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Purification and Biochemical Characterization of Deoxythymidine Kinase of Deoxythymidine Kinase-Deficient Mouse 3T3 Cells Biochemically Transformed by Equine Herpesvirus Type 1

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A line of mouse 3T3 cells lacking deoxythymidine kinase (dTK$^-$) was stably transformed to the dTK$^+$ phenotype after exposure to ultraviolet-irradiated equine herpesvirus type 1 (EHV-1). Deoxythymidine kinase (dTK) was purified from the biochemically transformed mouse cells by affinity chromatography on deoxythymidine-Sepharose. The purified dTK from EHV-1-transformed 3T3 cells was identical to the dTK purified from dTK$^-$ 3T3 cells lytically infected with EHV-1 with respect to its electrophoretic mobility, molecular weight, substrate specificity, phosphate donor specificity, and immunological specificity. The sedimentation velocity of the purified dTK from the transformed 3T3 cells was similar to that previously reported for the enzyme in lytically infected dTK$^-$ 3T3 cells, and its molecular weight was estimated to be 87,000. Antiserum prepared against the EHV-1 dTK induced in horse cells inactivated the dTK purified from the transformed mouse cells. The K_m for deoxythymidine (5 μM) of purified dTK from the EHV-1-transformed cells was the same as that reported for the EHV-1-induced dTK. These results further support the notion that the dTK acquired by dTK$^-$ mouse 3T3 cells after transformation by EHV-1 is of viral and not of cellular origin.

Recent studies from this laboratory have demonstrated that mouse 3T3 cells lacking deoxythymidine kinase (dTK; dTK$^-$ cells), lytically infected with equine herpesvirus type 1 (EHV-1), acquire a new dTK activity which is easily distinguished from cellular dTK's by biochemical, immunological, and chromatographic criteria (1, 3). We have also shown that dTK$^-$ 3T3 cells are susceptible to biochemical transformation by ultraviolet light-inactivated EHV-1 (2), thus demonstrating that biochemical transformation can be achieved with a herpesvirus other than herpes simplex virus. Other studies have demonstrated biochemical transformation of cells to the dTK$^+$ phenotype by herpes simplex virus types 1 and 2 using dTK$^-$ mouse (3T3, LM) and human (HeLa) cells (5, 6, 9, 11, 22-24). These virus (herpes simplex types 1 and 2 and EHV-1)-transformed cell lines possess virus-specific antigens (2, 4, 26, 29) and express a dTK activity with immunological, electrophoretic, and biochemical properties similar to those of the dTK induced by herpesviruses during a productive infection (2, 5-7, 9, 10, 13, 16, 23). The herpes simplex virus-transformed cell lines also contain herpes simplex virus-specific deoxyribonucleic and ribonucleic acids (8, 13, 16, 28). These data indicate that the dTK acquired by the transformed cell is specified by the herpesvirus genome.

We have recently used the affinity column chromatography procedures developed by Kowal and Markus (15) to partially purify and identify the new dTK induced in dTK$^-$ 3T3 cells lytically infected with EHV-1 (3). Using a modification of this chromatographic procedure of Kowal and Markus (15) as described by Lee and Cheng (17), we have now purified the dTK present in EHV-1 biochemically transformed mouse dTK$^-$ 3T3 cells. In this communication we report the physical, biochemical, and immunological properties of this purified transformed cell kinase and show it to be identical to the dTK purified from dTK$^-$ 3T3 cells lytically infected with EHV-1.

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MATERIALS AND METHODS

Media and cell cultures. dTK$^-$ 3T3 cells from random-bred Swiss mice (21) were obtained from H.

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Green, Massachusetts Institute of Technology, Cambridge, Mass., and were grown in Eagle minimal essential medium supplemented with 5% fetal bovine serum (Flow Laboratories, Rockville, Md.). The dTK$^-$ 3T3 cells transformed by EHV-1 were isolated in Eagle minimal essential medium containing 10^{-4} M hypoxanthine, 6×10^{-7} M aminopterin, and 2×10^{-5} M thymidine (HAT medium) and were then grown in HAT medium (to maintain selective pressure) for more than 60 passages. Further characterization of these biochemical transformants has been previously reported (1).

Extraction and assay of dTK. Cytosol fractions of cells were prepared by the method of Cheng and Ostrander (6) and were assayed for dTK activity as described by Lee and Cheng (17). Electrophoretic migration of dTK in polyacrylamide gels was determined by the procedure of Lee and Cheng (17).

Antibody inhibition assays. Antiserum to EHV-1 dTK induced in lytically infected KyED cells was prepared as previously reported (1–3). Inhibition of dTK by antibody was performed by incubation of the enzyme for 12 h at 2°C with dilutions of the immune serum, using preimmune serum as the diluent. A 40-μl portion of each enzyme antisera mixture was then assayed for dTK activity as described above.

Glycerol gradient centrifugation. The molecular weight of the dTK purified from EHV-1-transformed dTK$^-$ 3T3 cells was estimated by centrifugation in glycerol gradients using the method of Martin and Ames (20). A 0.1-ml sample of purified enzyme was layered onto a 4.75-ml, 15 to 30% (vol/vol) glycerol gradient prepared in a buffer containing 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5), 2.4 mM dithiothreitol, and 50 μM deoxythymidine (dT). Samples of lactate dehydrogenase and hemoglobin were added to the same gradient as molecular weight markers. The gradients were centrifuged for 36 h at 40,000 rpm at 4°C in an SW50.1 rotor (Beckman Instruments, Palo Alto, Calif.). Fractions (0.1 ml) were collected and assayed for dTK activity as described. Lactate dehydrogenase activity was assayed by the method of Reeves and Fimognari (27), and hemoglobin was located by its absorption at 555 nm.

Preparation of affinity gel for dTK. The procedures were essentially the same as those described by Kowal and Marcus (15) with the alterations of Lee and Cheng (17). Briefly, 120 mg of *p*-nitrophenylthymidine 3′-monophosphate was passed through a Dowex 50 column (H^+ 200–400 mesh, 3 by 8 cm) with water as the eluting solvent. The activated compound was reduced to *p*-aminophenylthymidine 3′-monophosphate under 1.5 atm of H_2 in the presence of palladium on carbon. The unreacted compound was eluted from the reduced compound by passage through a Dowex 50 column (H^+, 200–400 mesh, 3 by 13 cm). The reduced compound was lyophilized and linked to activated CH-Sepharose 4B, and unreacting ester groups were blocked as described by the manufacturer (25).

Protein determination. Protein was determined by the method of Lowry et al. (19) with bovine serum albumin as a standard.

Reagents. Radioisotopes were purchased from Schwarz/Mann, Orangeburg, N.Y. All unlabeled nucleosides and nucleoside triphosphates were obtained from Calbiochem, La Jolla, Calif. Arabinosylthymine was kindly provided by Jack Fox, Sloan-Kettering Cancer Research Institute. Phosphocreatine, creatine phosphokinase, hemoglobin, lactate dehydrogenase, thymidine 3′-monophospho-*p*-nitrophenyl ester, activated CH-Sepharose 4B, and bovine serum albumin were all obtained from Sigma Chemical Co., St. Louis, Mo. Dowex 50 was obtained from BioRad Laboratories, Richmond, Calif.

RESULTS

Purification of dTK from EHV-1-transformed 3T3 cells. Previously it was shown that dTK$^-$ 3T3 cells transformed by EHV-1 possess a new dTK activity that migrates in polyacrylamide gels with the same mobility at the cytosol enzyme from dTK$^+$ 3T3 cells, but distinguishable from the cellular dTK by its sensitivity to anti-(EHV-1) serum. To substantiate the viral origin of this new dTK, the enzyme was purified from the EHV-transformed dTK$^-$ 3T3 cells by affinity chromatography on dT-Sepharose and compared with the partially purified enzyme from lytically infected dTK$^-$ 3T3 cells (3, 5, 7). A typical elution profile for the EHV-1-transformed cell dTK is presented in Fig. 1. The concentrations of dT, tris(hydroxymethyl)-aminomethane-hydrochloride, and KCl required to elute the transformed cell dTK from the affinity column (0.45 M, 300 μM, and 0.8 M, respectively) were different from those required for column elution of the cytosol dTK from dTK$^+$ 3T3 cells but the same as those required

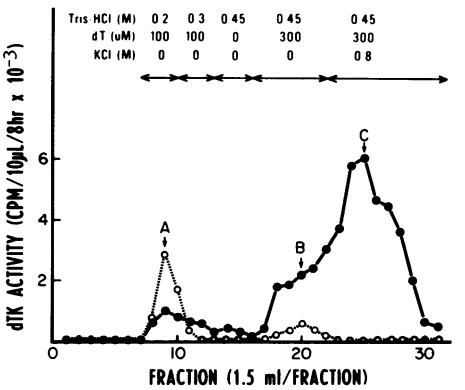

FIG. 1. *dT-Sepharose affinity chromatography of dTK from control dTK$^+$ 3T3 cells* (○- - -○) *or from EHV-1-transformed 3T3 cells* (●——●). *Cell extracts were loaded onto the affinity columns, which were then eluted in a stepwise fashion with buffers containing the indicated concentrations of tris-(hydroxymethyl)aminomethane-hydrochloride, dT, and KCl. Fractions of 1.5 ml were collected and assayed for dTK activity. Arrows mark the elution positions of dTK$^+$ 3T3 cytosol dTK (A), 3T3 mitochondrial dTK (B), and EHV-1 dTK (C) (3).*

for the dTK from dTK⁻ 3T3 cells lytically infected with EHV-1 (3). The partially purified dTK from activity peak C (see Fig. 1) was used in all subsequent studies in characterization of the enzyme. Enzyme present in this peak of activity represented a 79-fold purification with an overall recovery of 11% of the enzyme activity (Table 1).

Polyacrylamide gel electrophoresis. Figure 2 shows that when crude and purified dTK's were electrophoresed as described in Materials and Methods, the electrophoretic mobility (R_f) of the crude EHV-1 enzyme was 0.25, whereas that of the purified enzyme was 0.21. This is in agreement with the values found for the EHV-1 dTK in crude extracts of EHV-1-infected dTK⁻ 3T3 cells (3) and that previously reported for the dTK from crude extracts of EHV-1-transformed 3T3 cells (1).

Glycerol gradient centrifugation. The sedimentation velocity of the purified dTK from the transformed 3T3 cells was determined by centrifugation in 15 to 30% (vol/vol) glycerol gradients with the internal marker proteins hemoglobin and lactate dehydrogenase (Fig. 3). The sedimentation rate of the EHV-1-transformed 3T3 cell dTK (5.2S) was similar to that previously reported for the lytic enzyme (3) and results in a computed molecular weight of 87,000. This value is in agreement with value of 85,000 reported by Allen et al. (3) for the dTK induced in dTK⁻ 3T3 cells lytically infected with EHV-1.

Nucleoside triphosphates as phosphate donors for dTK activity. Table 2 shows that adenosine 5'-triphosphate and cytidine 5'-triphosphate acted as the best phosphate donors for the affinity chromatography-purified dTK from the EHV-1-transformed 3T3 cells. It can be seen that these data are in agreement with those obtained using purified dTK from dTK⁻ 3T3 cells lytically infected with EHV-1 (3). The other nucleoside triphosphates could also serve as phosphate donors for the transformed cell dTK but to a lesser degree. Deoxythymidine triphosphate inhibited the dTK reaction (data not shown).

TABLE 1. *Purification of EHV-1 dTK from transformed 3T3 cells*

Purification step	Total units[a]	% Recovery	Sp act (units/mg of protein)	Purification (fold)
Cell extract	3.4		0.038	
Ammonium sulfate	2.8	82	0.058	1.5
Peak C, affinity chromatography	0.36	11	3.0	79.0

[a] 1 unit of activity is defined as the conversion of dT to deoxythymidine monophosphate at a rate of 1 nM/min at 37°C.

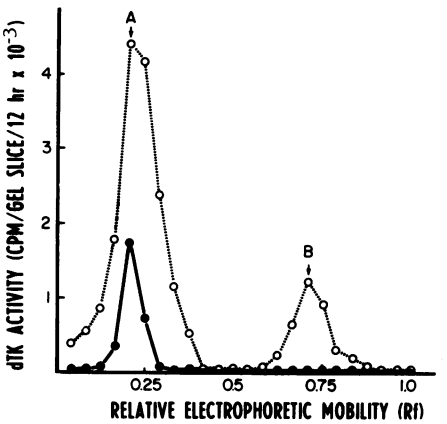

FIG. 2. *Polyacrylamide gel electrophoresis of the dTK purified by dT-Sepharose affinity chromatography from EHV-1-transformed 3T3 cells (●——●) or the dTK from crude cytoplasmic extracts of EHV-1-transformed 3T3 cells (O- - -O). Arrows mark the R_f's of EHV-1 dTK (A) and 3T3 mitochondrial dTK (B) (3).*

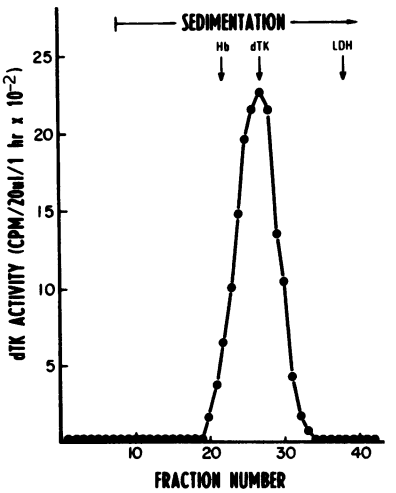

FIG. 3. *Glycerol gradient centrifugation of dT-Sepharose-purified, EHV-1-transformed 3T3 cell dTK. Hemoglobin (Hb; 68,000) and lactate dehydrogenase (LDH; 140,000) were used as molecular weight markers. The conditions for centrifugation and fractionation have been described.*

K_m of the EHV-1-transformed 3T3 cell dTK. A Lineweaver-Burk plot of initial reaction velocities versus the concentrations of dT substrate was constructed from reactions catalyzed by purified dTK from the EHV-1-transformed cells. The results (Fig. 4) indicate that the K_m of the transformed dTK for dT was 5 μM, which

TABLE 2. *Activity of various nucleoside triphosphates as phosphate donors in the dTK reaction*[a]

Nucleoside triphosphate[b]	dTK activity (percent of control)	
	EHV-1 dTK[c]	EHV-1-transformed 3T3 dTK
ATP	100	100
CTP	100	114
UTP	94	74
GTP	49	58
dATP	82	89
dCTP	89	91
dGTP	65	54

[a] The assays were performed under the conditions described in the text, except that adenosine 5'-triphosphate was replaced by the indicated nucleoside triphosphate (2 mM). The results are expressed as percent of ^{14}C-labeled deoxythymidine monophosphate formed when adenosine 5'-triphosphate was used.

[b] ATP, Adenosine 5'-triphosphate; CTP, cytidine 5'-triphosphate; UTP, uridine 5'-triphosphate; GTP, guanosine 5'-triphosphate; dATP, dCTP, dGTP, deoxy-forms of ATP, CTP, and GTP, respectively.

[c] Data from Allen et al. (3).

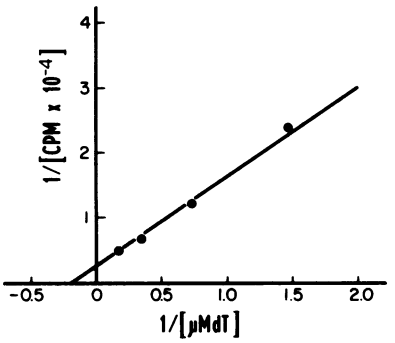

FIG. 4. *Lineweaver-Burk double-reciprocal plot of dTK activity versus dT substrate concentration for determination of the K_m of the transformed dTK. The reaction mixtures were allowed to incubate for 30 min before processing for the amount of ^{14}C-labeled deoxythymidine monophosphate formed.*

agrees with the value previously reported for the purified viral enzyme (3). Deoxycytidine was not a substrate for the transformed dTK (data not shown).

Antiserum inhibition of purified EHV-1-transformed dTK. Antiserum to EHV-1-induced dTK prepared in rabbits as previously described (1) was tested for its ability to inhibit the dTK activity of the dTK purified from EHV-1-transformed cells. The activities of the various purified dTK's were made equal by dilution, and antiserum inhibition was carried out by incubating the enzyme extract with an equal volume of immune serum. Figure 5 demonstrates that 10 μl of antiserum inhibited the purified viral dTK from dTK$^-$ 3T3 cells lytically infected with EHV-1 (3) and from dTK$^-$ 3T3 cells biochemically transformed by ultraviolet-inactivated EHV-1 by at least 90%. In contrast, the anti-EHV-1 serum did not inhibit the purified host cytosol or mitochondrial dTK (3; Fig. 5). Incubation of the purified enzymes with dilutions of preimmune serum resulted in no loss of any of the purified dTK activity (data not shown).

DISCUSSION

We have used the dT-Sepharose affinity chromatography technique to purify the dTK from EHV-1-transformed dTK$^-$ 3T3 cells. The elution profile of the enzyme from the affinity column was similar to that of the dTK induced by lytic infection of dTK$^-$ 3T3 cells by EHV-1 (3). The salt concentration required to elute the dTK was higher than that reported for elution of other herpesviruses dTK's (5, 6).

Electrophoresis of the affinity column-purified dTK from the EHV-1-transformed cells demonstrated a single peak of enzyme activity migrating to approximately the same position in polyacrylamide gels as the EHV-1 dT from lytically infected cells (R_f = 0.21) (3).

The dTK purified from cytosol fractions of the transformed 3T3 cells was unable to phos-

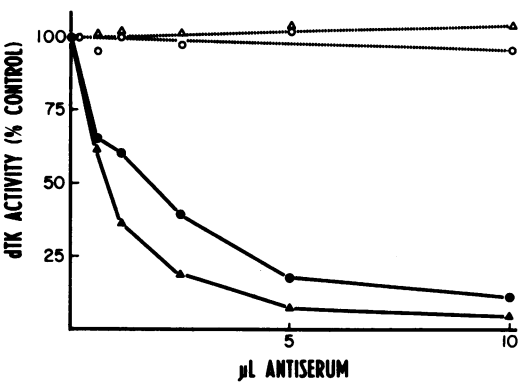

FIG. 5. *Antiserum inhibition of the dTK's purified by dT-Sepharose affinity chromatography. Mixtures of equal amounts of enzyme activities and increasing amounts of rabbit anti-EHV-1 serum were preincubated at 2°C for 12 h. A 40-μl sample of each mixture was then assayed for dTK activity as described in the text. (●——●) dTK purified from EHV-1-transformed dTK$^-$ 3T3 cells; (○- - -○) cytosol dTK purified from control dTK$^+$ 3T3 cells; the specific activities of these two dTKs were similar (3 and 2.4 units/mg, respectively). (△- - -△) Mitochondrial dTK purified from control dTK$^+$ 3T3 cells; (▲——▲) dTK purified from dTK$^-$ 3T3 cells lytically infected with EHV-1; data from Allen et al. (3).*

phorylate deoxycytidine, had a K_m of 5 μM for dTK, and could use a variety of nucleoside triphosphates as phosphate donors in the dTK reaction. The molecular weight of the purified dTK, estimated to be 87,000, was in agreement with that reported for the dTK in cells lytically infected with EHV-1 (3) and was in the same range as that reported for most other dTK's of mammalian cell or herpesvirus origin (5, 6, 12, 14).

We have shown by the results reported in this paper that the new dTK acquired by dTK$^-$ 3T3 cells upon transformation by ultraviolet light-inactivated EHV-1 is identical to the dTK expressed in cells lytically infected with the virus with respect to its electrophoretic mobility, phosphate donors, molecular weight, K_m for dT, and immunological specificity. These results provide further evidence that the dTK acquired by dTK$^-$ 3T3 cells after transformation with EHV-1 is coded by the viral genome.

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