Cloning of mouse mitochondrial thymidine kinase 2 cDNA[1]

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Abstract Phosphorylation of anti-viral nucleoside analogs by mitochondrial thymidine kinase 2 (TK2) has been implicated as a mechanism for the mitochondrial toxicity caused by several of these compounds. We have cloned the cDNA of mouse TK2 and shown that the enzyme is targeted to the mitochondria when expressed in fusion with the green fluorescent protein. This is the first report on the cloning of a mitochondrial TK2 and will contribute to elucidate the role of TK2 in the pharmacological activation of nucleoside analogs.

Key words: Nucleoside analog; Nucleoside kinase; Mitochondrial toxicity; Anti-viral therapy; Mitochondrial import signal

1. Introduction

Several pyrimidine nucleoside analogs are potent inhibitors of viral replication. These analogs include 3′-azido-2′,3′-dideoxythymidine (AZT), 2′,3′-dihydro-2′,3′-dideoxythymidine, 2′,3′-dideoxycytidine and 2′-deoxy-3′-thiacytidine, that are used in therapy of HIV infection. Pyrimidine nucleoside analogs are also active towards hepatitis B and C virus infections and investigations to elucidate their role in anti-hepatitis therapy are currently initiated.

Nucleoside analogs are transported into cells by nucleoside transporters and become phosphorylated intracellularly to their triphosphate derivatives by nucleoside and nucleotide kinases [1]. The nucleoside analog triphosphates subsequently inhibit the viral polymerases that replicate the viral genome. Although the nucleoside analogs preferentially inhibit viral polymerases, they also interfere to some extent with cellular DNA replication. Inhibition of nuclear DNA replication causes cell cycle arrest or cell death, resulting in adverse clinical effects such as anemia, neutropenia and gastrointestinal disturbances. In addition to interfering with nuclear DNA replication, certain pyrimidine nucleoside analogs also interfere with replication of mitochondrial DNA [2]. Mitochondrial DNA damage, and subsequent mitochondrial dysfunction, induced by nucleoside analogs ranges from mild myopathy and neuropathy to severe multi-organ failure [2].

Mitochondrial DNA replication, catalyzed by DNA polymerase γ, is performed in the mitochondrial matrix. Nucleoside analogs that interfere with this process must therefore be present in this subcellular compartment. The inner mitochondrial membrane prevents free transport of nucleotides between the cytosol and the mitochondrial matrix. This separates the mitochondrial deoxyribonucleotide pool from the cytosolic/nuclear pool [3,4]. Mammalian cells contain two different thymidine kinases that phosphorylate thymidine and uridine nucleoside analogs [3,5–7]. The nucleoside kinases, named thymidine kinase 1 and 2 (TK1 and TK2), are located in the cytosol and mitochondria, respectively [4,7]. The existence of a mitochondrial thymidine kinase has lead to the hypothesis that the mitochondrial location of this enzyme is correlated to the mitochondrial DNA damage caused by several pyrimidine nucleoside analogs.

We have previously cloned the cDNA of human TK2 [8]. However, the predicted amino acid sequence did not exhibit properties of a mitochondrial import signal and the enzyme was not targeted to mitochondria when expressed in mammalian cells [9]. Recently, Wang and co-workers reported the cloning of a human TK2 that differed in the N-terminal region from the previously cloned cDNA, although it was truncated and lacked a translation start codon [10]. The complete cDNA sequence of a mitochondrial TK2 is thus not known, which prevents further studies on the role of this enzyme for the activation of nucleoside analogs in vivo. In the present study, we report the cloning of a mouse TK2 cDNA. The N-terminal predicted amino acid sequence of this protein had properties similar to those of a mitochondrial import signal and we further showed that the enzyme is targeted to mitochondria when overexpressed in mammalian cells.

2. Materials and methods

2.1. Cloning of mouse TK2 cDNA

We searched the expressed sequence tag (EST) library of the GenBank database at the National Institute for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) with the Basic Local Alignment Search Tool [11] to identify murine cDNA clones homologous to human TK2. EST clones were obtained from Research Genetics (Huntsville, AL, USA). The complete DNA sequences of the clones were determined using the BigDye terminator sequencing kit (Perkin-Elmer) and an ABI310 automated DNA sequencer.

Rapid amplification of cDNA ends PCR was used to clone the lacking 5′-cDNA region from a mouse liver cDNA library (Marathon Ready cDNA, Clontech). The Advantage-GC Genomic PCR mix (Clontech) was used according to the manufacturer's protocol and PCR primers were designed based on the EST cDNA sequence (5′-CCCAGCCTGTGCTGTGCTTCTCCAGGGTAT and 5′-AGTCAACCTCGGGCATCTTCCCACTTCTAT). The amplified DNA fragment was cloned into the pGEM-T plasmid vector (Promega) and the DNA sequence was determined as described above.

2.2. Construction of green fluorescent protein (GFP) plasmid vectors

The open reading frame of mouse TK2 cDNA was PCR-amplified with primers containing engineered 5′-*Eco*RI sites (5′-ACAGAATTCACCATGCTGCTGCGGTCGCTGCGT and 5′-ACAGAATTCGTCCATGCTTCCAGTTCTCTGG) and the cDNA fragment was

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[1] The nucleotide sequence reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number AF105217.

Abbreviations: TK1, thymidine kinase 1; TK2, thymidine kinase 2; GFP, green fluorescent protein; EST, expressed sequence tag

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cloned into the *Eco*RI site of the pEGFP-N1 vector (Clontech). The cDNA sequence encoding the 31 amino acids N-terminal mitochondrial import signal of cytochrome *c* oxidase subunit VIII (cytC) was cloned upstream of GFP to create the cytC-GFP fusion [12]. The plasmids were purified with the NucleoSpin kit (Clontech) and their sequences verified by DNA sequencing.

2.3. Culture and transfection of cell lines

A Chinese hamster ovary (CHO) cell line was obtained from the American Type Culture Collection. The cells were cultured at 37°C in McCoy 5A modified medium supplemented with 10% (v/v) fetal calf serum (Gibco BRL), 100 U/ml penicillin and 0.1 mg/ml streptomycin. The cells were transfected using Lipofectamine (Gibco BRL) as described in the Gibco protocol and GFP fluorescence was visualized in the living cells 24 h after transfection by fluorescence microscopy [9].

3. Results

3.1. Cloning of mouse mitochondrial TK2 cDNA

We used the sequence of the cloned human TK2 cDNA [8,10] to search the GenBank EST sequence library to identify mouse TK2 cDNA clones. Several mouse EST clones ≈95% identical to human TK2 were identified. An EST clone derived from a mouse macrophage cDNA library (IMAGE clone ID 1311311) [13] showed the longest 5′-extension and its full-length DNA sequence was determined. However, the cDNA sequence of that clone was truncated in the 5′-region compared to the human TK2 cDNA. We used a PCR with primers designed from the sequence of the EST clone to amplify and clone the lacking 5′-region. Using this strategy, we obtained several cDNA fragments of mouse TK2, but none of the fragments contained an ATG start codon upstream of the open reading frame (data not shown). The sequences of these PCR amplified fragments were instead used for new searches of the GenBank EST library to identify cDNA clones further extended in the 5′-region. One clone was identified (AU035388) with a long 5′-extension and it contained an ATG codon in frame with the TK2 open reading frame.

The complete 1.6 kbp sequence of mouse TK2 contained an open reading frame encoding a 270 amino acid protein with a predicted molecular mass of 31.4 kDa (Fig. 1). The predicted protein sequence showed ≈85% identity to human TK2, but contained a 36 amino acid extended N-terminal region (Fig. 2A). Mitochondrial import signals are usually located in the N-terminal part of the protein, form an amphipathic α-helical structure that contains positively charged and hydrophobic amino acid residues and lack negatively charged residues [14]. Sequence analysis of the extended N-terminal region of mouse TK2 showed that the region was rich in positively charged arginine residues as well as in hydrophobic amino acids (Fig. 2B). No negatively charged amino acids were present among the first 30 N-terminal residues. Furthermore, a schematic sketch of the N-terminal region as a putative α-helix showed that the positively charged amino acid residues were located on one side opposite to several hydrophobic residues (Fig. 2C). These properties strongly suggest that the N-terminal region of mouse TK2 constitutes a mitochondrial targeting sequence. After import of the protein into the mitochondria, the mitochondrial targeting peptides are cleaved by mitochondrial pre-peptide peptidases. The consensus motif for cleavage according to the two protease model [15] was identified in the mouse TK2 N-terminal region and suggested that the putative signal is cleaved between amino acids 24 and 25 (Fig. 2B)

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                                                                5'-…ACA   -1
ATG CTG CTG CGG TCG CTG CGT AGT TGG GCT GCC CGG TCC CTG CGT    45
 M   L   L   R   S   L   R   S   W   A   A   R   S   L   R    15

AGC GTG GGC CCG GGG AGC TCC CGG AGC CCC GGG AGC CTC GAT TCG    90
 S   V   G   P   G   S   S   R   S   P   G   S   L   D   S    30

GGC GCC GGG CCG CTG TGG GCA CCT CGC CGC GCC TGG CCT CCG GAT   135
 G   A   G   P   L   W   A   P   R   R   A   W   P   P   D    45

AAA GAT AGA GAA AAT GAT AAG GAG AAA AAA GCA GTG GTT TGT ATT   180
 K   D   R   E   N   D   K   E   K   K   A   V   V   C   I    60

GAG GGC AAT ATT GCA AGT GGG AAG ACG ACA TGC CTG GAG TTC TTC   225
 E   G   N   I   A   S   G   K   T   T   C   L   E   F   F    75

TCC AAT ACA ACA GAC GTC GAG GTG TTA ATG GAG CCT GTG CTC AAG   270
 S   N   T   T   D   V   E   V   L   M   E   P   V   L   K    90

TGG AGA AAT GTC CAT GGC CAT AAC CCT CTG AGC CTC ATG TAC CAT   315
 W   R   N   V   H   G   H   N   P   L   S   L   M   Y   H   105

GAT GCC AGC CGA TGG GGC CTC ACA CTG CAG ACG TAC GTG CAG CTC   360
 D   A   S   R   W   G   L   T   L   Q   T   Y   V   Q   L   120

ACC ATG CTG GAC CAG CAC ACG CGC CCT CAG ATG TCA CCT GTA CGG   405
 T   M   L   D   Q   H   T   R   P   Q   M   S   P   V   R   135

TTG ATG GAA AGG TCA ATT TAC AGC GCA AGA TAC ATT TTT GTA GAA   450
 L   M   E   R   S   I   Y   S   A   R   Y   I   F   V   E   150

AAC CTG TAT AGA AGT GGG AAG ATG CCC GAG GTT GAC TAC GCG ATT   495
 N   L   Y   R   S   G   K   M   P   E   V   D   Y   A   I   165

CTG TCT GAG TGG TTT GAC TGG ATC GTC AGG AAC ATT GAT GTC TCT   540
 L   S   E   W   F   D   W   I   V   R   N   I   D   V   S   180

GTT GAT CTG ATA GTT TAT CTG CGA ACC ACT CCC GAA ATC TGC TAC   585
 V   D   L   I   V   Y   L   R   T   T   P   E   I   C   Y   195

CAG AGA TTA AAG ATG AGG TGC CGG GAA GAG GAG AAA GTC ATT CCG   630
 Q   R   L   K   M   R   C   R   E   E   E   K   V   I   P   210

ATG GAA TAC CTC CAT GCT ATT CAC CGC CTC TAC GAG GAG TGG CTG   675
 M   E   Y   L   H   A   I   H   R   L   Y   E   E   W   L   225

GTC AAC GGG AGC CTC TTC CCA GCT GCA GCC CCT GTT CTG GTG ATT   720
 V   N   G   S   L   F   P   A   A   A   P   V   L   V   I   240

GAG GCT GAC CAC AAC TTG GAG AAA ATG TTA GAA CTC TTT GAA CAA   765
 E   A   D   H   N   L   E   K   M   L   E   L   F   E   Q   255

AAC CGG GCC CGG ATA TTA ACT CCA GAG AAC TGG AAG CAT GGA CCC   810
 N   R   A   R   I   L   T   P   E   N   W   K   H   G   P   270

TAG GACTGAGTGATCTACAAGACAGCGCCAGGAACAGCCCAGCCAGGGCCAGATGTCA   868
TGGACCACGAGCATGAAGAAGTCATCTCTATTGGATCAGAAAATACCTTAGCTTTGCTG   927
AGCCGTAACTGTGCACTGAAGGTGCAGCTGTTACTTGAATCAGTCATGACCAAGAGAGG   986
CCGGCCATTCCTCAGGACCGTGAGACTGGAAAGTCATGGTTTGCCAACAAAGACAATTT  1045
AAGCCTTGCTGCTCTTGACATTCTAGAATGGATGGAGTGCATCTGGAAAAGACTGGACA  1104
CAGGTGATACAGGGGAATGATGACCGTATACCCTGGAGAAGCACAGCACAGGCTGGGGA  1163
GGGGGCTGCAGGGGAGCCCTGCTCCCCTGTTTAGCTGCCTGGGAGCTAGAAAGAGGGCC  1222
AAGAGTCCAGCTCTTGGTCCTTTCTGTTTGGACCAATTCTTGGCCCTTTTGGTAGTATG  1381
AAGAGTAGCATCCAGGGAGATTGTCCATCAGCACCTGCAGTCCAGCAATGGATAAACCT  1440
GAGAAAGGCAGGAACTTTGAACCAGCAGTACTCATGGGGGCATCTTTAGAATTTGCAAA  1499
CGAACAGTCTAGTACTTGCCCTTTGCTCTACCAGAACACTTGTTTTGGGGAGAAATGTG  1558
CTGTTTCTCTTTCTCTCTAGTAGCCAATAAAATTCTACAGCTAAACCATAAAAAAAAA… 1616
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Fig. 1. The cDNA and predicted amino acid sequences of mouse TK2. The start and stop codons are printed in bold. A possible poly-adenylation signal is underlined.

3.2. Expression of mouse mitochondrial TK2 in cancer cell lines

We decided to express the cloned mouse TK2 cDNA in mammalian cells in order to verify the mitochondrial location of the enzyme. TK2 was expressed as a fusion protein to GFP to visualize the protein in living cells. To avoid interference by GFP with the mitochondrial import signal, TK2 cDNA was cloned upstream of GFP (Fig. 3A). CHO cells were transfected with plasmids expressing GFP alone, a fusion of the mitochondrial targeting sequence of cytC and GFP or the TK2-GFP fusion (Fig. 3B). Cells expressing GFP alone showed homogenous green fluorescence both in the nucleus and in the cytosol. This distribution is expected because the low molecular mass of GFP allows for free diffusion between the cytosol and nucleus across the nuclear envelope. The cells expressing cytC-GFP showed a dotted fluorescence pattern located around a non-fluorescent nucleus. This pattern has been shown to be typical for the mitochondrial location of GFP [12]. The cells expressing TK2-GFP showed a similar fluorescence pattern as the cells that expressed cytC-GFP. Accordingly, we conclude that the cloned mouse TK2 cDNA is targeted to mitochondria.

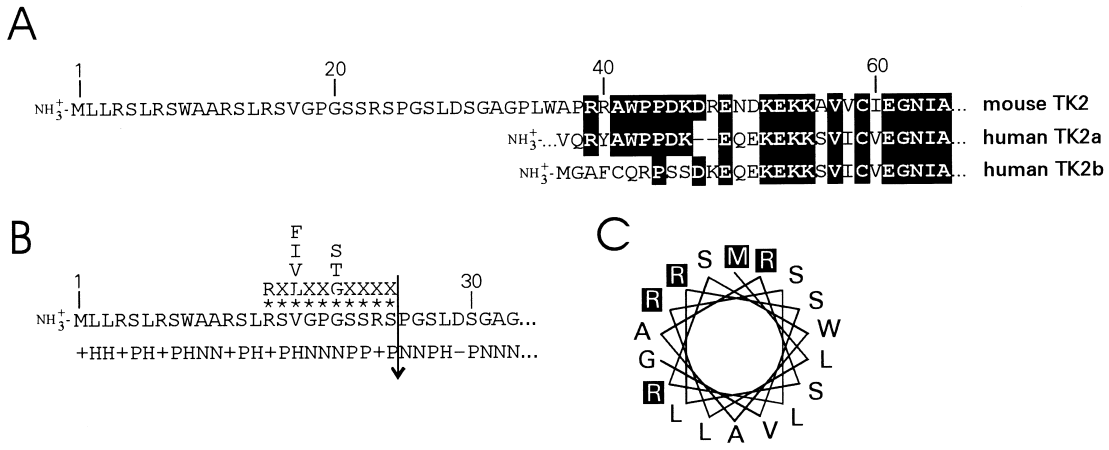

Fig. 2. The N-terminal putative mitochondrial import signal of mouse TK2. (A) Alignment of mouse TK2 N-terminus and the previously cloned human TK2 isoforms, named TK2a [10] and TK2b [8]. Black boxes indicate conserved amino acid residues compared to the mouse TK2 sequence. (B) Physical properties of the N-terminal amino acid residues. H, hydrophobic; N, neutral; P, polar; +, positively charged. The arrow indicates the probable mitochondrial pre-peptide peptidase cleavage site [15]. (C) The predicted location of the amino acid residues in an α-helix structure.

4. Discussion

In the present study, we have cloned the mouse TK2 cDNA and shown that the enzyme is targeted to the mitochondria. This is, to our knowledge, the first report on the cDNA cloning of a mitochondrial TK2. Two human TK2 cDNAs, that differ in their 5′-regions, have been cloned but neither of these cDNAs encode a mitochondrial targeting signal [8–10]. We have made attempts using several different methodological strategies to clone the 5′-region of human TK2 containing the mitochondrial import signal (unpublished results). However, these attempts have not yet been successful. It is possible that structural features of the mRNA 5′-region, such as hairpin loops, prevent generation of the full-length cDNA. Another possibility is that the previously cloned human TK2 cDNA encodes a cytosolic TK2 and that the cDNA of the mitochondrial TK2 isoform has not yet been identified. The existence of a cytosolic TK2 has been suggested by studies on subcellular fractionated crude protein extracts that appear to have a low level of enzyme activity of TK2 in the cytosolic fraction [7]. However, there are also experimental evidences that TK2 is exclusively located in the mitochondria. Berk and Clayton showed that a cell line deficient in cytosolic TK1 activity exclusively incorporated dThd into mitochondrial DNA [3]. These data strongly suggest that the major TK2 activity is located in the mitochondria.

The physiological consequences of a mitochondrial location of TK2 appear to be that nucleosides and nucleoside analogs that are phosphorylated by the enzyme become trapped within the mitochondria [3,16]. The nucleosides and nucleoside analogs can subsequently be incorporated into mitochondrial DNA but cannot access the cytosolic/nuclear deoxyribonu-

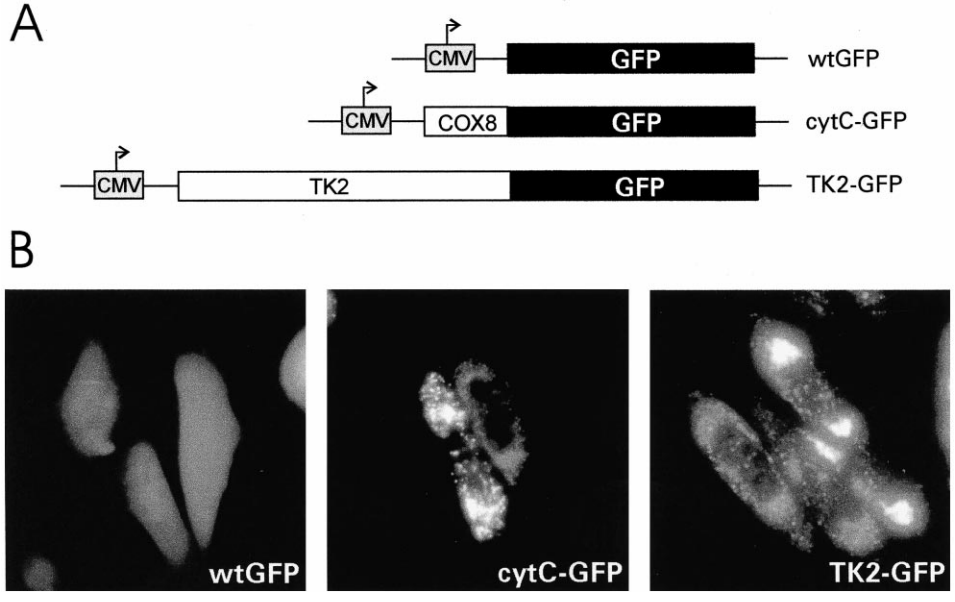

Fig. 3. Expression of mouse TK2 as a fusion to GFP. (A) Plasmids used to express wild-type GFP (wtGFP), mitochondrial cytC-GFP and mouse TK2 fused to GFP (TK2-GFP). CMV, cytomegalo virus promoter. (B) Fluorescence microscopy images of CHO cells transfected with the GFP plasmids.

cleotide pool. On the contrary, there is evidence that nucleoside analogs phosphorylated in the cytosol or nucleus can be imported into the mitochondria [17] and a mitochondrial deoxyribonucleotide transporter protein was recently identified [18]. Deoxyribonucleotides and phosphorylated nucleoside analogs thus appear to be able to be imported into the mitochondria, but once within this compartment, they become trapped. It is therefore likely that nucleoside analogs such as the anti-HIV thymidine analog AZT and the anti-hepatitis nucleoside analog 1-(2-deoxy-2-fluoro-B-D-arabinofuranosyl)-5-iodouracil (FIAU) will stay within the mitochondria once phosphorylated by TK2. These compound are not likely to have therapeutic effects in the mitochondrial compartment, since viral replication occurs in the cytosol. However, both AZT and FIAU interfere with mitochondrial DNA replication and cause delayed adverse effects due to mitochondrial dysfunction [2,19]. We recently showed that overexpression of the mitochondrial purine nucleoside kinase, deoxyguanosine kinase, enhances the sensitivity of cancer cells towards several purine nucleoside analogs [20]. These data suggest that the mitochondrial nucleoside kinases contribute to the pharmacological effects of nucleoside analogs. The cloning of the mitochondrial isoform of TK2 will be the basis to elucidate the role of the subcellular compartmentalization of TK2 and its correlation to therapeutic and toxic effects of pyrimidine nucleoside analogs.

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