Molecular Cloning of Human Phosphomevalonate Kinase and Identification of a Consensus Peroxisomal Targeting Sequence*

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Two overlapping cDNAs which encode human liver phosphomevalonate kinase (PMKase) were isolated. The human PMKase cDNAs predict a 191-amino acid protein with a molecular weight of 21,862, consistent with previous reports for mammalian PMKase (Mr = 21,000-22,500). Further verification of the clones was obtained by expression of PMKase activity in bacteria using a composite 1024-base pair cDNA clone. Northern blot analysis of several human tissues revealed a doublet of transcripts at approximately 1 kilobase (kb) in heart, liver, skeletal muscle, kidney, and pancreas and lower but detectable transcript levels in brain, placenta, and lung. Analysis of transcripts from human lymphoblasts subcultured in lipid-depleted sera (LDS) and LDS supplemented with lovastatin indicated that PMKase gene expression is subject to regulation by sterol at the level of transcription. Southern blotting indicated that PMKase is a single copy gene covering less than 15 kb in the human genome. The human PMKase amino acid sequence contains a consensus peroxisomal targeting sequence (PTS-1), Ser-Arg-Leu, at the C terminus of the protein. This is the first report of a cholesterol biosynthetic protein which contains a consensus PTS-1, providing further evidence for the concept that early cholesterol and nonsterol isoprenoid biosynthesis may occur in the peroxisome.

The pathway of cholesterol and nonsterol isoprenoid biosynthesis provides a variety of products necessary for growth of mammalian cells, including cholesterol, bile acids, heme A, dolichol, and ubiquinone. This pathway is subject to multiple levels of regulation at several sites, at both the post-translational and transcriptional level (1). The key regulatory site is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)1 reductase, which catalyzes the reaction producing mevalonate. When levels of cholesterol are high, HMG-CoA reductase is subject to down-regulation at the transcriptional level, increased turnover of protein, and inactivation of existing enzyme. Conversely, when cholesterol levels are low, transcription of HMG-CoA reductase increases, protein turnover decreases, and the existing protein can be reactivated. In addition, the levels of low density lipoprotein receptors, which are responsible for cellular uptake of cholesterol, are regulated at the level of transcription. Through these mechanisms, mammalian cells have the capacity to precisely regulate cholesterol and nonsterol isoprenoid metabolites, thereby avoiding overaccumulation of potentially toxic metabolites.

Although HMG-CoA reductase is a key regulatory site in the pathway, enzymes and genes further along the pathway are also important in regulation of pathway function. Activities of several enzymes involved in mevalonate disposition, mevalonate kinase (MKase), phosphomevalonate kinase (PMKase), and mevalonate diphosphate decarboxylase (MDDase), are responsive to cholesterol intake in animals (2–8). The transcription of the genes encoding farnesyl-diphosphate synthase and squalene synthase are also responsive to cholesterol levels, but the mechanisms are different from those involved in the control of transcription for the HMG-CoA reductase and low density lipoprotein-receptor genes (9, 10). MKase is subject to feedback inhibition at the protein level by pathway intermediates including geranyl and farnesyl diphosphates (3, 4). Preliminary data suggest that MKase responds to cholesterol levels via a sterol regulatory element localized in the promoter, in a fashion similar to that observed for other cholesterol biosynthetic genes (11).

The cholesterol biosynthetic pathway contains a unique series of three sequential ATP-dependent enzymes which convert mevalonate to isopentenyl diphosphate: MKase, PMKase, and MDDase. Since mevalonate is necessary for cholesterol and isoprenoid synthesis, as well as a precursor for isoprenylation of various proteins, the regulation of its disposition in animal cells is of great interest. Although cDNAs encoding MKase have been published, and some regulatory properties revealed, little is known about the regulation and control of the latter two ATP-dependent genes involved in mevalonate disposition, primarily because of a lack of the corresponding mammalian cDNAs. A cDNA encoding PMKase from yeast has been presented, which encodes a protein with a predicted Mr = 48,000. However, the purified PMKase from porcine liver has been reported as a monomeric protein of Mr = 22,000 (12, 13). This divergence from yeast to mammals seems unusual.

In order to undertake analysis of the regulation of mammalian reaction; EST, estimated sequence tags; MOPS, 4-morpholinepropanesulfonic acid.
lian PMKase, a cDNA encoding this protein was isolated and the discrepancies in protein structure were addressed. In the present report, the molecular cloning of human PMKase is described. Our data indicate that human PMKase is subject to regulation by sterol at the level of transcription. The human gene contains a consensus peroxisomal targeting motif (Ser-Arg-Leu) (PTS-1) at the C terminus of the protein, the first identified in a cholesterol biosynthetic enzyme. This finding provides additional evidence that early cholesterol biosynthesis may occur in the peroxisome.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Sequencing—Porcine liver PMKase was purified as described previously (13). N-terminal and tryptic peptide amino acid sequence was determined as described (14).**

**Amplification of Porcine Phosphomevalonate Kinase cDNA—**The mixed oligonucleotides primed amplification of cDNA procedure was used to generate a DNA probe for library screening. First strand cDNA was synthesized with murine Moloney leukemia virus reverse transcriptase using a downstream antisense primer (GGCTCGAGGGNAGNGTYTNGNTA) according to Kawasaki (15). Thirty cycles of PCR were performed on the cDNA using the upstream primer GGAATCTCGGNAAGAAARGAAGGG and the above downstream primer with annealing for 45 s at 92°C, extension for 2 min at 72°C, and a 2 min heat denaturing for 45 s at 94°C. A second round of PCR was carried out using 1 μl of the first PCR as template. The same upstream primer was used, but a nested downstream primer with the sequence GGCTCGAGGYTCYTGGRAACCATYG replaced the previous downstream primer. The same temperature profile and cycle number were employed. The PCR product of 317 bp was gel-purified with Qiaex beads (Qiagen) and subcloned into the pBluescript II SK+ vector (Stratagene) using the EcoR1 and Xho1 restriction sites engineered onto the ends of the primers. Five isolates of the clone were sequenced and proven to be identical. The clone contained an open reading frame which encoded all of the sequenced porcine tryptic peptides.

**Isolation of Human Phosphomevalonate Kinase cDNA—**The 317-bp porcine liver PMKase fragment was used to screen ∼5 × 10⁶ plaques from a human liver λZAP cDNA library (Stratagene) using standard procedures. One 827-bp cDNA clone was isolated and sequenced. The human clone was 94% identical with the 317-bp porcine clone in the aligned region and contained a poly(A) tract at its 3′ end; however, the human clone did not contain the complete N-terminal coding region. The remaining 5′ end of the human cDNA was amplified from a SuperScript human liver cDNA library (Life Technologies, Inc.) using a set of nested, nondegenerate PMKase-specific antisense primers and an anchored sense primer in the library vector. The first amplification was carried out with the anchored primer (TAATACGACTCACTATAGGG) and the most downstream gene specific primer (TGCCGAAGGCTTCCTTAGAG) using a standard amplification profile with the exception of an annealing temperature of 55°C and an elongation time of 1 min. A second amplification was performed with the same anchored sense primer and the nested antisense primer TCTGGAGTTCAAGCTCATGC, using 1 μl of the first amplification reaction as template. The same temperature profile and cycle number were used. The amplified product (341 bp) was treated with the Klenow fragment of T4 DNA polymerase to create blunt ends and digested at the library vector Xho1 site. The resulting 255-bp fragment was cloned into the Xho1 and Smal sites of pBluescript II SK+ and sequenced. DNA and Protein Sequence Analysis—DNA sequencing was determined by the dideoxyxanucleotide chain termination method (16). Double-stranded DNA sequencing was performed for the Sequenase version 2.0 T7 DNA polymerase (U. S. Biochemical Corp.) following the manufacturer’s protocol. DNA and protein sequences were aligned and compared using the MacDNA/s computer program (Hitachi). The EMBL/GenBank data bases were searched for homologies to the DNA and protein sequences of PMKase.

**Expression of Human Phosphomevalonate Kinase cDNA in Bacteria—**A composite human liver PMKase cDNA clone was constructed in the glutathione S-transferase fusion vector pGEX-4T-1 (Pharmacia Biotech Inc.) for the purpose of protein expression. The 255-bp amplification described above which contained 35 bp of 5′-untranslated region and 220 bp of coding region was excised from the pBluescript plasmid with Not1 and ligated into the Not1 site of the pGEX vector. The 5′-untranslated region, which contained two in-frame stop codons, was excised using Nol1 and Smal restriction endonucleases. A Stul/Xbal restriction fragment from the 827-bp human liver PMKase cDNA, which contained the remainder of the coding region and 116 bp of 3′-untranslated region, was then ligated into the construct. The resulting plasmid was transformd into Escherichia coli strain DH5α which were grown and harvested as described previously (14). Cell pellets were resuspended in 0.1 M MOPS/KOH buffer (pH 7.0) supplemented with 0.1 mM dithiothreitol and sonicated on ice for 3 min in 1-min intervals with 1 min between intervals. Cell debris was removed by centrifugation at 12,000 × g for 10 min at 4°C. Various amounts of the cleared sonicates were assayed for PMKase activity using a radiometric assay which was a modification of that employed for MKase activity determination (17). The substrate was R-[2-14C]mevalonate-5-phosphate, specific activity 45.5 mCi/mmol (DuPont NEN). Each assay contained 0.015 μCi of substratum final concentration of 0.1 μM. Incubation was carried out at 37°C for 10 min. For additional verification, PMKase/glutathione S-transferase fusion protein was isolated from cleared sonicates with glutathione-Sepharose 4B as described by the manufacturer. Purified protein obtained using the fusion and vector only constructs was analyzed by SDS-polyacrylamide gel electrophoresis and exhibited protein bands of the expected molecular weight (M₀ = 48,000 for the fusion and 26,000 for vector only). The purified fusion protein was assayed spectrophotometrically as described previously (3). Mevalonate 5-phosphate was produced in situ in this assay system using recombinant human PMKase (18) which was produced and purified using conditions identical to those described for PMKase.

**Northern and Southern Blot Analysis—Probing of human liver cDNA—**A composite human liver PMKase fragment was used to screen a SuperScript human liver cDNA library (Life Technologies, Inc.) for the purpose of protein expression. The 255-bp amplicon was cloned and shown to encode the amino acid sequence predicted from the sequence. The probe was used to hybridize a blot of Northern blots (Clontech Inc.) at a hybridization temperature of 72°C, and the blot was washed at 65°C. The resulting 317-bp amplicon was cloned and shown to encode the amino acid sequence predicted from the clone. The 317-bp fragment was also used to screen a human liver cDNA library using a downstream antisense primer with the sequence GGCTCGAGGTYTCYTGGRAACCATYG for the purpose of protein expression. The 255-bp amplicon was cloned and shown to encode the amino acid sequence predicted from the clone. The 317-bp fragment was also used to screen a human liver cDNA library using a downstream antisense primer with the sequence GGCTCGAGGTYTCYTGGRAACCATYG for the purpose of protein expression. The 255-bp amplicon was cloned and shown to encode the amino acid sequence predicted from the clone.
**DISCUSSION**

This report describes the first mammalian cDNA encoding PMKase, one of three ATP-dependent enzymes which convert mevalonate to isopentenyl diphosphate. A genomic DNA clone encoding PMKase (ERG8) from yeast has been described (19).
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However, the DNA and predicted amino acid sequence of the yeast ERG8 gene has no significant homology to the cDNA which we isolated. Further, the deduced amino acid sequence encoded by the human liver PMKase cDNA does not contain any part of three conserved regions identified in mammalian MKase, bacterial and yeast galactokinase, and yeast PMKase (18). One of these conserved regions is believed to encode part of a conserved ATP binding sequence. Because of this apparent divergence from yeast to man, we sought further verification of the cDNA isolated through expression analysis in bacteria.

Several lines of evidence strongly support the identity of the isolated cDNA as one which encodes PMKase. The deduced Mr of the protein encoded by the cDNA is 21,862, which is in good agreement with the Mr of 26,000 (data not shown), in agreement with the deduced amino acid sequence (Fig. 1). Our protein and enzymatic data, therefore, supported the identity of the cDNA as that encoding human liver PMKase.

The human PMKase cDNA isolated in the current study does not contain the putative ATP binding amino acid sequence which has been detected in other kinases (18). This sequence, GXGXXGX_{15–22}AXK (where X represents any amino acid), is detected in MKase but not PMKase. The recently cloned cDNA encoding mevalonate diphosphate decarboxylase (MDDase) from human and yeast also lacks this amino acid sequence (20), yet both PMKase and MDDase utilize ATP as cosubstrate. Although this putative ATP binding site sequence is not mandatory, it may provide insight into the regulation of the three ATP-dependent mevalonate catabolic enzymes. Geranyl and farnesyl diphosphates are potent competitive inhibitors of MKase at the ATP binding site. PMKase is not subject to similar competitive inhibition (4). The amino acid sequence of the MKase ATP binding pocket may enable selective inhibition of MKase by various pathway intermediates, which does not occur in PMKase and MDDase.

Our results provide the first evidence for regulation of mammalian PMKase gene expression at the level of transcription in response to sterol. Earlier studies of PMKase activity in the liver of animals fed a high cholesterol diet or diets containing cholesterol sequestering reagents, such as cholestyramine, were extended in the present study by demonstrating that PMKase messenger RNA increases upon removal of sterol from cultured cell growth medium and further increases when the medium is supplemented with the HMG-CoA reductase inhibitor lovastatin (Fig. 2B). It will be of interest to determine if a larger induction of transcription than reported here might be detected in the livers of animals maintained on different dietary regimens, supplemented with cholesterol or cholesterol-lowering agents. The data suggest that PMKase is subject to transcriptional regulation in a fashion similar to other cholesterol biosynthetic genes in terms of sterol responsiveness, consistent with the concept of coordinate pathway regulation.

It appears that many steps of cholesterol and nonsterol isoprenoid biosynthesis may be localized to the peroxisome, a mammalian organelle involved predominantly with fatty acid β-oxidation, plasmalogen biosynthesis, and respiration. Krisans et al. (21) have provided several elegant cell fractionation studies which indicate that the steps from acetoacetyl-CoA thiolase through farnesyl-diphosphate synthase occur within the peroxisome. These studies have relied upon both enzymatic analysis and immunoblotting with specific antisera made against the respective proteins. Recent work from this group provides strong evidence that MKase, PMKase, and MDDase are found in the peroxisome (22). The sterol carrier protein SCP-2 has been at least partially shown to associate with the peroxisome, and additional data reveal that dolichol synthesis from farnesyl diphosphate may occur in the peroxisome (23–25). Although the evidence that these enzymatic activities are peroxisomally associated is substantial, until the present report there has been no evidence to indicate the exact mechanisms by which these proteins may be imported into the peroxisome.

Subramani and co-workers have presented evidence concerning the mechanisms of protein import into peroxisomes (for a review, see Ref. 26). These investigators, and others, have defined peroxisomal targeting sequences at the C terminus (PTS-1; Ser-Lys-Leu and variants) and N terminus (PTS-2, (Arg/Lys)-(Leu/Val/Ile)-(X)_{15–22}-(His/Gln)-(Leu/Ala)) of some peroxisomally localized proteins. Although these sequences are by
themselves sufficient to facilitate peroxisome import, they are not mandatory elements, and other undefined targeting sequences and import mechanisms do exist. Of the cholesterol and nonsterol isoprenoid biosynthetic enzymes which have been reported to have peroxisomal locations, none have been shown to have PTS-1 or PTS-2 sequences until now. The PMKase sequence reported here is the first enzyme of the pathway shown to contain a consensus PTS-1 element, Ser-Arg-Leu, at the C terminus. Analysis of expressed sequence tags (ESTs) using the Blastn program revealed homology of our PMKase cDNA with several human, mouse, and rat ESTs. One mouse EST encoded the complete PMKase open reading frame and revealed the consensus PTS-1, Ala-Arg-Leu, at the C terminus of the deduced amino acid sequence, showing evolutionary conservation of the PTS-1 element in PMKase. Sequence analysis of the MKase polypeptide sequence reveals a consen-

sus PTS-2 motif of Lys-Val-(X)5-His-Ala within the first 40 amino acids of the mature N terminus, making MKase potentially only the third protein known with a PTS-2 sequence, in addition to peroxisomal thiolase from various species and malate dehydrogenase from watermelon (27). Using computer analyses, the polypeptide sequences of other cloned cholesterol biosynthetic genes were screened, and it was found that human isopentenyl-diphosphate isomerase has a close match to the consensus PTS-2 motif with the sequence His-Leu-(X)5-Gln-Leu. Of the four enzymes which convert mevalonate to isopentenyl diphosphate, only MDDase appears to lack a consensus PTS-1 or PTS-2 element, although convincing evidence indicates that this protein is located in the peroxisome (22). Although these targeting sequences support the concept of a peroxisomal location for these enzymes, to our knowledge none of the sequences in these proteins have been experimentally mutated to verify their role in peroxisomal import.

A peroxisomal location of the cholesterol biosynthetic enzymes may provide further insights into the pathophysiology of the peroxisomal biogenesis disorders (28). It has long been known that patients with these disorders, including Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease, hyperperipnic acidemia, and rhizomelic chondrodysplasia punctata, have decreased circulating levels of cholesterol. Cultured cells derived from these patients also invariably demonstrate peroxisome ghosts, in addition to decreased activities of several cholesterol biosynthetic activities (21, 28). Identification of putative peroxisome targeting motifs in MKase, PMKase, and isopentenyl-diphosphate isomerase is consistent with decreased levels of circulating cholesterol in patients with peroxisomal biogenesis disorders. The isolation and character-

ization of a cDNA encoding human PMKase is an important first step in understanding the regulation of PMKase gene expression and its role in the regulation of cholesterol and nonsterol isoprenoid biosynthesis.

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