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Biochemical and Biophysical Research Communications 307 (2003) 41–46
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Identification of a novel human uridine phosphorylase☆,☆☆

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Received 19 May 2003

Abstract

Uridine phosphorylase catalyzes the reversible phosphorylytic cleavage of uridine and deoxyuridine to uracil and ribose- or deoxyribose-1-phosphate. The enzyme has an important role in the metabolism of pyrimidine analogs used in cancer chemotherapy. The cDNA of a novel 317 amino acid human uridine phosphorylase ≈60% identical to the previously identified human uridine phosphorylase was cloned. The novel enzyme, named uridine phosphorylase-2 (UPase-2), showed broad substrate specificity and accepted uridine, deoxyuridine, and thymidine as well as the two pyrimidine nucleoside analogs 5-fluorouridine and 5-fluoro-2′-deoxyuridine. The human UPase-2 gene was mapped to chromosome 2q24.1 and the 2.2-kb mRNA was predominantly expressed in kidney. The mouse UPase-2 cDNA was also identified and shown to be predominantly expressed in liver. The identification of a novel uridine phosphorylase with broad substrate specificity is important for studies on both nucleoside metabolism as well as for studies on the pharmacological mechanisms of therapeutic pyrimidine nucleoside analogs.

Keywords: Nucleoside metabolism; Pyrimidine phosphorylase; Nucleoside analog; Thymidine phosphorylase; Cancer chemotherapy

Two pyrimidine nucleoside phosphorylases have been identified in human cells: uridine phosphorylase (UPase) and thymidine phosphorylase (TPase) [1,2]. The enzymes have overlapping substrate specificities and catalyze together the phosphorylytic cleavage of uridine, deoxyuridine, and thymidine to the corresponding free bases and ribose-1-phosphate or deoxyribose-1-phosphate [3–5]. The reaction catalyzed by the enzymes is freely reversible and the pyrimidine nucleoside phosphorylases can also convert thymine and uracil to nucleosides in the presence of ribose- or deoxyribose-1-phosphate [6,7]. The role of the enzymes in salvage of nucleoside bases to nucleosides appears to be tissue-dependent, but the enzymes predominantly catalyze phosphorylysis of the naturally occurring nucleosides in most tissues under normal physiological conditions [1,2].

In addition to their natural substrates, the pyrimidine phosphorylases also have an important role in the activation and metabolism of several nucleoside analogs used in chemotherapy of cancer [8–12]. These nucleoside analogs include 5-fluoro modified uracil and uridine derivatives such as 5-fluorouracil (5-FU) and its prodrugs 5-fluoro-2′-deoxyuridine (5-FdUrd) and 5′-deoxy-5-fluorouridine that are used in treatment of several types of solid tumors [13]. The nucleoside prodrugs are enzymatically cleaved by the pyrimidine phosphorylases to generate 5-FU, a reaction predominantly catalyzed by TPase. 5-FU is subsequently converted intracellularly to several cytotoxic metabolites such as 5-FdUMP that inhibit thymidylate synthase, 5-FUTP that is incorporated into RNA and interferes with both transcription and translation, and 5-FdUTP that is incorporated into DNA and causes DNA damage [13]. 5-FU is converted to its cytotoxic metabolites by several different pathways, but studies suggest that both TPase and UPase are important for this process [8–12]. Accordingly, the pyrimidine phosphorylases are involved in both the conversion of the prodrugs to 5-FU as well as the further

☆ *Abbreviations:* 5-FU, 5-fluorouracil; 5-FUrd, 5-fluorouridine; 5-FdUrd, 5-fluoro-2′-deoxyuridine; TPase, thymidine phosphorylase; UPase, uridine phosphorylase; MNGIE, mitochondrial neurogastrointestinal encephalomyopathy.

☆☆ *Sequence deposition:* The cDNA sequence reported in this paper has been deposited in GenBank Accession No. AY225131.

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doi:10.1016/S0006-291X(03)01062-3

activation of this compound to its cytotoxic metabolites. Increased levels of TPase and UPase have been found in many types of solid tumors and the level of expression of the enzymes may also correlate with disease progression [14–17]. The high level of pyrimidine phosphorylase expression in tumor cells compared to normal tissues is likely one of the mechanisms that results in high sensitivity of cancer cells to the cytotoxic pyrimidine nucleoside analogs.

The pyrimidine nucleoside phosphorylases have been purified from human and murine tissues [3–5,18,19]. The cDNAs of human and murine UPase and TPase have been cloned and the recombinant enzymes characterized [18–22]. Liu and co-workers [19] cloned the human UPase cDNA and also purified the enzyme from normal and malignant human tissues. In head-neck squamous carcinoma and breast tumors, they identified a pyrimidine phosphorylase activity with biochemical properties distinct from both the previously characterized UPase and TPase. It has been unclear whether this enzyme activity was due to a modified variant of one of the previously identified pyrimidine phosphorylases or whether it was due to a novel enzyme. I have in the present study used UPase and TPase sequence similarity to identify and clone the cDNA of a novel human pyrimidine nucleoside phosphorylase. The enzyme, named UPase-2, was predominantly expressed in human kidney and showed broad substrate specificity accepting uridine, deoxyuridine, thymidine as well as 5-fluoro-modified uridine and deoxyuridine analogs.

Experimental procedures

Cloning of human and mouse UPase-2 cDNAs. The expressed sequence tag library of the GenBank 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 and mouse expressed sequence tag cDNA sequences encoding proteins similar but not identical to the human UPase and TPase [18–22]. The expressed sequence tag clones identified were produced by the IMAGE consortium and obtained from Research Genetics (IMAGE ID 1534053, 4818300, and 4606868) [24]. The DNA sequences of the plasmids were determined using the BigDye sequencing reagent (ABI) and an ABI310 automatic fluorescent DNA sequencer (ABI).

Expression of recombinant enzymes. The human UPase-2 cDNA was expressed in *Escherichia coli* as a fusion protein to glutathione-*S*-transferase. The open reading frame of the cDNA was PCR-amplified with oligonucleotide primers containing *Bam* HI and *Xho*I restriction enzyme sites (5′-GGGATCCCCGAATTCATGGCTTCAGTTATAC CTGCCTC-CAATAG and 5′-AACTCGAGGTCACAAAGTCCA AGCCGCCGTCTGATG) and the PCR-amplified DNA fragment was cloned into the *Bam*HI–*Xho*I sites of the pGEX-5X-1 plasmid vector (Amersham Biotech). The protein was expressed in BL21(DE3)pLysS *E. coli* (Invitrogen). The bacteria were grown at 37 °C to $OD_{600} = 0.6$ in LB medium supplemented with 100 μg/ml ampicillin and 0.5% glucose. Protein expression was induced at 37 °C with 1 mM 1-thio-β-D-galactopyranoside for 3 h. The cells were harvested by centrifugation at 3000*g* for 15 min and the pellet resuspended in phosphate-buffered saline, pH 7.4, with 1 mg/ml lysozyme. The bacteria were incubated on ice 20 min and sonicated 3×15 s. The lysate was cleared by centrifugation 15 min at 17,000*g* and loaded onto a glutathione-4B affinity chromatography column (Amersham Biosciences). The column was washed with phosphate-buffered saline and the recombinant protein was eluted in 10 mM reduced glutathione in phosphate-buffered saline. The protein was stored at −80 °C in the elution buffer supplemented with 10 mM dithiothreitol, 300 mM NaCl, and 10% glycerol. The protein concentration was determined with Bradford Protein Assay (Bio-Rad) and bovine serum albumin was used as the concentration standard. The purity of the preparation was determined by SDS–PAGE (Novex gel, Invitrogen).

Enzyme assays. All nucleosides and nucleoside analogs were obtained from Sigma. The substrates were dissolved in phosphate-buffered saline, pH 7.4, and 20–200 ng/ml recombinant UPase-2 was added. The samples were incubated 30 min at 37 °C and the reactions terminated by heating to 95 °C for 3 min. The samples were analyzed by reversed-phase HPLC on a Supelcosil LC-18 150×4.6 mm column (Supelco). Fifty millimolar KH_2PO_4/K_2HPO_4 (pH 4.0) was used as a mobile phase with a linear 2.5–20% methanol gradient. The free bases and nucleosides were detected by UV light absorbance at 254 nm and quantified by comparison to concentration standards. The data were analyzed and fitted to Michaelis–Menten plots using Statistica (StatSoft).

Northern blot analysis. Human multiple tissue Northern blot, human tumor Northern blot, and mouse multiple tissue Northern blot were obtained from Clontech. The human and mouse UPase-2 cDNAs were labeled with [α-^{32}P]dCTP (3000 Ci/mmol, Prime-A-Gene, Promega) and the probe hybridized at 68 °C using the ExpressHyb hybridization solution (Clontech) as described in the manufacturer's protocol. Human actin cDNA was used as a control probe (Clontech).

Results

Cloning of human and mouse UPase-2 cDNA

A biochemical study on tumor tissues suggests that an enzyme with UPase activity, in addition to UPase and TPase, may be present in human cells [19]. I hypothesized that this enzyme activity, if distinct from the UPase previously cloned, would exhibit sequence similarity to either of the two previously identified human pyrimidine nucleoside phosphorylases. The expressed tag sequence library was searched with the predicted protein sequences of both human UPase and TPase, and a cDNA encoding a novel human protein similar but not identical to the human UPase was identified. The 2070-bp cDNA encoding this novel enzyme was completely sequenced. The cDNA contained an open reading frame of 317 amino acid residues corresponding to a protein with a predicted molecular mass of 35.6 kDa (GenBank ID AY225131). Alignment of the predicted amino acid sequence of the new enzyme with human UPase showed that it was ≈60% identical to human UPase at the amino acid level (Fig. 1). Based on the high level of sequence similarity, the novel enzyme was named uridine phosphorylase-2 (UPase-2) and the previously cloned human uridine phosphorylase was named UPase-1. A mouse homolog of the human UPase-2 was also identified in the expressed sequence tag database.

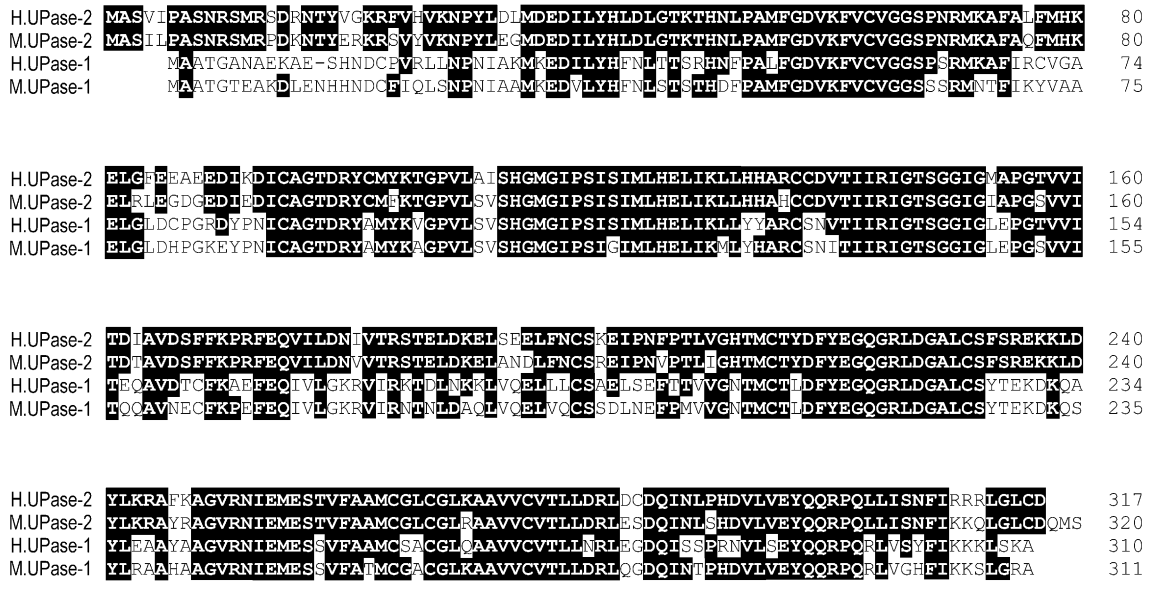

Fig. 1. Alignment of the human and mouse UPase-2 predicted amino acid sequences with human and mouse UPase-1. Black boxes indicate conserved amino acid residues compared to the human UPase-2 sequence.

The mouse enzyme was ≈85% identical to the human UPase-2, but had a three amino acid extension compared to the human enzyme in the C-terminal region of the open reading frame (Fig. 1). Both the human and mouse cDNA sequence had an ATG translation start codon in the same location of the open reading frame and showed no sequence similarity 5′ of this codon, indicating that the cDNA clones contained the complete open reading frame of the enzyme.

Expression and enzyme activity of human UPase-2

Human UPase-2 was recombinantly expressed to investigate the substrate specificity and kinetic properties of this novel putative nucleoside phosphorylase. The enzyme was expressed as a fusion protein to glutathione-*S*-transferase to facilitate purification (Fig. 2A). The enzyme was efficiently overexpressed and purified to homogenicity by one-step glutathione affinity chromatography with a yield of ≈3 mg pure recombinant protein per liter of bacterial culture (Fig. 2B). The fusion protein was used to investigate if the enzyme catalyzed cleavage of different naturally occurring pyrimidine and purine nucleosides at 100 μM concentration. Under these conditions, the enzyme accepted uridine, deoxyuridine as well as thymidine as substrates. In contrast, no activity was detected when the enzyme was incubated with adenosine, cytidine, guanosine, deoxyadenosine, deoxycytidine, or deoxyguanosine (data not shown). A factor Xa protease site was incorporated between the UPase-2 and glutathione-*S*-transferase proteins in the expression vector to allow separation of the two proteins after purification by proteolytic cleavage. However, incubation of the recombinant protein with factor Xa at different conditions resulted in unspecific cleavage of the fusion protein and loss of enzyme activity (data not shown). Subsequent studies were therefore performed using the uncleaved fusion protein.

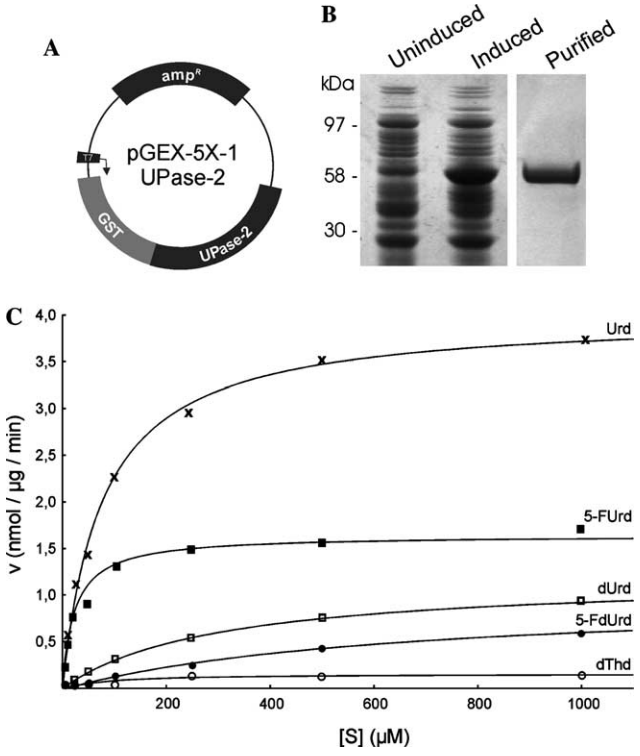

Fig. 2. Expression and enzymatic activity of human UPase-2. The enzyme was expressed in pGEX-5X-1 fused to GST (A). The overexpressed enzyme was purified from crude *E. coli* protein extracts by one-step glutathione affinity chromatography (B), and the nucleoside phosphorylase activity determined for uridine (×), deoxyuridine (□), thymidine (○), 5-FUrd (■), and 5-FdUrd (●) (C).

Table 1
Kinetic properties of recombinant human UPase-2 for pyrimidine nucleosides and nucleoside analogs (mean ± SE)

	K_m (μM)	V_{max} (nmol/μg/min)	V_{max}/K_m (×1000)
Urd	76 ± 6	4.0 ± 0.08	53
dUrd	300 ± 25	1.2 ± 0.04	4.0
dThd	73 ± 18	0.18 ± 0.01	2.5
5-FUrd	24 ± 4	1.6 ± 0.06	67
5-FdUrd	427 ± 67	0.8 ± 0.04	1.9

The kinetic properties of the recombinant human UPase-2 were determined with uridine, deoxyuridine, thymidine, 5-FUrd, and 5-FdUrd as substrates (Fig. 2C). The phosphorolytic cleavage of these nucleoside and nucleoside analogs were consistent with Michaelis–Menten kinetics in the concentration range investigated (5–1000 μM). Among the natural substrates, the enzyme showed preference for uridine (Table 1). The efficiency for uridine phosphorylysis, calculated as V_{max}/K_m, was ≈13- and 25-fold higher compared to the efficiency for deoxyuridine and thymidine, respectively. The efficiency of 5-FUrd cleavage was in the same range as the efficiency for uridine, whereas the efficiency for 5-FdUrd was ≈28-fold lower.

Tissue expression pattern

Northern blot analysis was used to determine the tissue expression of UPase-2 mRNA. A major ≈2.2 kb mRNA transcript was detected in human kidney (Fig. 3). A weak band of similar size was also detected in spleen and liver. The size of this transcript is consistent with the 2070-bp size of the cloned human cDNA. Expression of human UPase-2 mRNA in samples of eight human tumors was also studied. However, no expression of human UPase-2 mRNA was detected in any of the tumors investigated.

The expression of mouse UPase-2 mRNA was also investigated (Fig. 3). A ≈2.5-kb mRNA transcript was detected predominantly in liver with a weak band of similar size detected in kidney and brain. Two additional bands at ≈4.7 and 1.7 kb were also detected in mouse liver. These data suggest that the expression pattern of UPase-2 mRNA differs in human and mouse tissues.

Analysis of the expression pattern based on expressed sequence tag clones present in GenBank showed that 32 of the 35 human cDNA clones present in the database were derived from kidney cells. The three remaining human clones were from hippocampus, a total brain mRNA pool, and gall bladder. Accordingly, both the Northern blot analyses as well as the analysis of the expressed sequence tag clones suggest that the expression of human UPase-2 is largely restricted to kidney cells. In contrast, analysis of mouse expressed sequence tag clones showed that these clones were derived from other tissues. Out of 11 clones encoding mouse UPase-2, seven were derived from liver and the remaining clones were derived from lung, muscle, testis, and brain. No mouse expressed sequence tag clones from kidney encoding UPase-2 cDNA were identified. In conclusion, both Northern blot analysis and analysis of expressed sequence tag clones suggest that human and mouse UPase-2 exhibit differences in tissue-specific expression.

Gene structure

The human genome sequence database was searched with the human UPase-2 cDNA to identify the corre-

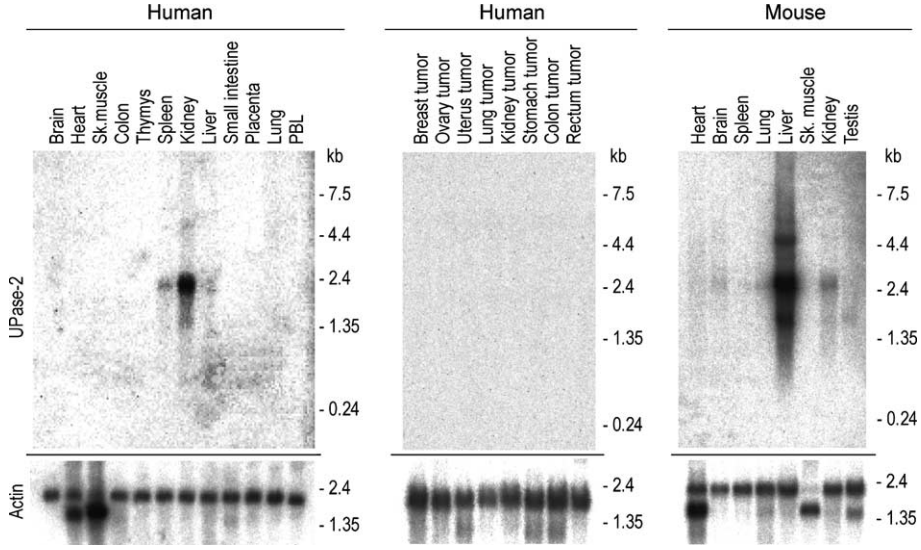

Fig. 3. Northern blot analysis of human and mouse UPase-2 mRNA expression in normal and malignant human tissues. An actin probe was used as a hybridization control.

Fig. 4. Structure of the human UPase-2 gene located at chromosome 2q24.1. Exons are shown as black boxes with roman numbers.

sponding genomic sequence. A bacterial artificial chromosome clone (RP11-335J18) containing the complete genomic sequence corresponding to the human UPase-2 cDNA was identified. The genomic clone was located at chromosome 2q24.1. Alignment of the UPase-2 cDNA with the genomic sequence showed that the gene was divided in nine exons distributed over ≈150 kbp (Fig. 4). The predicted ATG translation start codon was located in exon three.

Discussion

The identification and cDNA cloning of human UPase-2 shows that a third pyrimidine phosphorylase is present in mammalian cells, in addition to UPase-1 and TPase. The enzyme had broader substrate specificity compared to human UPase-1 and also accepted thymidine as a substrate in addition to uridine and deoxyuridine. The physiological role of the novel UPase-2 in nucleoside metabolism is not presently known. In human tissues, UPase-2 mRNA expression was largely restricted to kidney cells. Uridine sugar derivatives are required for protein glycosylation in the biosynthesis of basal membrane components in the glomerulus. High levels of UPase activity have been detected in glomerular cells and it is possible that the enzyme contributes to uridine pool homeostasis in kidney [25]. However, the mouse UPase-2 mRNA was predominately expressed in liver with low or absent expression in most other tissues. Therefore, both the physiological role of UPase-2 in nucleoside metabolism and why this enzyme shows this marked difference in tissue-specific expression in different species are presently unclear.

Fluorogenated uracil and uridine analogs are used in chemotherapy of several types of malignant tumors [13]. Their therapeutic efficiency is limited by toxicity to normal tissues. One strategy investigated to improve the therapeutic index of uridine nucleoside analog chemotherapy involves manipulation of the uridine pool by combining the cytotoxic nucleoside analogs with uridine and uridine phosphorylase inhibitors [26–29]. An increase in cellular uridine levels protects normal non-malignant tissues from nucleoside analog cytotoxicity but does only to a minor extent reduce nucleoside analog sensitivity of cancer cells. Administration of uridine alone, without a uridine phosphorylase inhibitor, results in rapid degradation of uridine and the therapeutic strategy requires the presence of a uridine phosphorylase inhibitor. The differences in substrate specificity of UPase-1 and UPase-2 suggest that it may be possible to design selective inhibitors of these enzymes. If UPase-2-mediated catalysis contributes to uridine clearance from plasma, selective inhibition of either UPase-1 or UPase-2 should be evaluated as a strategy to further improve the therapeutic index of the combination chemotherapy. Because of the difference in tissue expression of the enzyme, these experiments should not be performed in mice models.

Mutations in the human TPase gene resulting in loss of enzyme activity have been shown to cause mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) [30,31]. Patients with MNGIE exhibit increased levels of thymidine in plasma and an almost complete loss of TPase activity in fibroblasts and peripheral blood leucocytes [32]. Several features of MNGIE suggest that the disease is primarily caused by mitochondrial dysfunction and MNGIE patients exhibit multiple mitochondrial DNA deletions. However, the mechanism by which TPase deficiency results in mitochondrial dysfunction and DNA deletions is not known. Haraguchi and co-workers [33] recently reported the generation of TPase deficient mice to study the mechanism of MNGIE in a mouse model. However, in contrast to human UPase-1, the murine UPase-1 efficiently catalyzes cleavage of thymidine [5], and the TPase$^{-/-}$ mice retained TPase activity predominantly in the intestine. Double knockout mice with targeted deletion of both the TPase gene as well as the UPase-1 gene were therefore generated [33]. The double knockout TPase$^{-/-}$ UPase-1$^{-/-}$ mice had increased levels of thymidine and uridine in plasma, but did not exhibit signs of mitochondrial DNA damage or other features common in MNGIE patients. These investigators hypothesized that another genetic defect rather than TPase deficiency was the primary cause of MNGIE in humans. However, the existence of a third pyrimidine nucleoside phosphorylase reported in the present paper shows that the TPase$^{-/-}$ UPase-1$^{-/-}$ mice are not completely deficient in pyrimidine nucleoside phosphorylase activity. It is possible that UPase-2 protects these mice from the toxicity of thymidine accumulation and that the difference in tissue expression causes the difference in humans and mice TPase deficiency phenotype.

Acknowledgments

This work was supported by grants from the Swedish Medical Research Council and the Medical faculty of the Karolinska Institute.

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