## Cloning and expression of human deoxyguanosine kinase cDNA

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ABSTRACT A human cDNA sequence homologous to human deoxycytidine kinase (dCK; EC 2.7.1.74) was identified in the GenBank sequence data base. The longest open reading frame encoded a protein that was 48% identical to dCK at the amino acid level. The cDNA was expressed in Escherichia coli and shown to encode a protein with the same substrate specificity as described for the mitochondrial deoxyguanosine kinase (dGK; EC 2.7.1.113). The N terminus of the deduced amino acid sequence had properties characteristic for a mitochondrial translocation signal, and cleavage at a putative mitochondrial peptidase cleavage site would give a mature protein size of 28 kDa. Northern blot analysis determined the length of dGK mRNA to 1.3 kbp with no cross-hybridization to the 2.8-kbp dCK mRNA. dGK mRNA was detected in all tissues investigated with the highest expression levels in muscle, brain, liver, and lymphoid tissues. Alignment of the dGK and herpes simplex virus type 1 thymidine kinase amino acid sequences showed that five regions, including the substrate-binding pocket and the ATP-binding glycine loop, were also conserved in dGK. To our knowledge, this is the first report of a cloned mitochondrial nucleoside kinase and the first demonstration of a general sequence homology between two mammalian deoxyribonucleoside kinases. Our findings suggest that dCK and dGK are evolutionarily related, as well as related to the family of herpes virus thymidine kinases.

Purine deoxyribonucleoside analogs are extensively used in treatment of lymphoproliferative disorders (1). These compounds are administered as prodrugs, and their efficiency is dependent on intracellular phosphorylation to the corresponding triphosphates. In mammalian cells, the phosphorylation of purine deoxyribonucleosides is mediated predominantly by two deoxyribonucleoside kinases; the cytosolic deoxycytidine kinase (dCK; EC 2.7.1.74) and the mitochondrial deoxyguanosine kinase (dGK; EC 2.7.1.113) (2). The cDNA sequence of human dCK is known, and both tissue-purified and recombinant enzymes have been extensively studied (3-6). Previous studies of dGK are based on enzyme purified and characterized from different tissues (7-11). Purified dGK phosphorylates deoxyguanosine (dGuo) and deoxyinosine (dIno) with high affinity ( $K_m < 25 \ \mu$ M) and deoxyadenosine (dAdo) with lower affinity ( $K_m > 60 \ \mu$ M) (7–9). The clinically important nucleoside analogs 2-chloro-2'-deoxyadenosine (CdA) and 9- $\beta$ -D-arabinofuranosylguanine (araG) are also phosphorylated by dGK (9, 12). All of these nucleosides and nucleoside analogs are also substrates for dCK, although the kinetic properties differ (2). The effects of CdA and araG are generally considered to be mediated by dCK activation, but little is known about the relative contribution of dGK to therapeutic and toxic effects of these nucleoside analogs (13).

We have in the present study used the human dCK cDNA sequence to find homologous cDNA sequences in an attempt to identify novel dCK-related enzymes. Several similar cDNA clones with 40-50% DNA sequence identity to human dCK were found in GenBank sequence data base, and the cDNA

with the longest open reading frame was expressed in *Escherichia coli*. This protein was purified and demonstrated to have the same substrate specificity as shown for purified native dGK. The N-terminal sequence contained a possible mitochondrial translocation signal, and thus the putative intracellular localization is in accordance with previous reports on dGK (8–10). Northern blot analysis revealed a unique expression pattern for dGK mRNA with no cross-hybridization to dCK mRNA. We conclude that these two distinct human deoxyribonucleoside kinases are closely related, and our data thereby introduce the concept of a family of human nucleoside kinases with at least two members.

## **EXPERIMENTAL PROCEDURES**

**Cloning of Human dGK cDNA.** The GenBank sequence data base at the National Center of Biotechnology Information was accessed via internet World Wide Web at http://ncbi.nlm. nih.gov/and the Basic Local Alignment Search Tool (BLAST) (14) algorithm was used to identify sequences homologous to the open reading frame of human dCK cDNA (3). Clones homologous to dCK deposited by the I.M.A.G.E. (Integrated Molecular Analysis of Genomes and their Expression) consortium (15) were obtained from Research Genetics (Huntsville, AL) and were resequenced as described below.

Two PCR primers (5'-TAAGGATCCAAGAGCCCACTC-GAGG and 5'-TTCAAGCTTCAGAAAGTCAGGGAGC-CCAG) with 5' restriction sites (BamHI and HindIII) were designed based upon the GenBank sequences obtained. A two-step hot-started PCR with 64°C annealing and extension (16) was performed on a human fetal brain cDNA library in lambda-ZAP vector (Stratagene). Human promotor DNA walking kit (CLONTECH) was used to clone the genomic region located upstream of the 5'-end of the cDNA clone. Nested primers designed from the 5'-end of the cDNA sequence (5'-CCTCGAGTGGGCTCTTGGCCATGGAAC and 5'-TGAAGGGTGCTCGAAGCCGACTTAGAAA) were used in the PCR as described in the CLONTECH protocol. PCR products from both cDNA and genomic PCR were cloned into pGEM-T plasmid vector (Promega). DNA sequences were determined by automatic laser fluorescent (A.L.F.) sequencer (Pharmacia).

**Protein Expression and Purification.** The Protein Fusion & Purification System (New England Biolabs) was used to express the cDNA-encoded protein as a fusion to maltosebinding protein. The cDNA open reading frame was subcloned into the *Bam*HI-*Hin*dIII sites of the pMAL-c2 plasmid (New England Biolabs) and transformed into the *E. coli* TB1 host strain. A single positive colony was inoculated in a large-scale culture [Luria-Bertani (LB) broth with 0.2% glucose and 100 mg of carbenicillin per ml] and grown at 37°C until OD<sub>600</sub> =

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Abbreviations: dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; CdA, 2-chloro-2'-deoxyadenosine; araG, 9- $\beta$ -D-arabino-furanosylguanine; HSV1.TK, herpes simplex virus type 1 thymidine kinase; dGuo, deoxyguanosine; dAdo, deoxyadenosine; dIno, deoxyinosine; dCyd, deoxycytidine; Thd, thymidine.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U41668).

0.5. Fusion protein expression was induced by addition of 0.3 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside and growth continued for 2 h. The cells were harvested by centrifugation at 4000 × g for 20 min and resuspended in 20 mM Tris, pH 7.6/200 mM NaCl/1 mM EDTA/1 mM dithiothreitol. The cells were then lysed by freeze-thawing and sonication six times for 15 s each on ice. The supernatant was cleared by centrifugation at 9000 × g for 30 min and loaded onto the amylose resin column included in the protein purification kit. The protein was eluted in the Tris buffer stated above with 10 mM maltose added. The fusion protein was cleaved with 1% factor Xa (New England Biolabs) for 24 h at room temperature. The size and purity of the cleaved product was determined by SDS/PAGE (Phast-system, Pharmacia).

**Enzyme Assays.** The substrate specificity of the enzyme was determined by a phosphoryl transferase assay with  $[\gamma^{32}P]$ -ATP as described (17). The assay was performed in 50 mM Tris, pH 7.6/100 mM KCl/5 mM MgCl<sub>2</sub>/1 mM unlabeled ATP/25 mCi of  $[\gamma^{32}P]$ -ATP (3000 Ci/mmol; Amersham) and indicated concentrations of deoxycytidine (dCyd), dAdo, dGuo, dIno, thymidine (Thd), CdA, araG, 2',3'-dideoxycytidine, 2',3'-dideoxyguanosine, 2',3'-dideoxyinosine, 9- $\beta$ -D-arabinofurano-syladenine, 9- $\beta$ -D-arabinofuranosylcytosine, 3'-azido-2',3'-dideoxyguanosine, and 3'-azido-3'-deoxythymidine. The purified factor Xa cleaved fusion protein was used in all assays. Polyethyleneimine-cellulose sheets were autoradiographed for 1–12 h and quantified with Image Master system (Pharmacia).

Northern Blot Analysis. Human multiple tissue Northern blot and human immune system multiple tissue Northern blot with poly(A)<sup>+</sup> RNA from different human tissues were purchased from CLONTECH. Human dGK and dCK open reading frame probes were labeled with  $[\alpha^{-32}P]dCTP$  (6000 Ci/mmol, Amersham; Prime-A-Gene, Promega) and hybridized at 42°C in 50% formamide hybridization buffer following the protocol provided by CLONTECH. The blots were scanned and quantified with Image Master system (Pharmacia).

## RESULTS

Cloning and Identification of Human dGK cDNA. Several DNA sequences that were homologous but not identical to human dCK cDNA were found in the GenBank data base (accession nos. H93270, H93271, R51942, R53612, R79540, R79541, R07506, R07560, R70464, R70551, H14563, H14562, and R86014). The sequences were from cDNA clones from breast, placenta, and fetal liver-spleen deposited by the I.M.A.G.E. consortium (ref. 15; I.M.A.G.E. clone numbers: 125722, 141772, 145937, 154206, 159331, 187211, and 234450). Alignment of the sequences showed three distinct cDNA isoforms (Fig. 1). Isoforms 2 and 3 lacked region A or region B, which were present in isoform 1. Using PCR on a human



FIG. 1. Isoforms of human cDNA clones homologous to human dCK. The black regions are present and identical in all isoforms. The gray areas A and B are present in the isoforms as indicated. In isoform 5, an unrelated sequence C replaces sequence A. Lines represent truncated regions. The open reading frame (ORF) is defined as the length from the first possible translation start codon until the first stop codon (arrow).

fetal brain cDNA library with primer sequences derived from the I.M.A.G.E. clones, we found two additional isoforms (isoforms 4 and 5, Fig. 1). Isoform 4 had the same truncation pattern as isoforms 2 and 3. In isoform 5 region A was replaced by a shorter unrelated sequence (5'-GTAACATCCTCAAG-CAAATCAGAATGAGAGCACCCATTCAGGAAACCTG-AACAGGGAAGGTGAACCCCTGTCCTGACCAAAAT-TGG). Only isoforms 1 and 2 had open reading frames >250 bp.

Isoform 1 of the cDNA clones described above had the longest open reading frame and was homologous to dCK in all regions. We therefore chose to express it and characterize the encoded protein. The cDNA was expressed fused to maltosebinding protein in the pMAL-c2 expression vector. After induction of bacterial fusion protein expression and purification on amylose resin column, a major band of a  $\approx$ 75-kDa protein was detected by SDS/PAGE. The fusion protein was cleaved with factor Xa to the expected 43-kDa maltose-binding protein and the 30-kDa cDNA product (data not shown). The yield was  $\approx$ 10 mg of purified fusion protein per liter of bacterial culture.

A phosphoryl transferase assay was used to determine the substrate specificity of the purified recombinant enzyme. The enzyme efficiently phosphorylated dAdo, dGuo, dIno, CdA, and araG, whereas Thd, 9-B-D-arabinofuranosyladenine, and 9- $\beta$ -D-arabinofuranosylcytosine were not substrates for the enzyme (Table 1). Low levels of phosphorylated dCyd were also detected by this assay. A 50-fold excess of dCyd had, however, no inhibitory effect on CdA phosphorylation, indicating a very low affinity for dCyd as compared with CdA as a substrate (data not shown). The 3'-modified dideoxyribonucleoside analogs 2',3'-dideoxycytidine, 2',3'-dideoxyguanosine, 2',3'-dideoxyinosine, 3'-azido-2',3'-dideoxyguanosine, and 3'-azido-3'-deoxythymidine were not phosphorylated by the enzyme. The substrate specificity of the recombinant enzyme and the substrate specificity reported for purified native dGK (7-10) are the same. Our conclusion is therefore that the cloned cDNA encodes human dGK.

Sequence Analysis and Comparisons. By determining the genomic DNA sequence upstream of the cDNA sequence available from GenBank data base, we identified an additional in-frame translation start codon 51 bp upstream of the one identified in isoform 1. The DNA and predicted amino acid sequences of dGK based upon the cDNA and the 5'-genomic sequence are shown in Fig. 2. The N-terminal region of the protein lacked negatively charged residues, had high contents of positively charged residues, and had a secondary structure prediction indicated a potential  $\alpha$ -helix (Fig. 3). These features are common to most mitochondrial targeting signal peptides (18) and an algorithm analysis of partial amino acid composition indicated the intracellular location to be mitochondrial (19). Of the two possible translation start codons in the

Table 1. Nucleoside substrates of the enzyme encoded by cDNA isoform 1 determined by  $[\gamma^{-32}P]ATP$  phosphoryl transferase assay

Substrate	Relative phosphorylation	
	5 μM	100 µM
dAdo	$0.2 \pm 0.1$	$0.7 \pm 0.1$
dCyd -	$0.1 \pm 0.03$	$0.1 \pm 0.05$
dGuo	1.0	1.0
dIno	$0.4 \pm 0.1$	$0.8 \pm 0.2$
Thd	< 0.01	< 0.01
araA	<0.01	< 0.01
araC	<0.01	< 0.01
araG	$0.7 \pm 0.2$	$0.6 \pm 0.06$
CdA	$0.9 \pm 0.1$	$1.1 \pm 0.3$

The levels of phosphorylated products are expressed in relation to dGuo phosphorylation. araA, 9- $\beta$ -D-arabinofuranosyladenine; and araC, 9- $\beta$ -D-arabinofuranosylcytosine.



FIG. 2. Nucleotide and predicted amino acid sequence of human dGK. The two possible translation start codons are underlined. Amino acid residues in black boxes are identical to the human dCK sequence at the best sequence alignment. The two boxes outlining base pairs 143–255 and base pairs 445–708 indicate regions A and B shown in Fig. 1. The probable polyadenylylation signal is shown in lowercase letters. Numbering of the nucleotide sequence starts at the first methionine residue.

5'-region, the upstream codon is the one we therefore suggest as the translation start site. A motif for mitochondrial prepeptide proteases was found with the cleavage site between Arg-39 and Arg-40 (20) (Fig. 3). This putative cleavage site gave a 28-kDa mature protein, which has been shown to be the size of a purified dGK monomer (8, 9).

The cDNA sequence of isoform 1 included a 780-bp open reading frame (Fig. 2, base pairs 52-831) encoding a protein with 48% and 57% identity to human dCK at amino acid and nucleotide levels, respectively. The amino acid sequences of dCK and dGK were 51% identical in the region of the putative mature dGK.

Aligning the amino acid sequences of human dGK, human dCK, and herpes simplex virus type 1 thymidine kinase (HSV1.TK) showed that five of the six regions conserved in different herpes virus thymidine kinases (21) were also partially conserved in both dGK and dCK (Fig. 4). The first site in the N-terminal region was homologous to the glycine loop motif that is conserved in many kinases and other nucleotide-binding enzymes (22). These amino acid residues form in the



FIG. 3. N-terminal part of the predicted dGK amino acid sequence. The two possible N-terminal methionine residues are underlined. Physiological properties of the amino acid residues are indicated as follows. H, hydrophobic; N, neutral; P, polar; +, positively charged; and -, negatively charged. Secondary structure prediction of the region suggests an  $\alpha$ -helix as indicated. The arrow indicates the probable mitochondrial peptide protease cleavage site determined by the consensus motif for cleavage by the two protease models (20). HSV1.TK, an anion hole that coordinates the  $\alpha$ ,  $\beta$ , and  $\gamma$  phosphates of ATP (23). The ATP molecule is complexed with Mg<sup>2+</sup>, and the sequence alignment suggests Asp 147 in dGK to be responsible for the coordination of Mg<sup>2+</sup>. The other conserved regions have been shown for HSV1.TK to be involved in substrate binding (23).

Northern Blot Analysis of dGK and dCK. Multiple tissue Northern blots were used to determine the size and tissue distribution of dGK and dCK mRNA (Fig. 5). dGK mRNA was detected in all tissues investigated as a major band at 1.3 kbp with the highest levels in muscle, liver, brain, and lymphoid tissues. A shorter form of dGK mRNA at  $\approx 1.0$  kbp was also detected in several tissues. The human dCK probe hybridized with a single band at  $\approx 2.8$  kbp, with the highest levels in thymus, skeletal muscle, fetal liver, bone marrow, and brain. Major differences between dGK and dCK mRNA levels were found in adult liver and heart muscle. The levels of dCK mRNA in these tissues were very low, whereas dGK mRNA levels were high as compared with the levels in other tissues. Although the dCK mRNA level was very low in adult liver, it was high in fetal liver, probably explained by the role of fetal liver in hemopoiesis during fetal development. The levels of dGK mRNA in lymphoid tissues were fairly equal in the samples examined, whereas dCK had predominant expression in thymus, bone marrow, and lymph node. Surprisingly, no dCK mRNA was detectable in peripheral blood leukocytes, and the dGK mRNA level was also relatively low in these cells. No cross-hybridization between dCK and dGK was observed.

## DISCUSSION

By identification of a cDNA clone homologous to human dCK, we have cloned and expressed an enzyme with the same substrate specificity and size as reported for homogeneously purified mitochondrial dGK. Previous studies demonstrate overlapping substrate specificities between dCK and dGK, and sequence homology was therefore not surprising. Although the overall amino acid homology to dCK was ≈48%, the N terminus had fewer conserved residues and was extended in dGK as compared with dCK. The N-terminal part of dGK had characteristic properties of a mitochondrial translocation signal peptide, and the proposed mitochondrial protease cleavage site gave a mature protein of 28 kDa, which corresponds well to the estimated size of purified dGK (8, 9). Because dGK previously has been purified from mitochondria (8-10), whereas dCK is a cytosolic enzyme (10), the N-terminal differences are in agreement with the different intracellular locations of the two enzymes.

One important physiological function of dCK is considered to be in providing cells with deoxyribonucleotides for DNA repair (24). The role of dGK would be to provide deoxyribonucleotides for mitochondrial DNA synthesis together with the other mitochondrial nucleoside kinase; thymidine kinase 2. Previous studies show that cell extracts from liver and brain have very low dCK activity (25, 26). Although these tissues mainly consist of resting cells, there should be a demand for deoxyribonucleotides for DNA repair. Whether the high expression level of mitochondrial enzymes in these tissues is sufficient to provide phosphorylated nucleosides is not yet clarified.

The contribution of dGK in mediating cytotoxicity of nucleoside analogs is unknown. A report on secondary loss of dGK activity without loss of dCK activity in purine nucleoside phosphorylase-deficient mice (27) suggests that dGK nucleoside phosphorylation can mediate cytotoxic effects. These findings may be important in studies of the purine nucleoside phosphorylase resistant nucleoside analog araG, a substrate of both dCK and dGK (6, 9, 12, 28). In contrast to the dCK-phosphorylated 9- $\beta$ -D-arabinofuranosylcytosine, which is equally toxic to B and T cells, araG is selectively toxic to T cells



FIG. 4. Comparison of dGK, dCK, and HSV1.TK amino acid sequences in regions conserved in several herpes simplex virus thymidine kinases. S1–S5 are the sites described by Balasubramaniam *et al.* (21). Black boxes indicate conserved amino acid residues in dGK, dCK, and HSV1.TK. Asterisks indicate HSV1.TK amino acid residues conserved in all or most of other known herpes thymidine kinases (21).

(28, 29). The metabolic basis of the selective araG toxicity appears to be that T cells accumulate higher levels of araGTP than other cells (29). araG may therefore be useful in treatment of T-cell malignancies, and clinical trials of araG therapy are in progress (30). The cloning and expression of human dGK will hopefully contribute to the clarification of its role in both DNA precursor synthesis and nucleoside analog activation.

We do not know the function of the truncated dGK mRNA isoforms. That they are artifacts seem unlikely, because (i) the isoforms are present in four different cDNA libraries, (ii) at least two bands are seen on Northern blots, and (iii) truncations are in exactly the same regions in all clones. dGK



FIG. 5. Human multiple tissue Northern blots hybridized with human dGK and dCK cDNA probes. The estimated size of the mRNAs are 1.3 kbp and 2.8 kbp for dGK and dCK, respectively. In dGK blots, additional shorter bands at  $\approx$ 1.0 kbp are detected in several tissues. An actin probe was hybridized to the same blots as a control of the mRNA levels. PBL, peripheral blood leukocytes.

expression may be regulated by splicing of the full-length mRNA to nonfunctional products, as the isoforms lack functional motifs present in HSV.TK1. Another possibility is that translated products of the isoforms can associate to the full-length dGK subunits and alter its kinetic properties. We have so far expressed and characterized the cDNA isoform with the expected length of dGK and with all nucleoside and nucleotide binding sites that are conserved in other kinases. The expression and characterization of the other isoforms is presently in progress.

The high level of sequence conservation between dGK and dCK suggests a close evolutionary relationship. The highest degree of identity is in regions identified as conserved sites in herpes virus thymidine kinases, and the evolutionary connection between human dCK and this group of viral enzymes has been suggested (31). The crystal structures of HSV1.TK in complex with deoxythymidine and the nucleoside analog ganciclovir have recently been solved (23). The HSV1.TK structure provides information of amino acids involved in nucleoside recognition in dCK and dGK, because many of the active site residues are conserved in all these enzymes. One marked difference, however, between HSV1.TK and dCK/dGK is that the latter enzymes do not recognize thymine as a nucleoside base. The HSV1.TK Tyr-172 is conserved in many herpes virus thymidine kinases (21), and the crystal structure demonstrates how this residue is involved in positioning of the thymine base. In dCK, the corresponding residue is also a tyrosine, but in dGK it is a phenylalanine (Phe-156), the only amino acid that can functionally replace the tyrosine at this position as determined by a random mutagenesis study on HSV1.TK (32). Several of the regions described above as conserved between herpes virus thymidine kinases and dCK/dGK are also conserved in the heterodimeric deoxyguanosine/deoxyadenosine kinase of Lactobacillus acidophilus R-26 (33). Further comparative studies on these related enzymes may help to identify the motifs that determine substrate specificity of nucleoside kinases. Engineered nucleoside kinases with optimized kinetic properties for selected nucleoside analogs may be of value as prodrug activators in anti-cancer gene therapy.

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