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Phosphorylation of nucleoside analogs by equine herpesvirus type 1 pyrimidine deoxyribonucleoside kinase

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Summary

Replication of equine herpesvirus type 1 (EHV-1) was sensitive to 9-(1,3-dihydroxy-2-propoxymethyl)guanine(DHPG) but relatively resistant to *E*-5-(2-bromovinyl)-2′-deoxyuridine (BVDU). Likewise, plaque formation by EHV-1 was inhibited by DHPG, but not by BVDU. Plaque formation by a thymidine kinase-negative (tk^-) mutant of EHV-1 was not inhibited by DHPG. In order to investigate biochemical mechanisms determining the differential sensitivity of EHV-1 to these drugs, the EHV-1-encoded thymidine kinase enzyme activity (TK)[1] was partially purified from EHV-1-infected cells and analyzed. The EHV-1-induced enzyme utilized both ATP and CTP as phosphate donors and differed in relative electrophoretic mobility from the TKs of mock-infected and HSV-1-infected cells. Phosphorylation of ^{3}H-dThd by the EHV-1 TK was inhibited by AraT, IdUrd, BVDU, and DHPG. The EHV-1 TK phosphorylated ^{125}I-dCyd and ^{3}H-ACV. The results indicate that EHV-1 encodes a pyrimidine deoxyribonucleoside kinase with broad nucleoside substrate specificity. These observations suggest that the failure of BVDU to inhibit EHV-1 replication is not attributable to an inability of the EHV-1 TK to phosphorylate BVDU, but may result from the incapacity of the viral TK to convert BVDU monophosphate to the triphosphate or from lack of inhibitory effect of BVDU triphosphate on viral DNA polymerase reactions.

pyrimidine deoxyribonucleoside kinase; iododeoxycytidine kinase; acyclovir phosphorylation; inhibition of thymidine phosphorylation; equine herpesvirus type 1 (EHV-1); inhibition of EHV-1 replication

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Abbreviations: tk, thymidine kinase gene; TK, thymidine kinase enzyme activity.

Introduction

Replication of equine herpesvirus type 1 (EHV-1) is sensitive to 9-(2-hydroxyethoxymethyl)guanine (acyclovir, ACV) and to 9-(1,3-dihydroxy-2-propoxymethyl)-guanine (DHPG), 2′-NDG, BIOLF-62, BW B759U) [6,34], but relatively resistant to *E*-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) [8]. This is surprising and paradoxical because: (i) previous studies have reported that the EHV-1-induced thymidine kinase enzyme (TK) catalyzes the phosphorylation of 1-β-D-arabinofuranosylthymine (araT), but no deoxycytidine (dCyd) [1,2,29]; (ii) herpes-virus-specific TKs which phosphorylate ACV and DHPG generally exhibit dCyd kinase activity; (iii) herpesvirus TKs which are devoid of dCyd kinase activity are not active with ACV and DHPG [7,8,11,15,17,22,36]; and (iv) an EHV-1 TK which phosphorylates araT might be expected to phosphorylate BVDU. To resolve the paradox, the TK induced by a Vero-adapted EHV-1(A183) strain has been partially purified and compared with partially purified TKs induced by herpes simplex virus type 1 (HSV-1) and bovine cytomegalovirus (bovine herpesvirus type 4 (BHV-4). Enzyme kinetic studies have been carried out to analyze the ability of various nucleoside analogs to inhibit competitively the phosphorylation of [^{3}H]thymidine (dThd). Enzyme assays utilizing [^{125}I]deoxycytidine (IdCyd) and [^{3}H]ACV as substrates were also carried out. The data presented clearly demonstrate that EHV-1 encodes a pyrimidine deoxyribonucleoside kinase with broad nucleoside substrate specificity and suggest that the failure of BVDU to inhibit EHV-1 replication is not attributable to lack of BVDU phosphorylation by the EHV-1 TK.

Materials and Methods

Cells

Vero (African green monkey kidney), Georgia bovine kidney (BK), and rabbit skin (RAB-9) cells were grown in Auto Pow Eagle's minimal essential medium (APMEM, Flow Laboratories, McClean, VA) supplemented with 10% bovine fetal serum (BFS), 20 mM bicarbonate, 2 mM glutamine, 10 mM Hepes buffer (pH 7.3), and 0.005% neomycin. Bromodeoxyuridine (BrdUrd)-resistant, TK$^-$RAB(BU) cell mutants were grown in the same medium as the parental rabbit skin (RAB-9) cells, but were supplemented with BrdUrd (25 μg/ml) (Sigma Chemical Co., St. Louis, MO), except for the passage preceding each experiment [25].

Viruses

A seed stock of the Vero-adapted EHV-1(A183) strain [33] was obtained from Professor Stewart McConnell, Department of Veterinary Microbiology and Parasitology, Texas A&M University System, College Station, TX. The Army 183 strain of EHV-1 was originally isolated from a horse at Front Royal, VA, in 1941. It was maintained by inoculation of horses for 27 passages by army veterinarians and by the Grayson Foundation Laboratories, University of Maryland [16]. The

virus was also passaged by direct inoculation of equine fetuses at the University of Kentucky. Subsequently, it was adapted to equine, rabbit, and monkey (Vero) tissue culture cells [3,4,9,10,33]. Stocks of EHV-1(A183) were prepared at the Baylor College of Medicine by inoculating Vero cells at an input multiplicity of infection (MOI) of about 0.01 PFU/cell and incubating for about 3 days, at which time extensive cytopathic effects (CPE) were observed. Plaque titrations were performed in Vero cells at 34.5°C, as described [18,19,23,25]. Previous studies [1] and the data presented indicate that EHV-1(A183) is a thymidine kinase-positive (tk^+) herpesvirus.

EHV-1(H6b) is an araT-resistant, BrdUrd-resistant, tk^-EHV-1 mutant derived from EHV-1(A183) by serially passaging EHV-1(A183) at 34.5°C in Vero cells with gradually increasing concentrations of araT (from 2.5 to 200 μg/ml) and BrdUrd (from 5 to 20 μg/ml), as previously described for the isolation of BrdUrd-resistant, tk^-bovine herpesvirus type 1 (BHV-1) mutants [25]. The virus was plaque-purified twice in Vero cells at 34.5°C, first in the presence of 100 μg/ml araT and next in the presence of 20 μg/ml BrdUrd, and once in RAB(BU) cells in the presence of 20 μg/ml BrdUrd. Then virus stocks were prepared for experiment in Vero cells. Absence of TK-inducing activity by EHV-1(H6b) was demonstrated by TK assays using cytosol extracts from EHV-1(H6b)-infected RAB(BU) cells, by studying the putative incorporation of ^{3}H-dThd into nuclear DNA of virus-infected cells, and by thymidine plaque autoradiography (Kit, S., Kit, M. and Ichimura, H., unpublished experiments).

EHV-1(pEP) is a tk^+ recombinant virus obtained by cotransfecting infectious tk^-EHV-1(H6b) DNA and tk^+EHV-1(A183) DNA fragments in Vero cells. Progeny tk^+viruses were then selected in RAB(BU) cells with growth medium containing 10^{-4} M hypoxanthine, 10^{-6} M aminopterin, 4×10^{-5} M thymidine, and 10^{-5} M glycine (HATG medium) [22]. After plaque purification in Vero cells, stocks of EHV-1(pEP) were prepared for experiment and plaque-titrated in Vero cells at 34.5°C.

Stocks of HSV-1(KOS) and bovine herpesvirus type 4 were propagated at low multiplicity in owl monkey kidney and in BK cells, respectively, as described [19,21].

Neutralization of EHV-1 infectivity by EHV-1 antisera

Virus neutralization tests were done by incubating equal parts of horse anti-equine herpesvirus rhinopneumonitis type 1 (Kentucky D strain) sera (Wellcome Research Laboratories, Kansas, MO) (1:8, 1:32, and 1:128) plus virus (EHV-1(A183) or EHV-1(pEP), each diluted to 4×10^3 PFU/ml in APMEM containing 10% BFS), and diluent (PBS dilution fluid) plus virus for 1 h at 37°C. The samples were then inoculated onto monolayer cultures of Vero cells in 6-well tissue culture cluster plates (Costar, Cambridge, MA) at 0.1 ml/well, and incubated for 1 h at 37°C. The cultures were overlaid with 2.5 ml growth medium containing 1% agar, and further incubated at 34.5°C for 3–4 days, at which time a second 1% agar overlay with 0.005% neutral red was added. Plaques were counted the following day.

Inhibition of plaque formation by antiviral drugs

Plaque reduction tests were performed on monolayers of Vero cells in 6-well tissue culture cluster plates. The cells were infected with 0.1 ml of EHV-1 (about 100 or 200 PFU/well), and thereafter incubated for 3 days at 34.5°C in growth medium containing a 1% agar overlay, and DHPG or BVDU. The virus inocula also contained DHPG and BVDU at the appropriate drug concentrations. After the addition of a second agar overlay, plaque counts were made as above.

Effect of DHPG on the replication of tk^+ and tk^-EHV-1 strains

One-day-old monolayer cultures of Vero cells (4.1×10^6 cells in 4-ounce prescription bottles) were infected at a multiplicity of infection (MOI) of 0.05 PFU/cell with tk^+EHV-1(A183), tk^+EHV-1(pEP), or tk^-EHV-1(H6b) for 1 h at 37°C. The virus inocula contained DHPG at the appropriate drug concentration. The virus inocula were then removed, the monolayers were washed with growth medium containing DHPG, and 10 ml of fresh growth medium containing either 0, 10, or 100 μM DHPG were added. The cultures were further incubated at 34.5°C until 4, 24, and 38 h postinfection, and virus harvests were prepared and titrated in Vero cells at 34.5°C.

Preparation and analysis of EHV-1 DNA

The DNA from tk^+ and tk^-EHV-1 strains were isolated from infected Vero cells using the Triton X-100 method of Pignatti et al. [32], and purified by equilibrium centrifugation in CsCl gradients as described [24]. The purified DNA preparations were digested with EcoRI, BamHI, or Bg1II (New England BioLabs, Inc., Beverly, MA); the DNA fragments were separated on 0.6% agarose gels, stained with 0.5 μg/ml of ethidium bromide, visualized over a long-range UV-illuminator, and photographed.

Enzyme assays

Cytosol extracts were prepared from mock-infected and from virus-infected cells and assayed for nucleoside phosphorylating activities with either ATP or CTP as the phosphate donors and [^{3}H]thymidine (about 50 Ci/mmol) (ICN Pharmaceuticals, Inc., Irvine, CA), [^{125}I]deoxycytidine (2200 Ci/mmol) (New England Nuclear, Boston, MA) or [^{3}H]acyclovir (19.8 Ci/mmol) (New England Nuclear, Boston, MA) as the nucleoside acceptors [23]. When [^{3}H]thymidine was the substrate, the phosphorylated products were separated from the [^{3}H]thymidine by chromatography on Whatman DE-81 diethylaminoethyl cellulose paper (0.4 mEq./g) with 4 M formic acid and 0.1 M ammonium formate as the solvent. The radioactive dTMP spot was cut from the paper and counted with a Packard Tri-Carb liquid scintillation spectrometer [26]. In enzyme assays using [^{125}I]deoxycytidine and [^{3}H]acyclovir as substrates, the reaction products were separated by thin-layer chromatography on PEI-cellulose F precoated TLC plastic sheets (EM Laboratories, Elmsford, N.Y.) with sodium formate as the solvent [20]. With 0.2 M sodium formate, pH 3.2, as the solvent, the R_F values of dCMP, dUMP, IdCMP, and IdUMP were 0.40, 0.35, 0.32, and 0.27, respectively. With 0.5 M sodium for-

mate, pH 3.4 as the solvent, the R_F values of acyclovir and acyclovir monophosphate were about 1.00 and 0.39, respectively.

The virus-induced TK activity was partially purified by slowly adding ammonium sulfate (24.3 mg/ml) to cytosol extracts to precipitate the enzyme activity, stirring for 30 min at 4°C, and collecting the precipitate by centrifugation. The precipitate was dissolved in one-fourth the original volume of the cytosol extract in a solution consisting of 0.15 M KCl, 0.01 M Tris-HCl buffer (pH 8.0), 0.003 M 2-mercaptoethanol, 0.0025 M ATP, 0.00125 M $MgCl_2$, 0.05 M ε-aminocaproic acid, and 5% (v/v) glycerol, and utilized for enzyme experiments. This procedure resulted in a 3–5-fold increase in the specific activity of the TK enzymes. Polyacrylamide gel electrophoresis analyses of TK enzyme activities were carried out as described previously [23], except that 5% polyacrylamide slab gels (14 × 16 × 0.075 cm) were used, and the samples were electrophoresed at 185 V and about 40 mA for about 3 h at 4°C. At the conclusion of the run, the gel was cut vertically with a razor blade to separate the lanes and 0.2 cm slices were assayed for 1 h at 38°C with TK reaction mixture containing [^{3}H]thymidine as a nucleoside substrate.

Results

EHV-1 strains

To verify that an authentic equine rhinopneumonitis virus (equine herpesvirus type 1) was being used in these experiments, restriction endonuclease analyses of viral DNAs and virus neutralization experiments with EHV-1-specific antisera were carried out. The analyses with *Eco*RI, *Bam*HI, and *Bgl*II on the DNAs of the EHV-1(A183), EHV-1(H6b), and EHV-1(pEP) strains demonstrated that the restriction nuclease profiles were similar to those previously published for the aborto-

TABLE 1

Effect of BVDU and DHPG on plaque formation by EHV-1(A183) strains in Vero cells[a]

Drug concentration (μM)	Number of plaques formed by				
	tk$^+$ EHV-1(A183)		tk$^+$ EHV-1(pEP)		tk$^-$ EHV-1 (H6b)
	BVDU	DHPG[b]	BVDU	DHPG[b]	DHPG
0	76	76	170	170	>175
1	–	51	–	150	>175
2	–	–	–	–	–
5	–	39	–	116	>175
10	76	24	171	87	–
20	–	14	–	44	>175
25	67	–	–		
50	70	0	146		

[a] No toxicity on uninfected Vero cell monolayers was observed at 125 μM DHPG or at 50 μM BVDU.

[b] Plaques formed on dishes with DHPG were very small compared with plaques formed in absence of the drug.

genic (fetal, subtype 1) EHV-1 (Army-183) [3,35] and differed from the restriction nuclease profiles of the respiratory EHV-1, subtype 2 strain. The virus neutralization tests showed that anti-EHV-1 (KyD) sera at a dilution of 1:128 inhibited plaque formation of EHV-1(A183) and EHV-1(pEP), respectively, by 100% and 98%. No plaques were observed with antisera used at a dilution of 1:32. These experiments definitely indicate that the viruses were indeed authentic EHV-1 strains.

Effect of antiviral drugs on the replication of EHV-1

Plaque formation by tk^+ EHV-1(A183) and tk^+EHV-1(pEP) in Vero cells was inhibited by DHPG, but not by BVDU (Table 1). Likewise, replication of tk^+EHV-1(A183) in Vero cells was inhibited by DHPG (Fig. 1). On the other hand, plaque formation and replication of tk^-EHV-1(H6b) was not inhibited by concentrations of DHPG which inhibited the replication of the tk^+ viruses. These results confirm previous studies [7,8,34] and suggest that the EHV-1-specific TK has a role in the inhibition by DHPG of virus replication.

Properties of EHV-1-induced TK

To verify that the EHV-1-induced TK has a role in the inhibition by DHPG of tk^+EHV-1 replication, and to investigate whether the EHV-1-induced enzyme might catalyze the phosphorylation of BVDU, the EHV-1-induced enzyme was partially purified and characterized. RAB(BU) cells, which lack cytosol TK activity, were employed as host cells for virus infections. The TK activity partially purified from cytosol extracts at 16 h after infection of RAB(BU) cells with EHV-1(pEP) or EHV-1(A183) utilized ATP and CTP equally well in the [^{3}H]dThd (0.01 mM) phosphorylation reaction. With either 8 mM ATP or CTP as phosphate donor, the TK activity expressed as picomoles of [^{3}H]dTMP formed in 10 min at

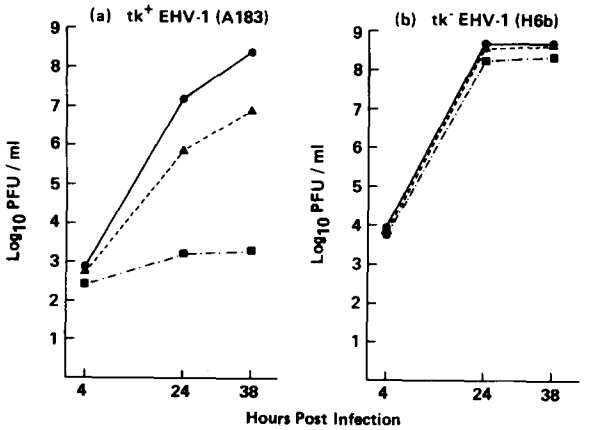

Fig. 1. Effect of DHPG on the replication in Vero cells of (a) tk^+ EHV-1(A183) and (b) tk^- EHV-1(H6b). Symbols are: no drug control (●——●), 10 μM DHPG (▲----▲), and 100 μM DHPG (■-·-·-■).

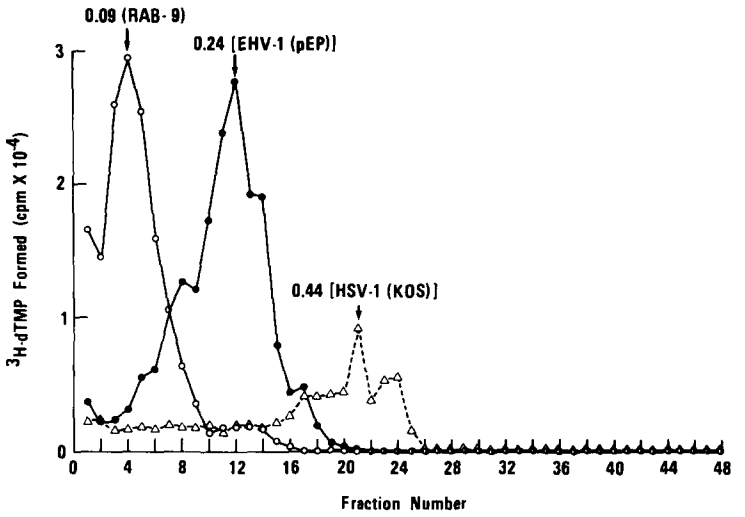

Fig. 2. Electrophoresis on 5% polyacrylamide slab gels at pH 8.9 of cytosol TK activities from mock-infected rabbit skin (RAB-9) cells (○——○), EHV-1(pEP)-infected RAB(BU) cells ((●——●), and HSV-1(KOS)-infected RAB(BU) cells (△----△). Numbers above the peaks signify electrophoretic mobilities (R_m relative to the tracking dye.

38°C/μg protein was about 3.1. This confirms previous studies which have shown that the EHV-1-induced TK, like many other herpesvirus-induced TKs, and unlike the cytosol TKs of unifected cells, efficiently utilize CTP in place of ATP as a phosphate donor [1,14,17,21,23,29].

Analyses using 5% polyacrylamide slab gels under nondenaturing conditions demonstrated that the relative electrophoretic mobility (R_m) of the EHV-1(pEP)

TABLE 2

Iododeoxycytidine (IdCyd) kinase, acyclovir (ACV) kinase, and thymidine kinase activities of partially purified cytosol extracts of uninfected rabbit skin (RAB-9) and herpesvirus-infected RAB(BU) cells[a]

Source of enzyme	IdCyd kinase activity[b]	ACV kinase activity[c]	dThd kinase activity[d]
RAB-9(mock-infected)	110	<20	7.4
HSV-1(KOS)-infected	14 000	4 120	9.1
EHV-1-infected	2 400	272	3.4
BHV-4(N124)-infected	73	27	6.6

[a] Cytosol TK extracts were prepared as described and partially purified by $(NH_4)_2SO_4$ precipitation at 40% saturation. The ^{125}I-dCyd kinase reaction mixture contained 3.2×10^{-3} μM ^{125}I-dCyd as phosphate acceptor in a total volume of 0.125 ml. Carrier-free ACV (side chain - 2 ^{3}H); 19.8 Ci/mmol was used for the ACG kinase reaction.

[b] cpm ^{125}I-dCMP formed in 10 min at 38°C per μg protein.

[c] cpm ^{3}H-ACV monophosphate formed in 40 min at 38°C per μg protein.

[d] pmol dTMP formed in 10 min at 38°C per μg protein.

TABLE 3

Inhibition by nucleoside analogs of the phosphorylation of ^{3}H-dThd by EHV-1-induced thymidine kinase (TK) activity[a]

Drug concentration (μM)	% inhibition of ^{3}H-dThd phosphorylation in presence of		
	BVDU	AraT[b]	IdUrd[b]
0	0 (control)[c]	0 (control)[c]	0 (control)[c]
10	–	–	86
20	36	–	92
40	60	18	97
50	74	35	99
160	83	59	–
320	–	79	–

[a] Partially purified enzyme from EHV-1(pEP)-infected RAB(BU) cells was assayed with ^{3}H-dThd at 10 μM for 10 min at 38°C. The specific activity of the ^{3}H-dThd substrate was 527 cpm/pmol.

[b] AraT was obtained from Yamasa Shoyu Co., Choshi, Japan. IdUrd was purchased from Sigma Chemical Co., St. Louis, MO.

[c] Control (no drug) TK activity was 233 cpm per μg protein.

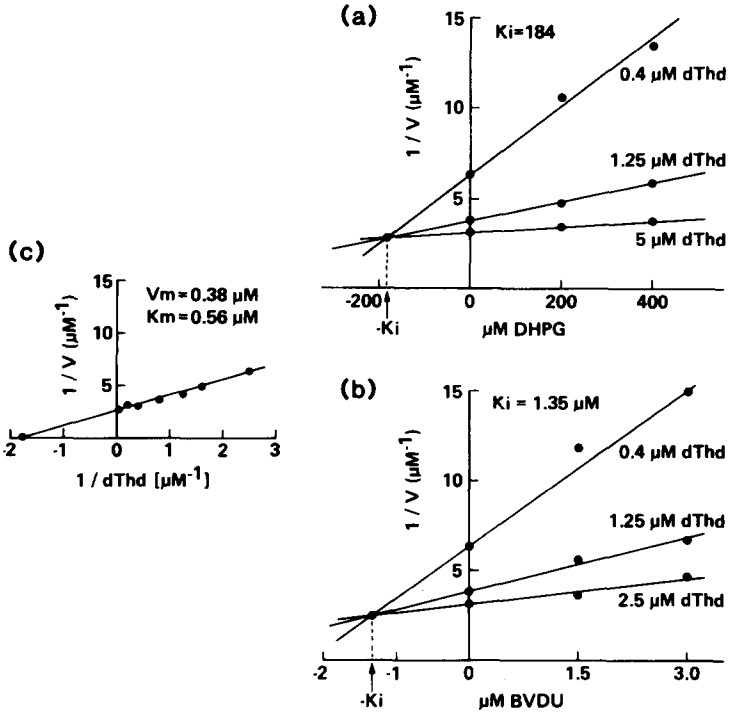

Fig. 3. Dixon plots showing the inhibition by (a) DHPG and (b) BVDU of the thymidine phosphorylation reaction catalyzed by a partially purified EHV-1(pEP)-induced enzyme. Initial reaction rates were measured by incubating different concentrations of ^{3}H-dThd and nucleoside analogs plus ATP (8 mM) and Mg^{2+} (4 mM) for 3 min at 38°C. The inset (c) also shows a Lineweaver-Burk plot for the determination of the dThd K_m value.

TK was greater than that of the cytosol TK of uninfected RAB-9 cells, but less than the R_m value of the HSV-1-induced TK (Fig. 2). This observation likewise demonstrates the virus specificity of the EHV-1-induced TK activity [1,22,23,25].

The capacity of the partially purified enzyme activity from the cytosol fraction of EHV-1(A183)-infected RAB(BU) cells to phosphorylate ^{125}I-dCyd and [^{3}H]-acyclovir (ACV) was next compared with the activities of enzymes partially purified from mock-infected RAB-9 cells and from HSV-1- and BHV-4(N124)-infected RAB(BU) cells (Table 2). The enzymes from the mock infected RAB-9 and the BHV-4(N124)-infected RAB(BU) cells failed to phosphorylate ^{125}I-dCyd and ^{3}H-ACV [21]. In contrast, the enzymes from HSV-1-infected RAB(BU) cells effectively phosphorylated the nucleoside analogs. Furthermore, the phosphorylation of ^{3}H-ACV by the partially purified enzyme induced by EHV-1(pEP) was inhibited 70% by nonradioactive (10 μM) dThd, indicating that the EHV-1 TK activity was primarily responsible for ^{3}H-ACV phosphorylating activity [30]. All of the enzymes exhibited high levels of ^{3}H-dThd phosphorylating activity.

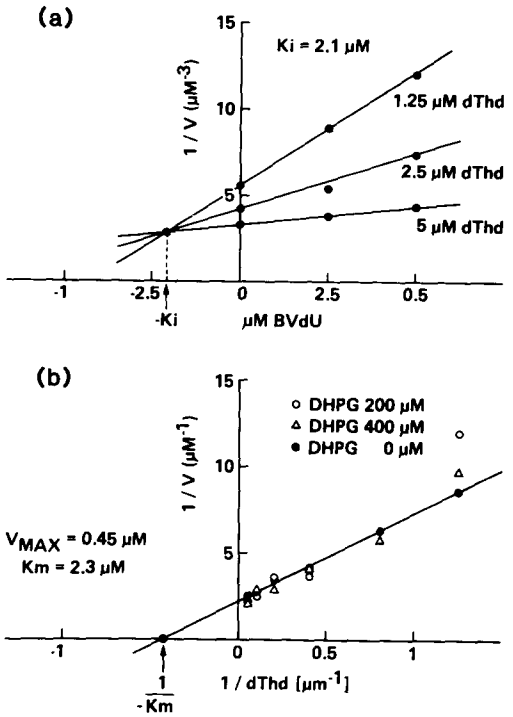

Fig. 4. Dixon plot showing the inhibition by (a) BVDU of the thymidine phosphorylation reaction catalyzed by a partially purified BHV-4(N124)-induced enzyme. Initial reaction rates were measured by incubating different concentrations of ^{3}H-dThd and BVDU plus ATP (8 mM) and Mg^{2+} (4 mM) for 3 min at 38°C. Lineweaver-Burk plots (reciprocal of velocity versus reciprocal of dThd concentration) are also shown in (b). The plot in (b) illustrates results obtained with different ^{3}H-dThd concentrations plus no DHPG (•——•), 200 μM DPHG (○——○), and 400 μM DPHG (△——△).

Inhibition of ^{3}H-dThd phosphorylation by nucleoside analogs

To investigate further the nucleoside substrate specificity of the EHV-1-induced TK activity, the effect of nucleoside analogs on the phosphorylation of ^{3}H-dThd by the EHV-1(pEP)-induced enzyme was studied (Table 3). Nonradioactive IdUrd strongly inhibited the phosphorylation of ^{3}H-dThd. Unlabeled AraT, although less effective than IdUrd, also inhibited the ^{3}H-dThd phosphorylation (14). Significantly, nonlabeled BVDU likewise proved to be a concentration-dependent inhibitor of ^{3}H-dThd phosphorylation.

K_m and K_i values for partially purified TKs

Dixon plots showing the inhibition by DHPG and BVDU of the phosphorylation of ^{3}H-dThd by the EHV-1(pEP)-infected RAB(BU) cells and a Lineweaver-Burk plot for the determination of the K_m value for dThd are presented in Fig. 3. Similar Dixon and Lineweaver-Burk plots for TK activities partially purified from BHV-1(N124)- and HSV-1(KOS)-infected RAB(BU) cells are also given in Figs. 4 and 5, respectively. Table IV summarizes data on the kinetic constants for these

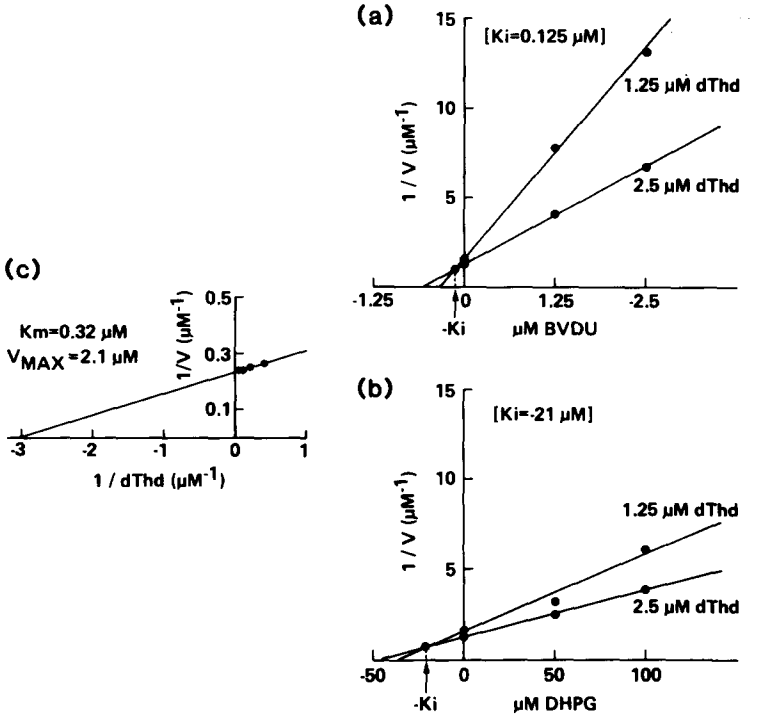

Fig. 5. Dixon plots showing the inhibition by (a) BVDU and (b) DHPG of the thymidine phosphorylation reaction catalyzed by a partially purified HSV-1(KOS)-induced enzyme. Initial reaction rates were measured by incubating different concentrations of ^{3}H-dThd and nucleoside analogs plus ATP (8 mM) and Mg^{2+} (4 mM) for 3 min at 38°C. The inset (c) also shows a Lineweaver-Burk plot for the determination of the dThd K_m value.

TABLE 4

Kinetic constants of various nucleosides as substrates and inhibitors of partially purified TK activities from herpesvirus-infected RAB(BU) cells

Herpesvirus TK	K_m(dThd)	V_{max}(dThd)	K_i(BVDU)	K_i(DHPG)
HSV-1(KOS)	0.32[a]	2.10	0.09	15
BHV-4(N124)	2.10	0.45	2.20	b
EHV-1(A183)	0.58	0.69	1.35	340
EHV-1(pEP)	0.54	0.37	1.32	184

[a] Kinetic constants (expressed as μM) are the means from two experiments with the HSV-1(KOS)-, EHV-1(pEP)-, and BHV-4(N124)-induced TKs and from one experiment with the EHV-1(A183)-induced TK.

[b] No inhibition detected (see Fig. 4).

enzymes. The data show that: (i) BVDU is a competitive inhibitor of dThd for the HSV-1-, BHV-4-, and EHV-1-induced ^{3}H-dThd phosphorylating enzyme; (ii) DHPG is a competitive inhibitor of HSV-1- and EHV-1-induced ^{3}H-dThd phosphorylating enzyme, but not the BHV-4(N124)-induced enzyme; (iii) the dThd K_m values for the HSV-1-induced TK is about 57% of that of the EHV-1-induced TK, but about one-seventh that of the BHV-4-induced TK; (iv) the V_{max} (dThd) value for the HSV-1-induced TK is about 4 times as great as those of the BHV-4- and EHV-1-induced TKs; (v) the K_i (BVDU) values for the EHV-1- and BHV-4-induced TKs are more than 10 and 20 times as great as the K_i (BVDU) value for the HSV-1-induced TK; and (vi) the K_i (DHPG) value for the EHV-1-induced TK is more than 10 times as great as that of the HSV-1-induced TK.

Discussion

The experiments show that replication of tk$^+$EHV-1 in Vero cells is sensitive to DHPG, but relatively resistant to BVDU. On the other hand, replication of BrdUrd-resistant and AraT-resistant tk$^-$EHV-1 in Vero cells is insensitive to DHPG inhibition, indicating that DHPG inhibition requires a functional virus-specific TK activity.

The TK activity induced by tk$^+$EHV-1 was partially purified and its properties were compared with those of TK enzymes induced by other herpesviruses. The EHV-1-induced TK activity resembled other herpesvirus-induced TKs in electrophoretic mobility and in phosphate donor specificity. It had an electrophoretic mobility on polyacrylamide gels distinctively different from the major cytosol TK of uninfected cells and it utilized ATP and CTP equally well as phosphate donors [17]. Recent experiments have also shown that the molecular weight of the EHV-1 TK polypeptide predicted from nucleotide sequencing data is about 36000 (Kit, S., Sheppard, M. and Kit, M., unpublished experiments). Since the molecular weight of the native enzyme is about 80000 [2], this shows that the EHV-1 TK, like other herpesvirus-encoded TKs, is composed of two subunits [17].

Phosphorylation of ^{3}H-dThd by the EHV-1 TK was inhibited by AraT and IdUrd,

as expected. The phosphorylation of ^{3}H-dThd by the EHV-1 TK partially purified from RAB(BU) cells was also competitively inhibited by DHPG. In addition, the partially purified enzyme catalyzed the phosphorylation of ^{3}H-ACV and ^{125}I-dCyd. Allen and coworkers [2] have previously reported that phosphorylation of ^{3}H-dCyd by EHV-1 could not be demonstrated, even with dCyd concentrations (100 μM) equivalent to 20 times the K_m value of the enzyme for dThd. McGowan et al. [29] reported that dCyd was not a substrate for the EHV-1 TK of biochemically transformed mouse cells. The same research group [28] found that the EHV-3 TK could not phosphorylate deoxycytidine, as determined by a filter disk assay method with ^{3}H-dCyd, but that some phosphorylation was detectable with a γ-^{32}P assay of enzyme activity. It seems likely that the use of the highly sensitive ^{125}I-dCyd system for the assay of dCyd kinase activity [36] accounts for the successful demonstration of an EHV-1-induced dCyd kinase activity in the present study, as opposed to the negative results reported by Allen and associates [2]. The present findings that the EHV-1-induced enzyme catalyzes the phosphorylation of IdCyd, DHPG, and ACV is consistent with the observations that herpesvirus TKs which phosphorylate DHPG and ACV generally exhibit dCyd kinase activity, while herpesvirus TKs which are devoid of dCyd kinase activity are not active with DHPG and ACV [15,17]. The experiments indicating that the EHV-1 TK exhibits IdCyd, ACV, and DHPG phosphorylation activities are also consistent with the finding that EHV-1 replication is sensitive to DHPG [34] (Table 1 and Fig. 1). Thus, replication of HSV-1, HSV-2, and varicella zoster virus are all sensitive to DHPG inhibition; these viruses, like EHV-1, encode TKs which phosphorylate IdCyd, ACV, and DHPG. On the other hand, pseudorabies virus, BHV-1, BHV-4, and *Herpesvirus saimiri* induce TKs which fail to utilize dCyd, ACV, and DHPG efficiently as nucleoside substrates, and replication of these herpesviruses is relatively insensitive to DHPG and ACV [7,8,15,21].

The present experiments clearly show that phosphorylation of ^{3}H-dThd by the EHV-1-induced TK activity is competitively inhibited by BVDU. The K_i value for the EHV-1-induced enzyme was about 13 times as great as that of the HSV-1-induced TK activity, but less than that of the BHV-4-induced TK activity (Figs. 3–5). The data summarized in Table 4 further illustrate the generalization that the herpesvirus TKs are often less fastidious than cellular TKs with regard to nucleoside substrate specificities, but, nevertheless, exhibit individual differences in their ability to phosphorylate nucleoside analogs [17]. For example, the K_i(BVDU)/K_m(dThd) ratios for the HSV-1-, BHV-4-, and EHV-1-induced enzymes were about 0.3, 1.0, and 2.4, respectively. The K_i(DHPG)/K_m(dThd) ratios for the HSV-1- and EHV-1-induced TKs were 47 and about 341–600, respectively. The K_i(DHPG)/K_m(dThd) ratio for the BHV-4-induced TK was indeterminate.

The data presented in this report indicate that EHV-1 induces a pyrimidine deoxyribonucleoside with broad nucleoside substrate specificity. The EHV-1-induced enzyme resembles the TKs induced by HSV-1, HSV-2, and varicella zoster virus more closely than the TKs induced by pseudorabies virus, BHV-1, BHV-4, and *Herpesvirus saimiri*. The results further suggest that the failure of BVDU to inhibit EHV-1 replication is not attributable to a lack of a BVDU phosphorylation

activity by the EHV-1 TK. How then is the inactivity of BVDU against EHV-1 replication to be explained?

The selective antiherpes activity of BVDU depends, in part, on a specific phosphorylation by a virus-induced TK. However, to inhibit virus replication and/or infectivity, the BVDU monophosphate must also be phosphorylated to the triphosphate [7,8]. In its triphosphate form, BVDU might interfere with DNA synthesis at the DNA polymerase level [5] by: (i) competing with dTTP for incorporation into DNA; (ii) binding to viral DNA polymerases forming inactive complexes [12]; (iii) being incorporated into replicating DNA, thereby affecting its stability and/or function [27]; and (iv) inducing unbalanced deoxyribonucleoside triphosphate pools through allosteric effects on ribonucleoside diphosphate reductase and/or dCMP deaminase. In addition, BVDU triphosphates may inhibit virus replication or infectivity by interfering with the formation of glycosylated viral envelope glycoproteins [31]. Thus, the intensity of EHV-1 replication to BVDU inhibition may result from an inability of enzymes in EHV-1-infected cells to phosphorylate BVDU monophosphate or from lack of inhibitory effects of BVDU triphosphate at the level of EHV-1 DNA polymerase function or viral glycoprotein synthesis. It is well known that about 150–200-fold higher concentrations of BVDU are required to inhibit HSV-2 replication than HSV-1 replication [7]. This differential sensitivity of HSV-1 and HSV-2 tot BVDU has been at least partially explained by a failure of HSV-2-infected cells to convert the 5′-monophosphate of BVDU to the 5′-diphosphate and 5′-triphosphate [13]. It remains to be seen whether the insensitivity of EHV-1 replication to BVDU also results from the fact that phosphorylation stops at the 5′-monophosphate level. Experiments utilizing ^{125}I-labeled (*E*)-5-(2-iodovinyl)-2′-deoxyurdine (IVDU) have been initiated to determine whether the triphosphate of IVDU is efficiently formed in cells infected with tk$^+$EHV-1 strains and will be the subject of a future report.

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