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Human mitochondrial pyrophosphatase: cDNA cloning and analysis of the gene in patients with mtDNA depletion syndromes

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Abstract

Pyrophosphatases (PPases) catalyze the hydrolysis of inorganic pyrophosphate generated in several cellular enzymatic reactions. A novel human pyrophosphatase cDNA encoding a 334-amino-acid protein ≈60% identical to the previously identified human cytosolic PPase was cloned and characterized. The novel enzyme, named PPase-2, was enzymatically active and catalyzed hydrolysis of pyrophosphate at a rate similar to that of the previously identified PPase-1. A functional mitochondrial import signal sequence was identified in the N-terminus of PPase-2, which targeted the enzyme to the mitochondrial matrix. The human pyrophosphatase 2 gene (PPase-2) was mapped to chromosome 4q25 and the 1.4-kb mRNA was ubiquitously expressed in human tissues, with highest levels in muscle, liver, and kidney. The yeast homologue of the mitochondrial PPase-2 is required for mitochondrial DNA maintenance and yeast cells lacking the enzyme exhibit mitochondrial DNA depletion. We sequenced the PPA2 gene in 13 patients with mitochondrial DNA depletion syndromes (MDS) of unknown cause to determine if mutations in the PPA2 gene of these patients were associated with this disease. No pathogenic mutations were identified in the PPA2 gene of these patients and we found no evidence that PPA2 gene mutations are a common cause of MDS in humans.

Keywords: Human pyrophosphatase; Inorganic pyrophosphate; Mitochondrial DNA; Mitochondrial depletion syndrome; Mitochondrial localization; Mitochondrial enzyme

Introduction

Inorganic pyrophosphate is generated as a by-product in several enzymatic reactions in cells. These reactions include DNA and RNA synthesis, activation of fatty acids and amino acids, as well as synthesis of the cyclic nucleotides cAMP and cGMP [1,2]. Pyrophosphate cannot be exported efficiently across the cell membrane and rapid intracellular hydrolysis of pyrophosphate is important for the progression and thermodynamic pull of the reactions. Pyrophosphatases (PPases)[1] catalyze the intracellular hydrolysis of pyrophosphate to inorganic phosphate. A family of soluble PPases has been identified and members of this enzyme family are present in both prokaryotic and eukaryotic cells [1,3]. *Escherichia coli* contain one major soluble PPase, which has been shown to be essential for cell growth [4,5]. The yeast *Saccharomyces cerevisiae* contains two sequence-related PPase genes: PPA1 and PPA2 [6,7]. The PPA1 gene encodes a cytosolic PPase that, similar to the *E. coli* PPase, has been shown to be required for cell growth [7]. The yeast PPA2 gene contains an N-terminal mitochondrial import signal sequence and the enzyme is localized in the mitochondrial matrix. The PPA2 gene product has been shown to be required for mitochondrial DNA (mtDNA) maintenance. Yeast cells

Abbreviations: GFP, green fluorescent protein; GST, glutathione *S*-transferase; PPi, inorganic pyrophosphate; PPase, pyrophosphatase; mtDNA, mitochondrial DNA; MDS, mtDNA depletion syndrome.

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deficient in expression of the mitochondrial PPase exhibit a ρ^0 phenotype, with no detectable mtDNA, and require growth medium that supports anaerobic metabolism [7].

In mammalian cells, both a soluble cytosolic and a mitochondrial PPase have been detected in biochemical studies [8–12]. Yang and co-workers purified a cytosolic PPase from bovine retina and cloned the cDNA encoding the enzyme [12,13]. The enzyme was shown to be sequence related to the family of soluble PPase's of yeast and bacteria. Recently, the cloning and recombinant expression of the human homologue of the bovine cytosolic PPase were reported [14,15]. Less is known about the molecular properties of the mammalian mitochondrial PPase. The enzyme is located in the mitochondrial matrix and biochemical studies suggest that the enzyme may be associated with mitochondrial inner membrane proteins such as components of the respiratory chain [2,10,11]. Several different PPase-containing protein complexes with different molecular masses have been purified from mammalian mitochondria, but it appears that the catalytically active subunit in each complex has a size similar to that of the yeast mitochondrial PPase [10,11].

The ρ^0 phenotype of yeast cells lacking the mitochondrial PPase suggests that deficiency in the expression of the human mitochondrial PPase could result in mtDNA depletion. Inherited mtDNA depletion syndromes (MDS) are a heterogeneous group of diseases that usually present with severe symptoms of mitochondrial dysfunction in early childhood [16,17]. For many of these patients the genetic defect causing MDS remains undetermined. We cloned the cDNA and gene encoding the human mitochondrial PPase to investigate the role of this enzyme in mitochondrial pyrophosphate metabolism and mtDNA maintenance. To investigate if PPase-2 is involved in MDS in humans, we sequenced the PPA2 gene from patients with MDS of unknown cause. In the present study, we report the molecular characterization of the human mitochondrial PPase and compare its properties to those of the human cytosolic PPase. We also report the sequencing of the PPA2 gene from 13 patients with MDS.

Results

cDNA cloning of the human mitochondrial pyrophosphatase

We planned to clone the cDNA encoding the human mitochondrial PPase based on sequence similarity to other PPases. Two separate genes with a high level of sequence similarity encode the yeast cytosolic and mitochondrial PPase. We hypothesized that the human mitochondrial PPase would exhibit sequence similarity to mammalian cytosolic PPases as well as to the yeast enzymes. Since the cDNAs encoding the human and bovine PPases have been cloned [17,18], we used the predicted amino acid sequences of these enzymes to search the GenBank expressed sequence tag database to identify human cDNAs that encoded proteins similar, but not identical, to the cytosolic PPase. Several cDNA clones that encoded a protein ≈60% identical to the cytosolic human PPase were identified. The 1214-bp cDNA encoding this novel enzyme was completely sequenced. The cDNA contained an open reading frame of 334 amino acid residues corresponding to a protein with a predicted molecular mass of 38 kDa (GenBank ID AF217187). We also identified and resequenced the cDNA encoding the human cytosolic PPase. This cDNA was 1204 bp and encoded a 289-amino-acid protein with a molecular mass of 32.7 kDa (GenBank ID AF217186). Based on the high level of sequence similarity, the novel enzyme was named PPase-2 and the previously cloned cytosolic enzyme was named PPase-1. Alignment of the predicted amino acid sequences of human PPase-1 and PPase-2 with the yeast cytosolic and mitochondrial PPases showed that human PPase-2 was ≈60% identical to human PPase-1 and ≈40–50% identical to the yeast enzymes at the amino acid level (Fig. 1). Human PPase-2 was extended 31 amino acids at the N-terminus compared to human PPase-1. This extension showed similarity to the N-terminal mitochondrial import signal of yeast PPase-2, suggesting that the human PPase-2 protein was targeted to the mitochondria.

Expression and enzyme activity of human PPase-1 and PPase-2

Human PPase-1 and -2 were recombinantly expressed to determine their enzymatic activity. The enzymes were expressed as fusion proteins to GST to facilitate purification. Both PPase-1 and PPase-2 were efficiently overexpressed and purified to homogeneity by one-step glutathione affinity chromatography with a yield of ≈3 mg pure recombinant protein per liter bacterial culture. A factor Xa protease site was incorporated between the PPase and GST in the expression vector to allow separation of the two proteins after purification by proteolytic cleavage. However, incubation of GST–PPase-2 with factor Xa resulted in nonspecific cleavage of the fusion protein and loss of enzyme activity (data not shown). Subsequent studies were therefore performed using the uncleaved fusion proteins.

The rate of pyrophosphate hydrolysis was determined for the purified recombinant fusion proteins. The reaction requires the presence of three or four Mg^{2+} ions that bind the enzyme and form a complex with the pyrophosphate [1,19]. The Mg^{2+} ion binding to the enzyme is concentration dependent and the kinetic parameters of the enzyme reaction are accordingly dependent on the Mg^{2+} ion concentration. The average physiologic concentrations of free Mg^{2+} ions in the cytosol and the mitochondria of human cells are similar, about 0.5 mM. The total concentration of Mg inside a human cell is approximately 20 mM, but most Mg is bound to proteins and other substances, which is why the amount of free Mg^{2+} ions is much lower. Considering the physiological conditions, we chose to use 0.2–5 mM $MgCl_2$ in reactions containing 10–200 μM pyrophosphate (Fig. 2). The data were fitted to Michaelis–Menten plots. However, the method used for quantification of inorganic phosphate produced in the reaction had a detection limit of ≈1 μM and we were therefore not able to determine accurately the initial rate kinetics for pyrophosphate concentrations <10 μM. The K_m of PPase-1 is reported to be ≈1 μM [13], but due to the limitations of the assay we were able to state only that the K_m of the recombinant PPase-1 was <10 μM. The K_m for PPase-2 pyrophosphate hydrolysis was ≈15 μM

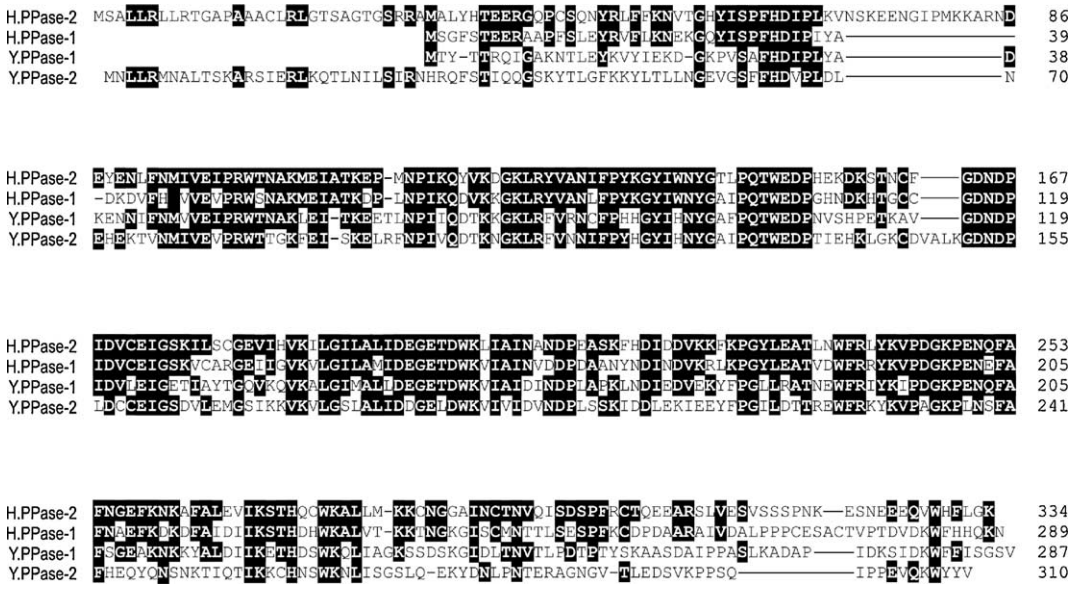

Fig. 1. Alignment of human and yeast PPase-1 and PPase-2 predicted amino acid sequences. Black boxes indicate conserved amino acid residues compared to the human PPase-2 sequence.

and was accordingly higher than the K_m for PPase-1. Higher Mg^{2+} ion concentrations (up to 20 mM) did not alter the kinetic properties of PPase-2 compared to 5 mM (data not shown). PPase activity is inhibited by Ca^{2+} ions and we compared the sensitivity of PPase-1 and -2 to Ca^{2+} inhibition (Fig. 3). Both enzymes were inhibited by Ca^{2+} ions with an $IC_{50} \approx 10$ μM. However, there was no difference between the PPase-1 and the PPase-2 sensitivity to Ca^{2+} inhibition.

PPA2 encodes an N-terminal mitochondrial import signal

The two yeast PPases that exhibit sequence similarity to both human PPase-1 and PPase-2 differ in their subcellular location [7]. The yeast PPase-1 is located in the cytosol, whereas the yeast PPase-2 is located in the mitochondria. Alignment of the yeast and human PPases showed that human PPase-2 has an N-terminal extension similar to the extended N-terminus of the yeast mitochondrial PPase (Fig. 1). This N-terminal extension contains positively charged and hydrophobic amino acid residues similar to a mitochondrial targeting signal. Subcellular localization prediction using the PSORT algorithm also suggested that the protein was targeted to the mitochondria [19].

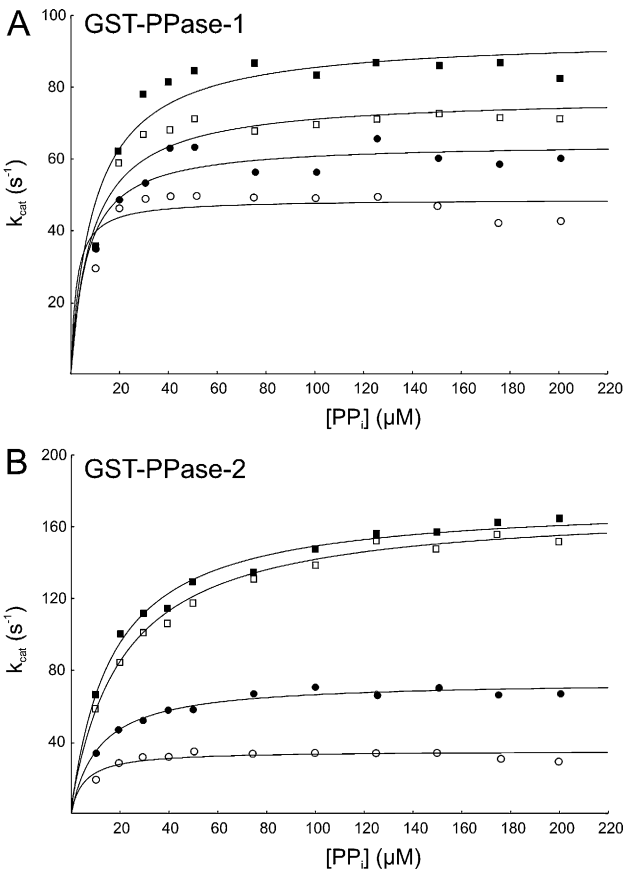

Fig. 2. Pyrophosphate hydrolysis catalyzed by recombinant human (A) PPase-1 or (B) PPase-2 in 0.2 (○), 0.5 (●), 2 (□), or 5 mM (■) $MgCl_2$. The recombinant enzymes used in the assays were fused to GST at their N-terminus.

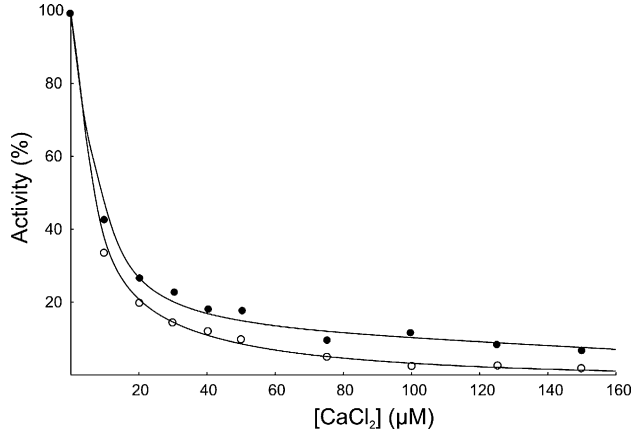

Fig. 3. $CaCl_2$ inhibition of PPase-1- and PPase-2-catalyzed pyrophosphate hydrolysis. 20 μM pyrophosphate and 0.5 mM $MgCl_2$ were used in the reaction and $CaCl_2$ was added to the indicated concentrations.

We expressed PPase-1 and PPase-2 fused to GFP in HeLa cells to verify the mitochondrial localization of PPase-2 in vivo (Fig. 4). Cells expressing PPase-1–GFP showed green fluorescence in both the cytosol and the nucleus. However, cells transfected with PPase-2–GFP did not exhibit any green fluorescence. Several different plasmids that included different parts of the untranslated 5′ region were constructed, but we were not able to detect expression of the full-length enzyme in fusion with GFP (data not shown). We therefore constructed a GFP fusion protein containing only the N-terminal 31 aa of PPase-2 corresponding to the putative mitochondrial import signal. This construct would allow verification that the mitochondrial import signal was functional, without introducing the full-length enzyme, which may be toxic or poorly expressed in the cells. The HeLa cells expressing the N-terminal region of PPase-2 in fusion with GFP showed a dotted fluorescent pattern indicating mitochondrial targeting of the fusion protein. The mitochondrial localization of the fluorescent protein was verified by counterstaining the cells with the MitoTracker mitochondrial dye.

Tissue expression pattern of human PPases

Northern blot analysis was used to determine the tissue expression of PPase-1 and PPase-2 mRNA (Fig. 5). A major mRNA transcript of ≈1.4 kb was detected in all 12 investigated tissues for both PPase-1 and PPase-2. The sizes of these transcripts are consistent with the 1204- and 1214-bp cDNAs. Compared to human PPase-1, the PPase-2 mRNA was expressed at higher relative levels in heart and skeletal muscle, kidney, and liver. Analysis of the expression pattern based on expressed sequence tag clones deposited in GenBank confirmed that mRNAs of both pyrophosphatase genes were ubiquitously expressed in most tissues (data not shown).

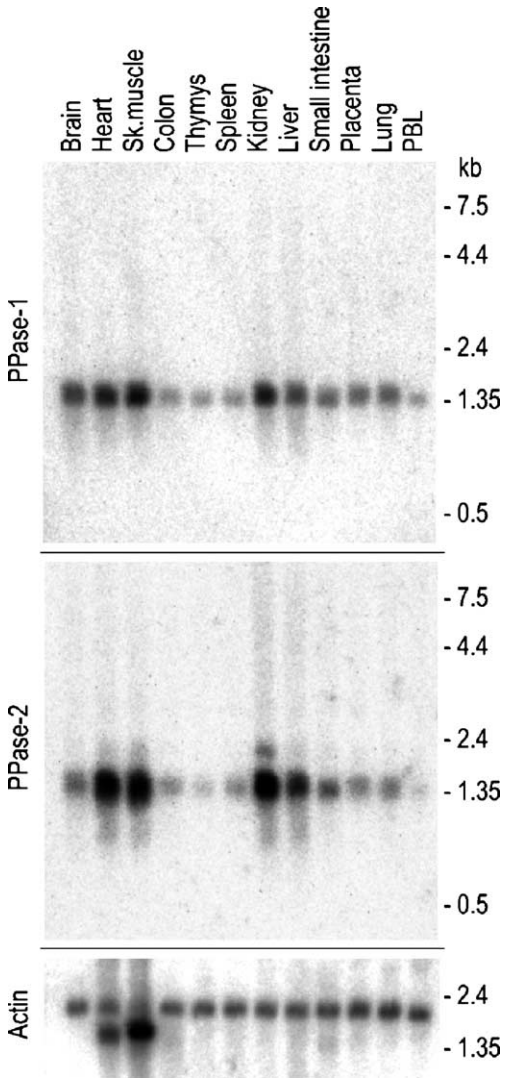

Fig. 5. Northern blot analysis of PPase-1 and PPase-2 mRNA expression in human tissues. An actin probe was used as a hybridization control.

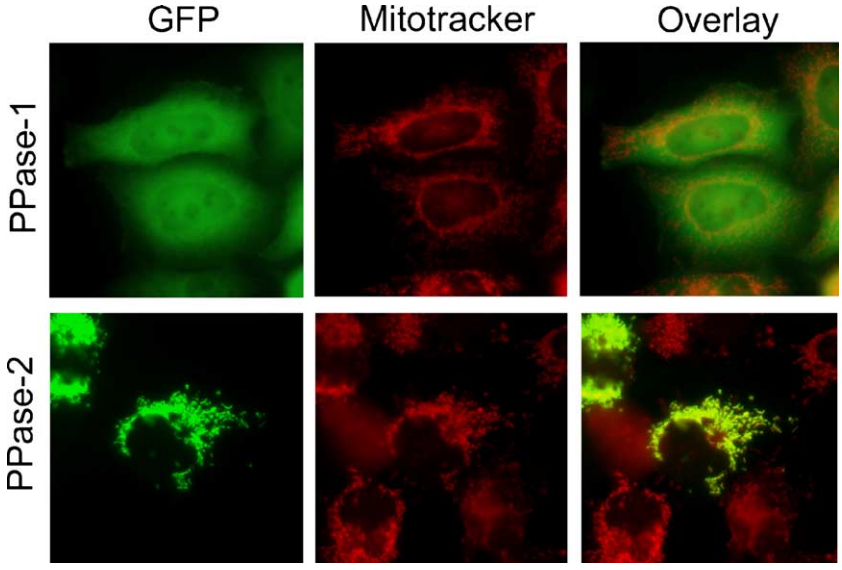

Fig. 4. Subcellular localization of PPase–GFP fusion proteins. The full-length PPase-1 protein or the N-terminal putative mitochondrial import signal of PPase-2 was expressed in HeLa cells fused to GFP (left). The cells were counterstained with MitoTracker red to visualize the mitochondria (center). Overlay images of GFP and MitoTracker fluorescence verified colocalization of the dyes in the cells transfected with PPase-2 (right).

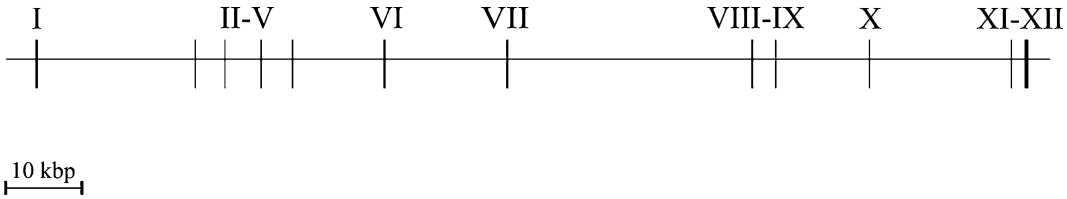

Fig. 6. Structure of the human PPA2 gene located at chromosome 4q25. Exons are shown as black boxes with roman numbers.

Chromosome localization and gene structure of human PPA2

The human genome sequence database was searched with the human PPA2 cDNA to identify the corresponding genomic sequence. Two bacterial artificial chromosome clones (B35C18 and CTD-2006P5) containing the complete genomic sequence corresponding to the human PPA2 cDNA were identified. The genomic clones were located at chromosome 4q25. Alignment of the PPA2 cDNA with the genomic sequence showed that the gene was divided into 12 exons distributed over ≈100 kb (Fig. 6). The predicted ATG translation start codon was located in the first exon.

SiRNA suppression of PPase-2 expression

To study the importance of PPase-2 for mtDNA integrity in human cells we tried to silence the expression of PPase-2 by single interference RNA. We transfected HeLa and osteosarcoma cells with seven different plasmids containing siRNA constructs and we selected for stable expression by using neomycin. RNA from cells that had survived in the presence of neomycin for 2 weeks or more was collected and reverse transcribed before being subjected to SQ-PCR. Although seven different constructs were tested, no decrease in PPase-2 mRNA expression was detected in any cell line (data not shown).

Analysis of the PPA2 gene in MDS patients

Yeast cells lacking expression of mitochondrial PPase exhibit mtDNA depletion. Therefore, based on analogies with other MDS-related etiologies (dGK, TK2), we hypothesized that mutations in PPA2 could be a cause of MDS in humans. The coding sequences of the PPA2 genes from 13 patients with MDS were sequenced. The locations of gene regulatory elements for the PPA2 gene are not yet identified and the sequencing was therefore limited to the coding region of the gene. The 12 exons containing the open reading frame were PCR amplified by primers flanking the exons (Table 1). No pathogenic mutations were identified among these patients; however, four polymorphisms were found. In one patient exon 1 had a silent heterozygous change, C to T, at nucleotide 41 (mRNA AF217187) at amino acid 9 (arginine). This polymorphism has been reported at the NCBI SNP database. In another patient exon 1 had a heterozygous change, A to G transition, at nucleotide 78 (mRNA AF217187), which changes amino acid 22 from threonine to alanine. In addition, this patient has a heterozygous change, C to G, in intron 5 at the 5th nucleotide after exon 5. In 8 patients, exon 9 showed a G-to-C transversion at nucleotide 846, which changes amino acid 282 from lysine to asparagine. The transversion is homozygous in 6 of these patients, while in the other 2 patients the change is heterozygous. This polymorphism is also listed in the NCBI SNP database. In 3 of these patients a change in intron 8 was also seen, IVS8−19 $(TTTG)_2$, whereas the normal sequence is IVS8−19 $(TTTG)_3$. These alterations were, however, also seen in 4 of the 5 controls and are therefore considered neutral polymorphisms. In summary, no mutations in the PPA2 gene were identified in these patients that suggest a role for PPase-2 deficiency in MDS.

Discussion

We have identified and cloned the cDNA of a novel human PPase and showed that the enzyme contains a functional N-terminal mitochondrial import signal. Although the biochemical properties of mammalian mitochondrial PPase have been studied on enzyme purified from tissues, the cloning of the

Table 1
The designed primer pairs used for sequencing of the PPA2 gene in humans

Exon	Forward primer (5′–3′)	Reverse primer (5′–3′)
1	GATGACTCCGGACGGTAGG	GGAAGTAAGGTGGCCTGGAC
2	GGTCCTTAGATGGTGGCAAG	CCTTTCAAGGTGCTTCCAAA
3	ACGAAGTGGGCATTTGGTAG	ACATCGGAATGCACATGAAA
4	CGGGGGAAAACATCTAAGGT	ATGCCAAACAACTTGCAACA
5	TGGTGTTTCCTTTCCAAACA	CCAAATGACATGTCCTCAGAA
6	TGGCCTTGGGCCTATGTAAT	AGGTGTTAGGCTGGCTTTGA
7	GCAAGGCAGACTTTGGCTAT	AACTCCCCTATGTGAACTTCTTTT
8	CAGTCCAATGCTCTGCCACT	TTCAGCCTTGTTGATTCACTT
9 set A	AAGCTTTGCCTGACTCTGGA	AAAATGCAAAAATCTGAAATCTG
9 set B	GGAGCTAGGCTTTTGCTCTTG	TCTTACCAATTTATAGCTCCTCCA
10	CTGGAGAGTGCTTTGCTGTG	TGCTTCTCAAAAGGACTTGACA
11	TCAGGTTAGAGATCCATTGTGTG	TCACCGTACAATCAAATCATTTTC
12	TTGGGACGAAAGTTTGAAGG	GGCTGGTCTTGAACTCCTGA

gene encoding the enzyme has not been reported previously. We found that the human cytosolic and mitochondrial PPases were both ubiquitously expressed in human tissues. The mitochondrial PPase-2 was expressed at highest levels in tissues rich in mitochondria, such as muscle, liver, and kidney. The ubiquitous expression of the human PPases suggests that these enzymes, similar to their microbial homologues, are involved in the "housekeeping" degradation of pyrophosphate generated by different cellular metabolic processes.

Inherited MDS are rare and usually severe diseases that often present in infancy or early childhood [16,17]. The symptoms of these disorders are related to mitochondrial dysfunction and decreased oxidative phosphorylation. The organs most affected are highly dependent on aerobic metabolism, such as muscle, liver, and brain. However, there is wide tissue specificity among MDS patients, likely associated with different genetic etiologies. Recently, mutations in the mitochondrial deoxyribonucleoside kinases, deoxyguanosine kinase, and thymidine kinase 2 have been identified in some patients with mtDNA depletion [20,21]. However, subsequent studies suggest that mutations in these genes account for only a minority of MDS cases and that other genetic defects are responsible for the syndrome in the majority of patients [22]. The importance of the yeast mitochondrial PPase in mtDNA maintenance suggests that the human PPase-2 also may be required for normal mtDNA integrity and patients with mtDNA depletion syndrome of unknown cause were therefore screened for mutations in the PPA2 gene. However, no pathogenic mutations were identified in the PPA2 gene of the 13 MDS patients studied. Although we found no evidence in the investigated patients that PPA2 gene mutations are likely to be a common cause of MDS in humans, additional patients with MDS are being investigated for PPA2 gene mutations. Accordingly, identification of further genes needed for mtDNA maintenance might pinpoint a major gene causing MDS.

Materials and methods

cDNA cloning and expression of human pyrophosphatases

The GenBank expressed sequence tag database at the National Institute for Biotechnology information (http://www.ncbi.nlm.nih.gov/) was searched with the Basic Local Alignment Search Tool (BLAST) [23] to identify human expressed sequence tag cDNA sequences that encoded proteins similar to the bovine cytosolic and yeast mitochondrial PPase [7,13]. The expressed sequence tag clones identified were generated by the IMAGE Consortium and obtained from Research Genetics (IMAGE ID 5193749 and 5205378) [24]. The DNA sequences of the plasmids were determined using an ABI310 automated DNA sequencer (Perkin–Elmer, Applied Biosystems).

We expressed the two human inorganic pyrophosphatases in *E. coli* fused to glutathione *S*-transferase. Oligonucleotide primers that flanked the open reading frame of the cDNAs were designed with *Bam*HI and *Xho*I restriction enzyme sites (PPA1: 5′-GGGATCCATATGAGCGGCTTCAGCACCGAGGAGCG and 5′-AACTCGAGTTAGTTTTTCTGGTGATGGAACCACT; PPA2, 5′-GGGATCCATATGGCCCTGTACCACACTGAGGAGCG and 5′-AACTCGAGTCACTTGCCAAGGAAGTGCCAC). The oligonucleotides were used in a PCR and the amplified DNA fragment was cloned in the *Bam*HI–*Xho*I sites of the pGEX-5X-1 plasmid vector (Amersham Biotech).

The plasmids were transformed into the *E. coli* strain BL21(DE3)pLysS (Stratagene). Protein expression was induced at OD_{600} 0.7 with 1 mM IPTG for 3 h at 37°C. The recombinant proteins were purified from crude bacterial extracts using glutathione affinity chromatography on glutathione-4B Sepharose (Amersham Pharmacia Biotech). The purified recombinant proteins were eluted in 50 mM Tris, pH 8.0, 250 mM NaCl, and 10 mM reduced glutathione (Sigma). The size and purity of the recombinant proteins were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Phast system; Amersham Biotech). The protein concentrations were determined with the Bradford Protein Assay (Bio-Rad) and bovine serum albumin was used as the concentration standard.

Enzyme assays

Hydrolysis of pyrophosphate (Sigma) was determined in 160 μl reaction buffer containing 50 mM Tris, pH 8.0, 0.1 mM EGTA, and indicated concentrations of $MgCl_2$. One to ten nanograms of recombinant PPase was added and the reactions were incubated 10 min at 22°C. Formation of inorganic phosphate was determined using a malachite green colorimetric assay [25,26]. Forty microliters of the malachite green assay mixture containing 1.3 μM malachite green, 3% (w/v) ammonium molybdate, 0.2% (v/v) Tween 20 dissolved in 2 M H_2SO_4 was added to terminate the enzyme reactions. The absorbance at 620 nm was determined and compared to a NaH_2PO_4 concentration standard. The data were analyzed and fitted to Michaelis–Menten plots using Statistica (StatSoft).

Subcellular localization

We used the pEGFP-N1 vector (Clontech) to express the human PPases fused to the green fluorescent protein in HeLa cells (American type culture collection). Oligonucleotides containing *Hin*dIII and *Bam*HI restriction enzyme sites (PPA1, 5′-TAAGCTTTCCGGCACTATGAGCGGCTTCAGCACC and 5′-TTGGATCCTTTTTCTGGTGATGGAACCACTTATC; PPA2, 5′-CGAAGCTTGTGTATGCTGAGCCGCTGCCGCA and 5′-ATGTCGACCAGCCTTCCTTGTCAAAAATCT) were used to clone the open reading frame of the cDNAs into the *Hin*dIII–*Bam*HI sites of the pEGFP-N1 vector. The HeLa cells were cultured in Dulbecco's MEM modified medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The plasmid was transfected with FuGENE (Boehringer Mannheim). The cells were counterstained with MitoTracker red (Molecular Probes). Microscopy was performed on a Nikon Eclipse E600 microscope equipped with a SPOT RT digital camera.

Northern blot expression analysis

A human multiple-tissue Northern blot containing poly(A) RNA from 12 human tissues was obtained from Clontech. DNA fragments comprising bp 1–895 of PPase-1 and bp 1–1002 of PPase-2 was PCR amplified and labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Biotech) using the Prime-A-Gene random labeling kit (Promega). The probe was purified by spin chromatography on ChromaSpin TE-100 (Clontech) and hybridized to the Northern blot filter in ExpressHyb hybridization solution (Clontech) at 68°C for 1 h. The filter was washed as described in the manufacturer's protocol and exposed to phosphoimaging plates. Human actin cDNA was used as a control probe (Clontech).

SiRNA analysis

Seven different plasmids that contained DNA templates for the synthesis of siRNAs under the control of the H1 promoter were generated using the pSilencer3.1-H1neo vector (Ambion). The selection of the coding sequences for the siRNA was determined using the siRNA target finder tool available on Ambion's home page (http://www.ambion.com). The sequences were analyzed by BLAST to ensure they had no significant sequence homology with other genes. Seven top-strand and seven bottom-strand oligos were ordered from TAG Copenhagen (the sequences are available by e-mail: magnus.johansson@mbb.ki.se) and used in the making of the plasmids according to the protocol. The generated plasmids were transfected into HeLa and osteosarcoma cells. The cells were cultured in Dulbecco's MEM modified medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 110 μg/ml sodium pyruvate, and 50 μg/ml uridine [27]. After 2 weeks of selection with neomycin, RNA from the cells was extracted using Trizol. The RNA was reverse transcribed (Invitrogen) and the resulting cDNA was subjected to semiquantitative PCR (PPase-2, forward 5′-AGCCAATGAATCCCATTAAACAA-3′ and

reverse 5′-TCCAAAGCAGTTCGTGCTCTT-3′; actin, forward: 5′-TCCTCCTGAGCGCAAGTACTC-3′ and reverse 5′-GCATTTGCGGTGGACGAT-3′). The PCR products were analyzed by gel electrophoresis.

Patient evaluations

At Columbia University College of Physicians and Surgeons in the United States, the patient samples were collected under Institutional Review Board (IRB)-approved protocols. Blood was collected for DNA analysis with an IRB consent form while muscle samples were studied under an IRB exemption protocol. Of the seven patients from Columbia, four had the myopathic form of MDS, two had the hepatic form, and the clinical feature of the last patient is still undetermined. All of the patients with myopathy were screened for TK2 mutations (MIM 188250), while both patients with hepatopathies were screened for dGK mutations (MIM 601465). The diagnosis of MDS was made by Southern blot analyses, which revealed <30% mtDNA in affected tissues relative to controls. At the Children's Hospital in Rome, Italy, the patient samples were collected after approval of the IRB. Of the six patients investigated in Rome, two had hepatopathy and significant involvement of the central nervous system, one had heart muscle involvement as the only clinical feature, and three patients had different degrees of myopathy, in some cases associated with encephalopathy. The level of mtDNA reduction in these patients varied from 42 to 95%. All the patients that were screened in Rome had also been sequenced for dGK (MIM 601465), TK2 (MIM 188250), the mitochondrial deoxynucleotide carrier (MIM 606521), and the 5′-deoxyribonucleotidase (MIM 605292). No mutations were detected.

Mutation analysis

Genomic DNA from patients with MDS was obtained as described [28]. PCR amplifications and DNA sequencing of PPase-2 were performed using intronic primers listed in Table 1. The PCRs were performed under standard conditions using 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM each primer, 100–200 ng DNA, and *Taq* polymerase (Roche). The amplification was done using a thermocycler (Applied Biosystems GeneAmp PCR System 7900) set at an initial denaturing step at 94°C for 5 min, followed by 30–35 cycles of 30 s at 94°C, 30 s at 59–60°C, and 30 s at 72°C, and in the end 5 min of extension at 72°C. Four exons required special conditions for amplification. Exon 1 was amplified by touchdown PCR from 70 to 60°C (annealing) and including 4 μl DMSO and *Taq* Platinum (Invitrogen) in the PCR. DMSO was used in the reaction of exon 6. To read the coding region of exon 9 in some individuals that showed intronic rearrangements, it was necessary to combine primer sets A and B. For the amplification of exon 12, touchdown PCR from 70 to 60°C (annealing) was used. The DNA sequencing of the PCR products was performed using the BigDye Terminator kit and the ABI Prism 310 system.

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