Cloning and characterization of full-length mouse thymidine kinase 2: the N-terminal sequence directs import of the precursor protein into mitochondria

Liya WANG[1] and Staffan ERIKSSON

Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, The Biomedical Centre, P.O. Box 575, SE-751 23 Uppsala, Sweden

The subcellular localization of mitochondrial thymidine kinase (TK2) has been questioned, since no mitochondrial targeting sequences have been found in cloned human TK2 cDNAs. Here we report the cloning of mouse TK2 cDNA from a mouse full-length enriched cDNA library. The mouse TK2 cDNA codes for a protein of 270 amino acids, with a 40-amino-acid presumed N-terminal mitochondrial targeting signal. *In vitro* translation and translocation experiments with purified rat mitochondria confirmed that the N-terminal sequence directed import of the precursor TK2 into the mitochondrial matrix. A single 2.4 kb mRNA transcript was detected in most tissues examined, except in liver, where an additional shorter (1.0 kb) transcript was also observed. There was no correlation between the tissue distribution of TK2 activity and the expression of TK2 mRNA. Full-length mouse TK2 protein and two N-terminally truncated forms, one of which corresponds to the mitochondrial form of TK2 and a shorter form corresponding to the previously characterized recombinant human TK2, were expressed in *Escherichia coli* and affinity purified. All three forms of TK2 phosphorylated thymidine, deoxycytidine and 2′-deoxyuridine, but with different kinetic efficiencies. A number of cytostatic pyrimidine nucleoside analogues were also tested and shown to be good substrates for the various forms of TK2. The active form of full-length mouse TK2 was a dimer, as judged by Superdex 200 chromatography. These results enhance our understanding of the structure and function of TK2, and may help to explain the mitochondrial disorder, mitochondrial neurogastrointestinal encephalomyopathy.

Key words: DNA precursor, mitochondrial, nucleoside analogues, nucleoside kinase.

INTRODUCTION

All mammalian cells possess two distinct thymidine kinases (TKs): one is found in the cytosol (TK1; EC 2.7.1.21), and the other is present in the mitochondria (TK2; no EC number) [1]. Both TK1 and TK2 phosphorylate thymidine (dThd) and 2′-deoxyuridine (dUrd) efficiently to their monophosphate forms, but TK2 also uses deoxycytidine (dCyd) as a substrate. These two enzymes are genetically distinct, being encoded by two different genes which are located on different chromosomes: chromosome 17 for TK1 and chromosome 16 for TK2 [2,3]. The expression of TK1 is cell-cycle-controlled, with the highest levels of both mRNA and protein being present in S-phase. There is a rapid decline of both mRNA and TK1 protein after mitosis [4,5]. TK1 levels are thus high in rapidly dividing cells, but low or zero in resting cells. The expression of TK2 is not cell-cycle-regulated and is similar in terminally differentiated cells and growing cells. Therefore, in non-proliferating tissues, TK2 is the only pyrimidine nucleoside salvage enzyme that is expressed [6,7].

Earlier studies have indicated that there is a mitochondrial form of TK, and that synthesis of mitochondrial DNA (mtDNA) is resistant to limitations in the cytosolic supply of dThd triphosphate [8]. A study by Bestwick and Mathews [9] also showed that mitochondria contain separate dNTP pools, and thus TK2 may play an important role in the synthesis of DNA precursors for mtDNA replication. Alterations in dThd levels in the cells can cause an imbalance of the dNTP pools [10], which may result in mutations or deletions in both newly synthesized nuclear DNA and mtDNA. A recent report on one type of mitochondrial disease, mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), is an example of this phenomenon, since it is related to aberrant dThd metabolism, which leads to deletions in the mtDNA in affected patients [11]. TK2 was suggested to be involved in this abnormality, but its role remains to be determined.

Most mitochondrial proteins are encoded by nuclear genes, synthesized as precursors in the cytosol, targeted to the mitochondria, and finally imported into their respective mitochondrial subcompartments. The N-terminal domains of mitochondrial proteins possess a targeting signal which directs the import of the precursor protein to the mitochondria [12]. For most matrix proteins the N-terminal presequences function as cleavable matrix-targeting signals that initiate translocation across both mitochondrial membranes in a membrane-potential-dependent fashion [13]. TK2 has, by several biochemical criteria, been shown to be located in the mitochondria (reviewed by Gentry [14]). However, there are studies suggesting a cytosolic form of TK2 in human cells [15]. The two earlier published

Abbreviations used: dThd, thymidine; dCyd, deoxycytidine; dUrd, 2′-deoxyuridine; DTT, dithiothreitol; AZT, 3′-azido-2′,3′-dideoxythymidine; MNGIE, mitochondrial neurogastrointestinal encephalomyopathy; FIAU, 1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil; mtDNA, mitochondrial DNA; TKs, thymidine kinases; mTK2, mouse TK2.

[1] To whom correspondence should be addressed (e-mail liya.wang@vmk.slu.se).

The nucleotide sequence data reported will appear in the GenBank®/EBI/EMBL/DDBJ/GSDB Nucleotide Sequence Databases under accession number AJ249341.

sequences of human TK2 did not include a mitochondrial targeting sequence as part of the cDNA [16,17], which makes the subcellular localization of this enzyme unclear.

Here we report the cloning of full-length mouse TK2 (mTK2) cDNA, clarify the mitochondrial location of the enzyme by using an *in vitro* mitochondria import assay, and characterize various forms of recombinant protein using the natural substrates and cytostatic nucleoside analogues. The mRNA and enzyme activity levels in different mouse tissues were also determined. Knowledge of the gene expression and properties of mTK2 will facilitate studies of the physiological role of this enzyme in the regulation of metabolism of mtDNA precursors.

MATERIALS AND METHODS

Materials

The radiolabelled substances [γ-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), [*methyl*-^{3}H]dThd (25 Ci/mmol), [5-^{3}H]dCyd (24 Ci/mmol) and L-[^{35}S]methionine (1000 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

cDNA cloning

A mouse cDNA clone was identified using the human TK2 sequence (GenBank accession no. Y10498) to search against the EST (Expressed Sequence Tag) database using the BLAST program. One clone (accession no. AU035388) was found to have the longest 5′-sequence, and was provided by Dr Katsuyuki Hasimoto (Division of Genetic Resources, National Institute of Infectious Diseases, Tokyo, Japan). The cDNA clone was resequenced on both strands using a Bigdye terminator cycling sequencing kit and an ABI prism 310 system (Perkin-Elmer). 5′-RACE (rapid amplification of cDNA ends) PCR was used to amplify the 5′-upstream region. The amplified PCR products were cloned into the TOPO-TA vector (Invitrogen) and sequenced. The nucleotide sequence was deposited in the GenBank/EBI databases under accession number AJ249341.

Northern blot analysis

A mouse multiple-tissue Northern blot (Clontech, Palo Alto, CA, U.S.A.) was used to examine the expression of mTK2 mRNA. The coding sequence (nucleotides 4–816) of mTK2 cDNA was labelled with ^{32}P (Random Primed DNA Labelling Kit; Boehringer Mannheim) and used as a probe. Hybridization was carried out at 65 °C in ExpressHyb® solution (Clontech) for 1 h, and then the filter was washed twice in 0.2 × SSC/0.1 % SDS (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) at 50 °C and autoradiographed. A human β-actin cDNA (supplied by Clontech) was labelled and hybridized to the same filter.

Expression and purification of mouse recombinant TK2 protein

The coding sequence for mTK2 cDNA was PCR-amplified and subcloned into the pET-14b vector (Novagene) using the *Nde*I and *Bam*HI cloning sites. The mTK2 construct was then transformed into *Escherichia coli* BL21(DE3) pLys S. mTK2 was expressed and purified as described previously [17], and analysed by SDS/PAGE. In order to stabilize the enzyme, dithiothreitol (DTT) and glycerol were added to the TK2 preparations to final concentrations of 1 mM and 15 % respectively. Two N-terminally truncated mTK2 proteins, starting at amino acids 39 and 55 respectively of the full-length sequence, were also generated using the same expression vector. These proteins were expressed and purified as described above for full-length mTK2.

Isolation of rat liver mitochondria, and *in vitro* import of mTK2

Mitochondria from the liver of a male Sprague–Dawley rat (approx. 250 g) were prepared essentially as described [18], and the mitochondrial pellet was resuspended in buffer containing 20 mM Hepes/KOH (pH 7.4), 250 mM sucrose, 1 mM ATP, 0.08 mM ADP and 2 mM K_2HPO_4 at a concentration of 1 mg of protein/ml. The full-length mTK2 cDNA (in the PET-14b vector) was transcribed and translated in the presence of L-[^{35}S]methionine in a cell-free rabbit reticulocyte lysate system according to the manufacturer's protocol (Promega). Lysates containing the labelled mTK2 protein were stored at −70 °C until needed. For the mitochondrial protein import reaction, 5 μl of labelled mTK2 was added to 20 μl of import buffer [250 mM sucrose, 20 mM Hepes/KOH (pH 7.4), 2 mg/ml BSA, 100 mM KCl, 2 mM magnesium acetate, 2 mM DTT, 2 mM ATP and 0.4 mM GTP] and 25 μl (50 μg) of purified mitochondria, and incubated at 30 °C or 37 °C for 120 min. The samples were then diluted with 200 μl of buffer [10 mM Hepes/KOH (pH 7.4), 250 mM sucrose and 12.5 μg/oligomycin] and a combined fraction was treated with proteinase K (50 μg/ml) by incubation on ice for 10 min. The reaction was stopped by the addition of 1 mM PMSF. The mitochondria were pelleted by centrifugation at 10000 ***g*** for 4 min, and the pellet was resuspended in SDS sample buffer and analysed by SDS/PAGE and autoradiography.

Enzyme assays

Nucleoside kinase activities were determined by radiochemical methods using the DE-81 filter paper technique with ^{3}H-labelled substrates [17], or the phosphoryl transfer assay with nucleoside analogues [19]. The DE-81 filter paper assay was performed in 50 mM Tris/HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM ATP, 10 mM DTT and 0.5 mg/ml BSA, with 10 μM ^{3}H-labelled substrate. The phosphoryl transfer assay was performed with 100 μM [γ-^{32}P]ATP (10 mCi/ml) in the same buffer as above, but with 0.1 M KCl added and 10 or 100 μM nucleoside, in a total volume of 50 μl. The phosphorylated products were separated by TLC [poly(ethyleneimine)–cellulose] and quantified as described [19].

Gel-filtration chromatography

Purified mTK2 was applied to either a Superose® 12 column or a Superdex® 200 column (Amersham Pharmacia Biotech) attached to an FPLC instrument (Amersham Pharmacia Biotech). Both columns were equilibrated with buffer containing 50 mM Tris/HCl (pH 7.6), 100 mM KCl, 1 mM $MgCl_2$ and 1 mM DTT. Fractions were collected and assayed for enzyme activity. Both columns were calibrated initially with the same standard molecular size markers (Sigma). The molecular size of active TK2 was determined with the aid of the calibration curves.

RESULTS

Cloning of mTK2

The cloning of mTK2 cDNA was achieved by using the human TK2 sequence to search the mouse EST database using the BLAST program. Three EST clones showed sequence similarity with the human TK2 sequence. The one with the longest open reading frame (accession no. AU035388) was obtained from Dr Katsuyuki Hasimoto. Both strands of this cDNA were re-

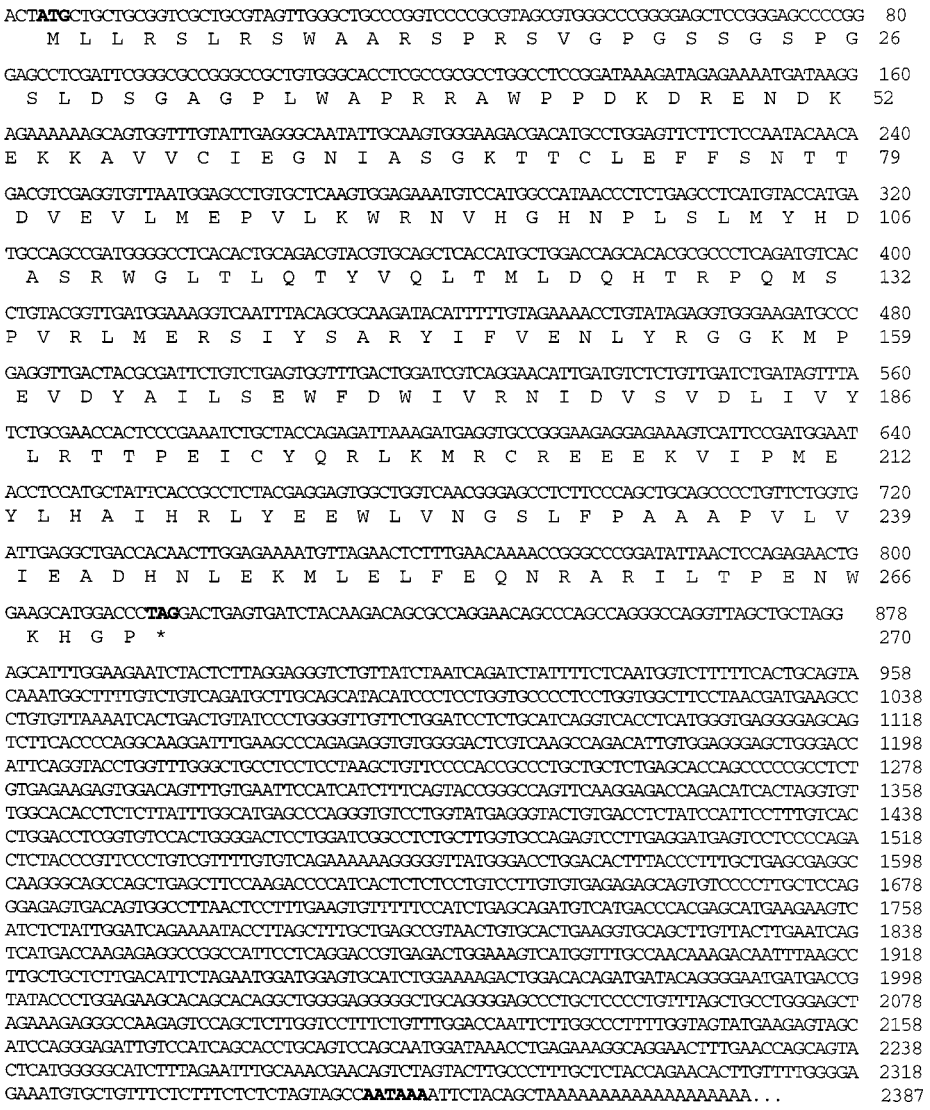

Figure 1 Nucleotide and deduced amino acid sequence of mTK2

The initiation and stop codons, as well as the polyadenylation site, are in **bold** type. The nucleotide sequence will appear in the GenBank/EBI databases under accession number AJ249341.

sequenced, and 5′-RACE PCR was used to amplify the 5′-upstream sequence. The amplified PCR products were then subcloned and sequenced. An mTK2 cDNA of 2387 bp was thus cloned, which contains an open reading frame of 813 bp that codes for 270 amino acids, with a 40-amino-acid putative mitochondrial targeting sequence (Figure 1). mTK2 shows 80% sequence identity with human TK2 [17] at the amino acid level, and only 50% identity at the nucleotide level. There was little sequence similarity in the 3′-untranslated region between the human and mouse sequences, but the functionally important sequence motifs, such as the ATP-binding loop and the substrate recognition sites [7], were all completely conserved.

Determination of mTK2 mRNA and enzyme activity levels

A mouse multiple-tissue Northern blot was used to examine the expression of TK2 mRNA in different tissues (Figure 2).There was only one transcript, of size 2.4 kb, expressed in most tissues, which corresponds to the size of the cloned cDNA. An additional transcript ($\approx$ 1 kb) was also observed in liver. The highest level of TK2 mRNA was found in liver, with much lower levels in spleen and lung. No detectable TK2 mRNA was expressed in skeletal muscle. As a control, a β-actin probe was hybridized to the same blot (Figure 2), and the levels of the 2.0 kb transcript and/or the 1.4 kb β-actin transcript were similar in all cases. Earlier studies have shown that levels of TK2 mRNA in human skeletal muscle are similar to those in other tissues [16,17], and the lack of TK2 mRNA in mouse skeletal muscle is at present unexplained.

In order to correlate TK2 mRNA expression with the levels of enzyme activity, we have determined the TK activity in different mouse tissue extracts (Table 1). Total TK activity was measured with [^{3}H]dThd as substrate. dCyd is also a substrate for TK2, but not for TK1. The measurement of activity with [^{3}H]dThd in the presence of excess dCyd is a selective measure of TK1 activity. We have used total TK activity minus the activity with dCyd to determine TK2 activity. As a control, recombinant mTK2 was assayed under the same conditions; 50% of total dThd-

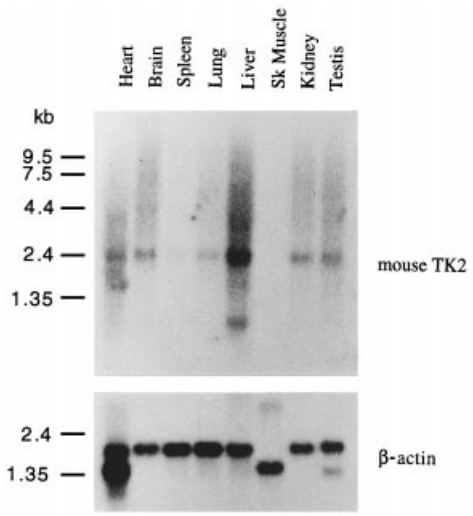

Figure 2 Northern analysis of mRNA expression

TK2 mRNA expression (upper panel) and β-actin mRNA expression (lower panel) in various mouse tissues was assessed using a multiple-tissue Northern blot (Clontech).

Table 1 Total TK activity and estimated TK2 activity in various mouse tissue extracts

The percentage inhibition of total TK activity caused by the addition of 500 μM dCyd is shown in parentheses.

Sample	Total TK activity (pmol/min per mg)		TK2 activity (% of total TK activity)
	No addition	+ 500 μM dCyd	
Spleen	1016	846 (16%)	< 15
Liver	7	4 (39%)	> 90
Brain	45	16 (64%)	≈ 100
Muscle	35	32 (4%)	< 5
Lung	26	8 (69%)	≈ 100
Kidney	45	13 (70%)	≈ 100
Heart	15	6 (57%)	≈ 100

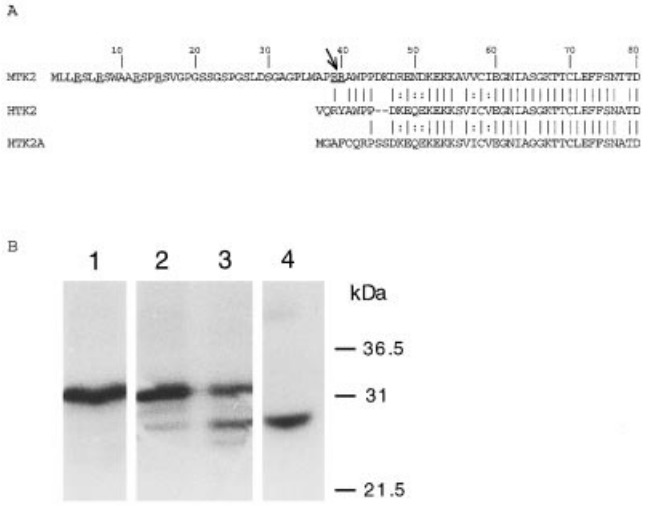

Figure 3 Functional analysis of the N-terminal amino acid sequence of mTK2

(**A**) Alignment of the N-terminal sequence of mTK2 with that of human TK2 (HTK2 [17] and HTK2A [16]). Vertical lines (|) indicate identical residues, and colons (:) indicate conservative substitutions. The positively charged residues in the first 40 amino acids of the mouse sequence are underlined. An arrow indicates the putative mitochondrial cleavage site. (**B**) Import of mTK2 into rat liver mitochondria. Full-length mTK2 was transcribed and translated *in vitro* in the presence of [^{35}S]Met (lane 1) and used in mitochondrial import assays. After incubation with mitochondria at either 30 °C (lane 2) or 37 °C (lane 3), aliquots from the two reactions were combined and treated with proteinase K to digest extramitochondrial precursor protein (lane 4).

phosphorylating activity was retained in the presence of an excess of dCyd. Total TK activity was found to be highest in spleen, while that in brain, kidney, muscle, lung, heart and liver was low. The TK activity in brain, kidney, lung, heart and liver extracts was derived mainly from TK2, while in spleen and muscle the activity appeared to be due to TK1. TK2 activity in spleen may be due to interference by the very high TK1 level; there was only a very low level of TK2 mRNA in this tissue. In muscle extracts total TK activity was not sensitive to dCyd inhibition, and therefore the level of TK2 is likely to be very low in this tissue.

Mitochondrial import of mTK2

The destination of a newly synthesized protein that is encoded by a nuclear gene relies on its primary sequence. Most mitochondrial proteins possess an N-terminal targeting signal sequence which directs the translocation of the protein into mitochondria. The N-terminal 40 amino acids of mTK2 contain six positively charged residues and nine hydroxylated residues, and the remaining amino acids are mostly hydrophobic residues (Figure 3A). This sequence is predicted to form a short α-helix followed by a β-sheet structure, which is characteristic of mitochondrial targeting sequences. The putative cleavage site for mTK2 is at amino acid 38, and the mature protein would be predicted to have a molecular mass of 27.6 kDa, which is similar in size and sequence to the previously purified human enzyme [17]. To investigate the function of the N-terminal sequence, we labelled the mTK2 protein with [^{35}S]methionine by using the T7 coupled *in vitro* transcription and translation system [20,21]. The labelled precursor protein was then used in an *in vitro* mitochondrial import experiment with purified rat liver mitochondria (Figure 3B). One major processed form of TK2 was observed, with an estimated molecular mass of 28 kDa. This form of TK2 was resistant to proteinase K treatment and thus represents intramitochondrial TK2. The intensity of the 28 kDa band in the proteinase K-treated sample could not be compared directly with the other two import reactions in this experiment. The efficiency of the import reaction was approx. 10% at 37 °C, and much lower at 30 °C. These results strongly suggest that the predicted mitochondrial cleavage site indicated in Figure 3(A) is the correct one.

Characterization of the enzymic properties of recombinant mTK2 with different N-terminal amino acid sequences

The open reading frame of mTK2 cDNA was PCR-amplified and subcloned into the PET-14b expression vector and transfected into BL21(DE3) pLys S bacteria. Three constructs were made: the entire open reading frame, giving a full-length enzyme (mTK2); one starting at the putative mitochondrial cleavage site (amino acid residue 39; mTK2-39); and a short version beginning with the putative ATP binding loop (starting at amino acid number 55; mTK2-55). The latter is similar to the recombinant

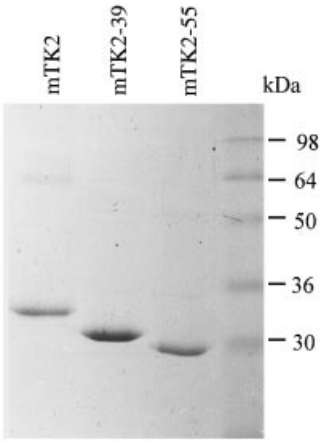

Figure 4 SDS/PAGE of purified recombinant mTK2 proteins

mTK2 is the full-length enzyme, mTK2-39 starts at residue 39, and mTK2-55 starts at residue 55.

form of human TK2 characterized previously [17]. All three forms of TK2 were expressed and affinity purified (Figure 4). The purified enzymes were stored in a stabilization buffer, but mTK2 lost enzyme activity when stored at 4 °C, while mTK2-39 and mTK2-55, like human recombinant TK2, were stable for up to 3 weeks. Therefore all characterizations were carried out within a few hours of purification.

As expected, mTK2 phosphorylated the pyrimidine deoxyribonucleosides dThd, dCyd and dUrd, and also a number of pyrimidine nucleoside analogues (Tables 2 and 3). The kinetic parameters for recombinant mTK2 were determined using the ^{3}H-labelled deoxynucleosides dThd and dCyd with all three forms of the enzyme. The kinetics of dThd phosphorylation were similar to those seen with human TK2, with negative cooperativity (Hill coefficient < 1). The kinetic constants presented here for dThd are apparent K_m and V_{max} values calculated from the standard Michaelis–Menten equation for simplicity. The length of the N-terminal fragment did not affect the K_m values, but the V_{max} values changed (Table 2). The phosphorylation efficiency (V_{max}/K_m) for dThd was 19 for mTK2, 58 for mTK2-39 and 214 for mTK2-55. Thus the most active form was mTK2-55 and the least active form was mTK2. The phosphorylation of dCyd followed classic Michaelis–Menten kinetics, and full-length mTK2 had much lower efficiency than the shorter forms (Table 2). Therefore the most active forms of the enzyme appear to be the processed forms.

We tested a number of nucleoside analogues using the phosphoryl transfer assay (Table 3). Recombinant mTK2 phosphorylated the arabinosyl nucleosides of dThd, dCyd and dUrd less efficiently than their natural substrates, with uracil arabinoside being the poorest substrate. At physiologically relevant concentrations (10 μM), phosphorylation of the anti-HIV nucleoside AZT (3′-azido-2′,3′-dideoxythymidine) was approx. 18 % that of dThd, and phosphorylation of the anti-cancer drugs cytosine arabinoside and 5-fluoro-2′-deoxyuridine was approx. 10 % and 70 % respectively that of dThd. Among the nucleoside analogues tested, 1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil (FIAU), an anti-hepatitis drug, was one of the best

Table 2 Kinetic parameters for various N-terminally truncated forms of recombinant mTK2

Values are the means of three independent determinations with $< 15\%$ variation. The K_m values were determined as the substrate concentration at $1/2 V_{max}$.

	dThd			dCyd		
Enzyme	K_m (app.) (μM)	V_{max} (app.) (nmol/min per mg)	V_{max}/K_m (app.)	K_m (μM)	V_{max} (nmol/min per mg)	V_{max}/K_m
mTK2	3.4	64	19	25.1	270	11
mTK2-39	4.9	284	58	23.6	1406	60
mTK2-55	7.3	1563	214	11.5	1971	171

Table 3 Phosphorylation of nucleoside analogues by various forms of recombinant mTK2 using the phosphoryl transfer assay

The values are the means of at least two independent determinations with $\pm 20\%$ variation, and are relative activity compared with that with dThd at 10 μM (= 1.00) for each enzyme. The specific activity with dThd at 10 μM was 35.4, 292 and 1110 nmol/min per mg for mTK2, mTK2-39 and mTK2-55 respectively. Abbreviations: AraT, thymine arabinoside; AraC, cytosine arabinoside; 5FdC, 5-fluoro-2′-deoxycytidine; AraU, uracil arabinoside; 5FdU, 5-fluro-2′-deoxyuridine.

	mTK2		mTK2-39		mTK2-55	
Substrate	10 μM	100 μM	10 μM	100 μM	10 μM	100 μM
dThd	1.00	2.74	1.00	2.09	1.00	1.59
AraT	0.57	1.51	0.73	1.68	1.17	1.68
AZT	0.18	0.21	0.19	0.25	0.14	0.20
dCyd	1.15	3.04	1.40	2.86	1.66	4.21
AraC	0.13	0.79	0.07	0.59	0.10	0.79
5FdC	0.46	1.09	0.30	0.86	0.46	1.32
dUrd	0.76	1.94	0.78	1.45	1.25	0.95
AraU	0.04	0.21	0.04	0.38	0.08	0.48
FIAU	1.03	3.00	1.12	3.23	1.44	2.72
5FdU	0.72	2.15	0.72	1.53	1.27	1.41

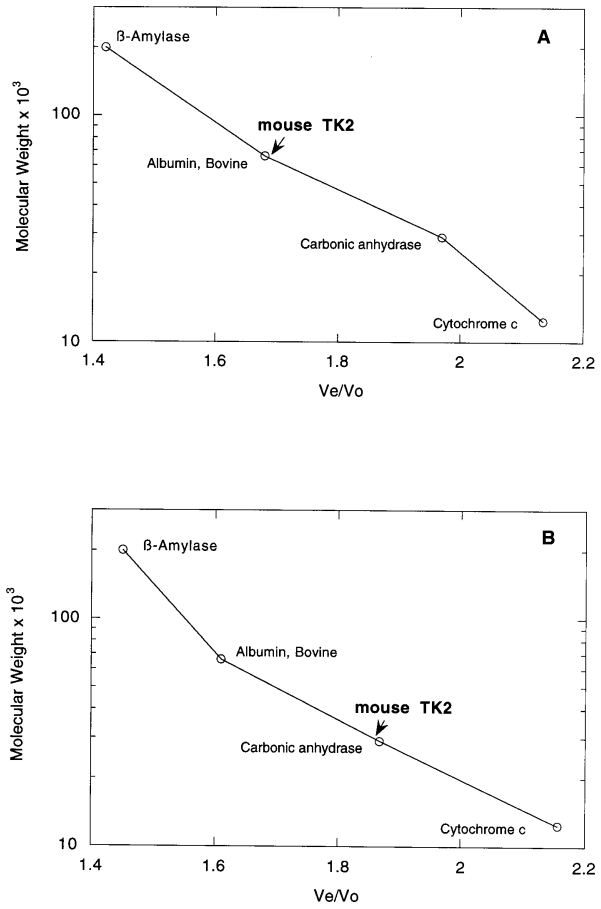

Figure 5 Determination of the molecular size of the active form of full-length mTK2

Gel-filtration chromatography was carried out on a Superdex 200 column (**A**) and a Superose 12 column (**B**). Arrows indicate the relative elution volume of active mTK2. Ve, elution volume. Vo, void volume.

substrates for the mouse enzymes, which is similar to results with human TK2 [19].

In order to determine the molecular size of the active enzyme, purified mTK2 was subjected to gel-filtration chromatography on both Superdex 200 and Superose 12 columns under similar conditions. Fractions were collected and assayed for TK2 activity. During Superdex 200 chromatography mTK2 eluted at a molecular size corresponding to approx. 60 kDa, while in case of Superose 12 chromatography mTK2 eluted at an apparently smaller molecular size corresponding to approx. 30 kDa (Figure 5). The possible reasons for this discrepancy will be discussed below.

DISCUSSION

TK2 was first identified in mouse cells that lacked cytosolic TK activity [1], and a study of bromodeoxyuridine incorporation into mtDNA in TK1-negative cell lines suggested a mitochondrial location for TK2 [8]. However, the human TK2 cloned by Johansson and Karlsson [16] did not contain a leader sequence that can direct the import of TK2 into mitochondria, and it also has a different N-terminal sequence than purified human brain TK2 [17]. We have previously cloned a human TK2 cDNA which codes for an N-terminal protein sequence identical with the one obtained for purified human brain TK2, but this sequence was not complete and also lacked a mitochondrial targeting signal [17]. Here we report the cloning of a full-length mTK2 cDNA. The open reading frame codes for 270 amino acids, and contains sequences typical of both the catalytic domain and a mitochondrial targeting sequence. The nucleotide sequence of the N-terminus shows 77.5% G+C content, and this high GC content may create problems in conventional reverse transcription procedures. The cloning of the full-length mouse cDNA could be achieved because the cDNA library was constructed using the oligo-capping method [22].

A 2.4 kb TK2 mRNA was expressed in most tissues, and the size of the mRNA thus corresponds to the length of the cloned cDNA. This was not the case in human tissues, where multiple transcripts were observed [16,17]. The expression of mTK2 mRNA was highly tissue-specific, with the highest expression in liver and very low levels in lung and spleen. However, TK2 activity in these tissue extracts did not correlate with the mRNA levels, and further studies are needed in order to clarify the mechanism involved in the regulation of the expression of TK2.

When the N-terminal 40 amino acids of mTK2 were analysed using the program 'Prediction of Protein Localisation Sites' (http://psort.nibb.ac.jp:8800), there were two cleavage sites suggested: one at amino acids 13–14 (-SPR-) and one at amino acid 38 (-WAPRR-). Cleavage at this latter site will result in a mature protein which is similar in both size and sequence to human TK2 [17]. We have performed *in vitro* mitochondrial import assays with ^{35}S-labelled full-length mTK2, and found that the N-terminal signal sequence directed the import of TK2 into isolated rat (Figure 3) or mouse (results not shown) mitochondria. The processed mitochondrial form of TK2 had a molecular mass of 28 kDa, which strongly indicates that mTK2 is cleaved at amino acid 38 as proposed above. Since only one type of mRNA is observed, we may conclude that there is just one form of mTK2 and that this enzyme is located in the mitochondria.

During the preparation of the present paper, Wettin et al. [23] reported the cloning of a cDNA coding for mTK2. There are several differences between that mTK2 sequence and the one reported here. Our full-length cDNA of 2.4 kb corresponds in size to the single mRNA transcript found in mouse tissues, while the sequence of Wettin et al. [23] is 1.6 kb in size, and lacks 865 bp in the middle of the 3′-untranslated region. Furthermore, the nucleotide and amino acid sequences reported in the publication of Wettin et al. [23] differed from those sequences in the GenBank entry (accession no. AF105217), and nucleotides 44 and 70 are also different from our sequence, leading to substitution of Pro-14 by Leu and of Gly-23 by Arg. Probably because of this difference, Wettin et al. [23] suggested a mitochondrial cleavage site at amino acid 24, which is not in agreement with results of the mitochondrial import reaction presented here. There was no determination of the enzyme activity of the product of the mTK2 cDNA cloned by Wettin et al. [23].

Characterization of the enzymic properties of TK2 has been performed previously using purified native human TK2 [24] or the N-terminally truncated form of the recombinant human enzyme [17]. We have expressed, purified and characterized full-length mTK2 protein, and also two shorter versions of the enzyme, in order to compare their properties [17,24]. mTK2 phosphorylated dThd with negative co-operativity, but the phosphorylation of dCyd followed classic Michaelis–Menten kinetics, as was observed previously with human TK2. The K_m and V_{max} values for dThd were lower than those for dCyd, and this resulted in similar overall efficiency with the two pyrimidine

substrates. Among the three forms of mTK2, the full-length enzyme showed the lowest efficiency, as measured by apparent V_{max}/K_m values. The phosphorylation of nucleoside analogues by the various forms of mTK2 was also examined, and there were no significant differences in overall substrate specificity. However, the N-terminally truncated forms of TK2 showed higher rates of phosphorylation of most of the analogues tested. mTK2 with truncation of the N-terminal 54 amino acids had similar properties to the corresponding human form of TK2. However, there were differences in the efficiency of phosphorylation of certain nucleoside analogues; e.g. AZT and cytosine arabinoside were somewhat better substrates for the mouse enzyme as compared with human TK2 [17].

Native human TK2 was reported to be a monomer in its active form, as judged by gel-filtration chromatography using a Superose 12 column [24]. Because TK2 shows high sequence identity with deoxycytidine kinase and deoxyguanosine kinase, one may expect that the active forms of these enzymes are similar, and both deoxycytidine kinase and deoxyguanosine kinase have been shown to be dimers [25,26]. Here we observed that the active form of recombinant mTK2 was indeed a dimer, when using gel-filtration chromatography on a Superdex 200 column. However, when the same enzyme preparation was analysed by Superose 12 chromatography, the TK2 activity eluted at a molecular size corresponding to 30 kDa, which is similar to what was reported previously with human TK2 [24]. The reason for this discrepancy is not known, but it may be that TK2 interacts with the matrix in the Superose 12 column, leading to an aberrant molecular size estimation.

As discussed above, the length of the N-terminal sequence of various forms of mTK2 affects not only the phosphorylation efficiency but also the stability of the enzyme. Full-length mTK2 was much less active and more unstable during storage as compared with the N-terminally truncated forms. One possible explanation is that the synthesis and translocation of a mitochondrial protein is a coupled process. Newly synthesized mitochondrial proteins may be transported directly into the mitochondria and processed with the help of chaperone proteins [27]. The N-terminal sequence of mTK2 not only directs the translocation of the enzyme, but also appears to regulate its stability. Any remaining TK2 in the cytosol is most probably degraded rapidly. The present study unequivocally demonstrates that both mitochondrially processed and non-processed TK2 forms have a broad substrate specificity with regard to pyrimidine deoxynucleosides. Therefore it may be more appropriate to name this enzyme pyrimidine deoxyribonucleoside kinase, and we are now applying for a separate EC number for this enzyme using the new name. The renaming of the enzyme will hopefully make the nomenclature and classification of the enzymes in the deoxynucleoside kinase family more clear-cut in the future.

In cells, dThd is either degraded to thymine or salvaged to dThd monophosphate by TK1 and TK2. Because mtDNA is continuously replicating, even in quiescent cells, a constant supply of dNTPs is vital for maintenance of the mitochondrial genome. We show here that the mitochondrial form of TK2 phosphorylates both dThd and dCyd with similar efficiency, but with different kinetic mechanisms. dThd inhibits efficiently the dCyd phosphorylation carried out by TK2, with a K_i value of 6 μM, while dCyd is a poor competitor of dThd phosphorylation, with a K_i value of 630 μM [17]. Under normal metabolic conditions the phosphorylation of dThd and dCyd by TK2 may be regulated by the availability of the substrate in the tissues and by feedback regulation by the end products, e.g. dTTP and dCTP. In this way, balanced dNTP pools in the mitochondria can probably be maintained. In patients with MNGIE, high levels of dThd were found, most probably due to the observed deficiency of the dThd-metabolizing enzyme, dThd phosphorylase [11]. The high levels of dThd should compete with dCyd for phosphorylation by TK2 *in vivo*. This would eventually result in large dTMP pools and small dCMP pools, which will lead in turn to altered dTTP and dCTP pools. Such pool inbalances may induce mutations or deletions in newly synthesized mtDNA. Thus this new information about full-length mTK2 will not only enhance our understanding of the physiological role of this enzyme, but may also help to explain the mechanism of some forms of mitochondrial diseases.

This work was supported by grant B95-03X-06230-14C from the Swedish Medical Research Council, and grant BMH4-CT96-0479 from the EU Commission. We thank Dr Jinping Li for preparation of mouse tissue extracts.

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Received 30 March 2000/22 June 2000; accepted 10 August 2000