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 (Asp92 → Glu) and R393H retain activity, indicating that a negative charge is important for Asp92 and a positive charge is required for Arg939. 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 Asp92 plays multiple roles in this region. His944 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 Phe860 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 Phe860 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.
Chen and T.-Y. Hsu, unpublished work). A biochemical study revealed that EBV TK has $K_m$ values of 22 $\mu$M for thymidine and 25 $\mu$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 mutagenic 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-iodoiodoxolane uracil) were gifts from Y. C. Cheng (Department of Pharmacology, Yale University School of Medicine, New Haven, CT, U.S.A.) [10]. [3H]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 BamHI primer, the other with the complementary mutated sense primer and the HindIII primer. These two PCR products were purified by electrophoresis, mixed in a 1:1 molar ratio in PCR buffer containing 10 $\mu$M Tris/HCl (pH 8.8), 1.5 $\mu$M MgCl₂, 50 $\mu$M KCl, 0.1% Triton X-100, 400 $\mu$M dNTP and 2 units of DynaZyme II DNA polymerase (Finzymes 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 BamHI and HindIII 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 BamHI and HindIII 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 seeded to Luria–Bertani broth containing 200 $\mu$g/ml ampicillin and 25 $\mu$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 $\mu$g/ml lysozyme (Sigma). After sonication (Ultrasonic, up400A), the lysate was used for Western-blot analysis and other assays or stored at −70°C.

| Table 1 Primers used in the construction of mutants with a single amino acid change |
| Mutants | Sense primer (5′ → 3′) |
| D392E | GATTTCGATGAAGCCATTTGC |
| D392H | GATTTCGATGAAGCGCCATTTGC |
| D392N | GATTTCGATGAATCGCCATTTGC |
| R393Q | GATTTCGATGAAGCCATTTGC |
| R393L | GATTTCGATGAAGCGCCATTTGC |
| R393H | GATTTCGATGAAGCGCCATTTGC |
| R393E | GATTTCGATGAAGCGCCATTTGC |
| H394D | GACATGTCGCAATTGCCATTTGC |
| H394F | GACATGTCGCAATTGCCATTTGC |
| H394K | GACATGTCGCAATTGCCATTTGC |
| H394R | GACATGTCGCAATTGCCATTTGC |
| H394L | GACATGTCGCAATTGCCATTTGC |
| H394N | GACATGTCGCAATTGCCATTTGC |
| V402C | GGCTCGTGTCTGCCTTTTCTAA |
| V402I | GGCTCGTGTCTGCCTTTTCTAA |
| V402S | GGCTCGTGTCTGCCTTTTCTAA |
| F402Y | CTGGGTGTATTACCCTCTAAGC |

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

Proteins of TK and mutants, which were expressed by pRsetA vectors, 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 Ni²⁺-nitrilotriacetate agarse 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

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overnight incubation, the tube was washed with two washing buffers (loading buffer plus 30 and 60 \( \mu \)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 \( \mu \)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. [\(^{3}\text{H}\)]Thymidine concentrations varied from 0.5 to 2.25 \( \mu \)M. Lineweaver–Burk plots were used to determine the \( K_m \) and \( K_{cat} \) (\( V_{max}/[E] \)) 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 M albumin, 12.6 mM phosphocreatine, 11.2 units/ml creatine kinase, 120 \( \mu \)M thymidine, 2.35 \( \mu \)M [\(^{3}\text{H}\)]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 \( \mu \)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 °C). The blot was washed and incubated with 1:5000 dilution, 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 \( \mu \)M [\(^{32}\text{P}\)]ATP (600 Ci/mmole), 50 mM Tris/HCl (pH 7.6), 0.5 mM MgCl\(_2\), 100 mM KCl, 0.5 mg/ml BSA and 0.2–100 \( \mu \)M nucleoside in a final volume of 50 \( \mu \)l. The reaction mixture was incubated at 37 °C for 30 min; the reaction was terminated by boiling for 1 min. The mixture was centrifuged and 2–4 \( \mu \)l of the supernatant was spotted on a polyethyleneimine)–cellulose F (Merck, Darmstadt, Germany) thin layer sheet. Chromatography was performed for 8–12 h using 99 % isobutyric acid/NH\(_4\)OH/water (66:1:33, by vol.) as the mobile phase. Products of the kinase reaction were detected by PhosphorImager (Molecular Dynamics, Sunnyvale, CA, 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 sequence of five human herpesvirus TKs, including \( \alpha \)-herpesviruses HSV-1, HSV-2, VZV and \( \gamma \)-herpesviruses EBV, HHV-8, using Biology 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 hydrophobic residues. The arginine at position 393 (Arg \(^{393}\)) is highly conserved among all TKs (the single amino acid and its position in the EBV TK was designated as Arg \(^{393}\)), 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 -VF- 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, Asp \(^{392}\), Arg \(^{393}\) and His \(^{394}\) in site 3 and Val \(^{401}\) and Phe \(^{402}\) 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 Asp \(^{392}\) mutants**

To investigate the putative role of Asp \(^{392}\) 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
Table 2 Kinetic parameters for thymidine of wild-type TK and site 3 and site 4 mutants

<table>
<thead>
<tr>
<th>EBV TK proteins</th>
<th>( K_m ) (( \mu M ))</th>
<th>( K_{cat} ) (s(^{-1}))</th>
<th>Mean relative ( K_{cat}/K_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>4.83 ± 0.48</td>
<td>0.065 ± 0.001</td>
<td>1.00</td>
</tr>
<tr>
<td>D392E</td>
<td>22.9 ± 4.99</td>
<td>0.045 ± 0.018</td>
<td>0.13</td>
</tr>
<tr>
<td>R393H</td>
<td>17.9 ± 1.63</td>
<td>0.022 ± 0.001</td>
<td>0.09</td>
</tr>
<tr>
<td>V401C</td>
<td>11.3 ± 0.81</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F402S</td>
<td>9.25 ± 0.24</td>
<td>0.044 ± 0.006</td>
<td>0.35</td>
</tr>
<tr>
<td>F402Y</td>
<td>8.77 ± 0.31</td>
<td>0.077 ± 0.011</td>
<td>0.03</td>
</tr>
</tbody>
</table>

–, not determined.

TK activities of Arg\(^{393}\) mutants

The involvement of Arg\(^{393}\) 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 Arg\(^{393}\) 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 \( \mu M \), 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 Arg\(^{393}\) is also involved in nucleoside binding.

TK activities of the His\(^{394}\) mutants

His\(^{394}\) was also found to be highly conserved in site 3 (Figure 1), even though its function was not very clear. His\(^{394}\) 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 His\(^{394}\) mutants, regardless of charge and structure, lost their enzymic activity, it seems that His\(^{394}\) plays a crucial role in enzymic activity.

Metal-ion usage of the site 3 mutants

An amino acid in adenylate kinase equivalent to EBV Asp\(^{392}\) was shown to fix Mg\(^{2+}\) 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 Asp\(^{392}\) in site 3 further, the metal-ion usage was assayed. Three metal ions, Mg\(^{2+}\), Mn\(^{2+}\) and Zn\(^{2+}\), 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], Mg\(^{2+}\) is the preferred ion for EBV TK, Mn\(^{2+}\) is less favoured and Zn\(^{2+}\) could inhibit its activity, as shown in Figure 3. Among the mutants tested,
K_{cat}/\text{K}_{m} values for D4T of D392E were increased, reflecting that their catalytic efficiency compared with the wild-type was better when compared with wild-type enzyme.

D4T were increased, reflecting that their catalytic efficiency compared with the wild-type was better when compared with wild-type enzyme.

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

<table>
<thead>
<tr>
<th>EBV TK proteins</th>
<th>Nucleoside drugs</th>
<th>IC_{50} (\mu M)</th>
<th>K_{m} (\mu M)</th>
<th>K_{cat} (s^{-1})</th>
<th>Mean K_{cat}/K_{m} (s^{-1} \cdot \mu M^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK</td>
<td>GCV</td>
<td>&gt; 200</td>
<td>&gt; 100</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>AZT</td>
<td>8.38 ± 0.47</td>
<td>3.35 ± 0.07</td>
<td>0.034 ± 0.002</td>
<td>0.01038</td>
</tr>
<tr>
<td></td>
<td>D4T</td>
<td>151 ± 0.5</td>
<td>6.97 ± 0.75</td>
<td>0.036 ± 0.007</td>
<td>0.00517</td>
</tr>
<tr>
<td>D392E</td>
<td>GCV</td>
<td>171.5 ± 29</td>
<td>&gt; 100</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>AZT</td>
<td>6.12 ± 0.78</td>
<td>4.25 ± 0.21</td>
<td>0.044 ± 0.026</td>
<td>0.01011</td>
</tr>
<tr>
<td></td>
<td>D4T</td>
<td>6.2 ± 0.55</td>
<td>5.67 ± 0.64</td>
<td>0.075 ± 0.011</td>
<td>0.00109</td>
</tr>
<tr>
<td>R292H</td>
<td>GCV</td>
<td>153 ± 3.75</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>AZT</td>
<td>5.58 ± 0.06</td>
<td>3.30 ± 1.27</td>
<td>0.059 ± 0.032</td>
<td>0.01622</td>
</tr>
<tr>
<td></td>
<td>D4T</td>
<td>13.6 ± 0.92</td>
<td>5.57 ± 0.45</td>
<td>0.075 ± 0.046</td>
<td>0.01600</td>
</tr>
<tr>
<td>F402S</td>
<td>GCV</td>
<td>&gt; 200</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>AZT</td>
<td>84.8 ± 0.21</td>
<td>63.3 ± 2.40</td>
<td>0.020 ± 0.032</td>
<td>0.00059</td>
</tr>
<tr>
<td></td>
<td>D4T</td>
<td>101 ± 5.66</td>
<td>60.5 ± 27.6</td>
<td>0.030 ± 0.009</td>
<td>0.00060</td>
</tr>
</tbody>
</table>

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, Asp^{392} also plays an important role in metal-ion binding.

Substrate specificity of the site 3 mutants

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, D4T, DDI and IdU 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_{m} 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 Val^{401} mutants

Substitution of Cys^{171} in HSV-1 TK (corresponding to EBV TK Val^{401}) 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 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 Val^{401} 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 Val^{401} in site 4 of the EBV TK may play some role in its activity and requires further study.

TK activities of the Phe^{402} 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 Tyr^{172} of HSV-1 TK (corresponding to EBV TK Phe^{402}) 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.

(1)

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.

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 Asp\textsuperscript{392} plays multiple roles in site 3 of EBV TK. From structural information for HSV-1 TK, the corresponding Asp\textsuperscript{162} is important for substrate and metal-ion binding. Results obtained from other studies suggested that it fixes the Mg\textsuperscript{2+} 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 Mg\textsuperscript{2+} > Mn\textsuperscript{2+} > Zn\textsuperscript{2+}, where, in the presence of Zn\textsuperscript{2+}, the enzyme is inactive. Interestingly, only the D392E substitution changed the usage pattern of metal ions, especially for Zn\textsuperscript{2+}, with which it exhibited the same activity as with Mg\textsuperscript{2+} and Mn\textsuperscript{2+} (Figure 3). Furthermore, the concentration of Mg\textsuperscript{2+} required for activity was much lower when compared with that of the wild-type (results not shown).

Arg\textsuperscript{393}, similar to its corresponding amino acid of HSV-1 TK, Arg\textsuperscript{163} [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 $K_a$ 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.

His\textsuperscript{394} 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 specific conformation for a functional TK activity, even though the precise role remains unclear [34–36].

It is clear that Phe\textsuperscript{402} 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 Tyr\textsuperscript{172} of HSV-1 TK. On the basis of the information obtained from the density functional studies on HSV-1 TK, the substrate–Tyr\textsuperscript{172} 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 Arg\textsuperscript{163} (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 Arg\textsuperscript{393}.

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 1dU, whereas EBV TK prefers to utilize pyrimidine nucleoside analogues only [43,49]. Our results agreed with these findings (Figures 4 and 6).

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 Phe\textsuperscript{402} 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 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 2TK in the Protein Data Bank. Structural modelling exploration was conducted using the program Swiss-PdbViewer v.3.6b3.

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 2TK in the Protein Data Bank. Structural modelling exploration was conducted using the program Swiss-PdbViewer v.3.6b3.
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 enzymatic mechanism and for application in the development of new antiviral drugs and creation of an effective tool for gene therapy.

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