. B, chromatogram of thiochrome from TDP on high-performance liquid chromatography. Crude extracts (10 μg of protein) of bacterial cells expressing GST alone (panel 2) or GST-mTPK1 (panel 3) were incubated for 30 min at 37 °C in the TPK assay mixture, and 100 μl of the solution, after conversion to the thiochrome, were injected onto the column. Panel 1 indicates the chromatogram from 1 nmol of authentic thiochrome. Blue staining. The position of the predicted GST-mTPK1 fusion protein is indicated by the arrow. lane 1 shows molecular standards proteins (Bio-Rad): phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000). MW, molecular weight. B, chromatogram of thiochrome from TDP on high-performance liquid chromatography. Crude extracts (10 μg of protein) of bacterial cells expressing GST alone (panel 2) or GST-mTPK1 (panel 3) were incubated for 30 min at 37 °C in the TPK assay mixture, and 100 μl of the solution, after conversion to the thiochrome, were injected onto the column. Panel 1 indicates the chromatogram from 1 nmol of authentic thiochrome. Blue staining. The position of the predicted GST-mTPK1 fusion protein is indicated by the arrow. lane 1 shows molecular standards proteins (Bio-Rad): phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000). MW, molecular weight. B, chromatogram of thiochrome from TDP on high-performance liquid chromatography. Crude extracts (10 μg of protein) of bacterial cells expressing GST alone (panel 2) or GST-mTPK1 (panel 3) were incubated for 30 min at 37 °C in the TPK assay mixture, and 100 μl of the solution, after conversion to the thiochrome, were injected onto the column. Panel 1 indicates the chromatogram from 1 nmol of authentic thiochrome. Blue staining. The position of the predicted GST-mTPK1 fusion protein is indicated by the arrow. lane 1 shows molecular standards proteins (Bio-Rad): phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000). MW, molecular weight. B, chromatogram of thiochrome from TDP on high-performance liquid chromatography. Crude extracts (10 μg of protein) of bacterial cells expressing GST alone (panel 2) or GST-mTPK1 (panel 3) were incubated for 30 min at 37 °C in the TPK assay mixture, and 100 μl of the solution, after conversion to the thiochrome, were injected onto the column. Panel 1 indicates the chromatogram from 1 nmol of authentic thiochrome. Blue staining. The position of the predicted GST-mTPK1 fusion protein is indicated by the arrow. lane 1 shows molecular standards proteins (Bio-Rad): phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000). MW, molecular weight.

AA981202 was identified as a potential homologue. The sequence of the AA981202 EST clone was 411 bp in length, and the predicted translation product had significant sequence similarity with the carboxyl-terminal moiety of the tnr3 protein. The F1 and R1 primers based on the sequence of AA981202 amplified a predicted 409-bp cDNA from a mouse embryo cDNA library, suggesting that the corresponding cDNA clone was present in this library. A two-step PCR was then performed to isolate the 5'—region of the cDNA, as described in Fig. 1. Because the R2 primer used in the second PCR involved the deduced stop codon of AA981202, the second PCR product was expected to contain the intact ORF and was inserted into the yeast expression vector pYES2 downstream of the yeast GAL1 promoter. Thus, the constructed library was employed to isolate the mouse TPK cDNA using functional complementation screening with the S. cerevisiae TPK-deficient thi80 strain. The expression of enzymes involved in yeast thiamin metabolism, such as thiamin-repressible acid phosphatase coded by the PHO3 gene (25), are repressed by thiamin in the medium via the thiamin regulatory system, in which TDP serves as a corepressor (19). However, the expression of PHO3 in the thi80 mutant is not repressed even under high concentrations of thiamin in the medium (Fig. 2), which are caused by an insufficient increase in the intracellular concentration of TDP be-

Fig. 4. Amino acid sequence of the mTPK1 protein aligned with other TPKs. Protein sequences of mTPK1, tnr3 (accession number X84147), THI80 (D14417), and TPK1 (E10547) were aligned using the ClustalW program. Identical residues are indicated by dark shading, and similar residues are indicated by light shading. Numbers on the right side of the figure indicate amino acid position. medium, suggesting that the intracellular concentration of TDP in the thi80 mutant was increased by the product of the mouse cDNA inserted in pYES2-MT.

We identified an 835-bp mouse cDNA in pYES2-MT that contained a single ORF whose ATG translation start codon conformed to Kozak's rules (26). The predicted protein encoded by the ORF contained 243 amino acid residues with a calculated molecular weight of 27,068. This numerical value was similar in size to the determined molecular mass of the purified TPK polypeptide from human red cells (28,000 Da) (13). To ascertain whether this mouse ORF actually encodes TPK, we attempted to express the ORF as a GST fusion protein in E. coli, in which TPK does not exist and TDP is synthesized from thiamin monophosphate by thiamin-monophosphate kinase (EC 2.7.4.16) (27). Fig. 3A shows the patterns of SDS-polyacrylamide gel electrophoresis of the crude extracts of bacterial cells expressing GST or the GST fusion protein. A 53-kDa band, which was almost consistent with the expected size, appeared in the crude extract of cells inducing the fusion protein. After the samples were dialyzed sufficiently, the TPK activities in the crude extracts were determined. As shown in Fig. 3B, marked TPK activity was detected in the cells expressing the GST fusion protein, whereas no enzyme activity was detected in the cells expressing GST alone. When thiamin monophosphate, for which the TPK is completely inactive (1), was used as substrate in place of thiamin, TDP formation was not observed in the cells. Moreover, potassium ions, which are required for thiamin-monophosphate kinase activity of E. coli (27), were not included in the mixture used for TPK activity. It was therefore concluded that this mouse ORF encodes a mouse TPK, and the predicted translation product had significant sequence similarity with other TPKs.
We compared the putative amino acid sequence of the mTPK1 protein with those of TPKs of other organisms such as <i>S. pombe</i>, <i>S. cerevisiae</i>, and <i>P. denitrificans</i> (Fig. 4). The amino acid sequence of mTPK1 showed 39% identity with the carboxyl-terminal half of tm3, which appears as a fusion protein (569 amino acids) with unknown function at the amino-terminal half (17). The mTPK1 protein was also similar to THI80 and pTPK1 over their full lengths (31% of amino acid identity). Amino acid sequence homology could not be detected between the conserved regions of TPKs and the TDP-dependent enzymes, suggesting that the structural feature in the interaction of amino acids with thiamin differs from that with TDP. It was also not possible to assign the ATP-binding site to any region of TPKs although many types of consensus sequences for peptide segments involved in nucleotide-binding sites are proposed (28). The determination of functional domains in TPK polypeptides may facilitate the comprehension of the transfer mechanism of pyrophosphate from ATP to thiamin.

To determine the expression pattern of the mTPK1 gene, we performed Northern blot analysis on various mouse tissues with the coding region as the probe. As shown in Fig. 5A, a 2.6-kilobase pair single band was detected predominantly in the kidney, followed by the liver, when the membrane was exposed to x-ray film for 2 weeks. The faint bands of the same size were detected in other tissues, including the brain, lung, and testis. The full-length mTPK1 mRNA was isolated using the 5′-RACE and 3′-RACE methods. The length of isolated transcript from mouse embryonic cells was 2,563 bp, which was almost consistent with that of the detected bands. The full-length mTPK1 cDNA contained a 5′-noncoding region of 56 bp and a long 3′-noncoding region of 1,738 bases, in which four putative polyadenylation sites, AATAAA (29), were found (nucleotides 1790, 1796, 1800, and 2410). These findings indicated that the mTPK1 gene was expressed in a tissue-specific manner. An unexpected finding was that a very low level of mTPK1 expression was observed in the brain, in which the glucose and energy metabolism are aggressive. However, the distinctive mode of expression of the mTPK1 gene may be present in some areas of the central nervous system.

It was previously demonstrated that the expression of the TPK gene (THI80) in <i>S. cerevisiae</i> is partially repressed by thiamin in the medium (16), in which intracellular TDP acts as a corepressor. In mammals, the relationship between the expression of TPK and the provision of thiamin are indistinct. Sanio et al. (30) reported that TPK activity was reduced in liver and heart muscle of thiamin-deficient rats, whereas Trebukhina et al. (31) reported that the activity was increased transiently in the livers of mice deprived of thiamin. To examine whether the expression of the mTPK1 gene is controlled by thiamin or its phosphorylated derivatives, the effect of thiamin content in the medium on the steady-state mRNA level in mouse neuroblastoma and normal liver cell lines was investigated. Fig. 5B shows the results of a reverse transcriptase-PCR experiment for mTPK1 in two cell lines growing for 2 weeks in thiamin-sufficient (10 μM) or -deficient (3.0 nM) medium. As expected from the results of the multiple tissue blot, the relative message level in neuroblastoma cells was about 20% of that in the liver cells by quantitation of radioactivity using the bioimaging analyzer system. However, the mRNA levels of both nerve and liver cells were unaffected by the thiamin concentration of the medium, suggesting that thiamin or a thiamin derivative does not participate in the regulation of mTPK1 in mouse cells. This finding was in contrast to TDP-dependent enzymes, such as transketolase and pyruvate dehydrogenase, whose mRNA levels in human cultured cells are decreased by thiamin deficiency (32).

In conclusion, this is the first report on identification of the primary sequence for mammalian TPK. It was not possible to isolate mammalian TPK cDNA using a conventional functional complementation strategy, possibly because of the very low levels of expression of TPK. The successful cloning revealed in this study appeared to be caused by selective enrichment of the desired clone using the two-step PCR procedure. This study suggests that, using the mTPK1 cDNA, progress will be made in understanding mammalian thiamin metabolism at the molecular level. Recently, we have isolated a human cDNA whose nucleotide sequence was nearly identical to mTPK1, and investigation to clarify the function of this human clone is in progress.

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