Functional Expression of a Multisubstrate Deoxyribonucleoside Kinase from Drosophila melanogaster and Its C-terminal Deletion Mutants

(Received for publication, October 20, 1998, and in revised form, November 24, 1999)

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The occurrence of a deoxyribonucleoside kinase in Drosophila melanogaster (Dm-dNK) with remarkably broad substrate specificity has recently been indicated (Munch-Petersen, B., Piskur, J., and Søndergaard, L. (1998) J. Biol. Chem. 273, 3928–3931). To prove that the capacity to phosphorylate all four deoxyribonucleosides is in fact associated to one polypeptide chain, partially sequenced cDNA clones, originating from the Berkeley Drosophila genome sequencing project, were searched for homology with human deoxyribonucleoside kinases. The total sequence of one cDNA clone and the corresponding genomic DNA was determined and expressed in Escherichia coli as a glutathione S-transferase fusion protein. The purified and thrombin cleaved recombinant protein phosphorylated the four deoxyribonucleosides with high turnover and $K_m$ values similar to those of the native Dm-dNK, as well as the four ribonucleosides and many therapeutical nucleoside analogs. Dm-dNK has apparently the same origin as the mammalian kinases, thymidine kinase 2, deoxycytidine kinase, deoxyguanosine kinase, and the herpesviral thymidine kinases, but it has a unique C terminus that seems to be important for catalytic activity and specificity. The C-terminal 20 amino acids were dispensable for phosphorylation of deoxyribonucleosides but necessary for full activity with purine ribonucleosides. Removal of the C-terminal 20 amino acids increased the specific activity 2-fold, but 99% of the activity was lost after removal of the C-terminal 30 amino acids.

DNA is made of four deoxyribonucleoside triphosphates, provided by the de novo and salvage pathways. The key enzyme of the de novo pathway is ribonucleotide reductase, and the key salvage enzymes are the deoxyribonucleoside kinases, which phosphorylate deoxyribonucleosides to the corresponding deoxyribonucleoside monophosphates (1). Deoxyribonucleoside kinases from various organisms differ in their substrate specificity, regulation of gene expression, and cellular localization. In mammalian cells, four enzymes with overlapping specificities (thymidine kinases 1 (TK1) and 2 (TK2), deoxycytidine kinase (dCK), and deoxyguanosine kinase (dGK)) phosphorylate purine and pyrimidine deoxyribonucleosides. All four enzymes have been cloned and are encoded by nuclear genes (2–6). TK1 and TK2 are pyrimidine specific and phosphorylate deoxuryridine (dUrD) and thymidine (dTId), and TK2 also phosphorylates deoxycytidine (dCyD) (7). dCK phosphorylates dCyD, deoxyadenosine (dAdo), and deoxyguanosine (dGuo) but not dTId (8). dGK phosphorylates dGuo and dAdo (9). TK1 is cytosolic, and TK2 and dGK are localized in the mitochondria, although recent reports indicate a cytoplasmic localization of TK2 as well (10, 11).

In procaryotic cells, the pattern of deoxyribonucleoside kinases is not very well clarified. In Escherichia coli, there seems to be only one deoxyribonucleoside kinase, which has been characterized as a TK with similarity to the mammalian TK1 (12). The ability to incorporate dCyD, dAdo, and dGuo seems to be lacking (13). In Lactobacillus acidophilus, which is deficient in ribonucleotide reductase, the four deoxyribonucleosides are phosphorylated by three enzymes (13, 14). In addition to a TK resembling the E. coli TK, there are two kinase complexes that phosphorylate dCyD, dAdo, and dGuo. Complex I is a dGk/dAK complex, and complex II is a dGk/dAK complex (14). Several viruses carry a gene for a TK. Herpesviruses have a TK that also can phosphorylate dCyD, as well as TMP and dCMP (15). The herpetic kinases with the relatively broad substrate specificity have many features in common with the mammalian TK2, dCK, and dGK. Poxviruses code for a TK very similar to the mammalian TK1 (16). So far, however, none of the known viral, bacterial, or eucaryotic deoxyribonucleoside kinases has been shown to phosphorylate all four deoxyribonucleosides.

Recently, we have isolated a deoxyribonucleoside kinase from Drosophila melanogaster and named it D. melanogaster deoxyribonucleoside kinase (Dm-dNK) (17). The isolated kinase appears to possess the ability to phosphorylate all four deoxyribonucleosides. However, the presence of more than one kinase with the same size in our highly purified enzyme prep-

1 The abbreviations used are: TK, thymidine kinase; (d)Ado, (deoxy)adenosine; AraC, 1-β-D-arabinofuranosylcytosine; AraT, 1-β-D-arabinofuranosylthymine; (d)CyD, (deoxy)cytidine; BVDU, 5-bromovinyldeoxyuridine; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; dCK, deoxycytidine kinase; dCyD, 2′,3′-dideoxycytidine; dGK, deoxyguanosine kinase; Dm-dNK, D. melanogaster deoxyribonucleoside kinase; rDm-dNK, recombinant Dm-dNK; (d)Guo, (deoxy)guanosine; dTId, thymidine; (d)UrD, (deoxy)uridine; dAdo, dCyD, dGuo, and dTId, 2′,3′-dideoxyribonucleosides; GST, glutathione S-transferase; HSV, herpes simplex virus; PCR, polymerase chain reaction; ΔCX, C-terminal truncation by X amino acids; bp, base pair(s); AZT, 3′-azido-2′,3′-dideoxythymidine; CdA, 2′-chlorodeoxyadenosine.
C-terminal Deletion Activates Drosophila Multisubstrate dNK

D. Harvey, L. Hong, M. Evans-Holm, J. Pendleton, C. Su, P. Brokstein, S. Lewis, and G. M. Rubin, personal communication.

Expression of rDm-dNK and Its C-terminal Truncated Mutants—After incubation of LB medium containing 50 μg/ml ampicillin with a transformed colony, the bacteria were grown to an OD600 = 0.6, and then the expression of the GST fusion protein was induced with 100 μM isopropyl-β-D-thiogalactopyranoside for 4 h at 37 °C. The cells were harvested by centrifugation, and the pellet was resuspended in ice-cold Buffer A (20 mM Tris/HCl (pH 7.4, 22 °C), 5 mM MgCl2, 1 mM dithiothreitol, 8% glycerol, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 50 mM α-aminoacrylic acid), and homogenized using a French press. The homogenate was centrifuged 15 min at 12,000 × g at 4 °C, and the remaining cell debris was removed by passing successively through a 1 μm Whatman glass microfiber filter and a 0.45 μm cellulose acetate filter.

Purification of the Recombinant Enzymes—Filtered cell homogenate of induced BL21 transformants was applied to a glutathione-Sepharose column (15 × 45 mm) preequilibrated with Buffer A. Unbound material was removed by washing with 50 column volumes of Buffer A. Strongly bound contaminating proteins were removed by recirculating twice for 1 h each time at room temperature, each time with two column volumes of Buffer A containing 3 mM ATP and 10 mM MgCl2. The column matrix was then equilibrated with 10 column volumes of Buffer B (140 mM NaClp, 2.7 mM KCl, 10 mM NaHPO4, 1.5 mM KH2PO4, pH 7.3). The expressed protein was cleaved from glutathione S-transferase by recirculating the column matrix with 4 column volumes of Buffer B containing 400 units of thrombin. Finally, the remaining glutathione S-transferase and uncleaved fusion protein were eluted with 3 column volumes of 50 mM Tris/HCl (pH 7.5, 22 °C) containing 10 mM reduced glutathione. Before storage of enzyme-containing fractions at −80 °C, glycerol, Triton X-100, and dithiothreitol were added to 8%, 1% and 1 μl, respectively.

Enzyme Assays—Nucleoside kinase activities were determined by initial velocity measurements based on four time samples by the DE-81 filter paper assay using tritium-labeled substrates (17). Alternatively, the enzyme activity was followed by the ADP production measured by the change in absorbance at 340 nm, caused by NADH oxidation in a coupled enzyme system with pyruvate kinase and lactate dehydrogenase in the presence of propionic acid. Cysteine was measured as thiol by reaction with 5,5′-dithiobis(2-nitrobenzoic acid). Asparagine and glutamine were not included due to high instability.

RESULTS

Sequence of an EST Clone Containing the Putative cDNA for Dm-dNK—Deposited EST sequences belonging to D. melanogaster were deposited in the GenBank™ data base with the accession number Y18048. The largest open reading frame encodes a protein of 250 amino acids, with a calculated mass of 28,055 Da. This is the same mass as that determined for the native Dm-dNK (about 30 kDa) (17).

Sequence of the corresponding genomic DNA showed that the primary transcript is 1246 bp long, that it has a 130-bp

were deposited in the GenBank™ data base from an embryonic D. melanogaster library screened for homology to deoxyribonucleoside kinases. The Bluescript SK+ phagemid clone LD 15983-5′, GenBank™ accession number AA441228, contained a 1.1-kilobase EcoRI-XhoI cDNA insert that showed a high homology to human TK2 and other known kinases.

Sequence of the Dm-dNK cDNA and Genomic DNA—Sequenceing by the Sanger deoxyribonucleotide method was performed manually using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech). Partially sequenced cDNA clones in the GenBank™ data base from an embryonic D. melanogaster for Dm-dNK—Partially sequenced cDNA clones in the GenBank™ data base from an embryonic D. melanogaster for Dm-dNK

Construction of the Expression Vectors—The largest open reading frame was overexpressed in E. coli using the GST gene fusion expression system (Amersham Pharmacia Biotech). The insert for subcloning was obtained by PCR amplification. The first primer, Dm-TK3 (5′-GCGGATCCATGACGGGCAAGCATCTC-3′), was designed to contain a BamHI site overhang, just upstream from the putative translation start site. The second primer, Dm-TK4 (5′-CGGGAATTTCTATGCTCG-3′), was designed to contain an EcoRI site overhang, just downstream from the putative translation stop site. C-terminal deletions (minus 10, 20, or 30 amino acids) were made using the primers Dm-TK4B (5′-ATACGGAATTCGAGGCCTGCAGGAGAATGCTGTGCA-3′). Dm-TK4C (5′-ATACGGAATTCGAGGCCTGCAGGAGAATGCTGTGCA-3′), and Dm-TK4D (5′-ATACGGAATTCGAGGCCTGCAGGAGAATGCTGTGCA-3′). Dm-TK4C (5′-ATACGGAATTCGAGGCCTGCAGGAGAATGCTGTGCA-3′), and Dm-TK4D (5′-ATACGGAATTCGAGGCCTGCAGGAGAATGCTGTGCA-3′) in combination with Dm-TK3. The PCR fragments were cut with BamHI and EcoRI and subcloned into the multiple cloning site of the pGEX-2T plasmid vector, allowing expression of the protein encoded by the open reading frame fused to the glutathione S-transferase (GST) gene. A specific thrombin cleavage site was engineered just after the GST gene. The sequences in the expression inserts were verified by sequencing. The E. coli strains BL21 was transformed with the expression plasmid vectors by the CaCl2 method. The expressed recombinant proteins were named rDm-dNK, rDm-dNKAC10, rDm-dNKΔC20, and rDm-dNKΔC30.

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Pairwise and Multiple Sequence Alignments—Pairwise sequence alignments were performed using the Smith-Waterman algorithm (20, 21), and multiple alignments were performed with PILEUP and CLUSTAL W (22).

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nontranslated 5’ end, and that the translated region is interrupted by two introns, one 75 bp long and another of 56 bp, located 331 and 679 bp downstream of the translation start, respectively. The sequence has the GenBank® accession number AF185268.

Amino Acid Composition of the Native Enzyme—The amino acid composition of the native Dm-dNK was determined and found to be very similar to the amino acid sequence predicted from the longest open reading frame (data not shown). As the similarity between the amino acid composition of the cloned and native protein was high, it was very likely that the cloned sequence represented the cDNA for Dm-dNK. For further confirmation, the largest open reading frame was expressed, and the recombinant putative kinase was purified and analyzed.

Expression and Purification of rDm-dNK—The longest open reading frame was overexpressed as a fusion protein with GST of about 56 kDa. TK activity reached maximum after about 5–7 h of induction. The recombinant protein was purified in one step to nearly homogeneity by glutathione-Sepharose affinity chromatography combined with in situ thrombin cleavage. The procedure was followed by SDS-gel electrophoresis (Fig. 1) and measurements of the TK activity. During the initial attempts to purify the cleaved protein, several high molecular mass proteins with subunit sizes about 60–70 kDa copurified consistently with the recombinant kinase. From the size of the bands and the firm association with the expressed protein, the contaminating proteins may be heat shock response proteins expressed by the E. coli host as a defense against abnormal proteins. Several heat shock proteins have been shown to release their target protein in the presence of ATP (29). Therefore, an additional step in the glutathione-Sepharose chromatography procedure with in situ incubation with ATP/MgCl₂ was added before the thrombin cleavage step. As can be seen from Fig. 1, the contaminating proteins were almost completely removed by two consecutive ATP/MgCl₂ incubations (fractions III and IV). The cleavage fraction (V), in which the TK activity was present, contained the expressed and cleaved recombinant protein, seen as a strong band of about 29 kDa. Remaining uncleaved fusion protein (56 kDa) was eluted by reduced glutathione together with glutathione S-transferase (27 kDa) in fraction VI. The results of the purification of rDm-dNK are summarized in Table I.

Kinetic Analysis of rDm-dNK—The kinetic relation of the rDm-dNK between the velocity and the substrate concentration was determined for the four deoxynucleosides. The results are shown in Fig. 2 as double reciprocal plots. v is the initial velocity (in µmol/min/mg) determined as described under “Experimental Procedures,” and s is the substrate concentration (in µM). Inset, the relationship between v and s.

| Table I Purification tables (measured with dThd) for rDm-dNK and the C-terminal truncated mutants with data for the crude bacterial extracts and the cleavage fractions. |
|-----------------|-----------------|-------------------|---------|---------|---------|
| Purification    | Total activity  | Total protein     | Specific activity | Yield   | % Fold  |
| rDm-dNK         | units          | mg                 | units/mg          |         |         |
| Crude extracts  | 333.0          | 601.0              | 0.56              | 100.0   | 1.0     |
| Pooled fractions| 61.0           | 3.6                | 17.9              | 18.3    | 32.0    |
| Peak fraction   | 9.5            | 0.26               | 36.5              | 2.9     | 65.0    |
| rDm-dNKΔC10     |                |                    |                   |         |         |
| Crude extracts  | 217.0          | 442.0              | 0.5               | 100.0   | 1.0     |
| Pooled fractions| 25.0           | 1.3                | 19.3              | 11.5    | 39.0    |
| Peak fraction   | 12.7           | 0.53               | 23.8              | 5.8     | 48.0    |
| rDm-dNKΔC20     |                |                    |                   |         |         |
| Crude extracts  | 1952.0         | 1390.0             | 1.4               | 100.0   | 1.0     |
| Pooled fractions| 1085.0         | 24.8               | 43.8              | 55.6    | 31.0    |
| Peak fraction   | 306.0          | 6.9                | 51.6              | 18.2    | 37.0    |
| rDm-dNKΔC30     |                |                    |                   |         |         |
| Crude extracts  | 4.0            | 356.0              | 0.01              | 100.0   | 1.0     |
| Pooled fractions| 0.12           | 0.42               | 0.29              | 3.0     | 29.0    |
| Peak fraction   | 0.09           | 0.26               | 0.35              | 2.2     | 35.0    |
concentrations of the phosphate donor and MgCl₂ were 29.4 units/mg with ATP and 28.1 units/mg with CTP. However, ATP exhibited substrate inhibition at concentrations above 0.1 mM with the native as well as the recombinant kinase, whereas no inhibition was observed with CTP (data not shown). These results proved that in D. melanogaster, a single enzyme has the ability to phosphorylate all four deoxyribonucleosides.

Sequence Analysis and Comparisons—From pairwise alignments using the Smith-Waterman algorithm (20, 21) Dm-dNK was found to have high identity with human TK2 (44%), human dGK (34%), and human dCK (31%). This indicates that Dm-dNK, together with TK2, dCK, and dGK belongs to the so-called “herpetic family” of deoxyribonucleoside kinases (24, 25). A multiple alignment of the amino acid sequences of Dm-dNK, human TK2, human dGK, human dCK and HSV1-TK is shown in Fig. 3. Dm-dNK contains five out of the six sites of strong sequence homology previously identified for most herpesviral TKs (24, 25). The crystal structure of HSV1-TK in complex with its substrates has been solved (26–28). As can be seen from the alignment in Fig. 3 nearly all of the specific residues closest to the bound nucleoside in the HSV1-TK structure, i.e. Glu-83, Trp-88, Tyr-101, Gln-125, Arg-163, Tyr-172 and Glu-225 (HSV1-TK numbering), are conserved in Dm-dNK. These amino acids were also reported to be conserved in human TK2 (29).

When the Dm-dNK amino acid sequence was examined by the signal prediction method for cellular transport signals (30), several motifs were found. The 15 N-terminal amino acids may function as a signal for import into mitochondria. However, so far, proteolytic processing of the native Dm-dNK N terminus could not be shown, because the N terminus apparently was blocked (data not shown). Two patches of amino acids within the open reading frame match classical nuclear signaling motifs and may be potential signals for nuclear import (31). The first, PTNKKLK, starts at residue 95, and the second, PSKRQVR, starts at residue 242. Additionally, a putative serine phosphorylation site, similar to the one found in human TK1 (32), was found in the C-terminal region at residue 241, SPSK (matching a cdc2 consensus recognition site) and overlapping with the second nuclear import signal. Phosphorylation of such consensus sites has been shown for lamin from Drosophila embryos, also influencing nuclear import (33). As can be seen from Fig. 3, the very C-terminal part of Dm-dNK shows no similarity and is extended compared with the mammalian kinases. Therefore, we decided to examine the role of the C terminus of Dm-dNK for substrate specificity and catalysis by engineering three C-terminal truncated mutants of rDm-dNK (deletion of the C-terminal 10, 20, or 30 amino acids).

Purification and Characterization of the C-terminal Truncated Enzymes—All three deletion mutants were expressed, cleaved, and purified as described for rDm-dNK. The purity of the preparations was compared by SDS-polyacrylamide gel electrophoresis (not shown) and judged to be the same as obtained with rDm-dNK. Comparison of the specific activities TABLE II Kinetic parameters of native Dm-dNK and rDm-dNK

| Substrate | Native Dm-dNK (data from Ref. 17) | rDm-dNK |
|-----------|---------------------------------|--------|
|           | Kₘ (μM) | Vₘax (units/mg) | Kₘ (μM) | Vₘax (units/mg) |
| dThd      | 0.9     | 29.4             | 29.9    | 1.2              |
| dCyd      | 1       | 28.7             | 28.7    | 1.2              |
| dAdo      | 109     | 35.5             | 225     | 34.2             |
| dGuo      | 654     | 37.7             | 665     | 13.1             |

Fig. 3. Multiple alignment of the predicted amino acid sequences of Dm-dNK, human TK2, human dGK, human dCK, and HSV1-TK. The site numbers are those used for the conserved functional sequences in herpesviral TKs (24) The consensus sequences referred to in the text are underlined. The TK2, dCK, dGK, and HSV1TK amino acid sequences were obtained from the SwissProt data base.
(Table I) shows that rDm-dNKΔC30 had about 1% of the specific activity of rDm-dNK. The specific activity of rDm-dNKΔC10 was in the same range as the specific activity of rDm-dNK. Surprisingly, the specific activity of rDm-dNKΔC20 was nearly twice as high as the specific activity of rDm-dNK.

Furthermore, as calculated from the specific activities of the enzymes in Table I, the expression of rDm-dNKΔC20 per liter of bacterial culture (38 mg) was more than 4-fold higher than that of Dm-dNK (9 mg). The Km values for thymidine were 1.7, 1.7, and 2.5 μM for rDm-dNKΔC10, rDm-dNKΔC20, and rDm-dNK respectively, and thus very similar to the Km value (1.2 μM) for rDm-dNK.

Finally, the substrate specificity of rDm-dNK and the two active deletion mutants rDm-dNKΔC10 and rDm-dNKΔC20 was determined (Table III). To enable measurement of the phosphorylation of unlabeled substrates, a modified version of the nonradioactive spectrophotometric assay of Valentin-Hansen (18) was used. rDm-dNK showed a clear preference for dideoxynucleosides, but also, all ribonucleosides were accepted as substrates, as much as 16% with cytidine as compared with thymidine. ddGuo was found not to be a substrate under our assay conditions, whereas all other dideoxynucleosides were accepted. Pyrimidine nucleosides were generally preferred over purine nucleosides.

Also several nucleoside analogs used or considered for use in therapy of cancer and viral infections were tested (Table III). BVVDU was a very good substrate. Acyclic guanosine was not accepted, and AZT and 3' deoxyadenosine were accepted, but to a low degree. Both arabinose pyrimidine-nucleosides, AraC and AraT, and CdA were good substrates for rDm-dNK. Much the same pattern was found with the truncated enzymes. It is noticeable that the relative phosphorylation of dCyd and CdA increases with the increasing C-terminal truncation.

As compared with rDm-dNK, the relative activities of rDm-dNKΔC10 and rDm-dNKΔC20 with dideoxynucleosides remained largely unchanged, whereas there was a substantial decrease in the phosphorylation of the purine ribonucleosides adenosine and guanosine, as well as of all dideoxynucleosides and AZT (Table III). The relative activities with the pyrimidine ribonucleosides and AraC and AraT were not affected by the C-terminal deletions.

### DISCUSSION

The results presented here establish the existence of a dideoxynucleoside kinase in D. melanogaster, associated to one polypeptide chain and with the ability to phosphorylate both purine and pyrimidine dideoxynucleosides with higher turnover than other known viral, bacterial, and mammalian kinases (Table IV). Recently, another group has independently reported that the same cDNA clone, AA441228, apparently codes for Dm-dNK (34). When the open reading frame was expressed in E. coli, the uncleaved GST fusion protein phosphorylated the four dideoxynucleosides but with 5–25-fold lower specificity constants as compared with both the native enzyme (17) and the recombinant enzyme presented in this paper.

Black and Hruby (35) suggested in 1990 to divide the thymidine-deoxypyrimidine kinases into two general classes on the basis of size, quaternary structure, substrate specificity, and feedback sensitivity. Class I kinases are dimeric enzymes phosphorylating both dThd and dCyd, and they are relatively insensitive to feedback inhibition with TTP. Class II kinases are tetramers of subunits of 20–25 kDa with a strict substrate specificity for dThd. HSV1 TK was proposed as the prototype for class I, and the term “herpetic kinase family” was suggested for this group. For class II, vaccinia virus TK was proposed as the prototype, with E. coli TK and the cytosolic mammalian TK1 as members.

The discovery of a new member of the dideoxynucleoside kinase family with a broader substrate specificity than the other members may justify a minor change of this classification. The multisubstrate character of Dm-dNK may be interpreted as if the enzyme represents a progenitor of dideoxynucleoside kinases. According to the three criteria for a “primitive” enzyme, proposed by Jensen (36), the original primitive enzyme would be encoded by a small genome, the substrate specificity would be relatively broad, and the enzyme would display simple kinetics. Dm-dNK is a relatively small, monomeric enzyme with a broader substrate specificity than any of the other dideoxynucleoside kinases (Table IV). The kinetics is straightforward Michaelis-Menten with both phosphate acceptors and donors, and in this respect, Dm-dNK is more primitive than dCK and TK1 and TK2 with their complex

### TABLE III

Substrate specificities of rDm-dNK and its C-terminal truncated variants

The activities were measured with the coupled spectrophotometric assay described under “Experimental Procedures.” The specific activity of each enzyme with 500 μM thymidine was set to 100. Relative activities from 3–5 measurements ± S.D. are given. ND, not detectable.

| Substrate (500 μM) | rDm-dNK | rDm-dNKΔC10 | rDm-dNKΔC20 |
|-------------------|---------|-------------|-------------|
| dThd              | 100     | 100         | 100         |
| dCyd              | 134 ± 10| 140 ± 7     | 144 ± 4     |
| dUrd              | 115 ± 2 | 123 ± 9     | 130 ± 4     |
| dAdo              | 96 ± 7  | 101 ± 3     | 109 ± 1     |
| dGuo              | 11 ± 1  | 11 ± 1      | 11 ± 1      |
| dFdUrd            | 92 ± 1  | 84 ± 3      | 91 ± 6      |
| Clyd              | 16 ± 2  | 17 ± 0.15   | 16 ± 0.19   |
| Urd               | 6.45 ± 0.5 | 6.90 ± 0.50 | 6.80 ± 0.20 |
| Ado               | 1.00 ± 0.26 | 0.41 ± 0.26 | ND          |
| Guo               | 1.18 ± 0.22 | 0.33 ± 0.26 | ND          |
| ddThd             | 4.44 ± 0.44 | 3.10 ± 0.24 | 3.10 ± 0.34 |
| ddCyd             | 11.1 ± 1.0 | 9.70 ± 0.52 | 8.80 ± 0.48 |
| ddAdo             | 0.90 ± 0.25 | 0.28 ± 0.12 | ND          |
| dGuo              | ND      | ND          | ND          |
| AraC              | 58.2 ± 5.6 | 54.4 ± 1.5  | 57.7 ± 2.1  |
| AraT              | 53.9 ± 5.4 | 49.4 ± 0.5  | 49.1 ± 1.6  |
| AZT               | 2.0 ± 1.0 | 1.30 ± 0.32 | 0.93 ± 0.54 |
| Acyclic guanosine | ND      | ND          | ND          |
| 3'-dAdo           | 1.9 ± 0.6 | 2.0 ± 0.9   | 1.9 ± 4.0   |
| CdA               | 126 ± 4 | 131 ± 8     | 149 ± 4     |
| BVDU              | 54 ± 4 | 57 ± 5      | 49.6 ± 0.6  |

### TABLE IV

Catalytic efficiencies kcat/Km (μM−1 s−1) for dideoxynucleoside kinases

Turnover kcat (s−1) values (in parentheses) are calculated assuming one active site per enzyme monomer.

|                     | dThd | dCyd | dAdo | dGuo |
|---------------------|------|------|------|------|
| rDm-dNK             | 1.2 × 10^7 (14.2) | 7.2 × 10^6 (16.5) | 9.2 × 10^6 (20.6) | 2.3 × 10^6 (15.1) |
| Dm-dNK (Ref. 17)    | 1.7 × 10^7 (15) | 1.4 × 10^7 (15) | 1.6 × 10^7 (17.8) | 2.9 × 10^6 (18.2) |
| hTK1 (Ref. 7)       | 8.0 × 10^6 (4.0) | 1.1 × 10^4 (0.4) | 2.6 × 10^3 (0.3) | 2.7 × 10^3 (0.4) |
| hTK2 (Ref. 7)       | 1.9 × 10^6 (0.3) | 1.1 × 10^4 (0.4) | 2.6 × 10^3 (0.3) | 2.7 × 10^3 (0.4) |
| hdCK (Ref. 8)       | 7.3 × 10^4 (0.07) | 2.6 × 10^3 (0.3) | 2.6 × 10^3 (0.3) | 2.7 × 10^3 (0.4) |
| hdGK (Ref. 9)       | 2.3 × 10^3 (0.001) | 2.3 × 10^3 (0.002) | 2.8 × 10^3 (0.002) | 2.8 × 10^3 (0.002) |
| HSV1-TK (Ref. 41)   | 3.8 × 10^6 (0.21) | 1.8 × 10^6 (1.6) | 1.8 × 10^6 (1.6) | 1.8 × 10^6 (1.6) |
| E. coli TK (Ref. 42) |      |      |      |      |
cooperative kinetics (7, 8, 37). In mammals, the progenitor enzyme with simple kinetics and broad specificity may have evolved into several more specialized and tightly regulated enzymes. Eventually, HSV TKs may have evolved from the eucaryotic kinase, dCK (38). Apparently, Dm-dNK has retained the main characteristics of the eucaryotic progenitor enzyme. However, the possibility cannot be ruled out that the multistructure character of Dm-dNK could be a consequence of evolutionary pressure to reduce the number of deoxyribonucleoside kinases, resulting in a single enzyme.

We propose that class I would comprise deoxyribonucleoside kinases with a size of about 30–40 kDa, monomers or dimers, with the ability to phosphorylate more than one of the deoxyribonucleosides. Because there are several nonheteroptic members of the class I kinases, with Dm-dNK probably representing the “oldest” member, we suggest Dm-dNK as the prototype for the class I deoxyribonucleoside kinases.

Mammalian deoxyribonucleoside kinases are found in different compartments of the cell (10, 11). For Dm-dNK, both putative mitochondrial and nuclear import signals were predicted. The simultaneous presence of mitochondrial and nuclear import signals has until now been postulated for any deoxyribonucleoside kinase and may be unique for Dm-dNK. Experiments to confirm this view are in progress.

An explanation at the molecular level for the multistructure character of Dm-dNK awaits crystallization and three-dimensional structural determination. However, the residues reported to be interacting with the nucleoside in the active site of the herpesvirus TK can nearly all be identified in Dm-dNK and the other class I kinases, as well as five out of the six conserved sites in herpesviral TKs (Fig. 3). When binding dThd to HSV1-TK, a void of 35 Å³ is left close to the C5 position of the nucleoside (20). When placing BVDU instead of dThd in the substrate character of the main characteristics of the eucaryotic progenitor enzyme.

-dNK, whereas the larger aspartate was present in dGK (Fig. 3). Here, alanine was present in TK2 and this enzyme, because the exchange to a larger side-chain disrupts the binding pocket. We have speculated further on this model when comparing the residues aligning with site 168 in HSV1-TK (see Fig. 3). Here, alanine was present in TK2 and Dm-dNK, whereas the larger aspartate was present in dGK and dCK. As predicted from the model, Dm-dNK and TK2 should show efficient phosphorylation of BVDU, whereas dCK and dGK should have no activity with this analogue. This is indeed the case. TK2 shows 20% activity with BVDU as compared with thymidine (39), and Dm-dNK shows 54%. dCK shows only 2% activity (39), and dGK is a purine deoxyribonucleoside kinase (9). These observations indicate that the structure of HSV1-TK can be used for modeling the other class I kinases.

When the amino acid sequence of Dm-dNK was aligned to other deoxyribonucleoside kinases, homologous amino acid patches were found to be distributed over the entire sequence, except the C-terminal part (Fig. 3). Apparently, the last 40 amino acids of Dm-dNK seemed to be unique. Therefore, the role of the C-terminal part of the enzyme was investigated in detail by construction and expression of deletion mutants. The very C-terminal part of the enzyme, the terminal 10 amino acids, harboring a putative phosphorylation and nuclear localization signal, did not influence catalytic efficiency for thymidine but reduced the phosphorylation of purine-dideoxyribonucleosides. The terminal truncation of 20 amino acids changed the thymidine turnover unexpectedly toward higher rates. This suggests that the C-terminal domain of Dm-dNK has an inhibitory effect on phosphorylation of deoxyribonucleosides, although on the other hand promoting phosphorylation of other substrates, such as purine ribo- and dideoxyribonucleosides. The terminal 20 amino acids seemed to be essential for phosphorylation of purine ribonucleosides and dideoxyadenosine. Thus, this deletion reduced the broad substrate specificity (Table III). Deletion of 30 amino acids at the C terminus decreased the activity to about 1% of rDm-dNK, indicating the loss of a domain essential for catalysis. Up-regulation of a TK activity parallel to the activity of polymerase α during proliferation has been reported for a D. melanogaster cell line (40). It is speculative whether the C terminus with the putative phosphorylation site is involved in this up-regulation, but our data clearly show the ability of this region to suppress deoxyribonucleoside phosphorylation, while providing broader substrate specificity. This finding may point to a different regulation of Dm-dNK compared with the other kinases, which are regulated by oligomerization, substrate concentrations, and subcellular localization (7, 8, 10, 11, 37). Studies to clarify possible regulation mechanisms of Dm-dNK are currently being initiated, as are studies to establish the importance of Dm-dNK for D. melanogaster development.

The broad substrate specificity and high catalytic efficiency (Table IV) compared with other kinases makes Dm-dNK, and especially rDm-dNKΔC20, with its even higher catalytic rates for deoxyribonucleosides, attractive for a number of applications. At the moment, ddNTPs used for sequencing and dNTPs used for PCRs are produced by chemical synthesis with toxic chemicals, leading to a number of unwanted byproducts. Efficient enzymatic synthesis of the monophosphates from (di)deoxyribonucleosides would be a key step that would reduce the number of byproducts and avoid the use of toxic chemicals. rDm-dNK, with its broad substrate specificity and high turnover, would be an obvious candidate for this task.

The prototype of genetic pharmacomodulation is the transduction of tumor cells with the gene encoding HSV1-TK and subsequent chemotherapy with nucleoside analogs. The unique kinetic properties of Dm-dNK makes it, therefore, an excellent candidate for use as a suicide gene in gene therapy of cancer or viral infections. Because the initial phosphorylation of a nucleoside analog is often the rate-limiting step in activation of the nucleoside analogs and thereby for the efficiency of the genetically modulated pharmacotherapy, the high catalytic rates compared with the cellular kinases and HSV1-TK (Table IV) would be advantageous.

Acknowledgments—The skillful and dedicated technical assistance of Anna Elisa Egholm is gratefully acknowledged. We are indebted to Liya Wang, Staffan Eriksson, and Hans-Georg Ihlenfeldt for valuable suggestions and to Susanne Jacobsen and Vibeke Barkholt for performing the amino acid analysis.

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*J. Biol. Chem.* 2000, 275:6673-6679.
doi: 10.1074/jbc.275.9.6673

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