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Production of herpes simplex virus type 1 thymidine kinase in the presence of thymidine analogues

Marcia L. Zucker*, William R. Mancini**, Michael J. Otto***, Jung-Ja Shim Lee and William H. Prusoff

Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, U.S.A.

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Summary

Treatment of herpes simplex virus type 1 (HSV-1) infected Vero, BHK, BHKtk$^-$ and LMtk$^-$ cells with 5-iodo-5′-amino-2′,5′-dideoxyuridine (AIdUrd) caused increased synthesis of ICP36 and an increase in HSV-1 thymidine kinase (tk) activity at late times of infection. The overproduced ICP36 was identified as the HSV-1 encoded tk protein by immunoprecipitation. Whereas the thymidine analogue 5′-amino-5′-deoxythymidine (AdThd) caused an increase in HSV-1 tk synthesis and activity in wild type Vero and BHK cells, 5-iodo-2′-deoxyuridine (IdUrd) caused a similar increase only in tk$^-$ cells (LMtk$^-$, BHKtk$^-$). In vivo and in vitro stabilization studies using a [^{35}S]methionine pulse-chase experiment or heat inactivation studies with purified HSV-1 tk revealed that stabilization of tk by the analogues could not account for the extent of the observed increase. Since overproduction of tk is observed only at late times of infection, it is suggested that the presence of these thymidine analogues in either the viral DNA or the cellular nucleotide pools is responsible for the observed differential effects.

HSV-1; thymidine kinase; iododeoxyuridine; aminonucleoside analogs

* To whom all correspondence should be addressed.

** Supported by Fellowship Grant F32 CA-06723 from the National Cancer Institute. *Present address*: Department of Pharmacology, University of Michigan Medical School, M6322 Medical Science Building 1, Ann Arbor, MI 48109, U.S.A.

*** *Present address*: Sterling-Winthrop Research Institute, Department of Microbiology, Rensselaer, NY 12144, U.S.A.

Introduction

Upon infection with herpes simplex virus (HSV) many mammalian cell biochemical processes become regulated by the virus. Among the enzymatic activities induced during HSV infection, thymidine kinase (tk) has been shown to be encoded by the viral genome [11,17]. Although much work has been done concerning the HSV tk gene, the precise regulatory mechanisms involved in tk production still need to be elucidated. Knowledge of the processes controlling the temporal expression of tk may lead to a further understanding of HSV-1 regulatory mechanisms. Leiden and coworkers [18] have found that regulation of the HSV-1 tk gene is controlled by at least two other HSV-1 gene products.

The thymidine analogues 5-iodo-2′-deoxyuridine (IdUrd), 5-iodo-5′-amino-2′,5′-dideoxyuridine (AIdUrd) and 5′-amino-2′,5′-dideoxythymidine (AdThd) are effective inhibitors of HSV-1 replication in vitro and in vivo [1,5,6,20–22]. Since AIdUrd and AdThd are phosphorylated only in HSV infected cells [2,3] and IdUrd exerts its antiviral effects at concentrations which reveal no apparent host cell toxicity [20,25], it is believed that the presence of the HSV-1 encoded tk is required for the analogue induced inhibition of HSV-1 replication.

In a recent report Otto, Lee and Prusoff [25] found that although HSV-1 α proteins were not affected, the production of β and γ proteins with three exceptions was reduced when HSV-1 infected Vero cells were treated with either IdUrd, AIdUrd or AdThd. Of the three exceptions only ICP #36 (M_r = 42000) was increased. This polypeptide migrates on SDS-PAGE to a position which has been identified as the HSV-1 induced tk [16,27].

In the present report we investigate in detail the overproduction of ICP 36, its stability and the production of HSV-1 tk in the presence of the nucleoside analogues IdUrd, AIdUrd and AdThd.

Materials and Methods

Cells and virus

The CL-101 strain of HSV-1 was propagated in Vero cells at a multiplicity of infection (MOI) of 0.01 pfu per cell. The virus yield was determined by plaque assay as described [22]. All cell lines were grown as monolayer cultures in DMEM (Gibco) supplemented with 5% fetal bovine serum (FBS) and 5% newborn calf serum (Gibco). BHK (baby hamster kidney) and BHKtk$^-$ cell lines were a generous gift of Dr. W.P. Summers.

Chemicals

5-Iodo-5′-amino-2′,5′-dideoxyuridine (AIdUrd) and 5′-amino-2′,5′-dideoxythymidine (AdThd) were synthesized by Dr. T.-S. Lin of this department [20,22]. 5-Iodo-2′-deoxyuridine (IdUrd), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), phosphocreatine, phosphocreatine kinase, dithiothreitol (DTT), bovine serum albumin (BSA), dThd and ATP were obtained from Sigma Chemical Co. *N,N*′-Diallyltar-

tardiamide (DATD) was obtained from Aldrich Chemical Co. L-[^{35}S]methionine (1300 Ci/mmol) and [2-^{14}C]dThd (56 Ci/mol) were obtained from Amersham Corporation and Moravek Biochemicals, Inc., respectively. [^{125}I]dCyd (2200 Ci/mmol) was obtained from New England Nuclear. Rabbit anti-HSV-1 tk antiserum was a generous gift of Dr. W.C. Summers and Dr. W.P. Summers.

Infection of cell monolayers

Confluent monolayers were infected with HSV-1 at a multiplicity of infection of 10 (Vero, LMtk$^-$ as noted in text) or 20 (LMtk$^-$, BHK, BHKtk$^-$). After one hour adsorption at 37°C, infected cells were washed once with phosphate buffered saline (PBS) before the addition of fresh medium containing 2% FBS with or without nucleoside analogue. Cells were then incubated at 37°C in a 5% CO_2 atmosphere until harvest. Cells were harvested by scraping with a rubber policeman, followed by two washes in PBS.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Gradient slab gel electrophoresis was performed using the method of Gibson and Roizman [13]. The gels were cast as a linear gradient of 5–15% acrylamide with a 4% stacking gel and run at a constant current of 25 mA per gel. Uniform slab gels were cast as 9% acrylamide with a 4.5% stacking gel. All gels were cast with DATD as crosslinker. After electrophoresis the gels were dried and analyzed by contact autoradiography [25] and densitometry. Alternatively, the HSV-1 tk band (ICP 36) which was identified by autoradiography, was cut from the gels, solubilized in 2% sodium periodate overnight at 37°C and the amount of radiolabel was determined by liquid scintillation spectrometry.

Immunoprecipitation of HSV-1 tk

Infected cells (MOI = 10 for Vero, 20 for LMtk$^-$ cells) to be extracted for immunoprecipitation were subjected to a starvation period of 2 h in methionine-free medium followed by a 2 h labelling period with [^{35}S]methionine (20 μCi/ml) after which the cells were harvested.

Two immunoprecipitation procedures were employed. One was essentially as described by Smiley et al. [26]. Infected cell monolayers were removed with a rubber policeman, pelleted by centrifugation and resuspended in RIPA buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% SDS, 1% Triton X-100 and 1% sodium deoxycholate). Cell debris was removed by centrifugation for 5 min in an Eppendorf microfuge. The anti-tk antiserum was added to the supernatant and the mixture was incubated at 25°C for 1 h. A preactivated (95°C, 30 min incubation in PBS, pH 7.2, 10% v/v, β-mercaptoethanol, 3% w/v SDS followed by 2 washes in PBS) *Staphylococcus aureus* cell suspension (in 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.5% Nonidet P40, 150 mM NaCl) was then added to a final concentration of 2.5% (w/v). After an additional 5 min incubation the immunoprecipitate was collected by centrifugation using a microfuge and the pellet was washed three times with RIPA buffer. The final precipitate was prepared for SDS-PAGE by boiling 5 min in SDS-sample buffer and centrifugation to remove the *S. aureus* cells.

Alternately, cells were resuspended in 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P40, 0.5 mM PMSF, and lysed by sonication for 2×5 s. A pre-precipitation of the cell lysate with activated *S. aureus* cells was performed to reduce any non-specific binding of antigen during the immunoprecipitation. Immunoprecipitations were performed on 200 µl cell lysate samples ($\approx 1 \times 10^6$ cells/sample). Anti-tk antibody was added to the cell extract and incubated on ice for 45 min. Activated *S. aureus* cells (0.1 ml) were then added and precipitation was allowed to proceed for 15 min on ice. Non-specific antigen was removed by 3 washes in 5% sucrose, 1% Nonidet P40, 0.5 M NaCl, 50mM Tris-HCl pH 7.4, 5 mM EDTA followed by one wash in 50 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA. The final pellet was prepared for SDS-PAGE as described above.

Preparation of cells for determination of tk activity in crude lysates

Confluent cell monolayers were infected with HSV-1 (MOI = 10 for Vero, LMtk$^-$, 20 for BHK, BHKtk$^-$) and collected at the indicated time as described for the preparation of purified HSV-1 tk up to the sonication step. In order to minimize potential heat inactivation of HSV-1 tk by extensive sonication, infected cells were uniformly disrupted while on ice with a 5-s pulse. Glycerol was added to a final concentration of 10% and the cell debris was removed by centrifugation in an Eppendorf microfuge at 5°C. The supernatant was immediately used for determination of tk activity (in duplicate) and the protein concentration was analyzed by the method of Lowry et al. [23].

Thymidine kinase (tk) assay

Two assay procedures were employed. The first assay procedure was essentially as described by Lin et al. [19]. The standard assay mixture contained 40 mM Hepes, pH 7.5 (at 37°C), 5.6 mM phosphocreatine, 6 units/ml phosphocreatine kinase, 0.1% BSA, 2 mM DTT, 2 mM ATP·Mg^{2+}, 25 mM NaF, 0.11 mM [2-^{14}C]dThd (56 Ci/mol) and the enzyme preparation. One unit of tk is defined as the amount of enzyme which catalyzes the formation of 1 nmol of dTMP from dThd per minute at 37°C. In studies using the purified enzyme NaF was omitted from the reaction mixture.

The second assay procedure was a modification of that described by Cremer et al. [10]. The assay mixture contained 0.1 M sodium phosphate, pH 6.0, 10 mM phosphocreatine, 25 U/ml phosphocreatine kinase, 0.6% BSA, 1 mM DTT, 10 mM ATP·Mg^{2+}, 10 mM $MgAc_2$, 0.2 mM IdCyd, 25 mM NaF, 2.5 µCi/ml [^{125}I]IdCyd and the enzyme preparation.

After 1 h incubation at 37°C, 10 µl of this mixture was diluted with 40 µl H_2O, boiled 2 min and centrifuged 2 min in an Eppendorf microfuge. The supernatant (45 µl) was applied to DE81 filters (Whatman 2.3-cm circles), and the filters were batch washed (20 ml/filter) twice in 0.04 M NH_4HCO_3/2 mM KI at 37°C with shaking. They were then washed once with water, twice with 95% ethanol, dried and counted in a Scientific Products AW 1450 Gamma Counter.

Purification of HSV-1 tk

LMtk$^-$ cells were infected with HSV-1 (MOI = 10), and incubated with fresh medium supplemented with 10% FBS for an additional 18 h after the adsorption

period. The infected cells were scraped from the flasks, collected by centrifugation and washed twice with phosphate-buffered saline. Four volumes of extraction buffer (10 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$ and 3 mM DTT) were added to the infected cell pellet and the mixture was frozen and thawed three times. Sonication, 1% streptomycin precipitation and ammonium sulfate fractionation steps were performed essentially as described by Williams and Cheng [28] except that the HSV-1 tk was present in the 20–50% ammonium sulfate precipitated fraction. This pellet was resuspended in 20 volumes of buffer (10 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM DTT) before being applied to the thymidine affinity column. The preparation of the affinity column and removal of the potentially interfering host enzymes has been described [24]. HSV-1 tk was eluted from the column as described by Cheng and Ostrander [8]. The thymidine and salt were removed by passing the purified HSV-1 tk through a Sephadex G-25 column which was equilibrated in a solution containing 25 mM Hepes, pH 7.5, 10% glycerol and 2 mM DTT. This procedure was performed at 5°C to minimize the loss of activity and the enzyme was stored at −70°C until use.

In vitro heat inactivation analysis

Purified HSV-1 tk was incubated at either 38°C or 41°C in a solution containing 50 mM Hepes, pH 7.5, 0.1% BSA, 2 mM DTT without or with nucleosides as indicated. At various times an aliquot was removed and assayed under standard conditions. An identically treated enzyme preparation was kept at 5°C during the incubation period and was used as the control condition, the catalytic activity of which was designated the original activity.

In vivo stability determination

One hour post HSV-1 infection (MOI = 10), [^{35}S]methionine (20 μCi/ml) was added to Vero or LMtk$^-$ cultures and the infected cells were allowed to incubate at 37°C for an additional 6 h. The radiolabelled medium was then removed and fresh medium containing 10% FBS with or without the indicated nucleosides was added. At the appropriate time infected cell samples were harvested and lysed with SDS-sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and 5% β-mercaptoethanol). The cell lysates were boiled for 5 min and stored at −20°C until analyzed by gradient SDS-PAGE.

Results

Identification of the HSV-1 tk polypeptide in analogue treated infected Vero cells

Since ICP 36 migrates on SDS-PAGE to a position corresponding to that of HSV-1 tk [16,27], it was necessary to determine if the overproduced polypeptide in analogue treated cultures reported by Otto and coworkers [25] was indeed HSV-1 tk. [^{35}S]Methionine-labelled infected cell proteins were isolated from HSV-1 infected Vero cells with or without nucleoside analogue treatment. The concentration used for each of the analogues was that which afforded a 2 log reduction in plaque yield. The HSV-1 thymidine kinase polypeptide was immunoprecipitated from the cell lysates and

quantitated after electrophoresis as described. No other polypeptides of apparent molecular weights of 15 000 to 60 000 daltons were apparent in these gels. The results, shown in Table 1, reveal a substantial increase in the amount of radioactivity associated with the tk band of the AIdUrd- and AdThd-treated cells. Furthermore, the observed increase compares quite well with the densitometer scans of ICP 36 from non-immunoprecipitated samples.

Time course of tk activity and synthesis in the presence and absence of analogues in HSV-1 infected LMtk⁻ cells

In order to examine the increase in HSV-1 tk production in more detail, the HSV-1 tk activity and synthesis during the course of infection in the presence of the analogues were determined. Since Vero cells have a cytoplasmic tk which could complicate the detection of HSV-1 tk, LMtk⁻ cells were employed.

The results, shown in Fig. 1A, reveal that synthesis of the HSV-1 tk protein in LMtk⁻ cells peaks at 12 h post infection (p.i.), with maximal enzyme activity apparent 8–12 h post infection (Fig. 1B). From the onset of infection through 12 h p.i., there are no significant differences in the rate of tk synthesis between drug treated and control cultures (Fig. 1B). At late times of infection, however, cultures treated with AIdUrd or IdUrd contained both a greater amount of the HSV-1 tk polypeptide as compared to control or AdThd treated cultures and an increased level of tk activity.

Treatment of cultures with 50–200 μM dThd resulted in production of HSV-1 tk in amounts similar to those observed with AdThd (data not shown). This suggests that the overproduction of thymidine kinase in HSV-1 infected LMtk⁻ cells observed in AIdUrd and IdUrd cultures is not solely due to substrate stabilization of tk by these analogues.

HSV-1 tk synthesis and activity in analogue treated and control Vero cells

Since initial studies in Vero cells indicated that HSV-1 tk synthesis was elevated late in infection in the presence of AIdUrd or AdThd but not IdUrd (Table 1), a time course

TABLE 1

Production of HSV-1 tk in the presence of nucleoside analogues in Vero cells

Analogue (μM)	% 18 h	untreated control
	ICP 36[a]	tk band[b]
None	100 ± 6	100 ± 4
IdUrd (25)	109 ± 7	104 ± 5
AIdUrd (800)	130 ± 10	156 ± 10[c]
AdThd (400)	117 ± 4	124 ± 7[c]

[a] ICP 36 was identified by apparent molecular weight according to the numbering system of Gibson and Roizman [13]. The relative amounts of ICP 36 were determined by densitometric scans of autoradiographs.

[b] Using rabbit antisera prepared against purified HSV-1 tk, the immunoprecipitate formed was electrophoresed and the tk band was excised and counted.

[c] $P<0.001$ by Bonferroni procedure for multiple comparisons using Student's t-test.

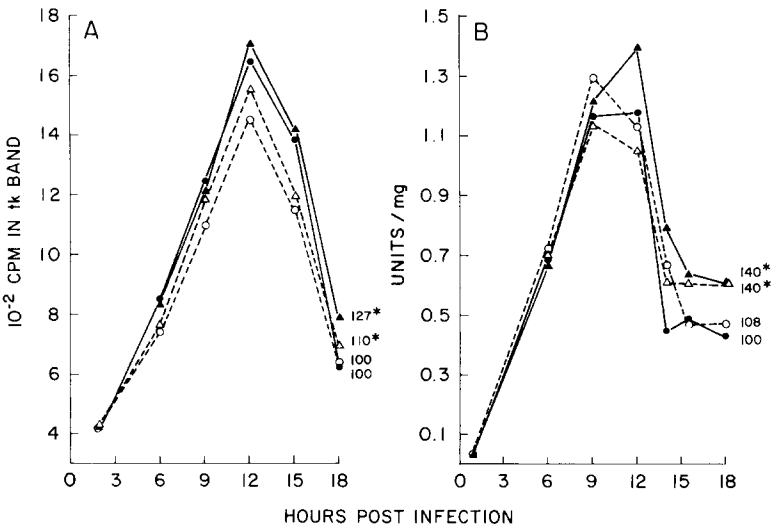

Fig. 1. Time course of HSV-1 tk synthesis and enzyme activity in LMtk$^-$ cells. LMtk$^-$ cells were infected with HSV-1 [(A) MOI = 20; (B) MOI = 10] in the absence (●——●) or presence of 400 μM AdThd (○---○), 800 μM AIdUrd (▲——▲) or 25 μM IdUrd (△---△). At the indicated time samples were harvested and (A) the tk polypeptide was immunoprecipitated from the cell lysates or (B) the amount of tk activity was immediately determined. Cell cultures for immunoprecipitation were [^{35}S]methionine labelled for 2 h prior to harvest. After SDS-PAGE the tk band was excised, solubilized, and the radioactivity was determined in a liquid scintillation counter. The tk activity in uninfected LMtk$^-$ cells was 0.0053 ± 0.0003 unit/mg. Numbers on right represent tk activity or synthesis at 18 h p.i. as percent of 18 h HSV-1 infected cells in the absence of nucleoside analogue. *$P < 0.005$ by Bonferroni procedure for multiple comparisons using Student's t-test.

of tk synthesis and activity in Vero cells was performed. To preclude the interference of endogenous cellular tk in the activity studies, [^{125}I]IdCyd was employed as substrate in all assays.

As with LMtk$^-$ cells, analogue treatment did not affect tk synthesis or activity in HSV-1 infected Vero cells until late times of infection (data not shown). Tables 2 and 3 present the results of immunoprecipitations and activity studies at 18 h p.i. Similar results were also seen at 15 h p.i. (data not shown). It is clear that in Vero cells AIdUrd and AdThd but not IdUrd induce increased tk production, while in LMtk$^-$ cells AIdUrd and IdUrd but not AdThd cause a similar overproduction of tk.

Stability of purified HSV-1 tk in vitro

Purified HSV-1 tk was incubated at either 38°C or 41°C in the absence or presence of various nucleosides. Sample aliquots were removed at the indicated times and assayed using the [^{14}C]dThd assay procedure. The conditions of the assay mixture were such that dThd was present at saturating concentrations, therefore, the final concentration of analogue in the assay mixture did not significantly affect the formation of [^{14}C]dTMP. The results, shown in Fig. 2, reveal distinct differences in the ability of the analogues to prevent heat inactivation of purified HSV-1 tk. Although the presence of nucleoside afforded more protection than its absence, clearly AdThd was the least

TABLE 2

Synthesis of HSV-1 thymidine kinase 18 h p.i. in the presence and absence of nucleoside analogues

HSV-1 infected	Nucleoside (μM)	Vero cells		LMtk⁻ cells	
		cpm[a]	% control[b]	cpm[a]	% control[b]
+	None	638	100	627	100
+	IdUrd (25)	678	106	690[c]	110
+	AIdUrd (800)	954[c]	149	795[c]	127
+	AdThd (400)	874[c]	137	628	100
-	None	98	15.4	126	20.1

[a] The immunoprecipitated tk band was cut from the gel, solubilized, and counted in a liquid scintillation counter as described in Methods.

[b] Synthesis of tk is expressed as the percent of the control condition which is HSV-1 infected cells in the absence of nucleoside treatment harvested at 18 h post infection.

[c] $P<0.005$ by Bonferroni procedure for multiple comparisons using Student's t-test.

TABLE 3

HSV-1 thymidine kinase activity 18 h p.i. in the presence and absence of nucleoside analogues

HSV-1 infected	Nucleoside (μM)	% Control[a]			
		Vero	BHK	BHKtk⁻	LMtk⁻
+	None	100[b]	100[b]	100[b]	100[b]
+	IdUrd (25)	101	101	137	141[c]
+	AIdUrd (800)	132[c]	138	133	140[c]
+	AdThd (400)	122[c]	134	104	108
-	None	1.4	2.9	1.9	3.0

[a] Thymidine kinase activity is expressed as the percent of the control condition which is HSV-1 infected cells in the absence of nucleoside treatment harvested at 18 h post infection.

[b] 100% = 1.38 units/mg for Vero cells; 0.73 units/mg for BHK cells; 0.51 units/mg for BHKtk⁻ cells; and 0.43 units/mg for LMtk⁻ cells.

[c] $P<0.005$ by Bonferroni procedure for multiple comparisons using Student's t-test.

effective in this regard. At equivalent concentrations, dThd and IdUrd had a similar effect but AIdUrd was consistently the best stabilizer of tk under these conditions.

Stability of the HSV-1 tk protein in vivo

Proteins labelled with [^{35}S]methionine during infection of Vero and LMtk⁻ cells in the presence and absence of nucleosides for 6 h were harvested at two hour intervals thereafter. Polypeptides were separated by SDS-PAGE, removed from the gel, dissolved and the amount of radioactivity associated with ICP 36 determined. The results (Fig. 3) reveal no significant difference in the rate of loss of the radiolabel with the exception of increased tk stability in LMtk⁻ cells in the presence of thymidine.

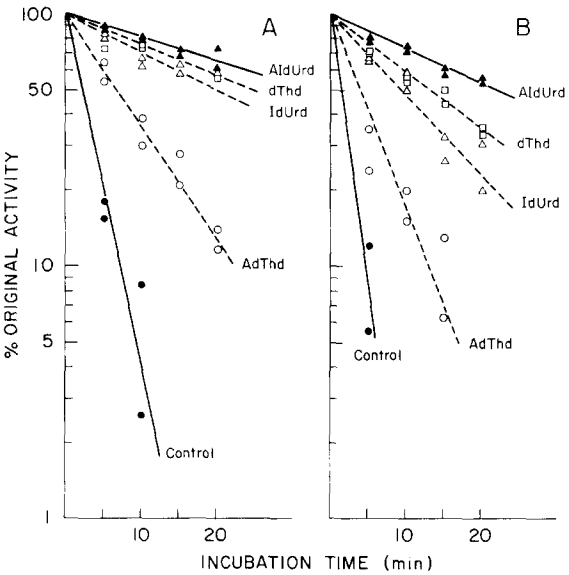

Fig. 2. Heat inactivation of purified HSV-1 tk in the absence and presence of thymidine and its analogues. HSV-1 tk was incubated at either (A) 38°C or (B) 41°C in the absence (●——●) or presence of 800 μM AIdUrd (▲——▲), 400 μM AdThd (○---○), 25 μM IdUrd (△---△) or 50 μM dThd (□---□). At the indicated time a sample aliquot was removed and incubated under standard assay conditions. The amount of remaining activity was plotted on a logarithmic scale as the percentage of the original activity.

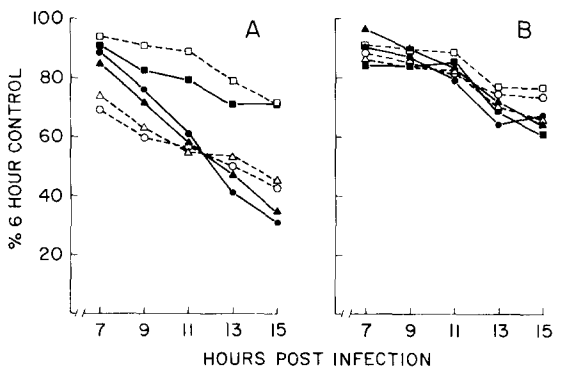

Fig. 3. Stability of labelled ICP 36 in vivo. HSV-1 infected (MOI = 10) LMtk$^-$ (A) or Vero cells (B) were labelled with [^{35}S]methionine for 6 h as described after which the label was removed and fresh non-labelled medium was added. Samples were harvested at the indicated time, polypeptides were separated using SDS-PAGE, ICP 36 was cut from the gel, solubilized and the amount of radioactivity was determined. The conditions were no drug (●——●), 800 μM AIdUrd (▲——▲), 400 μM AdThd (○---○), 25 μM IdUrd (△---△), 25 μM dThd (■——■) and 50 μM dThd (□---□).

Time course of HSV-1 tk activity in BHK and BHKtk⁻ cells

BHKtk⁻ cells and their parental BHK cell line were infected with HSV-1 (MOI = 20) in the presence or absence of nucleoside analogue. At various times post infection, cells were harvested and HSV-1 tk activity was assayed using [^{125}I]IdCyd as substrate. In BHK and BHKtk⁻ cells, as in both the Vero and LMtk⁻ cell cultures, analogue treated and control cultures exhibited parallel tk activities until late times after infection. Increases in tk activity in analogue treated samples became apparent at 15 h p.i. (data not shown).

The results (18 h p.i., Table 3) indicate that, as in Vero cells, the amino analogues AIdUrd and AdThd, but not IdUrd, cause increased tk activity at late times of HSV-1 infection in BHK cells. However, in BHKtk⁻ cells, the nucleoside analogue effects parallel those seen in LMtk⁻ cells, that is, AIdUrd and IdUrd but not AdThd treatment produced an increase in tk activity.

Discussion

It has been suggested by Cheng et al. [9] that the increased level of HSV-1 tk activity observed in the presence of 5-propyl-deoxyuridine (PdUrd) may be due to protection of the enzyme against inactivation, whereas Otto et al. [25] suggested that the overproduction of ICP 36 may be a result of a loss of regulation. Harris-Hamilton and Bachenheimer [14] found that in the presence of DNA synthesis inhibitors, the overproduction of HSV-1 tk is correlated with an overproduction of tk mRNA, which suggests the involvement of altered regulation. Whether tk overproduction in the present study is due to stabilization or altered regulation was addressed by two approaches.

In order to minimize complications that could arise in our analysis of the HSV-1 thymidine kinase polypeptide and enzyme activity, we initially selected LMtk⁻ cells. Cellular tk migrates to a position near HSV-1 tk in SDS-polyacrylamide gels, and could complicate quantitation of this protein. There also might be some overlap during antibody–antigen complex formation due to the known existence of cross-reactivity between the cellular tk and HSV-1 tk polypeptides [7]. The assay method for the determination of enzyme activity employed radiolabelled dThd which is a substrate for both the cellular and viral thymidine kinase. Because of these potential problems, LMtk⁻ cells were used initially for infection with HSV-1. This host cell possesses only a small amount of tk in the absence of infection (Fig. 1). Thus these potential problems were minimized and quantitation of HSV-1 tk polypeptide and tk activity was facilitated.

Analysis of tk activity in HSV-1 infected LMtk⁻ cells revealed an increased level of enzyme late in infection in the presence of IdUrd and AIdUrd (Fig. 1B). Similar results were obtained using in vivo accumulation experiments with [^{35}S]methionine (data not shown) and immunoprecipitation of pulse-labelled HSV-1 tk (Fig. 1A). Furthermore, the pulse-chase results lend support that there is no significant difference between analogue treated and control cultures in the rate of loss of label from ICP 36 (Fig. 3A).

The apparent discrepancies between which nucleoside analogues cause thymidine

kinase overproduction in LMtk$^-$ cells (AIdUrd and IdUrd, Fig. 1), and indications from initial studies in Vero cells (AIdUrd and AdThd, Table 1), led to a more complete characterization of HSV-1 tk production in analogue treated Vero cells. The complications arising from the presence of Vero cytoplasmic tk were circumvented by immunoprecipitation of all samples to separate the viral and cellular enzymes; by immunoprecipitations from uninfected Vero cells, which revealed the presence of small amounts of a polypeptide of lesser mobility than the HSV-1 tk which was present in analogue treated and control uninfected cells, but absent from infected cell samples; and by using [^{125}I]IdCyd as the assay substrate to preclude activity contributions by the cellular enzyme. Analysis of HSV-1 activity and polypeptide synthesis revealed that indeed, AIdUrd and AdThd, but not IdUrd, cause overproduction of thymidine kinase in HSV-1 infected Vero cells at late times of infection (Tables 1, 2, 3). As with LMtk$^-$ cells, pulse-chase studies revealed no significant differences in the rate of loss of labelled tk in Vero cells (Fig. 3B).

The second approach using purified HSV-1 tk in stabilization studies showed that although AIdUrd stabilizes best, IdUrd and dThd stabilize to approximately the same extent (Fig. 3). This is not surprising since IdUrd and dThd have similar binding affinities for HSV-1 tk [4]. Although IdUrd and dThd possess similar tk stabilizing ability, IdUrd consistently yields higher levels of HSV-1 tk in LMtk$^-$ cells. Also, at equivalent antiviral concentrations AIdUrd was better than IdUrd in protecting against heat inactivation of purified HSV-1 tk while the increase of enzyme activity over control cultures in LMtk$^-$ cells was essentially the same for these two analogues. The stabilization of purified HSV-1 tk by dThd is significantly greater than that observed with AdThd, yet in Vero cells AdThd consistently caused HSV-1 overproduction.

The differential effects of the thymidine analogues on HSV-1 tk production in Vero and LMtk$^-$ cells may be due to factors related to the species differences between these two cell lines. This difference might also be associated with the presence of a cellular thymidine kinase and tk regulatory factors in Vero cells, but an absence of this enzyme (and possibly additional factors) in LMtk$^-$ cells. Studies in BHK and BHKtk$^-$ cells (Table 3) support this latter explanation.

The BHKtk$^-$ cell line employed in this study was derived by Dr. W.P. Summers from the BHK line used (pers. commun.). The use of such a pair of cell lines precludes interspecies variations and minimizes any effects of differences in tissue culture lines. As was observed in the Vero–LMtk$^-$ comparison studies, HSV-1 tk overproduction was induced in the tk$^+$ cell line (BHK) by AIdUrd and AdThd, and in the tk$^-$ cell line (BHKtk$^-$) by AIdUrd and IdUrd (Table 3).

Collectively, these data provide good evidence that the increased level of HSV-1 tk seen in tk$^-$ cells in the presence of AIdUrd and IdUrd and in tk$^+$ cells in the presence of AIdUrd and AdThd cannot be explained solely on the basis of enzyme stabilization. Furthermore, these results suggest that the overproduction may be due, at least in part, to altered regulation of tk biosynthesis as suggested by Otto et al. [25]. This altered regulation appears to be determined to some extent, by cellular factors related to the presence or absence of a cell-encoded thymidine kinase.

Overproduction of thymidine kinase in HSV-1 infected cells has been observed

when cultures are treated with agents which block γ polypeptide synthesis [12,14], as well as during HSV-1 infection in the presence of PdUrd [9]. However, in the absence of γ polypeptide production many HSV-1 β polypeptides are overproduced [15], while in the presence of the nucleoside analogues employed in this study, the only overproduced polypeptide is tk [25]. HSV-1 infection in the presence of PdUrd leads to tk overproduction throughout infection [9], while this study reveals no analogue effect on tk production until late in infection, well after tk synthesis and activity peak (Fig. 1, Tables 2, 3). Clearly, in this study a novel mechanism is causing an alteration in the regulation of thymidine kinase production in thymidine analogue treated HSV-1 infected cells and this change in tk regulation is related in some unknown manner to the existence of a cellular tk gene.

In summary, the data presented in this report suggest that the overproduction of HSV-1 tk observed in the presence of nucleoside analogues is probably due to an altered regulation of the HSV-1 tk gene. Also apparent is a significant difference between wild type and tk^- cell responses to the presence of the thymidine analogues AdThd and IdUrd but not to AIdUrd. Further inquiry is required to determine the mechanism of tk overproduction, the cellular and/or viral effectors involved in the regulation of HSV-1 thymidine kinase synthesis, and the biochemical basis for the differential effects exerted by these thymidine analogues.

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References

1 Albert, D.M., Lahav, M., Bhatt, P.N., Reid, T.W., Ward, R.E., Cykiert, R.C., Lin, T.S., Ward, D.C. and Prusoff, W.H. (1975) Successful therapy of herpes hominis keratitis in rabbits by 5-iodo-5'-amino, 2',5'-dideoxyuridine (AIU): a novel analog of thymidine. Invest. Ophthalmol. 15, 470–478.

2 Chen, M.S., Shiau, G.T. and Prusoff, W.H. (1980) 5'-Amino-5'-deoxythymidine: Synthesis, specific phosphorylation by herpesvirus thymidine kinase, and stability to pH of enzymatically formed diphosphate derivative. Antimicrob. Agents Chemother. 18, 433–436.

3 Chen, M.S., Ward, D.C. and Prusoff, W.H. (1976) Specific herpes simplex virus-induced incorporation of 5-iodo-5'-amino-2',5'-dideoxyuridine into deoxyribonucleic acid. J. Biol. Chem. 251, 4833–4838.

4 Cheng, Y.-C. (1976) Deoxythymidine kinase induced in HeLa tk^- cells by herpes simplex virus type I and type II. Substrate specificity and kinetic behavior. Biochim. Biophys. Acta 452, 370–381.

5 Cheng, Y.-C., Neenan, J.P., Goz, B., Ward, D.C. and Prusoff, W.H. (1975) Synthesis and biological activity of some novel analogs of thymidine. Ann. N.Y. Acad. Sci. 255, 332–341.

6 Cheng, Y.-C., Goz, B., Neenan, J.P., Ward, D.C. and Prusoff, W.H. (1975) The selective inhibition of herpes simplex virus by 5'-amino-2',5'-dideoxyuridine. J. Virol. 15, 1284–1285.

7 Cheng, Y.-C., Chadha, K.C. and Hughes, R.G., Jr. (1977) Biochemical and immunological characterization of deoxythymidine kinase of thymidine kinaseless HeLa cells transformed by herpes simplex virus type 1. Infect. Immun. 16, 486–492.
8 Cheng, Y.-C. and Ostrander, M. (1976) Deoxythymidine kinase induced in HeLa tk^- cells by herpes simplex virus type I and type II. J. Biol. Chem. 251, 2605–2610.
9 Cheng, Y.-C., Grill, S. and Dutschman, G. (1979) Time-dependent action of 5-propyl-deoxyuridine as antiherpes simplex virus type 1 and type 2 agents. Biochem. Pharmacol. 28, 3529–3532.
10 Cremer, K., Bodemer, M. and Summers, W.C. (1978) Characterization of the mRNA for herpes simplex virus thymidine kinase by cell-free synthesis of active enzyme. Nucleic Acid Res. 5, 2333–2344.
11 Dubbs, D.R. and Kit, S. (1964) Mutant strains of herpes simplex deficient in thymidine-kinase-inducing activity. Virology 22, 493–502.
12 Garfinkle, B. and McAuslan, B.R. (1974) Regulation of herpes simplex virus-induced thymidine kinase. Biochem. Biophys. Res. Commun. 58, 822–829.
13 Gibson, W. and Roizman, B. (1974) Proteins specified by herpes simplex virus. X. Staining and radiolabelling properties of B capsid and virion proteins in polyacrylamide gels. J. Virol. 13, 155–165.
14 Harris-Hamilton, E. and Bachenheimer, S.L. (1985) Accumulation of herpes simplex virus type 1 RNAs of different kinetic classes in the cytoplasm of infected cells. J. Virol. 53, 141–151.
15 Honess, R.W. and Roizman, B. (1974) Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. 14, 8–19.
16 Honess, R.W. and Watson, D.H. (1974) Herpes simplex virus-specific polypeptides studied by polyacrylamide gel electrophoresis of immune precipitates. J. Gen. Virol. 22, 171–185.
17 Klemperer, H.G., Haynes, G.R. and Watson, D.H. (1967) A virus-specific thymidine kinase in BHK 21 cells infected by herpes simplex virus. Virology 31, 120–128.
18 Leiden, J.M., Buttyan, R. and Spear, P.G. (1976) Herpes simplex virus gene expression in transformed cells. I. Regulation of the viral thymidine kinase gene in transformed L cells by products of superinfecting virus. J. Virol. 20, 413–424.
19 Lin, T.-S., Gao, Y.-S. and Mancini, W.R. (1983) Synthesis and biological activity of various 3′-azido and 3′-amino analogues of 5-substituted pyrimidine deoxyribonucleosides. J. Med. Chem. 26, 1691–1696.
20 Lin, T.-S. and Prusoff, W.H. (1978) A novel synthesis and biological activity of several 5-halo-5′-amino analogues of deoxyribopyrimidine nucleoside. J. Med. Chem. 21; 106–109.
21 Lin, T.-S. and Prusoff, W.H. (1978) Synthesis and biological activity of several amino analogues of thymidine. J. Med. Chem. 21, 109–112.
22 Lin, T.-S., Neenan, J.P., Cheng, Y.-C. and Prusoff, W.H. (1976) Synthesis and antiviral activity of 5- and 5′-substituted thymidine analogs. J. Med. Chem. 19, 495–498.
23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
24 Mancini, W.R. and Lin, T.-S. (1983) Ribo- and deoxyribonucleoside effect on 3′-amino-2′,3′-dideoxycytidine induced cytotoxicity in cultured L1210 cells. Biochem. Pharmacol. 32, 2427–2432.
25 Otto, M.J., Lee, J.J. and Prusoff, W.H. (1982) Effects of nucleoside analogues on the expression of herpes simplex type 1 induced proteins. Antiviral Res. 2, 267–281.
26 Smiley, J.R., Wagner, M.J., Summers, W.P. and Summers, W.C. (1980) Genetic and physical evidence for the polarity of transcription of the thymidine kinase gene of herpes simplex virus. Virology 102, 83–93.
27 Summers, W.P., Wagner, M. and Summers, W.C. (1975) Possible peptide chain termination mutants in thymidine kinase gene of a mammalian virus, herpes simplex virus. Proc. Natl. Acad. Sci. U.S.A. 72, 4081–4082.
28 Williams, M.V. and Cheng, Y.-C. (1979) Human deoxyuridine triphosphate nucleotidohydrolase. J. Biol. Chem. 254, 2897–2901.