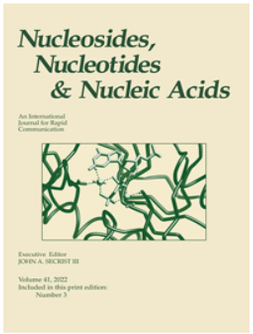

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Structural and functional analysis of human thymidylate kinase isoforms

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ABSTRACT

Thymidylate kinase (TMPK) phosphorylates deoxythymidine monophosphate (dTMP) and plays an important role in genome stability. Deficiency in TMPK activity due to genetic alterations of *DTYMK*, i.e., the gene coding for TMPK, causes severe microcephaly in humans. However, no defects were observed in other tissues, suggesting the existence of a compensatory enzyme for dTTP synthesis. In search for this compensatory enzyme we analyzed 6 isoforms of TMPK mRNA deposited in the GenBank. Of these, only isoform 1 has been characterized and represents the known human TMPK. Our results reveal that isoform 2, 3, 4 and 5 lack essential structural elements for substrate binding and, thus, they are considered as nonfunctional isoforms. Isoform 6, however, has intact catalytic centers, i.e., dTMP-binding, DRX motif, ATP-binding p-loop and lid region, which are the key structural elements of an active TMPK, suggesting that isoform 6 may function as TMPK. When isoform 6 was expressed and purified, it showed only minimal activity (<0.1%) as compared with isoform 1. A putative isoform 6 was detected in a cancer cell line, in addition to the dominant isoform 1. However, because of its low activity, isoform 6 is unlikely be able to compensate for the loss of TMPK activity caused by deletions and/or point mutations of the *DTYMK* gene. Thereby, future studies to identify and characterize the compensatory TMPK enzyme found in patients with *DTYMK* mutations may contribute to the understanding of dTTP synthesis and of the pathophysiological role of *DTYMK* mutations in neurodegenerative disorders.

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1. Introduction

Thymidylate kinase (TMPK) is a key enzyme in the synthesis of DNA. In the presence of magnesium ions and ATP, it catalyzes the transfer of the γ-phosphate group from ATP to the 5′-phosphate group of deoxythymidine monophosphate (dTMP), and the product, i.e., deoxythymidine

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5′-diphosphate (dTDP), will then be further phosphorylated to deoxythymidine triphosphate (dTTP) by nucleoside diphosphate kinases. This is a highly regulated process and the level of dTTP is critical for nuclear genome stability and mitochondrial DNA copy number maintenance.[1]

The expression of human TMPK mRNA and the levels of TMPK protein are highest during the S-phase the cell cycle in order to cope with rapid DNA synthesis, and once the cells enter mitosis the levels of TMPK protein decline rapidly from mitosis to early G1 phase. This cell cycle regulated proteolysis of TMPK protein is mediated by the anaphase promoting complex/cyclosome (APC/C)-complex since human TMPK contains several sequence motifs that are recognized specifically by the APC/C complex, which targeted TMPK for degradation.[2,3] Defects in enzymes involved in dTTP synthesis cause neurodegenerative disorders in humans.[4,5] For instance, genetic alterations of *DTYMK*, i.e., the gene coding for human TMPK, cause severe microcephaly in humans.[5,6] Notably, TMPK is up-regulated in cancer cells and tissues, implicating TMPK as a potential target for cancer treatment.[7] In line with this notion, it has been demonstrated that inhibition of TMPK activity sensitizes tumor cells to doxorubicin treatment, both *in vitro* and *in vivo*.[8]

As noted above, point mutations or deletions in *DTYMK* in humans lead to loss of function of TMPK and cause neurodegenerative disorders. However, two of the four reported patients were still alive at 2 respective 7 years old and there were no abnormalities observed in non-nervous tissues.[5,6] This suggests that there is a compensatory mechanism for dTTP production in tissues other than nervous tissue. Indeed, an earlier study performed on cancer cell line has identified a novel TMPK activity that is not recognized by an antibody against the *DTYMK*-encoded TMPK protein.[9] However, the identity of this alternative TMPK is still unknown.

To approach this issue, we here searched for TMKP isoforms in databases and assessed whether any of these could have TMPK enzymatic activity. In GenBank there are six isoforms of human TMPK mRNA deposited, and the encoded polypeptide ranges from 113 to 251 amino acids in length. Isoform 1, consisting of 212 amino acids, is well-characterized regarding enzyme function and 3D-structure and is considered as the human TMPK.[10–12] However, the structures and functions of isoforms 2 to 6 are still not known. To evaluate if any of these isoforms can function as the compensatory TMPK enzyme, we conducted structural and functional studies. Structural analysis revealed that isoform 2-5 lacked essential structure elements required for TMPK activity, whereas isoform 6 contains all necessary functional sequence motifs and may fold into an active enzyme. Therefore, isoform 6 was cloned and expressed and the recombinant protein was characterized. In addition,

the presence of the various TMPK isoforms in cell extracts was also studied.

2. Materials and methods

2.1. Sequence and structural analysis

Protein sequences of all 6 isoforms were extracted from GenBank and aligned by using the Clustal Omega algorithm at www.ebi.ac.uk. The 3D structure of human TMPK (isoform 1, PDB code: 1E2F) was extracted from the Protein Data Bank and analyzed by using PyMOL. Structure modeling of isoform 2 to 6 was performed by using the Swiss model (https://swissmodel.expasy.org/), with human TMPK structures in complex with dTMP, ADP and Mg^{2+} (PDB code: 1E2F) and in complex with AZTMP and ADP (PDB code: 1E98) as templates. The global model quality estimate (GMQE) is 0.83 and sequence identity is 99.06%. Local quality estimate of the newly built model is between 0.8 to 1.0 (1.0 is the highest grade), except for the loop region (residue 81–120 in isoform 6).

2.2. Protein expression, purification and enzyme assay

Human TMPK isoforms 1 (NP_036277.2) and 6 (NP_001307834.1) were cloned into the pET-14b vector with an N-terminal fusion 6x His tag and expressed in *E. coli* strain BL21 (DE3) pLysS. Recombinant proteins were expressed and purified as previously described.[13] The purity of the recombinant proteins were >95% as judged by SDS-PAGE. The recombinant proteins were used in TMPK activity measurement using [^{3}H]-dTMP as substrate, essentially as previously described.[9]

2.3. Size-exclusion chromatography

The Äkta Prime system was connected to a Superdex 200 10/300 GL column (GE healthcare). The column was washed extensively and equilibrated with buffer containing 10 mM Tris/HCl, pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$ and 5 mM DTT. Prior to loading, protein samples were filtered through a 0.22 µM filter (Merck). The flow rate was 0.2 ml/min. Fractions (0.4 ml) were collected and used for TMPK activity measurement and western blot analysis using a human TMPK specific antibody.[9] Blue dextran 2000 was used to determine the void volume and bovine serum albumin (66 kDa), ovalbumin (43 kDa) and cytoschrome c (12 kDa) were used as standards.

2.4. Cell culture and western blot analysis

Human fibroblasts, immortalized with hTERT,[14] provided by Prof Staffan Johansson, Uppsala University, were maintained in a 37 °C incubator with 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma Aldrich) containing 6% heat inactivated Fetal Bovine Serum (Thermofisher), 1% Penicillin-Streptomycin (Thermofisher) and 1 mM sodium pyruvate (Sigma Aldrich). Fibroblast cell cultures were harvested after reaching ~80 – 90% confluency. Total protein was extracted and used in western blot analyses using a polyclonal antibody against human TMPK, targeting all TMPK isoforms except isoform 4,[9] following standard protocols.

3. Results

3.1. Sequence and structural analysis

The amino acid sequences of the 6 human TMPK isoforms vary considerably in length, and sequence alignment shows that isoforms 2–5 have partial deletions, whereas isoform 6 has an insertion of 39 amino acids, as compared with isoform 1 (Figure 1). In all known TMPK structures there are three important sequence motifs: the p-loop, the DRX (X = Tyr or Phe) motif and the Lid region (Figure 1). The p-loop is involved in the binding and proper positioning of the phosphoryl group of ATP. The Asp residue of the DRX motif assists in binding and positioning the Mg^{2+} ion. The Arg residue of the DRX motif may aid in catalysis by bringing the phosphate donor (ATP) and acceptor (dTMP) into proximity. The Lid-region covers the ATP molecule and undergoes conformational changes during catalysis.[12]

To investigate if the corresponding deletions in isoforms 2-5 and the insertion in isoform 6 have any impact on the structure and function of the enzymes, the structure of human TMPK (isoform 1) in complex with ligands (dTMP, ADP and Mg^{2+}) was analyzed by using PyMol. As shown in Figure 2A, human TMPK (isoform 1) has a globular structure in which the ATP-binding domain is present on the surface, whereas the dTMP-binding domain is located in the interior of the enzyme. ATP binding is mediated by two flexible loops, i.e., the Lid region extending from helices α6 and α7, and the p-loop located between β1 and α1 (Figures 1 and 2). The binding of dTMP is mediated by a series of intricate interactions, including H-bonds with residues R76 in α3 and D96 in β3 and three pairs of hydrophobic π-π stacking mediated by F72 at α3, F105 at α4, and Y151 at the Lid region (Figure 2B, these residues are labeled in blue). Notably, this highlights that hydrophobic interactions are a driving force in protein and ligand interactions in human TMPK. The five β-strands make up the

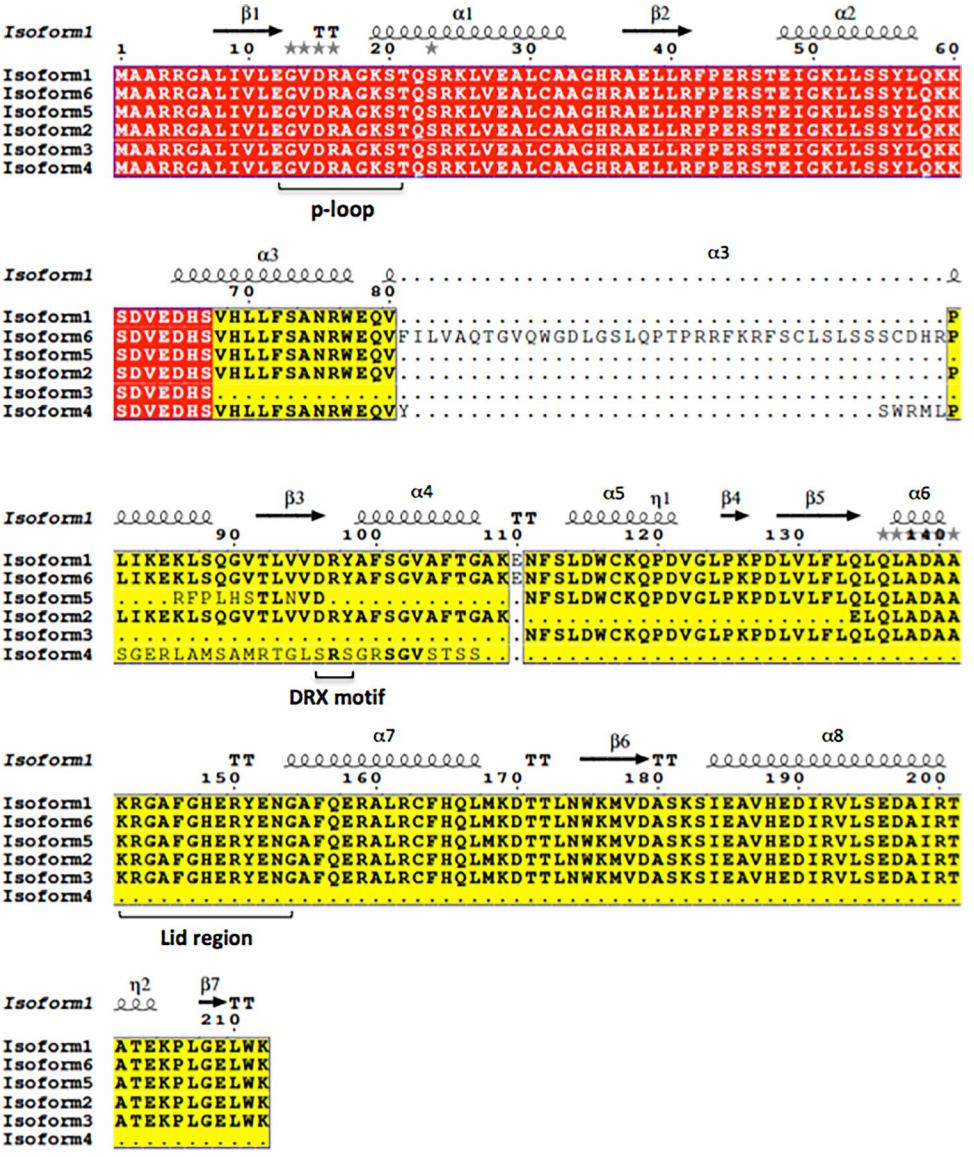

Figure 1. Sequence alignment of human TMPK isoforms. Accession numbers: Isoform 1 (Np_036277.2), isoform 2 (Np_001158503.1), isoform 3 (Np_001307831.1), isoform 4 (Np_001307832.1), isoform 5 (Np_001307833.1), isoform 6 (Np_001307834.1). Important functional motifs are marked. The structure-based sequence alignment was performed by using the ESPript 3.0 program.[15]

core structure of the enzyme. Each β-strand forms three to five H-bonds with adjacent β-strands, which is essential for folding and stability of the active site structure (Figure 2C).

Based on the key structural elements outlined above, the structure of the various human TMPK isoforms i.e., isoforms 2-6, was analyzed with the aim to identify variants that could possess catalytic activity.

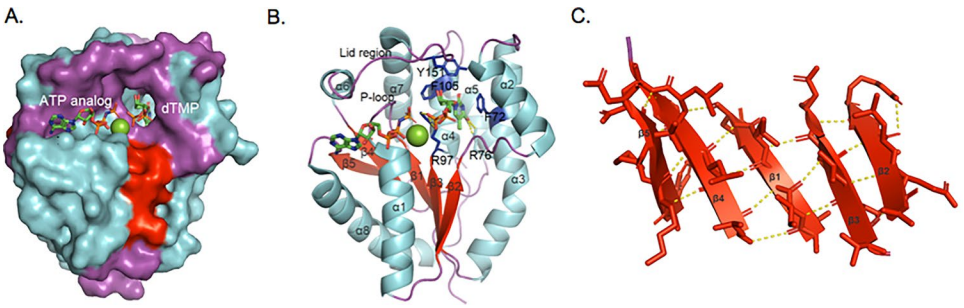

Figure 2. Human TMPK structure (isoform 1). (A) The human TMPK structure shown as surface model. The helices are labeled with cyan, the β-sheet with red and loops are labeled with magenta. In the ligand-binding pocket: ATP is located partly on the surface and dTMP binds in the interior of the protein. (B) Human TMPK structure shown as a cartoon. Residues interacting with dTMP: hydrophobic interaction with F72, F105 and Y151, labeled in blue, the side chain of R76 binds to the thymine ring and the side chain of R97 interacts directly with the phosphate group of dTMP via a H-bond. (C) The β-sheet structure of human TMPK. The five β-strands are placed in the same direction and form the backbone of the protein structure. The human TMPK structure used in the analysis was from https://www.rcsb.org/ (pdb code: 1E2F).

As shown in Figure 1, isoform 2 lacks α5, β4 and one connecting loop region. The lack of β4 leads to instability of the entire β-sheet. Since β4 makes direct contact with the Lid region (α6 and α7), this may pose constraints on the conformation of the Lid region. Thus, isoform 2 is most likely not capable of binding ATP, resulting in an inactive enzyme. Isoform 3 lacks α3, α4, β3 and two connecting loop regions. Both α3 and α4 are located in the dTMP-binding pocket, donating two pairs of π-π stacking (F72 and F105) and a H-bond (R76) to dTMP. With all these essential elements for dTMP binding missing, it is unlikely that isoform 3 can bind dTMP. Furthermore, β3 is important for the entire β-sheet stability and the loop that connects to β3 contains the DRX motif, which is crucial for substrate binding and catalysis. Therefore, isoform 3 is most likely catalytically inactive. Isoform 4 is the shortest of all isoforms and the deletions present in this isoform encompasses the entire active site, including α3, α4, α5, α6, α7, α8, β3, β4 and β5. Thus, isoform 4 cannot be an active enzyme. In isoform 5, two key helices (α3 and α4) are missing. Both of these are essential for dTMP binding, and thus isoform 5 is most likely not catalytically active.

Isoform 6 has an insertion of 39 amino acids, and has a calculated molecular weight of 28.2 kDa. The insertion is placed in the middle of α3. To clarify the effect of this insertion, it is important to understand the role of α3 in the TMPK structure and function. Helix α3 has three unique and essential roles in TMPK function: (1) active human TMPK is a homodimer (Figure 3A). Hydrophobic residues (L70, L71, A74 and W77) in α3 form the interface between the two monomers by hydrophobic

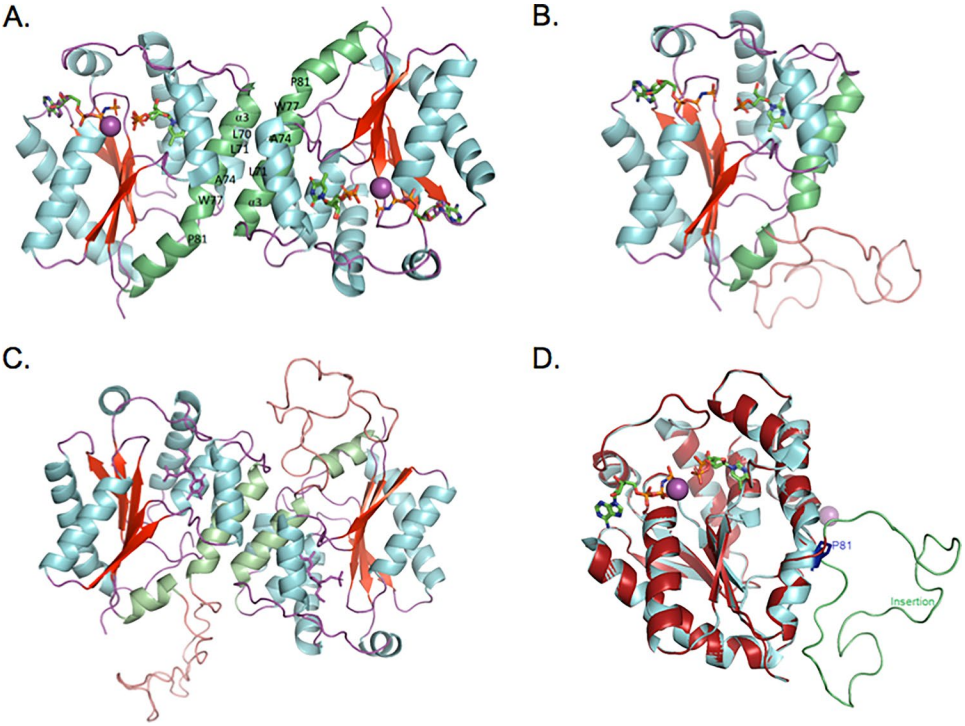

Figure 3. Structure model of TMPK isoform 6. (A) Human TMPK (isoform 1) homodimer. The α3 makes interface between the two monomers, through hydrophobic interactions (in green); (B) Homology model of the TMPK isoform 6 monomer. The 39-amino acids insertion is shown as a loop (salmon). (C) Structural model of the TMPK isoform 6 dimer. (D) Superimposed structures of isoform 1 and 6. Isoform 1 is in cyans and isoform 6 is in firebrick color. The 39 amino acid insertion is in green. Structural modeling was performed at https://swissmodel.expasy.org/ with human TMPK structure as template (PDB code: 1E2F).

interactions to the corresponding helix in the other monomer (Figure 3A); (2) residue F72 forms a hydrophobic interaction and R73 forms a H-bond with dTMP; (3) Residue P81 makes a kink of α3, which enables the interaction of F72 and R73 with dTMP (Figure 3A). The P81 residue is conserved in almost all TMPKs, including TMPK from viruses, bacteria and eukaryotes, although with one exception: *Mycobacterium tuberculosis* (*M. tuberculosis)* TMPK has a glycine instead of proline at this position (G76 in *M. Tuberculosis*, PDB code: 1N5I). Glycine has a similar function as proline, i.e., it can partly break a helix and make a kink. Mutation of P81 to P81L in human TMPK resulted in loss of TMPK function and severe microcephaly in human patients,[5,16] further demonstrated the essential role of this "kink" in TMPK function. How could this be achieved in isoform 6 with the 39 amino acid insertion prior to the conserved proline? As shown in Figure 3B, in the structural model of isoform 6 the 39 amino acids insertion folds into a flexible loop that may form the "kink" between α3 and α4 (labeled as "insertion" in the figure). This loop

Table 1. Summary of structural analyses of human TMPK isoforms.

		Functional motifs			Structure stability	
	MW (kDa)	dTMP binding	DRX motif	ATP binding	β-sheet	Kink in helix α3
Isoform 1	23.8	+	+	+	+	+
Isoform 2	21.1	+	+	+	–	+
Isoform 3	19.0	–	–	+	–	–
Isoform 4	12.5	–	–	–	–	–
Isoform 5	21.9	–	–	+	–	–
Isoform 6	28.2	+	+	+	+	+

+, present
–, absent

structure may provide the structure flexibility needed for dimerization (Figure 3C). Nonetheless, isoform 6 is predicted to have a preserved 3D-structure with all functional important motifs intact as shown in superimposed structures of isoform 1 and 6, and could therefore be catalytically active (Figure 3D). A summary of the predicted structural and functional features of the various human TMPK isoforms is presented in Table 1.

3.2. Expression, purification and characterization of isoform 6

Our molecular modeling approach suggested that isoform 6 might represent a catalytically active enzyme, although it cannot be excluded that the extra insertion may affect enzyme function. To address this, we cloned and expressed TMPK isoform 6 in *E. coli* and the recombinant protein was affinity purified to > 95% purity as judged by SDS-PAGE. The human TMPK (isoform 1) was also expressed and purified, and used as control (Figure 4A). Upon size-exclusion chromatography analysis isoform 1 is in dimer form,[16] however, isoform 6 eluted in various forms, from monomer, dimer to oligomer and the oligomer form was the dominant form (Figure 4B). The activity of the purified isoforms 1 and 6 were determined by using [^{3}H]-dTMP as substrate. This analysis revealed that the specific activity of isoform 6 is only 0.20 nmol/min/mg, which is < 0.1% of the activity of the human TMPK (isoform 1) (Figure 4C). Hence, the 39-amino acid insertion in isoform 6 has a major negative impact on the catalytic activity of the enzyme.

3.3. Expression of isoform 6 in cells

We next examined if isoform 6 is expressed in cells by using a human TMPK-specific antibody raised against the C-terminal sequence. This antibody will recognize all TMPK isoforms (except for isoform 4) if they are

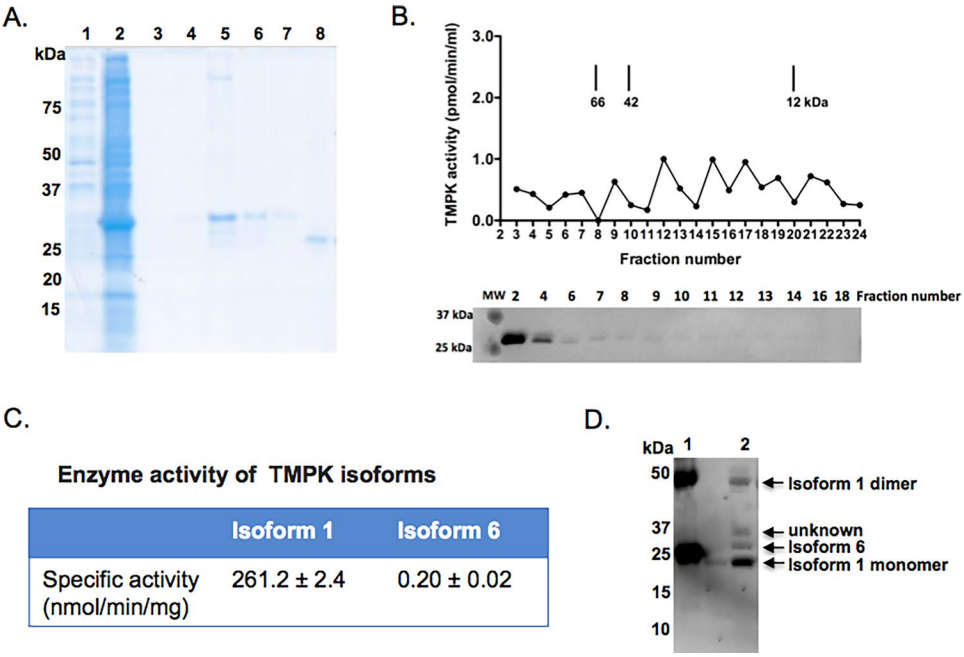

Figure 4. Functional characterization of TMPK isoform 6. (A) Purified TMPK isoform 6 and isoform 1. Lane 1, *E. coli* extracts of uninduced culture; lane 2: *E. coli* extracts of induced culture for isoform 6; lane 3 to 7, correspond to fractions 1-5 of the purified isoform 6; lane 8, purified isoform 1. (B) Size-exclusion chromatography of purified isoform 6. Fractions were collected and assayed for TMPK activity using [^{3}H]-dTMP as substrate and shown as pmol/min/ml. Selected fractions were analyzed by SDS-PAGE and western blot using a human TMPK specific antibody. The elution positions of molecular weight standards are indicated. (C) TMPK activity. Recombinant isoform 1 and 6 were assayed for enzymatic activity using [^{3}H]-dTMP as substrate; data are from three independent measurements and given as mean ± SD; (D) Western blot analysis using a human TMPK-specific antibody. Lane 1, recombinant human TMPK (isoform 1), Lane 2, extracts from fibroblasts.

expressed. Total protein from a cultured human fibroblast cell line was extracted and used in western blot analysis. As shown in Figure 4D, four protein bands were detected by the antibody. Of these, the 24 kDa and ~ 50 kDa bands correspond to the monomer and dimer forms of human TMPK (isoform 1), respectively, which represent the dominant form of TMPK in this cell line. In addition, a ~28 kDa TMPK band was observed, most likely representing isoform 6. Bands corresponding to isoforms 2, 3 and 5 were not detected. An additional band at ~37 kDa was also detected by the antibody. However, the identity of the latter band is uncertain.

4. Discussion

Alternative splicing is a mechanism of RNA maturation and can generate either degradable nonsense mRNAs or mature mRNAs of different splicing

variants, which may encode proteins with altered functions. For example, an alternative spliced dominant negative isoform of PPARγ (peroxisome-proliferator-activated receptor γ), which lacks the entire ligand binding domain, could impair PPARγ function.[17] In search for compensatory TMPK enzymes we here sought to characterize the isoforms of human TMPK mRNA that are deposited in GenBank, and to evaluate if any of these isoforms could exert TMPK enzymatic activity. Our sequence and structural analysis revealed that isoform 2, 3, 4 and 5 all lacked the structural elements that are essential for substrate binding and for formation of a globular structure. Thus, they are considered as nonfunctional isoforms. In contrast, isoform 6 has intact catalytic centers i.e., dTMP binding, DRX motif, ATP binding p-loop and Lid region, which are the key structural elements of an active TMPK. Therefore, isoform 6 may have TMPK activity. However, when isoform 6 was expressed and purified, it showed only minimal activity as compared with isoform 1. Furthermore, a putative isoform 6 was detected in a fibroblast cell line in addition to isoform 1, the latter being the dominant TMPK present in these cells. Since isoform 6 has minimal TMPK activity, it is unlikely that isoform 6 contributes significantly to dTTP synthesis *in vivo* but it cannot be excluded that it may have regulatory functions. For example, Isoform 6 may bind to both dTMP and ATP, and thus compete with isoform 1 for substrates, thereby interfering with dTTP synthesis in cells expressing isoform 6. It is also possible that isoform 1 and 6 form heterodimers, resulting in altered TMPK activity. At present, we do not have any experimental evidence for the presence of isoforms 2 to 5 in cells, however, if these inactive isoforms are expressed, they might interact with the active TMPK (isoform 1) and interfere with its function. To clarify this, further investigations are needed.

Studies of human patients with TMPK deficiency suggest that there is a compensatory TMPK in tissues other than nervous tissue.[5,16] In a fibroblast cell line, a high level of TMPK activity was detected in the mitochondrial outer membrane fraction and this TMPK activity had different biochemical properties as compared with the *DTYMK*-encoded TMPK.[9] Our results presented here suggest that the alternative TMPK identified in the fibroblast cell line, or present in TMPK-deficient patients, does not represent any of the TMPK isoforms characterized here, and is most likely encoded by a different gene. Since all other known nucleoside monophosphate kinases, i.e., AMPKs (adenylate kinases), CMP-UMP kinase, GMP kinase have narrow substrate specificity and none of them are able to phosphorylate dTMP.[18] In future investigations, identification and characterization of this alternative TMPK enzyme may contribute to the understanding of dTTP synthesis in different tissues, and may also provide further insight into the pathophysiological role of TMPK in neurodegenerative disorders.

5. Conclusion

For human TMPK, there are 6 mRNA isoforms present. Isoform 1 is the TMPK enzyme that is well-characterized. Our study revealed that, except for isoform 1, only isoform 6 might fold into an active enzyme. However, functional studies revealed that isoform 6 has minimal TMPK activity. Although isoform 6 may be expressed in cells, it is unlikely that it contributes significantly to dTTP synthesis, but may instead have regulatory functions. Therefore, the alternative TMPK activity that has been identified in human patients and cancer cell line is most likely not due to the TMPK isoform 6, but rather due to a hitherto unidentified enzyme encoded by a different gene.

Disclosure statement

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