Identification and characterization of the conserved nucleoside-binding sites in the Epstein–Barr virus thymidine kinase

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Thymidine kinase (TK), encoded by EBV (Epstein–Barr virus), is an attractive target for antiviral therapy and provides a novel approach to the treatment of EBV-associated malignancies. Despite the extensive use of nucleoside analogues for the treatment of viral infections and cancer, the structure–function relationship of EBV TK has been addressed rarely. In the absence of any structural information, we sought to identify and elucidate the functional roles of amino acids in the nucleoside-binding site using site-directed mutagenesis. Through alignment with other human herpesviral TK protein sequences, we predicted that certain conserved regions comprise the nucleoside-binding site of EBV TK and, through site-directed mutagenesis, showed significant changes in activity and binding affinity for thymidine of site 3 (-DRH-) and 4 (-VFP-) mutants. For site 3, only mutants D392E (Asp³⁹² → Glu) and R393H retain activity, indicating that a negative charge is important for Asp³⁹² and a positive charge is required for Arg³⁹³. The increased binding affinities of these two

mutants for 3'-deoxy-2',3'-didehydrothymidine suggest that the two residues are also important for substrate selection. Interestingly, the changed metal-ion usage pattern of D392E reveals that Asp³⁹² plays multiple roles in this region. His³⁹⁴ cannot be compensated by other amino acids, also indicating a crucial role. In site 4, the F402Y mutant retains full activity; however, F402S retains only 60 % relative activity. Strikingly, when Phe⁴⁰² is substituted with serine residue, the original preferred pyrimidine substrates, such as 3'-azido-3'-deoxythymidine, iododeoxyuridine and β -L-5-iododioxolane uracil (L-form substrate), have decreased competitiveness with thymidine, suggesting that Phe⁴⁰² plays a crucial role in substrate specificity and that the aromatic ring is important for function.

Key words: Epstein–Barr virus, nucleoside-binding sites, thymidine kinase.

INTRODUCTION

Thymidine kinase (TK) is an enzyme that catalyses the transfer of the γ -phospho group of ATP to thymidine to generate dTMP in the salvage pathway of pyrimidine synthesis. dTMP is phosphorylated further by a cellular enzyme to dTTP, which serves as a substrate for DNA polymerase during DNA replication. Herpesviral TK phosphorylates a broad range of nucleoside analogues [1], and serves as an important target for antiviral therapy. The TK enzymes convert nucleoside analogues, such as ACV (acyclovir) [2] and GCV (ganciclovir) [3] to their monophosphate form. Cellular enzymes then phosphorylate the prodrug to the active nucleoside triphosphate. The viral DNA polymerase incorporates the metabolites preferentially into the elongating viral DNA, resulting in DNA-chain termination [4,5]. Recently, the herpesviral TK, such as those of HSV (herpes simplex virus), VZV (Varicella Zoster virus), equine herpes virus 4 and EBV (Epstein–Barr virus) [6–9] also have been found to be useful suicide genes in gene therapy. The genes are introduced into tumour cells, which, subsequently, can be killed selectively by the appropriate nucleoside analogues. Much effort has been put into improving the selective killing effect by synthesizing new prodrugs [10] and constructing TK mutants [11].

EBV, a member of the herpesviridae family, is a ubiquitous virus that infects most of the human population during early life. EBV has been shown to cause infectious mononucleosis [12–14], fatal acute infectious mononucleosis, X-linked lymphoproliferative

syndrome and oral hairy leukoplakia [15–17]. EBV also has a close association with several types of malignancies, e.g. African Burkitt's lymphoma [18,19], nasopharyngeal carcinoma [20], Hodgkin's disease and T-cell lymphoma [21]. Anti-viral approaches have been developed based on the complex relationship between EBV and these diseases. EBV TK, an early lytic protein encoded by the BXLFI open reading frame [22,23], plays a major role in most of these anti-EBV strategies. Diseases involving lytic EBV infection, such as HIV-oral hairy leukoplakia, regress when treated with nucleoside analogues [15]. Recently, a new virus-specific therapy, involving induction of EBV TK by concomitant antiviral nucleoside treatment, was developed by Mentzer and co-workers [24–26], who conducted a clinical trial of the EBV-associated post-transplant lymphoproliferative disease. The expression of TK was increased by arginine butyrate, and GCV treatment caused regression of the tumour cells. Therefore a better understanding of EBV TK should permit the development of new approaches and new drugs for the treatment of EBV-associated diseases. However, unlike for the TK of α -herpesvirus, the characterization of EBV TK has been relatively slow because of the lack of a cell-culture system, which allows lytic viral replication.

EBV TK shares protein sequence and functional homology with HSV-TK but has an additional 243 amino acids at its N-terminus. The function of the N-terminal domain is unknown, but this hydrophilic region has strong antigenicity and has been shown to react with sera from nasopharyngeal carcinoma patients (P. Yeh, J.-Y.

Abbreviations used: ACV, acyclovir; AZT, 3'-azido-3'-deoxythymidine; D4T, 3'-deoxy-2',3'-didehydrothymidine; DDI, dideoxyinosine; DTT, dithiothreitol; EBV, Epstein–Barr virus; L-FMAU, 2'-fluoro-5-methyl- β -L-arabinofuranosyl uracil; GCV, ganciclovir; HHV-8, human herpes virus 8; HSV, herpes simplex virus; IdU, iododeoxyuridine; L-I-OddU, β -L-5-iododioxolane uracil; TK, thymidine kinase; VZV, Varicella Zoster virus.

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Chen and T.-Y. Hsu, unpublished work). A biochemical study revealed that EBV TK has K_m values of 22 μ M for thymidine and 25 μ M for ATP [27]. Substrate specificity has also been determined by several groups [27,28]. However, the structure–function relationship of EBV TK has been addressed rarely. In our previous study, we have shown that the C-terminus of EBV TK is important for its activity [29]. In the present study, we sought to identify and characterize the nucleoside-binding sites of the enzyme.

Multiple alignments reveal six highly conserved sites in the amino acid sequences of 12 herpesviral TKs [30]. Sites 1, 3 and 4 are supposed to be involved in substrate binding. HSV-1 TK has three substrate-binding sites, site 1 for ATP and sites 3 and 4 for thymidine binding, which have been confirmed by mutagenetic methods and structural analysis [31–36]. Thymidine-binding sites are also the regions that are responsible for binding to various nucleoside analogues. Attempts to engineer the thymidine-binding sites to modify the substrate specificities of viral TKs for clinical application have been reported [11]. In this study, the EBV TK nucleoside-binding sites were defined and the biochemical roles of amino acids in these regions were characterized.

EXPERIMENTAL

Chemicals

Nucleoside analogues, thymidine, ATP, phosphocreatine, creatine kinase, albumin, sodium fluoride, DTT (dithiothreitol) and metal chloride were purchased from Sigma. L-FMAU (2'-fluoro-5-methyl- β -L-arabinofuranosyl uracil) and L-I-OddU (β -L-5-iododioxolane uracil) were gifts from Y. C. Cheng (Department of Pharmacology, Yale University School of Medicine, New Haven, CT, U.S.A.) [10]. [3 H]Thymidine was obtained from NEN (Boston, MA, U.S.A.).

Site-directed mutagenesis

Mutants encoding proteins with single amino acid substitutions were generated by recombinant PCR. The template for PCR was the plasmid pET-TKB1B, which encodes a full-length, 607-amino-acid TK protein [37]. A mutated complementary set of primers was designed for each specific wild-type residue (Table 1). Two PCRs were set up at the same time, one with a mutated antisense primer and the *Bam*HI primer, the other with the complementary mutated sense primer and the *Hind*III primer. These two PCR products were purified by electrophoresis, mixed in a 1:1 molar ratio in PCR buffer containing 10 mM Tris/HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 400 μ M dNTP and 2 units of DynaZyme II DNA polymerase (Finnzymes Oy, Espoo, Finland), then annealed and extended with one cycle at 95 °C for 5 min, one at 52 °C for 5 min and another at 72 °C for 10 min without primers. The *Bam*HI and *Hind*III primers were then added to the reaction and a further 25 cycles of 95 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min were performed. The resulting products were digested with *Bam*HI and *Hind*III and ligated to the corresponding sites of the pRsetA vector (Invitrogen, Carlsbad, CA, U.S.A.) to obtain recombinant plasmids with point mutations in the EBV TK open reading frame. The sequences of all mutants were confirmed by DNA sequencing (ABI PRISM™ dye terminator cycle sequencing kits).

Expression of the TK protein

The recombinant plasmids obtained were used to transform the *Escherichia coli* strain BL21(DE3)pLysS [29]. One colony was

Table 1 Primers used in the construction of mutants with a single amino acid change

Mutants	Sense primer (5' → 3')
D392E	GATTTTGCATGAACGCCATTGCG
D392H	GATTTTGCATCATCGCCATTGCG
D392N	GATTTTGCATAATCGCCATTGCG
R393Q	GATTTTGCATGATCAGCATTGCG
R393L	GATTTTGCATGATCTGCATTGCG
R393H	GATTTTGCATGATCATATTGCG
R393E	GATTTTGCATGATGAGCATTGCG
H394D	GCATGATCGCGATTGCTGTCGG
H394F	GCATGATCGCTTTTGTGCTGCGG
H394K	GCATGATCGCAAGTTGCTGTCGG
H394L	GCATGATCGCCTTTGTGCTGTCGG
H394N	GCATGATCGCAATTGCTGTCGG
V401C	GGCCTCCGTGTGTTCCCTCTAA
V401L	GGCCTCCGTGCTTTTCCCTCTAA
V401S	GGCCTCCGTGTTCTTTCCCTCTAA
F402S	CTCCGTGGTTTCCCTCTAATGC
F402Y	CTCCGTGGTTTACCCTCTAATGC

seeded to Luria–Bertani broth containing 200 μ g/ml ampicillin and 25 μ g/ml chloramphenicol and grown until a value of 0.4–0.6 was reached for A_{600} . The culture was treated with 0.5 mM IPTG (isopropyl β -D-thiogalactoside) for 2.5 h at 25 °C with shaking. The bacterial suspension was centrifuged and lysed with 1/50 volume of lysis buffer [0.5 M NaCl/20 mM Tris/HCl, pH 7.9/10% (v/v) Nonidet P40], 1 mM PMSF (Sigma) and 200 μ g/ml lysozyme (Sigma). After sonication (Ultrasonic, up400A), the lysate was used for Western-blot analysis and other assays or stored at –70 °C.

TK activity assay

The TK activity assay was performed as described previously [38]. Crude lysate (10 μ l) was mixed with 75 μ l of reaction mixture containing 0.16 M Tris/HCl (pH 7.5), 0.14 mM albumin, 12.6 mM phosphocreatine, 11.2 units/ml creatine kinase, 120 μ M thymidine, 2.35 μ M [3 H]thymidine (6.7 Ci/mmol), 2.4 mM ATP, 2.4 mM $MgCl_2$, 9 mM NaF and 1.9 mM DTT. The mixture was incubated at 37 °C for 30 min. A 50 μ l sample was spotted on to a Whatman DE-81 filter, washed twice with alcohol and dried. The radioactivity on the filter was measured in a liquid-scintillation counter (LS-6000; Beckman, Fullerton, CA, U.S.A.). The relative-specific activity was calculated as the value of TK activity subtracted from the value of the vector control, which was almost identical with the water control, and normalized with the TK concentration calculated from enhanced chemiluminescence (ECL®, Amersham Biosciences, Little Chalfont, Bucks., U.K.) Western-blot analysis.

Determination of the kinetics of wild-type and mutant TK proteins for thymidine

Proteins of TK and mutants, which were expressed by pRsetA vector, had the histidine-tagged polypeptides at the N-terminus. The batch histidine beads' purification was used for TK protein purification according to the manufacturer's instruction (Qiagen, Hilden, Germany). The purification tube was prepared by loading 2 ml of Ni^{2+} -nitrilotriacetate agarose and washed with loading buffer (0.5 M NaCl/20 mM Tris/HCl, pH 7.9/10% Nonidet P40) and rotated for 10 min. All subsequent steps were performed at 4 °C. The crude lysate was clarified by centrifugation at 6000 g for 30 min and loaded on to a purification tube. After

overnight incubation, the tube was washed with two washing buffers (loading buffer plus 30 and 60 μ M imidazole respectively) each for 10 min. The protein was obtained by incubating three times each with 1 ml of elution buffers (loading buffer plus 100, 200 and 300 μ M imidazole respectively) for 20 min. Purified proteins were assessed on Coomassie Blue-stained gel and the protein concentration was quantitated with bicinchoninic acid kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and was stored at -70°C for determining the K_m value.

To determine the K_m values of the wild-type and mutant EBV TKs for thymidine, reaction conditions were similar to that for the TK activity assay described above. [^3H]Thymidine concentrations varied from 0.5 to 2.25 μ M. Lineweaver–Burk plots were used to determine the K_m and K_{cat} ($V_{\text{max}}/[E]$) values.

Metal-ion usage and substrate-competition assay

Metal-ion usage and substrate competition assays were performed using a procedure similar to that used for the TK activity assay. Several metal ions, including magnesium, manganese and zinc, were tested for their effect on enzyme activity. Assay conditions were 0.16 M Tris/HCl (pH 7.5), 0.14 mM albumin, 12.6 mM phosphocreatine, 11.2 units/ml creatine kinase, 120 μ M thymidine, 2.35 μ M [^3H]thymidine (6.7 Ci/mmol), 2.4 mM ATP, 9 mM NaF, 1.9 mM DTT and 2.4 mM metal chloride. Conditions of the substrate competition assay were the same as that for the TK activity assay except that the competitor was added to the reaction mixture to a 50-fold excess over the total thymidine concentration before adding the radioactive thymidine.

Western-blot analysis

The crude bacterial lysate was diluted 1:70 with loading buffer, electrophoresed by SDS/PAGE, blotted on to Immobilon-P (45 μ m; Millipore, Bedford, MA, U.S.A.), and blocked with 10 mM Tris/HCl (pH 7.4), 0.9% NaCl and 4% (w/v) skim milk (blocking buffer) for 1.5 h. Anti-TK monoclonal antibody, 5F4C (Y.-R. Chang, T.-Y. Hsu and J.-Y. Chen, unpublished work), was used as the primary antibody and it was allowed to react for 1 h at room temperature (25 $^{\circ}\text{C}$). The blot was washed and incubated with 1:5000 diluted, horseradish peroxidase-labelled goat anti-mouse antibody (Amersham Biosciences) at room temperature for 1 h. After incubation, the blot was washed three times in washing buffer, then developed with freshly prepared substrate for 1 min (ECL[®] Western blotting; Amersham Biosciences). The luminescence was detected by a short exposure to X-ray film. The relative concentration of each protein was determined by densitometry (UltraScan XL; Amersham Biosciences).

Phosphate transfer assay to determine the enzyme kinetics

Phosphate transfer assay was performed as described by Eriksson et al. [39] to determine the enzyme kinetics. Purified TK proteins were mixed with 1.6 μ M [γ - ^{32}P]ATP (6000 Ci/mmol), 50 mM Tris/HCl (pH 7.6), 0.5 mM MgCl_2 , 100 mM KCl, 0.5 mg/ml BSA and 0.2–100 μ M nucleoside in a final volume of 50 μ l. The reaction mixture was incubated at 37 $^{\circ}\text{C}$ for 30 min; the reaction was terminated by boiling for 1 min. The mixture was centrifuged and 2–4 μ l of the supernatant was spotted on a poly(ethyleneimine)–cellulose F (Merck, Darmstadt, Germany) thin layer sheet. Chromatography was performed for 8–12 h using 99% isobutyric acid/ NH_4OH /water (66:1:33, by vol.) as the mobile phase. Products of the kinase reaction were detected by PhosphorImager (Molecular Dynamics, Sunnyvale, CA,

Figure 1 Alignment of five human herpesvirus TKs

Multiple alignment of the amino acid sequence of five human herpesvirus TKs, including α -herpesviruses HSV-1, HSV-2, VZV and γ -herpesviruses EBV, HHV-8, using Biology Workbench 3.0. Black blocks indicate amino acid identity. Five conserved regions are noted.

U.S.A.). The intensity of spots was determined by ImageQuant. Lineweaver–Burk plots were used to determine the enzyme kinetics.

RESULTS

Alignment of five human herpesvirus TKs

We aligned the amino acid sequences of five human herpesvirus TKs: the α -herpesviruses HSV-1, HSV-2, VZV, γ -herpesviruses EBV and HHV-8 (human herpes virus-8), using Workbench 3.0 to identify conserved regions that might indicate functional domains. As shown in Figure 1, there are five conserved regions in these TK protein sequences. Among them, sites 3 and 4 are highly conserved and have been suggested to be involved in nucleoside binding through studies of crystal structure and mutation analysis of HSV-1 TK [32–36]. Site 3, consisting of the motif -DRH-, comprises three hydrophilic residues. The arginine at position 393 (Arg³⁹³) is highly conserved among all TKs (the single amino acid and its position in the EBV TK was designated as Arg³⁹³), suggesting its importance in the evolution of herpesviral TKs [40]. This site is important for TK activity and has been suggested to be involved in nucleoside recognition [32,35]. Site 4 comprises residues -(C/V)(Y/F)P- and is -CYP- in HSV-1 and HSV-2, -CFP- in VZV and -VFP- in EBV and HHV-8. Although site 4 is not as well conserved as site 3, it was still expected to participate in thymidine binding [33,36]. In the present study, Asp³⁹², Arg³⁹³ and His³⁹⁴ in site 3 and Val⁴⁰¹ and Phe⁴⁰² in site 4, which are predicted to be nucleoside-binding residues in EBV TK, were chosen for the investigation of their functional and biochemical roles.

TK activities of the Asp³⁹² mutants

To investigate the putative role of Asp³⁹² at site 3, a series of mutants were constructed, including D392E, D392H and D392N (Figure 2a). Protein expression was confirmed by Western blotting with EBV TK monoclonal antibody, and the activities of the

Figure 2 Mutational analysis of amino acids in the thymidine recognition site

(a) Mutations created within the conserved domain site 3 are indicated by * and blocked. (b) Protein expression in *E. coli* BL21(DE3)pLysS, transformed with plasmids D392E, D392N, D392H, R393Q, R393L, R393H, R393E, H394D, H394N, H394K, H394F, H394L, vector control and wild-type EBV TK, was detected using a mouse anti-TK monoclonal antibody 5F4C. (c) The relative TK activity was measured using [3 H]thymidine as the substrate. D392E and R393H retained approx. 60–70 % of the activity and the remaining mutants were inactive. The value of each mutant was normalized with the amount of relative expression in (b). Bars represent S.D. from duplicate assays.

Table 2 Kinetic parameters for thymidine of wild-type TK and site 3 and site 4 mutants

EBV TK proteins	K_m (μ M)	K_{cat} (s^{-1})	Mean relative K_{cat}/K_m
Wild-type	4.83 ± 0.48	0.066 ± 0.001	1.00
D392E	22.9 ± 4.99	0.043 ± 0.018	0.13
R393H	17.9 ± 1.63	0.022 ± 0.001	0.09
V401C	11.3 ± 0.81	–	–
F402S	9.25 ± 0.24	0.044 ± 0.006	0.35
F402Y	8.77 ± 0.31	0.077 ± 0.011	0.63

–, not determined.

mutants were determined by TK activity assay. As shown in Figure 2(c), D392E retained 68 % activity of the wild-type enzyme but D392H and D392N lost almost all of their activities. The function of Asp³⁹² could be compensated for by replacement with the negatively charged glutamic residue but not with the positively charged histidine residue, or the uncharged asparagine residue. Results indicate that the negative charge plays an important role at this position. The K_m and K_{cat} values (catalytic-centre activity) for thymidine of D392E were compared with that of the wild-type TK (Table 2). The K_m value for thymidine was 22.9 and 4.83 μ M for the D392E mutant and wild-type TK respectively. The increased K_m value indicated that the mutant has decreased affinity for thymidine. The decreased K_{cat} and relative K_{cat}/K_m values may reflect local structure perturbation caused by the substituted residue, which interferes with the catalytic process. The results suggest that Asp³⁹² is involved in nucleoside binding.

Figure 3 Metal-ion usage by the wild-type EBV TK and its mutants

Three metal ions, magnesium, manganese and zinc, were tested in the activity assays of EBV TK, D392E and R393H respectively. D290E and T298S, which were the mutants corresponding to the ATP-binding site of HSV-1 TK, were tested as controls. Relative activity was determined by comparing the value with wild-type TK in the presence of magnesium. Bars represent S.D. from duplicate assays.

TK activities of Arg³⁹³ mutants

The involvement of Arg³⁹³ residue in TK activity was tested by replacing it with a glutamine, leucine, histidine or glutamic residue (Figure 2a). Protein expression was confirmed by Western blotting with EBV TK monoclonal antibody (Figure 2b). TK activities were measured and calculated as shown in Figure 2(c). The R393H mutant, in which arginine was replaced by the positively charged histidine residue, retained 61 % activity relative to that of the wild-type. However, substitution of Arg³⁹³ residue with leucine, glutamine or aspartic residues, which are hydrophobic, uncharged and negatively charged residues respectively resulted in loss of activity. These results suggested that a positive charge may be required to maintain the functional activity of EBV TK. Furthermore, the K_m and K_{cat} values for thymidine of the R393H mutant were determined (Table 2). The K_m value for thymidine was 17.9 μ M, a 4-fold increase, and the K_{cat} value was reduced to one-third, resulting in a marked decrease in the K_{cat}/K_m values in comparison with that of the wild-type enzyme. These results revealed that Arg³⁹³ is also involved in nucleoside binding.

TK activities of the His³⁹⁴ mutants

His³⁹⁴ was also found to be highly conserved in site 3 (Figure 1), even though its function was not very clear. His³⁹⁴ was replaced with asparagine, lysine, phenylalanine, leucine or aspartic residue (Figure 2a). As shown in Figure 2(b), the TK protein expression was confirmed by Western blotting and all mutants lost their activities (Figure 2c). Since all the His³⁹⁴ mutants, regardless of charge and structure, lost their enzymic activity, it seems that His³⁹⁴ plays a crucial role in enzymic activity.

Metal-ion usage of the site 3 mutants

An amino acid in adenylate kinase equivalent to EBV Asp³⁹² was shown to fix Mg²⁺ via water molecules [41]. For HSV-1 TK, replacement of this aspartic residue with glutamine residue did not alter the requirement for a bivalent cation [42], but structural studies revealed that the negative charge of this aspartic residue may be important for co-ordination with a bivalent cation [35,36]. To evaluate the role of the Asp³⁹² in site 3 further, the metal-ion usage was assayed. Three metal ions, Mg²⁺, Mn²⁺ and Zn²⁺, were tested with D392E, R393H and wild-type TKs. D290E and T298S, which are in site 1, have been suggested to be involved in ATP binding and were tested as controls. Results were similar to those reported by Tung and Summers [27], Mg²⁺ is the preferred ion for EBV TK, Mn²⁺ is less favoured and Zn²⁺ could inhibit its activity, as shown in Figure 3. Among the mutants tested,

Figure 4 Determination of the substrate specificity of site 3 mutants

GCV, ACV, D4T, AZT, DDI and IdU were used at 50-fold excess over the total thymidine concentration to compete for the binding of thymidine by wild-type TK, D392E and R393H. Relative activity was determined by comparing the value with wild-type TK in the absence of the competitors. Bars represent S.D. from duplicate assays.

only D392E revealed an altered pattern of metal-ion usage, where nearly similar activities were found for all three metal ions. These results show that, in addition to its involvement in enzymic activity and nucleoside binding, Asp³⁹² also plays an important role in metal-ion binding.

Substrate specificity of the site 3 mutants

The importance of site 3 of the EBV TK in the nucleoside and metal-ion binding was deduced from the above results. We performed competition assays to determine the substrate specificities of the site 3 mutants. Six commonly used nucleoside analogues, GCV, ACV, AZT (3'-azido-3'-deoxythymidine), D4T (3'-deoxy-2',3'-didehydrothymidine), DDI (dideoxyinosine) and IdU (iododeoxyuridine), were chosen and thymidine was used as the control. These drugs were used at a 50-fold higher concentration when compared with that of thymidine to compete for binding. With wild-type EBV TK, thymidine usage could not be competed by ACV, GCV and DDI but could be competed partially by D4T, and almost completely by AZT and IdU (Figure 4). This result is similar to other studies, suggesting that EBV TK seems to favour pyrimidine nucleoside analogues such as D4T, AZT and IdU, but not purine nucleoside analogues such as ACV, GCV and DDI [43]. With the mutants D392E and R393H (Figure 4), similar to the wild-type, thymidine could not be competed by ACV, GCV and DDI but both mutants were competed for their activity by AZT and IdU. Interestingly, for both mutants, more than 50% of the activity was competed by D4T, suggesting that they had higher affinities for this nucleoside analogue when compared with the wild-type.

To address this phenomenon further, the IC_{50} and the kinetic parameters of wild-type TK and mutants for GCV, AZT and D4T were determined (Table 3). The K_m values for D4T of D392E and R393H were slightly decreased when compared with that of wild-type. Both K_{cat} and K_{cat}/K_m values of D392E and R393H for D4T were increased, reflecting that their catalytic efficiency for D4T was better when compared with wild-type enzyme. On the basis of these results, the residues at site 3 were supposed to play a role in the determination of substrate specificity.

TK activities of the Val⁴⁰¹ mutants

Substitution of Cys¹⁷¹ in HSV-1 TK (corresponding to EBV TK Val⁴⁰¹) with a glycine residue did not destroy its enzymic activity [44]. It was suggested that this residue is not involved in nucleoside binding and is not essential for the catalytic activity of the enzyme. No specific role could be assigned to this residue from

Table 3 Kinetic parameters for nucleoside analogues of wild-type TK and site 3 and site 4 mutants

EBV TK proteins	Nucleoside drugs	Kinetic parameters			Mean K_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
		IC_{50} (μM)	K_m (μM)	K_{cat} (s^{-1})	
TK	GCV	> 200	> 100	—	—
	AZT	8.38 ± 0.47	3.35 ± 0.07	0.034 ± 0.002	0.01038
	D4T	151 ± 8.5	6.97 ± 0.75	0.036 ± 0.007	0.00517
D392E	GCV	171.5 ± 29	> 100	—	—
	AZT	6.12 ± 0.78	4.25 ± 0.21	0.044 ± 0.026	0.01011
	D4T	16.2 ± 0.49	5.67 ± 0.64	0.075 ± 0.011	0.01039
R292H	GCV	153 ± 37.5	—	—	—
	AZT	5.58 ± 0.06	3.30 ± 1.27	0.056 ± 0.032	0.01622
	D4T	13.6 ± 0.92	4.57 ± 0.45	0.075 ± 0.046	0.01600
F402S	GCV	> 200	—	—	—
	AZT	84.8 ± 0.21	63.3 ± 2.40	0.020 ± 0.032	0.00059
	D4T	101 ± 5.66	60.5 ± 27.6	0.030 ± 0.009	0.00060

—, not determined.

three-dimensional studies of the HSV-1 TK [34,36]. To determine the functional roles of the amino acid residues at site 4 of the EBV TK, five mutants were constructed: V401C, V401L, V401S, F402S and F402Y (Figure 5a). Three of Val⁴⁰¹ mutants, those substituted with the hydrophilic amino acids cysteine or serine or the hydrophobic leucine, were used to determine the contribution of this residue to EBV TK activity. The activities of the mutants were determined after confirming the expression level of each protein (Figure 5b). The mutant V401C retained approx. 80% of the wild-type activity, whereas the V401L and V401S mutants were almost inactive (Figure 5c). EBV TK has a valine residue at this position, whereas HSV TK has a cysteine residue; therefore it is not surprising that the mutant V401C was active. However, EBV TK was inactive when this residue was changed to the hydrophilic residue serine. Similarly, the HSV-1 TK activity was lost when the equivalent residue was changed to serine [44]. The kinetic parameters of V401C mutant for thymidine were also different from that of the wild-type TK (Table 2). These findings suggest that Val⁴⁰¹ in site 4 of the EBV TK may play some role in its activity and requires further study.

TK activities of the Phe⁴⁰² mutants

The importance of this residue in HSV-1 TK was discussed extensively, especially the three-dimensional structural studies [34,36]. In a random mutational analysis, it was found that Tyr¹⁷² of HSV-1 TK (corresponding to EBV TK Phe⁴⁰²) could be replaced only by phenylalanine, without changing its activity [33]. The three-dimensional crystal structure of the HSV-1 TK also revealed that this residue is stacking on the thymidine and contacts it closely [36]. Two mutants were designed at this position, F402S and F402Y. After expression, the amounts of the mutant proteins and enzyme activities were determined (Figure 5b). As shown in Figure 5(c), the mutant F402Y was fully active but F402S was partially inactive. The F402Y had a 122% relative activity when compared with that of wild-type, whereas substitution with a serine residue retained 60% of the wild-type activity. These results revealed that the aromatic ring of phenylalanine and tyrosine residues at this position is important, although serine residue, without an aromatic ring in its structure, could compensate this function partially. The K_m values of these two mutants altered slightly and K_{cat}/K_m values were decreased

Figure 5 Mutational analysis of amino acids in the thymidine-binding site

(a) Mutations created within conserved domain site 4 are indicated by * and blocked. (b) Protein expression in *E. coli* BL21(DE3)pLysS, transformed with plasmids containing V401C, V401L, V401S, F402S, F402Y, vector control and wild-type EBV TK, was detected with mouse anti-TK monoclonal antibody 5F4C. (c) The relative activity was measured using [³H]thymidine as the substrate. Val⁴⁰¹ mutants varied in their activities. Phe⁴⁰² mutants retained at least 60 % of the wild-type activity. The value for each mutant was normalized with the relative expression amount in (b). Bars represent S.D. from duplicate assays.

(Table 2), reflecting that Phe⁴⁰² has a decreased catalytic efficiency and it also plays a role in nucleoside binding.

Substrate specificity of the site 4 mutants

To determine further the contribution of the amino acid residues at site 4 to substrate specificity, six commonly used nucleoside analogues were prepared as described in the Experimental section. The competition assay was performed and the results are shown in Figure 6. F402S altered the substrate specificity, whereas F402Y had the same specificity as the wild-type. For F402S, not only the purine, but also the pyrimidine analogues D4T, AZT and IdU, were much less efficient in competing with thymidine for binding. The significant difference in K_m and K_{cat}/K_m values for AZT and D4T indicated that the binding efficiency of this mutant to these drugs was affected severely (Table 3). It strongly suggested that Phe⁴⁰² is important in substrate selection.

Recently developed L-form nucleoside analogues L-FMAU and L-I-OddU have been shown to inhibit EBV replication [10]. To confirm further the role of site 4 in substrate usage, these two

Figure 6 Determination of substrate specificity of site 4 mutants

GCV, ACV, D4T, AZT, DDI and IdU were used at 50-fold excess over the total thymidine concentration to compete for the binding of thymidine by wild-type TK, V401C, V401L, F402S and F402Y. Unlabelled thymidine was used as a control. Relative activity was determined by comparing the value with wild-type TK in the absence of the competitors. Bars represent S.D. from duplicate assays.

Figure 7 Competition of L-form nucleoside analogues to thymidine in site 3 and site 4 mutants

L-FMAU and L-I-OddU were used at 50-fold excess over total thymidine concentration to compete for binding of thymidine by wild-type TK and site 3 and site 4 mutants. Relative activity was determined by comparing the values with that in the presence of DMSO. Bars represent S.D. from duplicate assays.

nucleoside analogues were also used to compete with thymidine for binding at sites 3 and 4. As shown in Figure 7, these two nucleoside analogues competed well with thymidine for the wild-type TK. This result is consistent with the reported results [10]. The competitive effects were also observed for site 3 and site 4 mutants, except for F402S and F402Y. Surprisingly, L-I-OddU was relatively incapable of competing with thymidine for binding, indicating that Phe⁴⁰² is critical in substrate selection.

DISCUSSION

Herpesviral TKs have been chosen as important targets for the development of anti-viral agents and gene therapies. Accordingly, EBV TK is considered a potential target for the treatment of EBV-associated disease and as a tool for anticancer therapies [9,24,26,45]. Understanding the structure–function relationship of TK enzymes is helpful in designing new antiviral drugs and gene therapy strategies. Site-directed mutagenesis, in combination with crystal structure studies, has provided significant insights in this direction. However, there are few studies on the structure–function relationship of EBV TK. To the best of our knowledge, this is the first study to describe the importance of individual amino acids in the conserved regions of EBV TK. In the absence of any structural information, we determined the nucleoside-binding sites and examined the roles of amino acid residues at these sites using site-directed mutagenesis. Following the alignment of the TK sequences of five human herpesviruses and comparing with the studies of HSV-1 TK, our results confirmed that sites 3 and 4 of EBV TK are involved in nucleoside binding, metal-ion binding and substrate specificity.

Our results indicated that Asp³⁹² plays multiple roles in site 3 of EBV TK. From structural information for HSV-1 TK, the corresponding Asp¹⁶² is important for substrate and metal-ion binding. Results obtained from other studies suggested that it fixes the Mg²⁺ ion via a water molecule through its negative charge, in addition to binding to the phospho group of TMP [35,36]. However, this function was not supported by another study [42], which showed that the requirement for a bivalent cation was not altered when it was changed to an uncharged glutamine residue. In our study, the metal-ion usage of all site 3 and site 4 mutants is similar to that of HSV-1 TK [46]. The catalytic activity of the bivalent cations tested for wild-type EBV TK was in the order Mg²⁺ > Mn²⁺ > Zn²⁺, where, in the presence of Zn²⁺, the enzyme is inactive. Interestingly, only the D392E substitution changed the usage pattern of metal ions, especially for Zn²⁺, with which it exhibited the same activity as with Mg²⁺ and Mn²⁺ (Figure 3). Furthermore, the concentration of Mg²⁺ required for activity was much lower when compared with that of the wild-type (results not shown).

Arg³⁹³, similar to its corresponding amino acid of HSV-1 TK, Arg¹⁶³ [32], was found to be crucial for the activity. According to the models of adenylate kinase and HSV-1 TK, this arginine residue is in a strained conformation and forms a hydrogen bond with the phosphate groups of AMP and TMP [35,36,41]. Further evidence of a functional role was provided by Sawyer et al. [47], who identified a substitution with glutamine at this position in an ACV-resistant virus, corroborating the role of this arginine residue in nucleoside binding and substrate specificity. The increase in *K_m* value for thymidine (Table 2) and binding affinity for D4T (Figure 4, Table 3) of the R393H mutant revealed that this arginine residue participates in nucleoside binding and substrate specificity, at least for D4T. The potential role of R393H TK in the nucleoside utilization is under further investigation.

His³⁹⁴ is a very important residue and cannot be substituted with other amino acids with different properties. By analogy, Black and Loeb [32] also found that the histidine residue at the corresponding position in HSV-1 TK could not be substituted with other amino acids. On the basis of the structural information of HSV-1 TK, it seems probable that this histidine contributes to the special conformation for a functional TK activity, even though the precise role remains unclear [34–36].

It is clear that Phe⁴⁰² plays an important role at site 4. Our results reveal that the special structures of phenylalanine and tyrosine residues are important for the activity of EBV TK. This finding was consistent with the study [32] that activity was retained only with phenylalanine at Tyr¹⁷² of HSV-1 TK. On the basis of the information obtained from the density functional studies on HSV-1 TK, the substrate–Tyr¹⁷² interaction is essentially an electrostatic force and was suggested to be involved in the substrate binding [48]. The three-dimensional structure of the HSV-1 TK showed the aromatic ring of the tyrosine residue at this position stacked on to the thymine ring, and Arg¹⁶³ (HSV-1 TK) formed the hydrogen bond with the hydroxy group of the tyrosine residue at this position [36]. Interestingly, F402Y of the EBV TK had higher activity when compared with that of the wild-type TK (Figure 5c). This phenomenon suggests that the hydrogen bond is important to fix thymidine, because the phenylalanine residue at this position in EBV TK could not form the hydrogen bonds with Arg³⁹³.

There are differences between EBV and HSV-1 TKs in their substrate specificities, which may have resulted from the divergence of protein sequences. HSV-1 TK can phosphorylate purine nucleoside analogues such as GCV, ACV and DDI, and pyrimidine nucleoside analogues such as D4T, AZT and IdU, whereas EBV TK prefers to utilize pyrimidine nucleoside analogues only [43,49]. Our results agreed with these findings (Figures 4 and 6).

Figure 8 Hypothetical model for the structure of the conserved nucleoside-binding region of EBV TK

This model is based on the structure for the nucleoside-binding region of HSV-1 TK. The position and geometry of dTMP and amino acids in the conserved nucleoside-binding sites are shown as labelled. The polypeptide shown in light blue is the non-conserved region. The co-ordinate template was indexed as 2VTK in the Protein Data Bank. Structural modelling exploration was conducted using the program Swiss-PdbViewer v.3.6b3.

Interestingly, comparison of the TK protein sequences of these human herpesviruses enables their separation into two types; HSV-1 and HSV-2 in one class and EBV and HHV-8 in the other. For HSV-1 and HSV-2, not only are the residues of the conserved nucleoside-binding sites completely identical, but the substrate diversity is also almost the same. For the conserved nucleoside binding sites 3 and 4, all herpesviruses have the same residues (-DRH-) in site 3, whereas EBV and HHV-8 both have -VFP- in site 4. They also prefer to utilize the pyrimidine analogues [50]. Furthermore, it is reasonable to speculate that Phe⁴⁰² may be involved in determining the difference between HSV-1 and EBV TK in substrate range.

In Figure 8, a model is proposed for the protein structure of the nucleoside-binding region spanning from site 3 to site 4 in EBV TK, based on structural information for HSV-1 TK [34,36]. We can find relevant residues forming the cavity to accommodate the nucleoside substrate for catalysis. At site 3, Asp³⁹² may provide its negative charge to fix the Mg²⁺ and phosphate group of dTMP through water molecules and Arg³⁹³ provides its positive charge to fix the phosphate of TMP. At site 4, Phe⁴⁰² stacks on the thymine ring to stabilize the substrate. Some other residues, which are not located in the conserved sites 3 and 4, may also contribute and co-operate with the conserved residues to execute the catalytic process.

EBV TK, similar to HSV-1 TK, has been proved sensitive to many nucleoside analogues, implying that it can be applied in gene therapy [9,43]. Although HSV-1 TK has broader substrate specificity, EBV TK has a specific advantage in using anti-HIV drugs, such as AZT and D4T, which may have an extra benefit in treatment of AIDS-associated cancer [43]. On the other hand, our results may also be applied in other herpesvirus TKs, to gain a useful mutant. Our next goal will be to create double or triple

mutations in these two regions to find a mutant with better TK activity and broader substrate range for usage *in vivo*.

While we try to build a plausible model by analogy with other TKs, the precise interpretation of these results, as well as the effects of the mutants, await a detailed structural analysis of EBV TK. We expect these results to provide information useful in understanding the enzymic mechanism and for application in the development of new antiviral drugs and creation of an effective tool for gene therapy.

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