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Biochemical and Immunological Characterization of Deoxythymidine Kinase of Thymidine Kinaseless HeLa Cells Biochemically Transformed by Herpes Simplex Virus Type 1

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Thymidine kinase (TK) from herpes simplex virus type 1 (HSV-1) biochemically transformed HeLa cells, purified by affinity chromatography, has been characterized with respect to its electrophoretic mobility, molecular weight, activation energy, substrate specificity, and immunological specificity. TK purified from HSV-1-transformed HeLa cells has the same electrophoretic mobility as TK purified from HeLa cells lytically infected with HSV-1. The sedimentation velocity of purified TK from transformed cells was similar to that previously reported for the lytic enzyme, and its molecular weight was estimated to be 70,000. The activation energy of purified transformed-cell TK was 18.3 kcal/mol. Antiserum prepared against purified HSV-1 TK, although it showed some cross-reactivity, preferentially neutralized homologous TK. The transformed-cell TK antiserum also neutralized the deoxycytidine kinase activity of HSV-1-infected cell extracts but had no effect on deoxycytidine kinase activity of HSV-2-infected cell extract. These results further support the notion that TK acquired by HeLa cells transformed by HSV-1 is of viral and not of cellular origin.

Mouse L cells (LTK^-) and HeLa cells (HeLa Bu) lacking the cytosol thymidine kinase (TK) have been changed to the TK^+ phenotype by ultraviolet-irradiated herpes simplex virus types 1 and 2 (HSV-1 and HSV-2, respectively) (4, 5, 20). This heritable change of TK^- cells to the TK^+ phenotype is defined as transformation for the purpose of this paper. With crude cellular extracts from these transformed cells, it was found that the TK present has similar properties to that derived from HSV-1 and HSV-2 lytically infected cells (5, 19, 23). These results suggest that the deoxyribonucleic acid in these transformed cells specifies the synthesis of at least one HSV-specific protein, TK.

Several studies (1, 3, 8, 9, 17, 24) have shown that HSV-induced TK could also phosphorylate deoxycytidine (CdR). Thus, genetic, immunological, and kinetic evidence supports the hypothesis that the same enzyme moiety could phosphorylate both thymidine (TdR) and CdR, although TdR is a much better substrate than CdR. Consequently, the enzyme, although it also possesses deoxycytidine kinase (dCK) activity, will be referred to as a TK in this paper.

We have recently described an affinity column chromatography procedure for the purification of TK derived from various sources (3, 15). Using this method, TK derived from HSV-1 lytically infected HeLa Bu cells and TK from HSV-1-transformed HeLa Bu (HeLa Bu_1) cells were purified. This allowed us to characterize this enzyme and compare its behavior with that from lytically infected cells. Antibody was made against the purified enzyme from the transformed cells. This communication will report some of the physical, biochemical, and immunological properties of this purified enzyme, indicating that TK isolated from HSV-1 lytically infected HeLa Bu and HeLa Bu_1 cells is the same enzyme, providing additional evidence that it is the viral gene for TK that is functional in transformed cells.

MATERIALS AND METHODS

Cells, media, and viruses. Eagle medium (6) containing nonessential amino acids and glycine and supplemented with 5% calf serum (EM5C) was used as the basic cell culture medium. African green monkey kidney (CV_1) cells were grown in EM5C with no additional supplements. LTK^- and HeLa Bu cell lines were grown in EM5C supplemented with 20 μg of 5-bromodeoxyuridine per ml. HeLa Bu_1 and HeLa Bu transformed by HSV-2 (HeLa Bu_2) lines were grown in EM5C supplemented with methotrexate, 6×10^{-7} M; TdR, 1.6×10^{-5} M; adenosine, 5×10^{-5} M; and guanosine, 10^{-5} M. The HeLa Bu, HeLa Bu_1, and HeLa Bu_2 cell lines were obtained from W. Munyon. The origin of the transformed lines has been described (5).

The virus strains used in this study were KOS, Sasha, HF, MacIntyre, and Cl 101 of HSV-1 and 333 and MS of HSV-2. Stocks of all these strains were grown and assayed in CV_1 cells. Sasha virus was isolated by W. Munyon and, with KOS and 333, was kindly provided by him. The remaining viruses were obtained from the American Type Culture Collection.

Infection of cells by viruses. Confluent monolayers of LTK^- or HeLa Bu cells were infected with the desired strain of either HSV-1 or HSV-2 at an input multiplicity of 10 to 20 plaque-forming units/cell. After an adsorption period of 1 h, EM5C was added and the cells were incubated for 18 h at 37°C. Infected cells were harvested by scraping them into the medium and were pelleted by low-speed centrifugation. The cell pellets were washed twice with cold phosphate-buffered saline.

Preparation of crude cell extracts for TK and dCK activities. The details of the procedure for extraction of TK from cells are described elsewhere (3). The procedures followed to obtain the organelle fractions from HeLa Bu cells were the same as described by Kit et al. (12). All operations were performed at 4°C and the samples were frozen at −70°C until assayed.

Enzyme assays. The enzyme assays were performed at 37°C for 30 min. The details of the reaction mixture and other procedures were as described previously (3, 15). Protein concentrations were determined by the method of Lowry et al. (18).

Purification of TK by affinity gel chromatography. The details of purification of TK from HSV-1-transformed HeLa Bu cells were essentially the same as described in an earlier publication (3). The affinity column matrix was first made by Kowal and Marcus (14).

Polyacrylamide gel electrophoresis and glycerol gradient centrifugation. The details of the procedures for polyacrylamide gel electrophoresis and glycerol gradient centrifugation used to characterize TK purified from HeLa Bu_1 cells were identical to those described earlier for TK from HeLa cells lytically infected with HSV-1 (3).

Antiserum preparation. Antiserum was prepared in New Zealand white rabbits against TK purified from HeLa Bu_1 cells. Animals were first injected in footpads with a 4:1 mixture of incomplete to complete Freund adjuvant 3 to 4 days before immunization. For the first injection equal volumes of antigen and Freund complete adjuvant were used. For subsequent injections Freund incomplete adjuvant was used. A properly emulsified antigen preparation was injected subcutaneously and around the stimulated lymph nodes once a week for 5 weeks. At this time, animals were rested for 2 weeks and then given a booster injection without any adjuvant. Animals were bled 1 week after the last injection. For each injection approximately 0.5 μg of purified TK was used. All animals were bled before immunization.

Gamma globulins from the serum were precipitated with ammonium sulfate according to the procedure of Spendlove (21). The final precipitate was dissolved in distilled water to its original volume and dialyzed extensively against phosphate-buffered saline in order to ensure complete removal of sulfate ions. Immunoglobulins partially fractionated with ammonium sulfate were used for all TK or dCK neutralization studies.

Antiserum neutralization of TK and dCK activities. Two parts of the enzyme preparation containing 4 mM adenosine 5'-triphosphate (ATP)-Mg^{2+} and 30 μM TdR were mixed with 1 part of the antiserum. The enzyme-antibody mixture was incubated for 40 min at room temperature. Under this condition, no inactivation of TK activity occurred. At the end of this time, 4 parts of goat anti-rabbit antiserum (Cappel Laboratories, Inc., Downingview, Pa.) was added, and the mixture was incubated for an additional 30 min at room temperature. The precipitate was collected after centrifugation at 2,000 × *g* for 10 min. The supernatant from this centrifugation step was used to assay for TK and dCK activities. Preimmune serum and cell extracts of HeLa Bu cells served as controls.

Reagents. The nucleotide triphosphates were purchased from Sigma Chemical Co. 5-Ethyldeoxyuridine, 5-vinyldeoxyuridine, 5-propyldeoxyuridine, 5-allyldeoxyuridine, and 5-vinyluridine were kindly provided by R. A. Sharma and M. Bobek of this department. 5-Iodouridine and 5-iododeoxyuridine were gifts from W. H. Prusoff. [^{14}C]deoxythymidine and [^{14}C]CdR were purchased from Nuclear Dynamics Inc.

RESULTS

Physical and biochemical characterization of the purified enzyme. TK was purified from HeLa Bu_1 cells by the same affinity chromatography procedure used for purifying TK from HSV-1 lytically infected HeLa Bu cells (3); a typical elution profile from the affinity column is presented in Fig. 1. The concentrations of TdR and tris(hydroxymethyl)aminomethane-hydrochloride required to elute TK from HeLa Bu_1 cells were different from that required for purifying TK from HeLa Bu_2 cells and human cell cytosol or mitochondria but the same as that required for TK from cells lytically infected with HSV-1 (3, 15). Figure 2 shows that when TK was electrophoresed as described in Materials and Methods, the electrophoretic mobility (R_f), relative to the bromophenol blue tracking dye, of the crude enzyme was 0.45, which is in good agreement with the value found for TK in crude extracts of HSV-1-infected HeLa Bu cells (3). However, after purification, the R_f of the enzyme from the transformed cells increased to 0.70. This has also been observed previously with TK from infected cells (3). Kit et al. have reported an increase in the R_f of HSV-1 TK when ATP was added to the upper-gel buffer and as an explanation have suggested that the enzyme is more negatively charged in the presence of ATP due

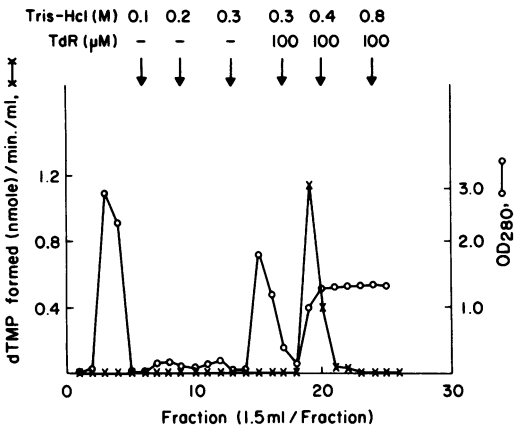

FIG. 1. *Elution profiles of HSV-1-transformed-cell TK from affinity column chromatography. The enzyme preparation after ammonium sulfate fractionation was loaded onto the affinity column (0.5 by 5 cm) and the column was eluted with gradually increasing concentrations (see graph) of tris-(hydroxymethyl)aminomethane(Tris)-hydrochloride buffer at pH 7.5 containing 10% glycerol, 3 mM dithiothreitol, and increasing concentrations of TdR.*

to the formation of an enzyme-phosphate intermediate (13). This is unlikely in our case for the following reasons: (i) ATP was not included in the upper-gel buffer and was not present during chromatographic purification, and (ii) when ATP was included in the upper-gel buffer, no change of R_f value was observed. It should be noted that the R_f of HSV-2 TK from infected HeLa Bu cells and the R_f of TK from the cytosol and mitochondria of acute myelocytic leukemic blast cells were unchanged after purification (3, 15).

The sedimentation velocity of purified TK from transformed cells was determined in glycerol gradients as shown in Fig. 2. The sedimentation rate of the transformed-cell enzyme was similar to that previously reported for the lytic enzyme (3), and the molecular weight was estimated to be about 70,000. This value is in reasonable agreement with the value of 74,000 reported by Leung et al. (17) using the same technique with enzyme from lytically infected cells.

The activation energy of purified transformed-cell TK was 18.3 kcal/mol, and is similar to that reported previously (3) for purified TK from lytically infected cells.

Nucleoside triphosphates as phosphate donors for and effectors of TK activity. Table 1 shows that ATP, guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP), and uridine 5'triphosphate (UTP) could act as phosphate donors for TK purified from transformed cells. These data are in agreement with those obtained using enzyme purified form HSV-1-infected HeLa Bu cells (1). Neither thymidine 5'triphosphate (TTP) nor 5-iododeoxyuridine triphosphate (IdUTP) could serve as a phosphate donor in the TK reaction. The deoxynucleoside triphosphates dATP, dGTP, and dUTP could also serve as phosphate donors, but were less effective in this regard than the corresponding ribonucleoside triphosphates. None of the compounds stimulated TK activity and only TTP and IdUTP inhibited the reaction.

Inhibition of TK activity by TdR analogues. Several recently synthesized analogues of TdR were examined for their ability to inhibit puri-

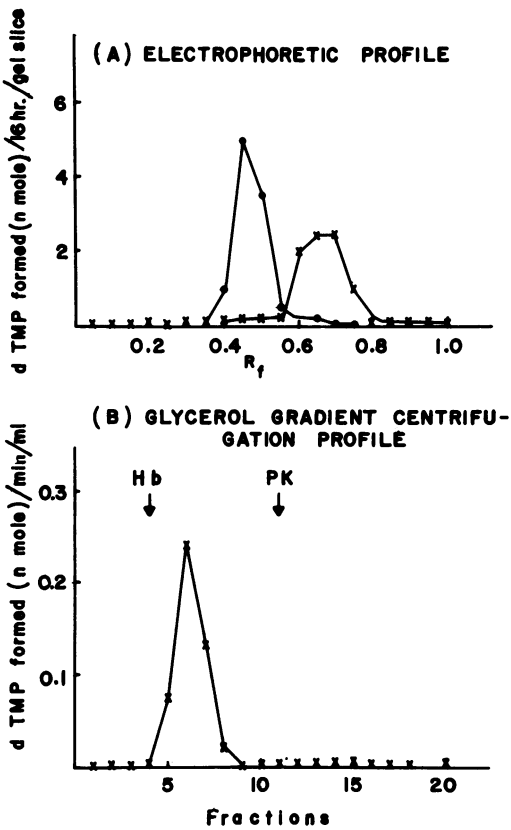

FIG. 2. *Biophysical properties of TK activity derived from HeLA Bu_1 cells. (A) Electrophoretic mobility profile. Mobility (R_f) was measured with respect to bromophenol blue. Symbols: ●, crude enzyme preparation; ×, purified enzyme preparation. (B) Glycerol gradient centrifugation of purified TK. Hemoglobin (Hb) and pyruvate kinase (PK) were used as molecular weight markers. Fractions of 0.6 ml were collected from the bottom of the tube. Conditions for electrophoresis, glycerol gradient centrifugation, enzyme purification, and TK assay have been described (3).*

fied transformed-cell TK. It has been shown that two of these analogues, 5-propyldeoxyuridine and 5-allyldeoxyuridine, are inhibitory for the growth of HSV-transformed cells but not for untransformed cells or TK$^+$ cells from several sources (2). Consequently, it was of interest to see if these analogues inhibited the TK of HSV-transformed cells. The analogues, 5-ethyldeoxyuridine, 5-vinyldeoxyuridine, 5-propyldeoxyuridine, 5-allyldeoxyuridine, or 5-iododeoxyuridine, were added to the TK reaction mixtures. The results shown in Table 2 indicate that all of these compounds, as well as the ribonucleoside derivatives 5-vinyluridine and 5-iodouridine, inhibited the TK reaction and that, where tested, the deoxyribonucleoside was more effective than the corresponding ribonucleoside. These results are similar to those obtained with HSV-1 TK from lytically infected cells (1). However, the same inhibitory pattern was not seen with TK from HSV-2-infected cells or human TK (1, 16), indicating that these enzymes are sufficiently different from HSV-1 TK that they have different substrate and inhibitor specificities.

TABLE 1. *Effects of nucleoside triphosphates (NTP) as phosphate donors or effectors of the TK reaction*

NTP	TK activity (%)[a]	
	NTP as phosphate donor[b]	NTP as effector[c]
None	0[d]	100
ATP	100	100
GTP	35	100
CTP	80	100
UTP	60	105
dATP	80	110
dGTP	15	100
dUTP	33	101
TTP	5	31
IdUTP[e]	3	15

[a] Enzyme (0.1 U) was used for each assay.
[b] ATP-Mg^{2+} was substituted by 2 mM NTP-Mg^{2+}.
[c] NTP-Mg^{2+} (2 mM) was added to a reaction mixture containing 2 mM ATP-Mg^{2+} and 100 μM TdR. The term effector is used to describe compounds that either stimulate or inhibit the TK reaction.
[d] Percentage of activity relative to that with ATP.

TABLE 2. *Effect of TdR analogues on TK activity*

Analogue added[a]	% Inhibition[b]
None	0[c]
TdR	62
5-Ethyldeoxyuridine	64
5-Vinyldeoxyuridine	68
5-Propyldeoxyuridine	64
5-Allyldeoxyuridine	41
5-Vinyluridine	37
5-Iodouridine	35
5-Iododeoxyuridine	77

[a] Compounds were added to the TK reaction mixture to a concentration of 0.2 mM.
[b] Purified enzyme (0.1 U) was used for each assay.
[c] Percentage of inhibition relative to that with no addition.

Serological properties of purified TK. Antibodies were raised in rabbits to the purified transformed-cell TK, and the antiserum was partially purified by ammonium sulfate precipitation as described in Materials and Methods. The antiserum was mixed with crude enzyme extracts from HeLa Bu or LTK$^-$ cells infected with five strains of HSV-1 or two strains of HSV-2. After incubation at 25°C for 40 min, rabbit immunoglobulins were precipitated and residual TK and dCK activities were measured and compared to enzyme samples incubated with preimmune rabbit serum. The results of these experiments are shown in Table 3. The antiserum neutralized the homologous HSV-1 TK and dCK activities efficiently but neutralized the HSV-2 TK activity to a lesser extent and had little, if any, inhibitory effect on the HSV-2 dCK activity. The antiserum was also not inhibitory to the TK and dCK activities of uninfected cells. Since the crude enzyme extracts contained mitochondrial TK and dCK in addition to cytosol dCK, these activities were detected in uninfected cell extracts (Table 3) and probably contribute to the residual activities observed in the virus-infected cell extracts after treatment with immune serum.

To obtain a clearer idea of the specificity of neutralization of the immune serum to transformed-cell TK, neutralization experiments were carried out on TK purified from HeLa Bu or LTK$^-$ cells infected with HSV or vaccinia, HeLa Bu cells transformed by HSV, and uninfected human cells. Both TK and dCK activities were determined after exposure of the purified enzyme to antiserum as before (Table 4). The data indicate that the antiserum to HSV-1-transformed-cell TK neutralized virtually all of the TK and dCK activities of purified HSV-1 TK but had very little effect in neutralizing the TK and dCK activities induced by HSV-2. The antiserum had no effect in neutralizing the TK activity found in human cell cytosol. Also, it had no effect on human mitochondrial TK. The specificity of the antiserum was further emphasized by the observation that a 1:150 dilution of the antiserum failed to neutralize HSV-2 TK activity, although it was still capable of neutralizing about 60% of HSV-1 TK activity derived from either lytically infected or transformed HeLa Bu cells.

TABLE 3. *Neutralization of crude HSV TK by antiserum to purified HSV-1-transformed-cell TK*[a]

Source of enzyme		Activity[b]					
		TK			dCK		
Cell type	Infecting virus	Preimmune	Immune	% Neutralized	Preimmune	Immune	% Neutralized
HeLa Bu	HSV-1 (KOS)	8,400	100	99	1,000	250	75
Ltk$^-$	HSV-1 (KOS)	7,400	150	98	820	30	96
HeLa Bu	HSV-1 (Cl 101)	4,900	200	96	450	270	40
Ltk$^-$	HSV-1 (Cl 101)	5,200	70	99	350	30	91
HeLa Bu	HSV-1 (Sasha)	6,000	100	98	800	300	63
Ltk$^-$	HSV-1 (Sasha)	7,400	100	99	820	30	96
HeLa Bu	HSV-1 (HF)	5,500	200	96	1,400	300	79
Ltk$^-$	HSV-1 (HF)	5,600	250	96	1,200	120	90
HeLa Bu	HSV-1 (MacIntyre)	5,100	200	94	1,000	250	75
Ltk$^-$	HSV-1 (MacIntyre)	5,100	300	94	1,250	80	94
HeLa Bu	HSV-2 (333)	10,000	7,100	29	2,400	2,100	13
Ltk$^-$	HSV-2 (333)	5,700	3,700	35	450	300	33
HeLa Bu	HSV-2 (MS)	7,000	4,400	37	780	800	-26
HeLa Bu[c]	None	1,800	1,300	28	450	400	11
Ltk$^-$[c]	None	1,500	1,100	27	900	900	0

[a] Antiserum to TK purified from HeLa Bu_1 cells was mixed with crude enzyme extracts from infected cells as described in the text. Enzyme assays were carried out as described previously (3).

[b] Counts per minute per microliter as phosphorylated [^{14}C]TdR or [^{14}C]CdR.

[c] In order to detect low levels of enzyme activity, 10 times as much cell extract was used in the uninfected cell assays as in the virus-infected cell assays.

TABLE 4. *Neutralization of purified HSV TK by antiserum to purified HSV-1-transformed-cell TK*[a]

Source of enzyme		Activity[b]					
		TK			dCK		
Cell type	Infecting virus	Preimmune	Immune	% Neutralized	Preimmune	Immune	% Neutralized
HeLa Bu	HSV-1 (KOS)	4,000	100	98	200	0	100
HeLa Bu	HSV-2 (333)	4,400	3,400	23	320	290	9
HeLa Bu	Vaccinia	4,000	3,200	20	100	100	0
Ltk$^-$	HSV-1 (Cl 101)	4,800	150	97	ND[c]	ND	
HeLa Bu_1	None	3,000	50	98	ND	ND	
HeLa Bu_2	None	5,900	3,600	39	ND	ND	
AML[d]	None	4,800	4,800	0	ND	ND	
AML[e]	None	900	1,00	-11	0	ND	

[a] Antiserum to TK purified from HeLa Bu_1 cells was diluted with an equal volume of phosphate-buffered saline. This solution was mixed with TK purified from infected, noninfected, or transformed cells as described in Materials and Methods.

[b] Counts per minute per microliter as phosphorylated [^{14}C]TdR or [^{14}C]CdR.

[c] ND, Not done.

[d] Mitochondria from the blast cells of an acute myelocytic leukemia patient.

[e] Cytosol from the blast cells of an acute myelocytic leukemia patient.

DISCUSSION

We have shown in this paper that TK purified from HSV-1-transformed HeLa Bu cells by affinity chromatography has properties that are very similar to those of TK induced in HSV-1 lytically infected cells (1, 3). The transformed-cell TK has been characterized with respect to its electrophoretic mobility, activation energy of the reaction, molecular weight, substrate specificity, and immunological specificity. The enzyme differs in these properties from TK purified from HSV-2- or vaccinia-infected HeLa Bu cells as well as purified TK from human cell cytosol and mitochondria (1, 3, 15, 16). These results support a substantial body of evidence (5, 7, 19, 20, 23) that the TK activity acquired by TK$^-$ cells after infection with ultraviolet-irradiated HSV is viral, not cellular, in origin.

The increase in electrophoretic mobility of

the enzyme after purification may result from the loss of certain cellular factors and this is under current investigation. It should be noted that antiserum prepared by using purified enzyme has equal capability in neutralizing crude or purified preparations of TK. The TK from transformed cells accepts the four ribonucleoside triphosphates, ATP, GTP, CTP, and UTP, as phosphate donors as does the lytic enzyme, and the enzymes are also similar in being inhibited by the same 5-substituted deoxyuridines, 5-ethyldeoxyuridine, 5-vinyldeoxyuridine, 5-propyldeoxyuridine, 5-allyldeoxyuridine, and 5-iododeoxyuridine.

Antiserum was prepared to the purified transformed-cell TK to determine its specificity in neutralizing TK from various sources. Enzyme was prepared from cells infected with several strains of HSV-1 and HSV-2. In every case, the antiserum neutralized the homologous HSV-1 TK activity to a high degree and the HSV-2 TK activity only slightly. These results are in accord with those of Thouless (22) and Kit et al. (11), which indicated that HSV-1 TK antiserum cross reacts with HSV-2 TK. The transformed-cell antiserum also neutralized the dCK activities of the HSV-1-infected cell extracts, but had no effect on the dCK activities of the HSV-2-infected cell extracts. Similar results have been obtained by Thouless and Wildy (24) and Kit et al. (10).

This report substantiates that the TK expressed in these transformed cells is the same as that induced by HSV-1 when used to produce lytic infection in HeLa cells. They share not only common physical and biochemical properties, but also a common immunogenicity. Various types of HSV-1 will induce TK with similar antigenicity which is different from TK induced by various types of HSV-2, and both of these are in turn antigenically different from the known human mitochondrial and cytoplasmic TKs.

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