Lack of stereospecificity of suid pseudorabies virus thymidine kinase

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We have partially purified suid pseudorabies virus (PRV) thymidine kinase from infected thymidine kinase$^-$ mouse cells, and cytosolic swine thymidine kinase from lymphatic glands, and we have found that PRV thymidine kinase, unlike the host enzyme, shows no stereospecificity for D- and L-β-nucleosides. *In vitro*, unnatural L-enantiomers, except L-deoxycytidine, function as specific inhibitors for the viral enzyme in the order: L-thymidine $\gg$ L-deoxyguanosine > L-deoxyuridine > L-deoxyadenosine. Contrary to human and swine thymidine kinases and like herpes simplex virus-1 and -2 thymidine kinases, PRV thymidine kinase phosphorylates both the natural (D-) and the unnatural (L-) thymidine enantiomers to their corresponding monophosphates with comparable efficiency. The kinetic parameters $V_{max.}/K_m$ for D- and L-thymidine are 3.7 and 2.3 respectively. Our results demonstrate that the lack of stereospecificity might be a common feature of the thymidine kinases that are encoded by human and animal herpes viruses. These observations could lead to the development of a novel class of antiviral drugs.

INTRODUCTION

Thymidine kinase (TK) catalyses the phosphorylation of thymidine with ATP as the conventional phosphate donor. Although TK is only involved in the salvage pathway of nucleotide biosynthesis and is therefore not strictly required for normal cell growth, it is a widespread and common enzyme that is absent only from selected mutants of cells and viruses.

Cellular TK has a high substrate specificity and only phosphorylates deoxyuridine (dU) and its 5-substituted derivatives such as thymidine (5-methyl-dU) and the 5-halogenated derivatives (5-chloro, 5-bromo, 5-iodo and 5-fluoro). Other deoxynucleosides and ribonucleotides are not phosphorylated to any significant extent. Herpes simplex virus (HSV) TKs differ from the cellular TK both in their substrate specificity and in the phosphate donors that they can use. In fact, the viral enzymes can also phosphorylate deoxycytidine and a wide range of pyrimidine and purine analogues such as acyclovir and (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (Prusoff, 1959; Elion et al., 1977; De Clercq et al., 1979). These and related substrate analogues of viral TKs, once phosphorylated to form triphosphates by cellular enzymes, interfere with viral DNA polymerase activity and therefore with viral DNA replication (Derse et al., 1981; De Clercq et al., 1986). More recently, non-substrate inhibitors of viral TKs have also been synthesized (Nutter et al., 1987; Focher et al., 1988, 1989; Martin et al., 1989a, 1989b; Spadari and Wright, 1989; Hildebrand et al., 1990; Nsiah et al., 1990; Gambino et al., 1992) and they were shown to prevent viral reactivation from latency (Leib et al., 1990). These peculiar biochemical properties made viral TKs an important target for the development of anti-herpetic drugs.

Recently, we have also demonstrated that HSV1 TK not only shows a low substrate specificity but also does not show stereospecificity for D- and L-β-nucleosides (Iotti et al., 1990; Spadari et al., 1992). In fact, unlike the human TK, which only recognizes the natural substrate D-thymidine (D-T), the HSV1 TK phosphorylates L-thymidine (L-T) and D-T with the same efficiency (Spadari et al., 1992). Similar results have been obtained in our laboratory with HSV2 TK (G. Maga, A. Verri, A. Garbesi, D. Niccolai, S. Spadari and F. Focher, unpublished work). We have also shown that L-T reduces the incorporation of exogenous [^{3}H]D-T into cellular DNA in HeLa TK$^-$/HSV1 TK$^+$ cells but not in wild-type HeLa cells, without affecting RNA synthesis, protein synthesis or cell growth and viability, and also reduces HSV1 multiplication in HeLa cells (Spadari et al., 1992). To determine whether the lack of stereospecificity is a common property of herpes virus TKs, we have extended our studies to the pseudorabies virus (PRV) TK. PRV (also known as Aujeszky's disease virus or suid herpes virus-1) is an α-herpes virus that is responsible for serious disease in domestic and wild animals (Baskerville et al., 1973). In swine, Aujeszky's disease causes severe neurological and respiratory syndromes, which lead to considerable economic losses in most of the world. The natural infection by PRV is similar to that of HSV1 in humans, since both viruses can establish latent infection in trigeminal ganglia. Viral TK is specifically required for HSV1 and PRV reactivation from neuronal ganglia (Leib et al., 1990; Nsiah et al., 1990; Volz et al., 1992), where endogenous TK is constitutively not expressed after birth. Furthermore several lines of evidence indicate that PRV TK could also be important for the virulence of PRV (Kit et al., 1985; McGregor et al., 1985; Shibata et al., 1991).

Chemotherapy against herpes virus infections, based on the use of substrate analogues for TK, is known to be successful. In the case of PRV infections this approach has been neglected for practical reasons, and the current eradication programmes are based on the development of vaccines. However several problems with the vaccine approach (such as mutation and genetic recombination resulting in the restoration of virulence in the vaccine) make it worthwhile to develop the chemotherapeutic

Abbreviations used: DMEM, Dulbecco's modified essential medium; DTT, dithiothreitol; HSV, herpes simplex virus; NP-40, Nonidet P-40; PMSF, phenylmethanesulphonyl fluoride; PRV, pseudorabies virus; D-, L-T, D, L-thymidine; TK, thymidine kinase.

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approach as well (Field, 1985; Veerisetty et al., 1990), in particular for domestic animals.

The low cytotoxicity and high specificity of L-T could make this compound a useful antiviral agent, not only for human but also for animal herpes viruses, if their TKs also show no stereospecificity.

METHODS

Chemicals

[^{3}H]D-T (30 Ci/mmol) was from Amersham. [^{3}H]L-T (6 Ci/mmol) was from Moravek Biochemicals. [^{14}C]TMP (40 mCi/mmol) was from ICN Flow. Phenylmethanesulphonyl fluoride (PMSF) was from Sigma. DEAE-Sepharose Fast Flow and Mono-Q columns were from Pharmacia. All other chemicals and reagents were purchased from local suppliers.

L-Nucleosides

L-Nucleosides were synthesized by the methods of Spadari et al. (1992).

TK assay

The viral, human and swine TKs were assayed as follows: 0.02 units of enzyme were incubated at 37 °C for 30 min in 25 μl of a mixture containing 30 mM Hepes-K^+ (pH 7.5), 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM dithiothreitol (DTT) and 3.3 μM [^{3}H]D-T (1500 c.p.m./pmol) or 10 μM [^{3}H]L-T (660 c.p.m./pmol). The reaction was terminated by spotting 20 μl of the incubation mixture on to a 25 mm DEAE paper disc (DE-81 paper; Whatman). The disk was washed twice in an excess of 1 mM ammonium formate, pH 5.6, to remove unconverted nucleoside, and then once in distilled water and finally once in ethanol. Radioactive TMP was estimated by scintillation counting in Omnifluor (NEN). One unit of TK is defined as the amount of enzyme that converts 1 nmol of thymidine into TMP per h under the above assay conditions.

Thymidylate-kinase assay

PRV TK (0.02 units) were incubated at 37 °C for 60 min in 15 μl of a mixture containing 30 mM Hepes-K^+ (pH 7.5), 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM DTT and 50 μM [^{14}C]TMP (1000 c.p.m./pmol). Aliquots of 3 μl were spotted at 0, 10, 20, 40 and 60 min on to a polyethyleneimine–cellulose F t.l.c. plastic sheet (Merck). The sheet was developed in 0.5 M NH_4 formate, pH 3.6, and the position of the labelled TMP and TDP was revealed by autoradiography. The sheet was cut and the amount of radioactive DTP that had been formed was estimated by scintillation counting in an Omnifluor (NEN) mixture.

Determination of kinetic parameters (K_m and $V_{max.}$)

Analysis of substrate kinetics was done using the Michaelis–Menten equation in the form: $v = V_{max.} - K_m (v/S)$. Values of K_m and $V_{max.}$ were calculated using the 'Enzyme Kinetics 1.0' program (Star Software) for the Apple Macintosh by a two-step procedure: firstly, a direct linear estimate was obtained (Cornish-Bowden and Eisenthal, 1974) and then a maximum likelihood estimate was made. K_i was determined according to the equation $K_i = I/[K_p/K_m) - 1]$, where I is the concentration of the inhibitor and K_p is the slope of the curve derived from the Hofstee plot at each I value.

Cells and viruses

The mouse cell line LM (TK$^-$) was used; the cells were maintained at 37 °C in Dulbecco's modified essential medium (DMEM) containing 10% (v/v) fetal-calf serum. The wild-type strain of PRV was used in all the experiments.

Culture of cells for the preparation of the viral TK

LM (TK$^-$) cells were grown in a monolayer culture in 75 cm^2 plastic flasks in DMEM containing 10% (v/v) fetal-calf serum in a CO_2 incubator at 37 °C. Confluent monolayers of TK$^-$ cells were incubated with virus for 1 h at 37 °C in DMEM in the absence of serum with 20 plaque-forming units of PRV/cell. After 14 h of incubation, the cells were rinsed and suspended in PBS using a rubber policeman and collected by centrifugation at 2000 g at 4 °C. The cells were kept at −80 °C until they were used.

Partial purification of cytosolic TK from swine tissue

Swine lymphatic glands (2.6 g) were homogenized in 8 ml of 20 mM potassium phosphate buffer, pH 6.8, containing 1 mM DTT, 1 mM PMSF and 0.5 mM EDTA using an Ultraturrax blender and then a potter. The crude homogenate was centrifuged for 15 min at 8000 g at 4 °C. The supernatant was collected and the pellet was resuspended in 8 ml of 20 mM potassium phosphate buffer, pH 6.8, containing 0.32 M sucrose, 1 mM DTT, 1 mM PMSF, 0.5 mM EDTA and 0.1% (w/v) Nonidet P-40 (NP-40). After potter homogenization, the extract was centrifuged for 15 min at 8000 g at 4 °C. The supernatant was collected and combined with the previous one. This fraction was centrifuged for 75 min at 100000 g at 4 °C and the supernatant was loaded on to a 5 ml phosphocellulose (P11) column equilibrated with 20 mM potassium phosphate buffer, pH 6.8, containing 1 mM DTT, 1 mM PMSF and 0.5 mM EDTA. Cytosolic TK activity was eluted in a single step at 0.1 M potassium phosphate and ultraconcentrated in a Centricon-10 (Amicon).

RESULTS

PRV TK induction

To determine the activity profile of the TK induced by PRV during viral infection, 2×10^7 cells of pelleted mouse TK$^-$ cells infected with PRV were collected at 0, 2, 4, 6, 8, 10, 14, 24 and 48 h post-infection, resuspended in 400 μl of 10 mM Tris/HCl, pH 7.5, 1 mM DTT, 400 mM KCl, 0.1 mM EDTA, 1 μM pepstatin, 4 mM sodium bisulphite and 1 mM PMSF, sonicated five times for 5 s at 100 W and centrifuged for 10 min in an Eppendorf centrifuge. Supernatants were tested for TK activity. Figure 1 shows the activity profile of viral TK under the conditions of infection that are described above: maximal TK activity is reached at about 12–14 hours post-infection.

Partial purification of PRV TK

Mouse TK$^-$ cells (2×10^8) infected by PRV, collected 14 h post-infection as previously described, were resuspended in 5 ml of 10 mM Tris/HCl, pH 8, 1 mM DTT, 0.2 mM PMSF, 1 μg/ml pepstatin, 10 mM KCl and 0.1 mM EDTA. After 10 min on ice, cells were homogenized with a Dounce homogenizer (pestle A) and nuclei were precipitated by centrifugation for 25 min at 15000 rev./min at 4 °C using a JA-20 rotor in a Beckman centrifuge. The supernatant was collected, made 10% (v/v) in glycerol and stored on ice. Nuclei were washed in a Dounce homogenizer

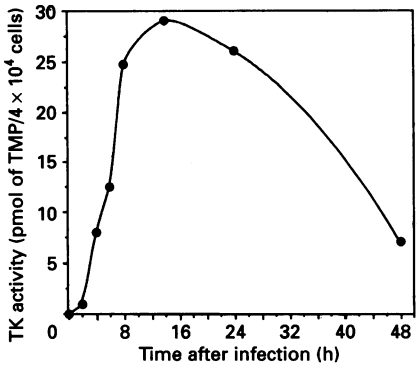

Figure 1 Activity profile of PRV TK during *in vitro* infection

Experimental details are in the text.

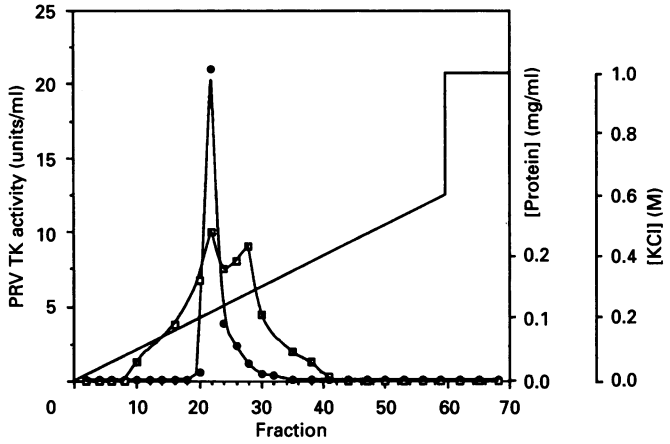

Figure 2 F.p.l.c. Mono-Q chromatography of PRV TK

(●) PRV TK activity (units/ml); (□) protein (mg/ml); solid line, KCl gradient. Experimental details are in the text.

Table 1 Partial purification scheme for PRV TK

Fraction	Amount of protein (mg)	Activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude extract	48.00	21.2	0.44	100.0	–
S-100	18.69	18.4	0.99	86.8	2.2
DEAE-Sepharose	4.02	20.0	4.97	94.3	11.3
Mono-Q	0.24	17.0	70.80	80.2	160.9

in 5 ml of 10 mM Tris/HCl, pH 8, 0.32 M sucrose, 1 mM $MgCl_2$, 0.3% (w/v) NP-40, 1 mM DTT, 0.2 mM PMSF, 0.1 mM EDTA and 1 μg/ml pepstatin. Nuclei were then centrifuged for 25 min, as described above, and the supernatant, after having been made 10% (v/v) glycerol, was combined with the previous one (crude-extract fraction) and centrifuged for 1 h at 100000 *g* at 4 °C (S-100 fraction). The supernatant was then loaded on to an f.p.l.c. DEAE-Sepharose Fast Flow column (with a volume of 10 ml) equilibrated with 10 column volumes of 10 mM Tris/HCl, pH 8,

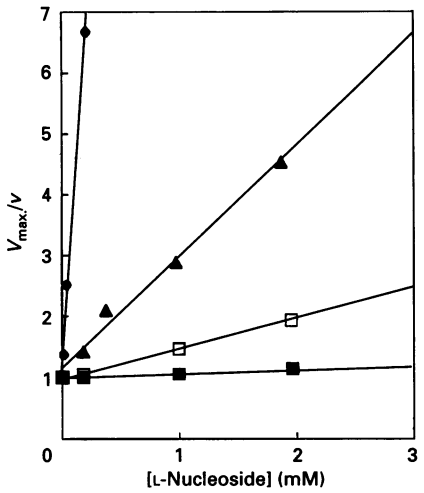

Figure 3 Effect of increasing amounts of (●) L-T, (▲) L-dG, (□) L-dU and (■) L-dA on the phosphorylation of the natural substrate [^{3}H]D-T by PRV TK

10% (v/v) glycerol, 1 mM DTT, 0.15% (w/v) NP-40, 0.2 mM PMSF, 0.1 mM EDTA and 1 μg/ml pepstatin (buffer A). The enzyme was eluted with a linear gradient of 0–600 mM KCl in buffer A. Active fractions were pooled (DEAE-Sepharose fraction), diluted 1:1 with buffer A and loaded on to a f.p.l.c. Mono-Q column (with a volume of 1 ml) equilibrated with 10 column volumes of buffer A. The column was eluted with a linear gradient of 0–1 M KCl in buffer A, and TK was eluted as a single peak at 250 mM KCl (Mono-Q fraction) as shown in Figure 2. Table 1 summarizes the partial purification described here. The final preparation had a specific activity of 70.8 units/mg.

L-Deoxynucleosides selectively inhibit D-T phosphorylation by PRV TK without having an effect on swine TK

We have screened five L-β-nucleosides, L-T, L-dC, L-dA, L-dG and L-dU, which are all known to be inactive against the human enzyme (Spadari et al., 1992), for their capacity to inhibit the phosphorylation of [^{3}H]D-T when catalysed by swine and by PRV TKs. All of the L-nucleosides tested were also found to be inactive against the swine TK that had been partially purified from lymphatic glands, as described in the Methods section. We determined the K_m (μM) and $V_{max.}$ (pmol/min per unit)/K_m values for D-T (1.2 and 17.8 respectively). We then studied both the effect of increasing concentration of L-T on the phosphorylation of [^{3}H]D-T, and the direct phosphorylation of [^{3}H]L-T. L-T neither acts as an inhibitor nor as a substrate for swine TK up to a concentration of 2 mM (the highest concentration tested). However, L-nucleosides, except L-dC, exerted differential inhibitory capacities against the viral enzyme in the following order: L-T $\gg$ L-dG > L-dU > L-dA. Inhibition curves, as shown in Figure 3, indicate that the ID_{50} values for PRV TK are 7.6, 460, 2000 and > 2000 μM for L-T, L-dG, L-dU and L-dA respectively.

L-T is a selective competitive inhibitor of PRV TK

To determine the mechanism of inhibition by L-T of D-T phosphorylation by PRV TK, the viral enzyme was incubated in the presence of increasing amounts of L-T at different concentrations of D-T in the assay conditions that are described in the Methods section. Velocity values were plotted according to

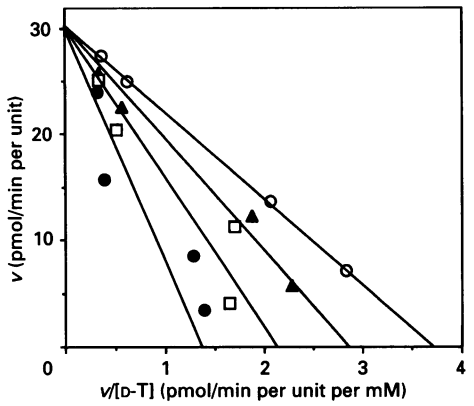

Figure 4 Hofstee plot of the effect of L-T on the activity of PRV TK in the presence of increasing concentrations of the natural substrate [^{3}H]D-T

The enzyme was assayed as described in the Methods section with different concentrations of L-T: (○) 0 μM; (▲) 0.5 μM; (□) 1 μM; and (●) 2.5 μM. Each point represents the mean of triplicate determinations.

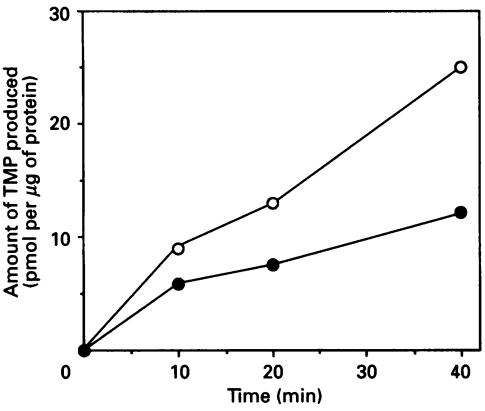

Figure 5 Time-dependent phosphorylation of [^{3}H]D-T (○) and [^{3}H]L-T (●) by PRV TK

The enzyme was assayed in presence of 5 μM of either [^{3}H]D-T or [^{3}H]L-T.

Table 2 Kinetic parameters for D-T and L-T as substrates for PRV TK

Values are given with S.D. from three experiments.

Substrate	K_m (μM)	$V_{max.}$ (pmol/min per unit)	$V_{max.}/K_m$
D-T	8.1±0.2	30.9±0.2	3.7
L-T	2.4±0.8	5.7±0.8	2.3

the method of Hofstee. As is shown in Figure 4, increasing concentrations of L-T affect the slope ($-K_m$) of the curves without changing the ordinate intercept ($V_{max.}$). Therefore, L-T acts as a competitive inhibitor: in fact it decreases the apparent affinity (K_m) of the substrate for the enzyme with no effect on the reactivity of the enzyme–substrate complex, once it has been formed. The K_i value for L-T, calculated as described in the Methods section, was 2.2±0.36 μM.

PRV TK phosphorylates both L- and D-enantiomers of thymidine with comparable efficiency

Figure 5 shows the time-dependent phosphorylation of D-T and L-T by PRV TK; under the assay conditions used, the rate of phosphorylation of L-T is about 50% of that observed with D-T. To determine the K_m and $V_{max.}$ both of D-T and of L-T using PRV TK, we incubated the enzyme in the presence of increasing concentrations of [^{3}H]D-T and [^{3}H]L-T respectively, under the assay conditions described in the Methods section. As is shown in Table 2, the calculated K_m values for D-T and L-T were 8.1±0.2 μM and 2.4±0.8 μM respectively. As expected for an inhibitor that is also a substrate, the K_m for L-T is also identical to its K_i (2.4 and 2.2 μM, respectively). The ratio $V_{max.}/K_m$ is very similar for both substrates, being 3.7 and 2.3 for D-T and L-T, respectively. This indicates that the PRV TK phosphorylates the two enantiomers with comparable efficiency.

PRV TK lacks an associated thymidylate-kinase activity

To determine whether PRV TK possesses an associated thymidylate kinase activity, similarly to the HSV1 enzyme, we incubated both HSV1 and PRV TKs with [^{14}C]TMP and analysed the reaction products, as described in the Methods section. No detectable amounts of [^{14}C]TDP were found even after 1 h of incubation of PRV TK with [^{14}C]TMP, whereas the HSV1 enzyme phosphorylated 30% of the substrate under the same assay conditions (data not shown). We therefore conclude that, under our assay conditions, PRV TK does not show an associated thymidylate kinase activity.

DISCUSSION

In the study described in this paper, we have checked whether PRV TK, similarly to HSV1 TK (Spadari et al., 1992) and HSV2 TK (G. Maga, A. Verri, A. Garbesi, D. Niccolai, S. Spadari and F. Focher, unpublished work), can recognize the L-enantiomers of the natural D-nucleosides thymidine, dA, dG, dC and dU. In particular, we focused our attention on L-T, the enantiomer of the natural D-T, which is the best substrate for the viral TK. Our results show that PRV TK, unlike human and swine TKs, does not show stereospecificity, and can not only recognize but also phosphorylate both enantiomers with comparable efficiency. This finding suggests that the overall configuration of the sugar ring has a very minor influence on the recognition of the substrate by the viral enzyme. Thus as has been found previously for HSV1 (Spadari et al., 1992) and HSV2 (G. Maga, A. Verri, A. Garbesi, D. Niccolai, S. Spadari and F. Focher, unpublished work) TKs, PRV TK is also very tolerant of the overall configuration of its natural substrate molecule, unlike the general behaviour of enzymes. However, the data obtained with swine TK have confirmed results obtained previously with human TK (Spadari et al., 1992); L-T neither functions as a substrate nor as an inhibitor for the mammalian TKs.

To the best of our knowledge, HSV1, HSV2 and PRV TKs are the first reported examples of viral enzymes that have no specificity for the overall configuration of the substrate. From a therapeutic point of view, the lack of stereospecificity that is shown by the herpes virus TKs that have been tested so far and the absence of toxic effects of L-T on mammalian cells (Spadari et al., 1992) suggest a possible use of L-T as a prototype for the design and development of a new class of drugs that may be valuable for the treatment of recurrent herpetic infections in man and in domestic animals. In fact, the different stereoselectivity for the substrate that is shown by viral and cellular enzymes supports the hypothesis that nucleoside analogues in which

effective modifications of the base moiety are associated with the presence of a sugar with an unnatural configuration could have promising therapeutic uses.

G. M. was supported by a fellowship from the National Research Council of Italy, special project RAISA. This work was supported by the National Research Council of Italy, special project RAISA, sub-project no. 3 paper no. 235.

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Received 21 January 1993/18 March 1993; accepted 25 March 1993

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