FEBS Letters 443 (1999) 170–174 FEBS 21452

Human thymidine kinase 2: molecular cloning and characterisation of the enzyme activity with antiviral and cytostatic nucleoside substrates

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Received 10 December 1998

Abstract Based on amino acid sequence information from purified mitochondrial thymidine kinase (TK2), a cDNA of 1930 bp was cloned, containing an open reading frame encoding 232 amino acid residues starting with the N-terminal sequence determined from the native human protein preparation. Northern blot analysis with the cDNA coding region demonstrated several TK2 mRNAs, with 2 and 4 kb forms present in many tissues. We also characterised N-terminally truncated (starting at position 18) human TK2 with pharmacologically important antiviral and cytostatic nucleoside analogues. Results were highly similar to those with the native TK2 preparation. The anti-leukaemic drug arabinosyl cytosine is phosphorylated. The antitumour drug difluorodeoxycytidine and its metabolite difluorodeoxyuridine are good substrates, with K_m values of 66 and 29 μM, respectively, and a relative V_{max} of 0.6 compared to that of thymidine. Negative cooperativity was found with thymidine and the anti-HIV drug 3′-azidothymidine, but the reaction followed Michaelis-Menten kinetics with deoxycytidine, arabinosyl cytosine, and arabinosyl thymine. The results demonstrate a broad substrate specificity and complex kinetics, and suggest a role for TK2 in the activation of chemotherapeutic nucleoside analogues.

Key words: Cloning; Thymidine kinase; Antiviral nucleoside; Cytostatic nucleoside; Metabolic conversion; Mitochondrial enzyme

1. Introduction

Mitochondrial thymidine kinase (TK2) phosphorylates pyrimidine deoxynucleosides and their analogues to the corresponding nucleoside monophosphates, using a nucleoside triphosphate as phosphate donor. There are two thymidine kinases in animal cells, a cytosolic (TK1) and a mitochondrial (TK2) enzyme, with different properties [1–3]. TK1, which was cloned in 1983 [4], is expressed only in S-phase cells, while TK2 is expressed in all tissues in proportion to the mitochondrial content of the cell type. In many resting cells, such as nerve and muscle cells, TK2 is the only pyrimidine deoxynucleoside phosphorylating enzyme expressed. In these tissues, where de novo synthesis of DNA precursors is undetectable [3], TK2 is likely to be responsible for the supply of deoxyribonucleotides required for mitochondrial DNA synthesis. The physiological role of TK2 is not well defined but in cells with no TK1 activity, TK2-mediated phosphorylation of thymidine (dThd) leads to a product apparently incorporated into mitochondrial DNA [5]. Similarly, phosphorylation of 3′-azidothymidine (AZT) in monocytes/macrophages and peripheral blood lymphocytes intrinsically lacking TK1 activity is dependent on TK2, which is important for the efficacy of anti-HIV nucleoside chemotherapy [6,7].

The TK2 enzyme has been purified from human spleen, brain and lymphoblasts [3,8], the gene has been localised to chromosome 16 [9] and a cDNA cloning has been reported [10]. However, the TK2 protein described from that clone [10] shows differences from the purified protein in an N-terminal segment and in substrate specificity. We have carried through another cloning based on sequence information from human and bovine brain TK2 and have cloned human TK2 cDNA in a form compatible with the native enzyme, expressed an active, truncated TK2 protein at high level in *Escherichia coli*, and characterised enzymatically both the recombinant and native TK2 forms with antiviral and cytostatic analogues not previously known to be substrates for TK2.

2. Materials and methods

2.1. Purification and amino acid sequence analysis of TK2

Brain tissues were homogenised and TK2 was purified [8,11]. Calf brain was obtained freshly frozen from a local slaughterhouse. Human brain was obtained from the Department of Pathology, Karolinska Hospital, with ethical permit. SDS-polyacrylamide gel electrophoresis of the fractions after hydroxylapatite revealed the presence of one polypeptide of 29 kDa, corresponding to TK2. Amino acid sequencer analysis (ABI 470) of the purified human TK2 yielded the N-terminal sequence VQRYAWPPDKEQEKEKKSVIXVEGNIAS. Internal sequence information for three peptides, YVFVENLYR, XXPETXYQR and MLEXF, of bovine TK2 was obtained from material excised from a Coomassie-stained SDS gel preparation, subjected to in-gel trypsin cleavage and subsequent peptide isolation by reverse phase liquid chromatography [12].

2.2. Cloning of TK2 cDNA

Parts of two of the TK2 amino acid sequences, EGNIA (from the human N-terminal segment) and YVFVENL (from one of the bovine internal segments), were highly similar to the N-terminal region of human deoxycytidine kinase [13] and selected for oligonucleotide synthesis. The sense primer mixture was 5′-ggaattccGTNGAA(G)GGNAAT(C)ATT(CA)GC, and the antisense primer mixture was 5′-gcgtcgACA(G)TTT(C)TCNACA(G)AANACA(G)TA, where N denotes T+A+G+C. An RT-PCR reaction was used to amplify a TK2-specific DNA fragment of 276 bp with bovine brain mRNA as template (prepared with a polyA tract mRNA isolation system from Promega). The amplified PCR fragment was purified, cloned into the Bluescript SK$^-$ vector, and sequenced. A human brain Lambda ZAP cDNA library (Stratagene) was screened with the 276 bp fragment as probe. One positive λ phage was isolated, the cDNA insert was subcloned into the Bluescript vector, and both strands were sequenced. Human brain mRNA (Clontech) was used in an RT reaction (5′/3′-RACE kit from Boehringer Mannheim) with a TK2-specific primer to

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PII: S0014-5793(98)01711-6

amplify the 5′ end of TK2 cDNA. The amplified PCR fragments were cloned into the PCR 2.1 vector (Invitrogen) and sequenced. The nucleotide sequence of the TK2 cDNA was deposited in GenBank/EMBL databases with accession number Y10498.

2.3. Northern blot analysis

Human Multi-Tissue Blots (Clontech) were probed with the coding region of the TK2 cDNA (680 bp) in 5×SSPE, 10×Denhardt's solution, 100 μg/ml freshly denatured salmon sperm DNA, 2% SDS and 50% formamide at 42°C for 20 h, washed in 0.2×SSC, 0.1% SDS twice at 60°C, and autoradiographed.

2.4. Expression of N-terminally truncated TK2 protein

TK2 cDNA, starting corresponding to Ser-18 in Fig. 4, was PCR amplified and subcloned into the pET-14b expression vector (Novagen). The fusion protein contains an N-terminal 6×His tag and a thrombin cleavage site. The resulting construct was transformed into *E. coli* BL21 (DE3) pLysS cells, and induction was performed for 2 h in the presence of 1 mM IPTG at 37°C. Bacteria were lysed in 50 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 0.5% NP-40, 140 μM phenylmethylsulphonyl fluoride by freezing and thawing. The lysate was centrifuged at 100 000×*g* for 90 min at 4°C. TK2 was purified by metal affinity (His Bind resin, Novagen) column chromatography and eluted with 0.15 M imidazole in 50 mM Tris-HCl, pH 7.9, and 0.5 M NaCl. Dithiothreitol (2 mM) and glycerol (20%) were added to the purified TK2. To remove the N-terminal His tag, thrombin (Novagen) was added in a ratio of 5 units to 1 mg protein, and incubated at 22°C overnight.

2.5. Enzyme assays and kinetic parameters

Nucleoside kinase activity was determined either by initial velocity measurements with four-time samples in a DE81 paper assay using tritium-labelled substrates [8] or by a phosphoryl transfer assay [14]. One unit is defined as 1 nmol dTMP formed per min. Substrate kinetics were analysed using a broad substrate range to identify deviation from hyperbolic kinetics. Cooperative kinetics were analysed by Hill plots, with the maximal velocity determined by non-linear regression analysis. The type of kinetics was identified using Hofstee plots for negative cooperativity and double reciprocal plots for hyperbolic or positive cooperativity.

3. Results

3.1. Molecular cloning of TK2 cDNA

TK2, more than 95% pure, was obtained from human and bovine tissue extracts by DEAE-chromatography followed by affinity chromatography and hydroxyl apatite chromatography [8,11]. Human brain TK2 gave an N-terminal 28 residue amino acid sequence. To obtain internal peptide sequence information, purified bovine brain TK2 was used and subjected to SDS-polyacrylamide gel electrophoresis. The protein band was excised, treated with trypsin and the resulting peptides were separated by reverse phase liquid chromatography. The human and bovine sequences obtained are given in Section 2.2. Based on two sequences that showed homology with a region of the human dCK sequence [13], two primer mixtures were designed and used in an RT-PCR reaction starting with bovine brain mRNA. A DNA fragment of the expected length, 276 bp, was obtained and then used as probe to screen a human brain cDNA library. There was only one positive plaque of 1×10^6 screened and its cDNA insert was 1325 bp. Cloning of 5′-RACE PCR products with mRNA from human brain and liver showed the same 1325 bp fragment sequence. Searches in the EST database revealed that there are more than 30 EST sequences showing nearly 100% identity to the first 365 bp of the 1325 bp TK2 cDNA. Later studies have shown that the 365 bp are part of an intron sequence containing repetitive elements (L. Wang et al., unpublished). PCR reactions performed using high temperature reverse transcription (to reduce the influence of mRNA secondary structure) and an oligonucleotide mixture deduced from the eight N-terminal amino acids of human brain TK2 gave a 150 bp fragment. This fragment was found, after cloning and sequence analysis, to encode the correct N-terminal segment of TK2. Thus, a TK2 cDNA with a total length of 1953 bp could be isolated, containing an open reading frame of 699 bp. All the characterised peptide sequences, from both human brain and bovine brain TK2, were identified in this TK2

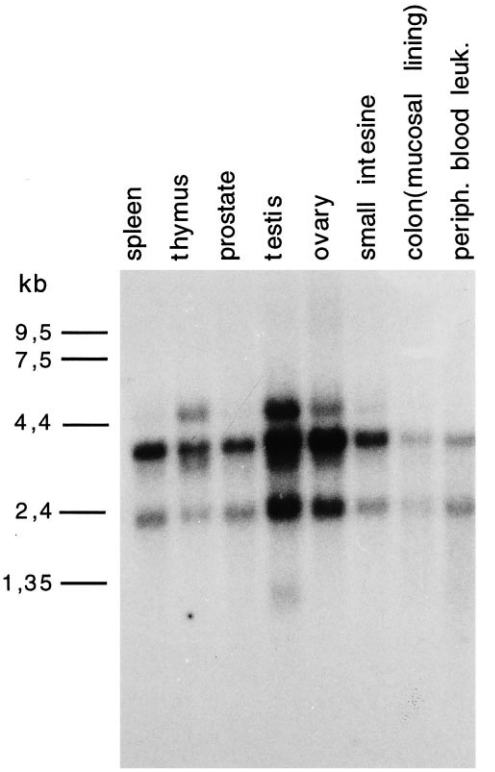

Fig. 1. Northern blot analysis of TK2 mRNA levels in human tissues, using a multiple tissue Northern blot from Clontech with 2 μg mRNA in each lane and a 680 bp TK2 cDNA as probe.

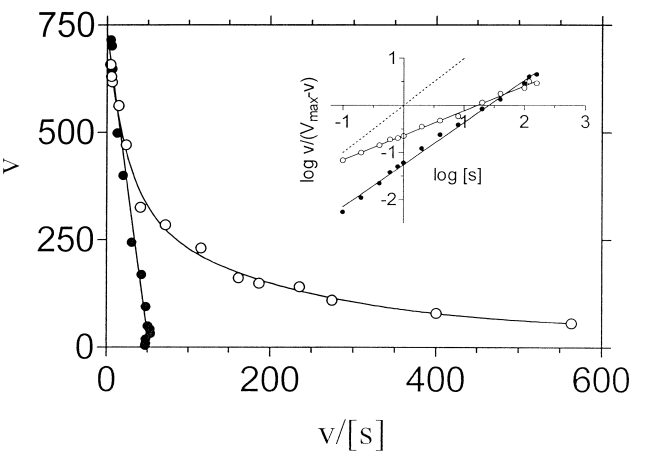

Fig. 2. The activity of recombinant TK2 at varied dThd (open circles) and dCyd (closed circles) concentrations. The data are plotted as *v* versus *v/s*, where *v* is the initital velocity in units/mg, and *s* is the deoxynucleoside concentration in μM. The inset shows Hill plots of the same data, where the dotted line indicates the Hill coefficient of 1.0.

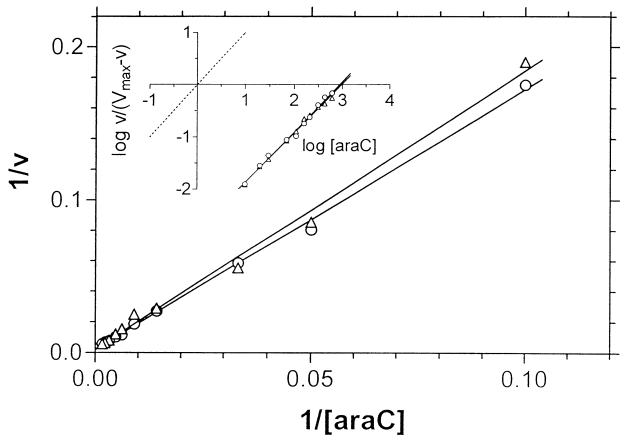

Fig. 3. The activity of recombinant TK2 (triangles) and human spleen TK2 (circles) at varied concentrations of AraC. The data are plotted as double reciprocal plots of 1/*v* versus 1/*s*. The inset shows Hill plots of the same data, where the dotted line indicates a Hill coefficient of 1.0.

cDNA (but with one species difference, Ile at position 109 in human TK2 and Val in bovine TK2). However, the present human TK2 cDNA (Y10498) differs from the TK2 cDNA reported earlier [10].

The human TK2 gene showed a complex transcription pattern as determined by Northern blot analysis using TK2 cDNA as probe (Fig. 1). Multiple transcripts exist in several tissues, with two major bands at 4.0 kb and 2.2 kb. In testis, ovary, and thymus, there is a longer transcript of about 5.0 kb and in testis also a shorter transcript of about 1.35 kb. The expression of the 4.0 kb transcript (Fig. 1) occurs at similar levels in several tissues, with the exception of low values in colon and peripheral blood leukocytes. The levels of the 2.2 kb transcript is about 20–50% of that of the 4.0 kb transcript in the same tissue, and the levels of all four TK2 transcripts are several-fold higher in testis and ovary.

3.2. *Enzymatic characterisation of recombinant and native TK2 with natural substrates and antiviral and cytostatic analogues*

Using the pET system, expression of N-terminally truncated TK2 (starting at position 18 of the native protein) resulted in a fully active enzyme. The kinetics of this recombinant TK2 and of the enzyme prepared from leukaemic spleen were investigated. As demonstrated by the results in Fig. 2 and Table 1, the recombinant TK2 phosphorylated both dThd and deoxycytidine (dCyd) but with negative cooperativity ($n < 1$) for dThd and hyperbolic ($n = 1$) for dCyd. CTP was an efficient phosphate donor with about 60% of the capacity of ATP, and dCyd was a poor inhibitor of the TK2 activity (K_i 630 μM), whereas dThd was an efficient inhibitor of the deoxycytidine kinase activity (K_i 6 μM). These characteristics were similar to those observed earlier with spleen TK2 [8]. Furthermore, recombinant TK2 also showed similar capacity to phosphorylate pyrimidine deoxynucleosides, pyrimidine arabinosides, and their analogues (Table 2). The anti-hepatitis B analogue 1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil (FIAU) is as good a substrate as dThd for both bovine brain TK2 and recombinant human TK2 [14]. The kinetic pattern with 1-β-*D*-arabinofuranosylcytosine (AraC) was identical for recombinant and spleen TK2 (Fig. 3), and hyperbolic as indicated by the straight lines in the double reciprocal plots and *n* values of 1 in the Hill plots (inset, Fig. 3). Because of lack of radioactive 2′-difluoro-deoxycytidine (dFdC) and 2′-difluorodeoxyuridine (dFdU), those analogues were tested with the phosphoryl transfer assay using [γ-^{32}P]ATP. This assay gave kinetic parameters similar to those with the radioactive nucleosides, although the V_{max} values were approximately half of those in Table 1. The K_m values for dThd, dCyd, dFdC and dFdU were 4, 7, 66 and 29 μM, respectively.

Table 1
Kinetic parameters of native and recombinant human TK2 with phosphate acceptors

Substrate	Recombinant TK2		Native TK2	
	K_m (μM)	V_{max}/K_m [a]	K_m (μM)	V_{max}/K_m
dThd	16 (*n*, 0.4)	55	16 (*n*, 0.5)	35
dCyd	26	34	36	25
AraC	820	0.7	800	0.8
AraT	16	41	39[b]	30
AZT	33 (*n*, 0.3)	1	90 (*n*, 0.4)	0.4

In all kinetic experiments, the variation is less than 20% and in those cases where the enzyme displays negative cooperativity, the Hill constant (*n*) is given.
[a] V_{max} is in U/mg protein and K_m in μM.
[b] Data from Arnér et al. [6].

The anticancer drug 5-fluorodeoxyuridine (FdUrd) was also phosphorylated, as well as the antiherpes compound 5-(2-bromovinyl)-2′-deoxyuridine (BVdU). Previous investigations with native TK2 have shown that the anti-HIV drug AZT is phosphorylated although with low efficiency [8]. This ability was also found with the recombinant TK2 (Table 1). The maximal velocities were nearly identical for the two enzymes. Furthermore, a biphasic Hofstee plot and a Hill coefficient below 1 with the recombinant TK2 showed negative cooperativity, as with native TK2 [8]. Both 2′,3′-didehydrothymidine (D4T) and 2′,3′-dideoxycytidine (ddCyd) have been recently registered as anti-HIV drugs [15] and as with purified TK2 [8] they showed very low activity with the recombinant TK2.

4. Discussion

The capacity of resting cells to phosphorylate pyrimidine deoxynucleosides relies on TK2 and the efficacy or side effects of antiviral therapy are in part governed by the properties of this enzyme. A human TK2 cDNA was now cloned using

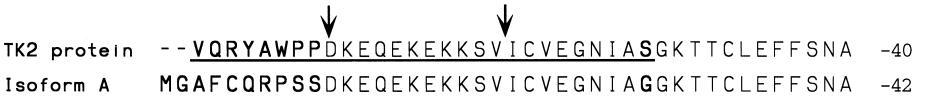

Fig. 4. N-terminal amino acid sequences of the present TK2 and a variant previously reported (isoform A in [10]). Underlined amino acids are those determined from human brain TK2 and the bold letters indicate differences between the published [10] TK2 and the TK2 described here. The arrows indicate positions of the exon intron boundaries.

Table 2
Activity of recombinant and native TK2 with deoxynucleoside analogues

Substrate	Recombinant TK2	Native TK2
dThd	1.0	1.0
FLT	0.03	0.0005[a]
AraT	0.5	0.75
D4T	0.005[b]	0.00005[a]
dCyd	1.5	1.3
dFdC	0.6	0.4
ddCyd	0.002[b]	0.002[b]
dUrd	1.4	1.7
AraU	0.19	0.29
FdUrd	2.3	3.0
FIAU	1.1	0.99
dFdU	0.6	0.4
BVdU	0.54	0.18

The values show the activity with 20 μM substrate analogue relative to the activity with 20 μM thymidine as determined with the radioactivity or phosphoryl transfer assays.
[a]Data from Munch-Petersen et al. [8].
[b]These analogues were tested with radioactive substrates yielding a high sensitivity in the assay as compared to the other nucleosides which were tested with the phosphoryl transfer assay.

amino acid sequence information from purified human and bovine brain TK2. This TK2 cDNA contains an open reading frame of 699 bp encoding 232 amino acid residues, and a 1264 bp 3′-untranslated sequence including a polyadenylation signal followed by a polyA tail. All the peptide sequences determined from purified TK2 were found in the deduced coding sequence. Another cDNA for human TK2 has been reported [10]. The cloning was then achieved by identification of EST sequences which showed homology to deoxycytidine kinase, and use of that information with RACE protocols to amplify 5′- and 3′-adjoining regions. Two forms of TK2 cDNAs were then found, one form claimed to encode full-length TK2. However, that TK2 cDNA shows a sequence identical to the one reported here only from the nucleotide which encodes Asp-9, while the present cDNA matches the protein sequence of native purified TK2. In addition, Ser-28 in the TK2 cDNA presented here matches the protein sequence but not the previously deduced sequence [10]. In spite of a cDNA corresponding to the full-length mature protein, the present TK2 cDNA is incomplete, most likely because of the fact that the 5′ part contains several unusual features with repetitive elements which make it unsuitable for reverse transcription. There is a splice site where the previous sequence [10] and the one reported here differ (Fig. 4; L. Wang et al., unpublished) and this may perhaps explain the different cDNA previously obtained and the absence of coding regions for a mitochondrial target sequence in both the previous and present TK2 cDNAs. Such a presequence is expected in order to direct the protein to the mitochondria.

The TK2 gene showed multiple transcripts in most tissues, including two major bands at 4.0 and 2.2 kb, and in testis, ovary and thymus longer and shorter transcripts of about 5.0 and 1.35 kb. The differential expression of human TK2 mRNAs with high levels of multiple transcripts in ovary and testis and low levels in colon and blood leukocytes was not observed in the earlier study [10]. The existence of several forms of TK2 mRNA may be derived from alternative usage of an additional 3′ polyadenylation signal or from alternative splicing of the TK2 mRNA.

Alignment of the human deoxynucleoside kinase sequences now known shows that the sequence identity of TK2 to deoxycytidine kinase and the other mitochondrial purine deoxynucleoside phosphorylating enzyme deoxyguanosine kinase is fairly extensive, about 40% at the amino acid level, while it is low to TK1, confirming that TK2, deoxycytidine kinase and deoxyguanosine kinase are the kinases evolutionarily most closely related [16].

The enzyme kinetic characterisation of recombinant and native TK2 demonstrates that the overall kinetic properties of the two enzyme forms are indistinguishable. A broad capacity of recombinant TK2 to phosphorylate pyrimidine analogues was found. Important anticancer and antiviral analogues, such as FdUrd, AraC, dFdC, FIAU and AZT, were found to be substrates for the enzyme. Notably, AraC was shown to be a phosphate acceptor for both recombinant and native TK2. In earlier studies AraC phosphorylation by TK2 was not observed [2,10] and the reason could be the high K_m value and the limited amount of pure enzyme then available. The implication from the fact that TK2 now is found to be capable of phosphorylating AraC is that the enzyme may play a role in activation of AraC.

Gemcitabine (dFdC) is a relatively new deoxycytidine analogue which has shown antitumour activity against ovarian cancer and non-small-cell lung cancer, and is now in frequent clinical use [17,18]. The mechanism of inhibition is assumed to be dependent on dFdCTP incorporation into tumour cell DNA, and deoxycytidine kinase is regarded to be the enzyme responsible for the first rate-limiting step in the anabolism of this drug. We now observe a relatively high capacity of native and recombinant TK2 to phosphorylate dFdC. Earlier studies have shown low expression of deoxycytidine kinase mRNA in ovary and lung [19], while we find high levels of TK2 mRNA in ovaries. These results suggest a role of TK2 in the metabolism and pharmacology of dFdC. The conversion of dFdC to dFdU occurs relatively efficiently in vivo because of the action of cytidine deaminase, and dFdU has been regarded as a catabolite that cannot be phosphorylated in cells or tissues [18]. In accordance with this assumption, TK1 shows no significant activity with dFdU (J. Wang and S. Eriksson, unpublished). However, we now find that TK2 is able to phosphorylate dFdU, and that the efficiency of this substrate with TK2 is approximately 10% of that of Thd. Thus, it is likely that TK2 contributes to the anabolism of dFdU and may be involved in the efficacy or side effects associated with these types of analogue. The characterisation of a highly active recombinant TK2 shows the importance of the enzyme in activation of pharmacologically valuable nucleosides.

Acknowledgements: This work was supported by grants from the Swedish Medical Research Council, the Swedish Cancer Society (Project 1806) and grants from the European Community (BM44-CT96-0479; BIO4-CT97-2123).

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