Substrate Specificity of Epstein-Barr Virus Thymidine Kinase

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Purified recombinant protein encoded by the BXLF1 open reading frame of the Epstein-Barr virus genome has thymidine kinase activity. The substrate behaviors of various nucleosides toward this enzyme were tested. Halogenated deoxynucleotides, zidovudine, and bromovinyldeoxyuridine are efficient substrates, while acyclovir and dihydroxypropylmethylguanine are relatively poor substrates for the Epstein-Barr virus thymidine kinase.

Like most other herpesviruses, Epstein-Barr virus (EBV) encodes a virus-induced protein with thymidine kinase (TK) activity. This enzyme catalyzes the transfer of the gamma phosphoryl group of ATP to the 5′ hydroxyl of a variety of deoxynucleosides to produce the corresponding nucleoside monophosphate. It is presumed that the further phosphorylations to the di phosphate and triphosphate forms are carried out by cellular kinases. In the case of herpes simplex virus type 1 (HSV-1) and HSV-2, the substrate specificity of the viral TK is much less restricted than that of the mammalian cell TK (12). Thus, in infected cells the viral enzyme initiates the conversion of nucleoside analogs to phosphorylated forms which either are inhibitors of the viral DNA polymerase or are relatively toxic when incorporated into the viral DNA (5). If the EBV TK has a similar broad substrate specificity, it may provide a route to the activation of nucleoside analogs for the production of useful antiviral nucleotides. To examine this possibility, we explored the use of various nucleoside analogs by EBV TK made in a bacterial expression system programmed with the cloned EBV DNA sequence encoding this enzyme.

Reagents. Nucleoside analogs, thymidine, ATP, morpholinolanesulfonic acid (MES), and bovine serum albumin (BSA) were purchased from Sigma. [14C]thymidine and [γ-32P]ATP were obtained from Amersham. [3H]bromodeoxyuridine ([3H]BrdU) was purchased from New England Nuclear. Water was from a Millipore MilliQ system. All other compounds were reagent grade or better.

EBV TK. The DNA sequence from nucleotides 144862 to 142745 of the B95-8 strain of EBV was cloned into the pET3a expression vector (20) at the NdeI and BamHI sites. This vector was introduced into Escherichia coli SY 211 (25), a TK-deficient derivative of E. coli BL21 (DE3) (20), so that the only TK activity in the cell comes from the expression of the EBV sequences. Cultures were grown at 22°C in 4 liters of LB medium containing 200 µg of ampicillin per ml. Once the cultures reached the mid-log phase of growth, isopropylthio-galactoside was added to 0.4 mM to induce the expression of EBV TK. Induction was allowed to continue for 16 h at 22°C. Twenty grams (wet weight) of cells was harvested, washed with STE (20 mM NaCl, 1 mM EDTA, 10 mM Tris [pH 8.0]), and resuspended in 50 ml of 10% sucrose-50 mM Tris-HCl (pH 7.5) before being frozen in liquid nitrogen. The frozen cells were defrosted in 100 ml of 10% sucrose-200 mM NaCl-30 mM spermidine-HCl (pH 7.5) and were lysed by the addition of 300 µg of lysozyme per ml. The lysed cells were sonicated briefly to reduce the viscosity before pelleting the cellular debris. The supernatant was loaded directly onto a DEAE Biogel column (4 by 30 cm), and the column was eluted with 1 liter of a linear gradient of salt (0 to 500 mM NaCl). Fractions (6 ml) were collected and assayed for TK activity (see below). Fractions containing TK activity were pooled and applied to a hydroxyapatite column (1 by 8 cm) equilibrated with 1 mM potassium phosphate buffer (pH 6.8). The column was washed in succession with one column volume of the equilibrating buffer, the equilibrating buffer containing 500 mM NaCl, and finally the equilibrating buffer. The column was eluted with a 100-ml linear gradient of 10 to 300 mM potassium phosphate (pH 6.8) (10). Fractions with TK activity were dialyzed against 0.2 mM thymidine—50% glycerol—50 mM Tris (pH 6.8) and were stored at −20°C. The enzyme was judged to be about 30% pure on the basis of the staining intensity of the TK polypeptide on polyacrylamide gels.

Standard TK assay. Enzyme activity was determined by the differential binding of phosphorylated versus unphosphorylated nucleoside to positively charged DEAE paper (26). All assays were stopped prior to the consumption of greater than 5% of the substrate to ensure the linearity of the assay.

Assay for inhibition by competition. To assess the binding of nucleoside analogs to the TK, cold nucleoside analogs were used to compete with [14C]thymidine as a substrate. To the reaction mixture was added 2 mM unlabeled nucleoside analog. The concentrations of the other reagents were kept the same. The assay conditions were identical to those described above for the standard TK assay; in particular, thymidine was present at 0.2 mM.

P transfer assay. Phosphorylation of the nucleoside analogs was determined by the transfer of radioactive phosphate from [γ-32P]ATP to the nucleoside. This assay was performed as described by Doberson and Greer (8). The specific activity of [32P]ATP used in these assays was 18 µC/mmol.

The open reading frame from nucleotides 144861 to 143038 on the EBV genome (BXLF1) has previously been identified as having some homology to the HSV-1 and HSV-2 TK genes and, hence, is likely to encode an EBV TK (3). As predicted, the expression of this open reading frame yielded a polypeptide of about 70 kDa. This protein was found to aggregate in concentrated solutions and in low-ionic-strength buffers, making purification difficult. In this respect the recombinant enzyme behaved similarly to the TK activity from induced, EBV-containing lymphoblastoid cells (7). Gel filtration of the soluble form supported the conclusion that the enzyme exists in solution as a monomer of the expected size.

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Initial characterization of the EBV TK activity was directed at determining the divalent metal ion requirements and the optimal pH for TK activity. EBV TK can use a variety of metals, with magnesium providing optimal activity (Fig. 1). Copper and iron are ineffective, while barium, calcium, cobalt, and manganese provide roughly the same activities. For subsequent study of substrate affinity, magnesium was the divalent ion used.

The apparent affinities of the EBV TK for the thymidine and ATP substrates were determined by measuring the reaction velocity as a function of each substrate concentration in the presence of an excess of the other. Lineweaver-Burk plots for both thymidine and ATP were linear (Fig. 2). This result suggests that the enzyme can be described by the simple Michaelis-Menten model for enzyme behavior. The apparent $K_m$ for thymidine is 22 $\mu$M, and that for ATP is 25 $\mu$M.

Competition assays between nucleoside analogs and radio-labeled thymidine showed that a variety of nucleoside analogs can compete with thymidine for EBV TK (Fig. 3). Several 5-halogenated deoxyuridine analogs (5-chlorodeoxycytidine [CldU], BrdU, and 5-iododeoxycytidine [IdU]) appear to be even more effective competitors than the normal substrate thymidine. Although these comparisons are not as quantitatively precise as complete $K_m$/affinity determinations, they allow preliminary estimations of the potential usefulness of a given analog as an alternate substrate by the EBV TK. Since the molar ratio of the test compound to $[^3]P$thymidine was 10:1, this experimental approach is most informative for inhibitors which compete well with thymidine.

To test if the competitive analogs were able to function as alternate substrates and not just as inhibitors of true substrate binding, we examined the formation of phosphorylated product by using the appearance of new $[^3]P$-labeled material as the basis for such activity. This assay is able to detect agents which are relatively efficiently phosphorylated; very low levels of phosphorylation may be masked by the unavoidable background of radioactive material which appears even in the absence of enzymatic activity. The following compounds exhibited detectable competition at concentrations 10-fold greater than the $[^3]P$thymidine concentration: 5′-aminodeoxyuridine (aminodU), 5-bromodeoxyuridine (BrdC), deoxyuridine (dU), methyluridine (meU), 4-thiothymydine (thiodU), 5-hydroxythymidine (HOMedU), 5-iododeoxyuridine (IdC), thymine arabinofuranoside (araT), dideoxythymidine (ddT), 3′-O-acetyltymidine, (acetylIdU), 5-fluorodeoxycytidine (FdU), 5-bromovinyldeneoxuridine (BrdVu), pseudouridine (AZT), 5-ethyldeneoxuridine (EtdU), ClidU, BrdU, and IdU (Fig. 3). All of these competitive nucleoside analogs were not recognized by EBV TK: deoxyribosyladenine (dA), deoxyribosylcytidine (dC), deoxyriboseguanine (dG), $\alpha$-deoxycytidine ($\alpha$dT), 2-deoxyribose, 5′-aminodeoxythymidine, azadoxycytidine, dihydroxypropylguanine (DHPG), acyclovir, and
TABLE 1. Comparisons of dA, dC, and dG with thymidine as competitors and substrates for the EBV TK

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Competition assay</th>
<th>Phosphorylation assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1,002</td>
<td>890</td>
</tr>
<tr>
<td>dT</td>
<td>190</td>
<td>3,777</td>
</tr>
<tr>
<td>dA</td>
<td>1,053</td>
<td>636</td>
</tr>
<tr>
<td>dC</td>
<td>1,259</td>
<td>647</td>
</tr>
<tr>
<td>dG</td>
<td>1,086</td>
<td>623</td>
</tr>
</tbody>
</table>

* For the competition assay each nucleoside was added to the standard assay reaction mixture at a 10-fold molar excess over the 14C-labeled thymidine substrate. For the phosphorylation assay, the production of phosphorylated nucleoside was assayed by the amount of 32P transferred from ATP to the indicated nucleoside.

The substrate behavior of BrdU was analyzed in more detail (Fig. 4). [3H]BrdU was used in place of [14C]thymidine to assay enzyme activity, and these results confirm the fact that EBV TK phosphorylates BrdU. With a measured Km of 15 μM for BrdU, EBV TK may have a slightly higher affinity for the nucleoside analog BrdU than for its presumptive natural substrate thymidine.

TK provides the pathway for phosphorylating various nucleoside analogs important in the treatment of herpes simplex virus (6) and human immunodeficiency virus. We characterized some aspects of EBV TK with an emphasis on its ability to use various nucleoside analogs. The requirement for a divalent metal ion is typical of most ATP-requiring enzymes since most such enzymes recognize Mg2+-ATP as the physiological substrate (18). Since, except for Mg2+, only one concentration of metal ion was examined in our survey, we cannot be sure that we have determined the optimal activity; we can be sure only that the metals tested function to some extent.

Linear double-reciprocal plots were obtained for both thymidine and ATP. This result indicates that EBV TK exhibits Michaelis-Menten kinetic behavior. For EBV TK the Km for thymidine is 22 μM, while for HSV-1 TK the Km is in the 0.5 μM range (5). Conversely, ATP binding is tighter for EBV TK, with a Km of 25 μM, as opposed to a Km of greater than 100 μM for HSV-1 TK. Since the intracellular pool concentration of thymidine is lower (21) than the Km of the EBV TK, this pathway probably is of minor importance except during times of lytic viral replication when the cellular DNA may be degraded, raising the thymidine concentration.

It is interesting that, on the basis of the amino acid sequence homology, EBV TK and HSV TK have more homologous ATP-binding sites than thymidine-binding sites (3, 17). HSV TK is known to function also as a thymidylate kinase (4), and it will be interesting to see in further studies if EBV TK, purified to homogeneity to eliminate the possibility of contaminating proteins, has such an activity.

As shown in Fig. 3 it is apparent that a variety of nucleoside analogs are used by EBV TK. Changes in the base or changes in the sugar of the nucleoside can be accommodated by this TK. On the basis of the competitive activities of these analogs, we can infer that there are regions of the nucleosides in which structural modifications can be tolerated without destroying substrate-binding activity. Substitution on the sugar is tolerated at the 3′ position, with the bulkiness and charge of the group possibly determining the acceptability by the enzyme (compare AZT with acetyldeT). Interestingly, dT with a hydrogen substituted at the 3′ position is not as effective a substrate as AZT or acetyldeT, which have much bulkier constituents. Only the substituents azide and acetyl, however, can function as hydrogen bond acceptors. This H bonding may be more important in TK substrate recognition than steric occupation.

The 2′ position of the sugar may also be substituted, although with limited acceptance by the TK. The ribose of methyl uridine and the arabinose of araT were both phosphorylated. Limited phosphorylation of araT by EBV TK indicates that the enzyme can distinguish the 2′ hydroxyl group in the beta orientation of the arabinose sugar.

Large changes in the nucleobase were least well tolerated. EBV TK only binds and phosphorylates pyrimidines. Of the naturally occurring nucleosides, thymidine is the best substrate, with some activity seen with deoxyuridine and no activity observed for dC. TK is thus dissimilar to HSV TK, which can phosphorylate most pyrimidines and some purines. However, Stinchcombe and Clough (23) reported that an activity in a DEAE column fraction, which seemed to be specific to lymphoblastoid cell lines expressing EBV lytic cycle genes, phosphorylated dC as efficiently as it did dT. Likewise, Litter and colleagues (17) have reported the ability of EBV TK to phosphorylate both deoxyuridines and deoxyuridines. The purine analogs acyclovir and DHPG were also phosphorylated at a very low level in the preparation of EBV TK described by Litter et al. (17). Since the enzyme used by Stinchcombe and Clough (23) was an impure preparation isolated from lymphoblastoid cells in culture, it may have contained several different activities, some of which were unrelated to EBV yet which may have modulated the substrate behavior of the EBV TK polypeptide or altered its stability in some way. Although our observations, too, are based on only partially purified EBV TK, such other contaminating activities may have been eliminated from our preparation or may be absent from material produced from recombinant genes in E. coli. Another possibility to explain the discrepancy between our results and those of Stinchcombe and Clough (23) may be the phosphorylation of dC by a virus-induced kinase analogous to the cytomegalovirus protein kinase which can use dC as an alternate substrate (16, 24).

Deoxyuridines substituted at the 5′ position were effectively used by the enzyme. Substituents at this position ranged from

![FIG. 4. Lineweaver-Burk plot varying BrdU. The Km value obtained was 15 μM. Assay conditions were 10 mM NaF, 10 mM MgCl2, 10 mM ATP, 0.6 mg of BSA per ml, 100 mM MES (pH 6.0), and various concentrations of [3H]BrdU (0.1 to 0.2 Ci/mM). Data from two separate experiments are plotted as open and closed circles.](http://aac.asm.org/Downloaded from http://aac.asm.org/ on July 8, 2017 by guest)
vinyl to halogenated compounds. The chloro, bromo, and iodo 5-substituted deoxyuridines were the most effective nucleoside analogs and were better even than the physiological substrate thymidine. A comparison of the van der Waals radii suggests that these halogens (Cl = 0.18 nm, Br = 0.195 nm, I = 0.215 nm) are very close to that of thymidine (0.20 nm). On the basis of steric factors, these analogs should bind as effectively as thymidine. With a somewhat smaller size, FdU (0.135 nm) was the least effective competitor among this series of 5-halodeoxyuridine analogs. However, the bromovinyl substituent is tolerated despite its larger size. The terminal bromomethene group on BrVdU may be oriented away from the active site and the proximal methene group may be situated to interact with the active-site pocket. This orientation would make BrVdU sterically similar to dT. A similar argument can be made for the proximal methene group of EdTdU.

Hydrogen bonding at the active site may also play an important role in substrate recognition. Because of the electron-dense character of the halogens, their effects on the nucleobase are to favor the enol tautomer as opposed to the keto tautomer favored by all other nucleobases, including thymidine (9). Such a change in hydrogen-bonding potential, along with steric factors, may provide augmented binding in the active site and result in a substrate with a higher affinity than that of the naturally occurring one. That electronegativity alone is insufficient to explain our results, however, was shown in the case of the fluoro derivative, which, although it was the most electro-negative, was a poor substrate. The steric effect in the case of the fluoropyrimidines may negate the increase in H bonding potential.

ACV has been reported to inhibit EBV replication in cell culture (13, 15) and has been used to treat EBV infections in a number of different clinical settings (1, 11). Our observations of the inability of EBV TK to phosphorylate this nucleoside analog are not incompatible with these findings. Although several studies have shown that ACV is inefficiently phosphorylated by cellular or virus-induced enzymes (14, 22), the action of ACV as a DNA chain terminator means that even very low levels of phosphorylation can be effective in stopping DNA replication. This interpretation is compatible with the literature reports of ACV efficiently inhibiting the production of EBV in culture and human patients while at the same time being poorly phosphorylated. While the clinical use of ACV has shown some success in the case of oral hairy leukoplaikia, an EBV-related condition, other sorts of EBV infections have been reported to be resistant to treatment with ACV (2, 19, 27).

In summary, we characterized some aspects of EBV TK function, including the use of various nucleoside analogs to functionally probe the enzyme's active site. Understanding of the active site of this enzyme will allow a better understanding of the enzyme's mechanism of action and, in turn, the design of better substrate analogs with possible therapeutic importance.

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REFERENCES


