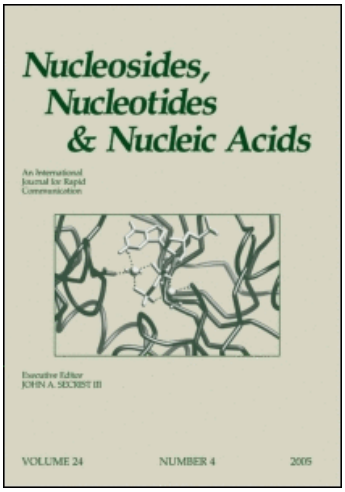

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Substrate Specificities, Expression and Primary Sequences of Deoxynucleoside Kinases; Implications for Chemotherapy

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SUBSTRATE SPECIFICITIES, EXPRESSION AND PRIMARY SEQUENCES OF DEOXYNUCLEOSIDE KINASES; IMPLICATIONS FOR CHEMOTHERAPY

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ABSTRACT: Deoxynucleoside kinases are key enzyme in deoxyribonucleoside salvage, phosphorylating many important anti cancer and anti viral drugs. There are four kinases in animal cells; cytosolic thymidine kinase (TK1) and deoxycytidine kinase (dCK) and the mitochondrial thymidine kinase (TK2) and deoxyguanosine kinase (dGK). The biochemical properties of the purified enzymes and the sequences of their cDNA;s have been determined. In case of TK2 and dGK this was done very recently and they show high homology to dCK and the herpes virus kinases but not to TK1. The evolutionary and functional consequences of this fact will be discussed.

This article attempts to summarise some of the important biochemical properties of the four cellular deoxynculeoside kinases: cytosolic thymidine kinase (TK1) and deoxycytidine kinase (dCK) and the mitochindrial thymidine kinase (TK2) and deoxyguanosine kinase (dGK) and relate that to their recently known primary structures. A comparison with the herpes virus deoxynucleoside kinases is also done.

Biochemical properties of the deoxyncucleoside kinases: TK1 is an enzyme that is present in almost all types of organisms. The expression of TK1 is tightly regulated during the cell cycle and active enzyme is found only in actively proliferating cell in S-phase. This is achieved through transcriptional, translational and post-translational regulatory mechanisms[1-5]. The TK1 cDNA is 1,421 base pairs long and has an open reading frame of 702 bp that encodes a protein of 25.5 kDa[2-3]. Active TK1 has a molecular mass ranging from 55 kDa to 110 kDa, respectively, with subunit molecular mass of 25.5 kDa. A dimer or tetramer is formed in the absence or presence of ATP[1,6].

ATP as well as other nucleoside triphosphates can be used as phosphate donors for TK1, but not dTTP or CTP. In addition to the natural substrates, thymidine and deoxyuridine, TK1 also phosphorylates a number of clinically important nucleoside analogues. For instance the anti-HIV compounds, 3'-azido-

2',3'-deoxythymidine (AZT) and FLT (3'-fluoro-2',3'-deoxythymidine [7,8]. Among 5-substituted deoxyuridine analogues, 5-fluoro, 5-bromo, and 5-ethyl were accepted but not bulkier substitutions, such as 5-propenyl, 5-(2-chloroethyl) and 5-(2-bromovinyl)[1,9,10].

dCK catalyses the phosphorylation of deoxycytidine to deoxycytidine 5'-monophosphate using a nucleoside triphosphate as phosphate donor. Unlike TK1, dCK is not cell cycle regulated, but constitutively expressed in many cells. There is a high dCK activity in resting lymphocytes and a modest (2- to 3-fold) increase of activity in stimulated lymphoblasts. The highest level of dCK was found in lymphoid tissues and the lowest in nerve, liver and muscle tissue. Tumour cells from different tissues show 2-5-fold increased levels as compared to the corresponding normal tissue[1,11].

Human dCK cDNA is 2460 bp long, including 780 bp which codes for a protein with a calculated molecular weight of 30.5 kDa[12]. Active dCK has an apparent molecular weight of 60 kDa, *i. e.* a dimer of two 30 kDa subunits. dCK phosphorylates deoxycytidine, deoxyadenosine and deoxyguanosine and their analogues with complex kinetics. dCTP is a competitive inhibitor with respect to dCyd and a non-competitive inhibitor with respect to ATP and dCTP probably acts as a bisubstrate analog-inhibitor. UTP was a better phosphate donor than ATP and UTP is likely to be the major intracellular phosphate donor for dCK[13-15].

In addition to the natural substrates, dCK also phosphorylates numerous analogs with both 2'- and 3'- modifications, as well as acyclic sugars, provided that the base is cytosine with minor substitutions at 5-position. However, 5-substitution with bulky group were not acceptable[1,10]. With uracil or thymine as base dCK showed very low activity. No purine nucleosides with 3'-modification or acyclis sugar were allowed, but arabinofuranosyl nucleosides were accepted[9-10].

TK2 is a mitochondrial enzyme which catalyses the transfer of gamma phosphate group from ATP to the 5'-hydroxyl group of deoxythymidine, the same substrate as for TK1. The level of TK2 is low as compared to TK1 in proliferating cells, but is significant in resting or terminaly differentiated cells where TK1 activity is undetectable. The expression of TK2 is most likely correlated to the mitochondrial content of the tissues but not to the growth state of the cell[1].

TK2 from human leukemic spleen had a subunit molecular mass of 29 kDa. The molecular weight of active enzyme was estimated to be 30 kDa[16]. TK2 utilises Thd, dCyd and dUrd as its substrate but with different efficiency and kinetic mechanisms. TK2 phosphorylates thymidine with negative co-operativity, where the affinity for substrate decreased with increasing substrate concentrations. The phosphorylation of dCyd and dUrd showed normal Michaelis-Menten mechanism. Both dTTP and dCTP inhibit the enzyme and ATP and CTP can be used as phosphate donors by TK2[16].

TK2 phosphorylates all the three pyrimidine deoxyribonucleosides and their analogues. Thymidine analogs with modification on the sugar moiety such as AZT, arabinofuranosyl thymine (ara-T), and ribothymine could be phosphorylated, but with lower efficiency. TK2 showed about 5-10 % of the activity with AZT as compared to that with thymidine. A large number of dUrd analoges can be phosphorylated by TK2 including 5-substitutions such as halogen, amino, ethyl, and some bulky groups as well as arabinosyl uracil[9,10,16]. The phosphorylation of FIAU by TK2 may in part explain the cytotoxic effect of this anti-hepatitis B compound[17]. Deoxycytidine analogues with 5-substitution of halogen, amino or even bulky groups, such as 5-(2-chloroethyl), 5-(2-bromovinyl) showed activity with TK2 and modification of the sugar moiety such as 2'-fluoro was also acceptable. Interestingly some of the 5-aryl substituted deoxycytidine analogues were phosphorylated by TK2 but not by dCK [9,10]. The cDNA for TK2 was recently cloned, sequenced and expressed in *E. coli*. The properties of the pure recombinant protein was very similar to those of the human spleen enzyme or the bovine brain enzyme[18].

dGK is a nuclear gene product, localised to the mitochondria, catalysing the phosphorylation of purine deoxyribonucleosides and their analogs, using a nucleoside triphosphate as phosphate donor. The expression of dGK is not cell cycle regulated and the activity of dGK is found in most tissues, including lymphoid tissues, spleen, skin, liver and brain, although in most cases at a low level compared to cytosolic dCK[1,19].

Active dGK protein is a dimer of two 28 kDa subunits. dGuo and dIno were efficiently phosphorylated by dGK, but the specific activity of this enzyme was low in all cases possibly because of inactivation of the enzyme during purification [1,20]. The end product dGTP behaved as a bisubstrate analog, and thus served as a feed-back inhibitor without the need for a separate allosteric site[1,20]. The cDNA for dGK was recently cloned, sequenced and expressed in *E. coli*. [21,22]. The properties of the pure recombinant protein was very similar to those of the bovine brain enzyme[21].

Most herpes viruses encode thymidine kinase genes which are considered to be virulence factors[23]. Herpesvirus TKs, like other TKs, catalyses the formation of thymidylate from thymidine using a nucleoside triphosphate as phosphate donor. Herpesvirus TKs, differ from cytosolic TK1, can utilise a broad range of substrates in addition to thymidine, including deoxycytidine and a large number of nucleoside analogs such as the antiherpes compound acyclovir and ganciclovir. Both ATP and CTP can be used as phosphate donor, and HSV TKs is less sensitive to feed back inhibition by dTTP[23].

Structure-function relationships among the deoxynucleoside kinases: Herpes virus TKs show similar characteristics as the host enzymes but they still have important differences in substrate specificity which is the basis for successful anti viral chemotherapy. Today a large number of thymidine kinase genes has been sequenced from various sources, including vertebrates, bacteria, viruses. In the review of Gentry[23] TKs has been classified into two main groups: the

poxviral and cellular thymidine kinases, and herpesviral thymidine kinase and eukaryotic deoxycytidine kinases. The sequence of the mitochondrial thymidine kinase was not known at that time, but it is clear now that the mitochondrial TK is a member of herpesviral TK and cellular dCK family because of their similar biochemical characteristics and gene sequences[18] (Fig. 1). Mitochondrial dGK is certainly a member of dCK family as well[21,22] (Fig. 1). The recent release of the 3-D structure of HSV-1 TK[24] and the identification of conserved sequence motifs among deoxyribonucleoside kinases enable a reasonable prediction of structure function relationships among deoxyribonucleoside kinases.

A comparison of herpesviral TKs with cellular dCK sequence was made and revealed a clear relationship (Fig. 1). Therefor, Harrison *et al.* [25] proposed that the TKs of herpesviruses of higher and lower vertebrates have evolved either independently or successively from a cellular dCK. The finding of great similarity of dCK sequence to the two newly cloned human dGK and TK2 sequences, thus establish a new family of deoxyribonucleoside kinases (Fig. 1). The sequence of human TK2 showed significant homology to the eukaryotic dCKs and dGK, as well as to the family of herpesviral TKs, but not to the family of bacterial, poxvrial and eukaryotic cytosolic TKs. The high homology of TK2 to dCKs, dGK and herpesviral TKs suggests that they are evolutionary related, and may originate from a common ancestor.

Alignment of the human deoxyribonucleoside kinases with selected herpesviral TKs revealed conservation of functional important sequence motifs. An overview of the overall arrangement of these sequence motifs along the entire sequences is shown in Fig. 2, suggesting that they are structurally and functionally related, but still the mammalian enzymes have an additional conserved feature that distinguish them from the herpesviral TKs. They contain a leucine zipper type of structure close to the C-terminal of the polypeptide which also could be found in several protein kinases such as CDK activating kinase[26]. This domain identified for the first time may have involved in protein-protein interactions.

The ATP binding site (Fig. 2) closed to the N-terminal has a conserved sequence fingerprint -G-X-X-G-X-G-K-T/S-T-. This motif is also found in adenylate kinase and a large number ATP/GTP binding proteins. The presumed loop structure facilitates interactions between the γ phosphoryl group of the nucleotide and a positively charged amino acid residue (lysine), a crucial part of the active centre of all kinases as demonstrated by the AK and HSV1-TK structural studies. The substrate specificity site , including residue 161-192 in HSV1-TK with a conserved sequence triplet -DRH- in all the herpesviral TKs has been shown to be important in substrate binding. In the mammalian deoxynucleoside kinases there is a similar triplet -E-R-S-, possibly with the same function. Channel catfish virus (CCV), a lower vertebrate herpes virus, encodes a TK which can be distinguished biochemically from TK1 and other herpes virus TKs because of the inability to use CTP as phosphate donor. CCV TK does not

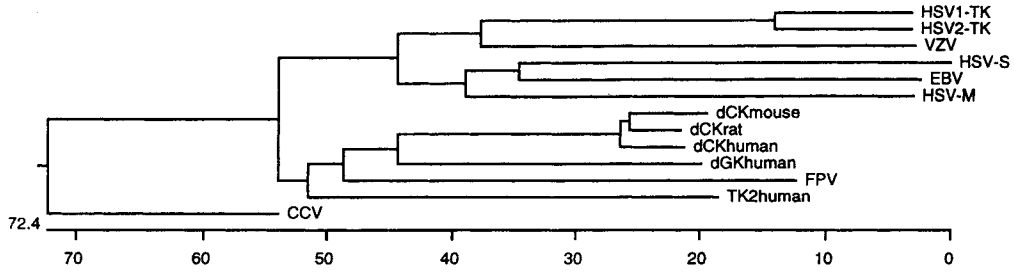

FIG. 1. Phylogenetic tree of the "dCK-family" of deoxynucleoside kinases using the Clustal method for pairwise alignments of the Genebank sequences (when available) in the DNASTAR program. The scale measures distances (the number of substitution events) between the sequences.

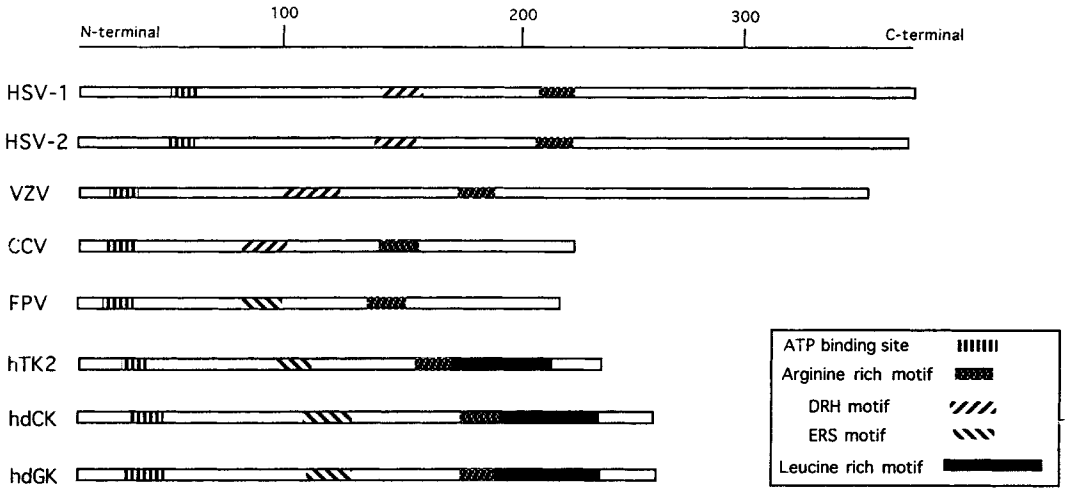

FIG. 2. Schematic representation of the structure of the deoxynucleoside kinases. Alignments were initially done with the DNA STAR program.

contain the consensus -DRH- motif but instead has the -ERS- triplet which is found in the mammalian dCK family.

The arginine rich motif, similar to the ATP binding site, is conserved among all the sequences and numerous ATP/GTP binding proteins. The conserved arginine residue is involved in the binding of the phosphoryl group as it does in adenylate kinase and HSV1 TK. The distance between substrate specificity site and the arginine rich motif of mammalian enzymes is only marginally longer than that of herpes enzymes which suggests that they probably have the same function.

The properties of poxviral TKs and bacterial TKs are similar to the cytosolic TKs of vertebrates, so are their amino acids sequences. The human, chinese hamster, mouse and chicken TK1 sequence showed great similarities and conservation, only a few substitutions is seen[23]. Poxviral TKs and eukaryotic cytosolic TKs are very similar, except that poxviral TKs are shorter both at the N-terminal and the C-terminal part. The bacterial TKs seemed to differ from their host TKs to some extend but still conserved sequence motif could be identified. The highly conserved ATP binding domain with the sequence finger print G/A-X-M-X-S/A-G-K-S/T closed to the N-terminal was found in of all the sequences, as well as an arginine rich motif. However the other substrate binding motifs could not be identified. Detailed knowledge of the structure function relationship of the TK1 family must await 3-D structure determinations.

Conclusions There are two distinct enzyme families among the so far known deoxy-nucleoside kinases and there are members in each families that are key enzymes in anti viral and anti tumour therapy. The similarity between dCK, TK2 and dGK and the herpes virus kinases may help medicinal chemists to target activation of deoxynucleosides analoges to certain tissues e. g. lymphocytes, nerve cells and liver cells. There is however a potential problem in that antiherpes nucleosides may have cytotoxic or mitochondrial side effects, but increased knowledge about the structure -function relationships among these enzyme families should aid future rational drug design projects.

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