Characterization of Three Essential Residues in the Conserved ATP-Binding Region of Epstein-Barr Virus Thymidine Kinase†

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ABSTRACT: The thymidine kinase encoded by Epstein-Barr virus (EBV TK) is an important target for antiviral therapy and the treatment of EBV-associated malignancies. Through computer-assisted alignment with other human herpesviral TK proteins, EBV TK was shown to contain a conserved ATP-binding motif as for the other TK enzymes. To investigate functional roles of three highly conserved residues (G294, K297, T298) within this region, site-directed mutagenesis was employed to generate various mutants. The TK enzyme activity and ATP-binding ability of these mutant TK enzymes were determined and compared with EBV wild-type TK (wtTK). Mutant G294V lost its ATP-binding ability and was inactive in enzyme activity assay. As the enzyme activity of G294A was reduced to 20% of that of wtTK, the K_m for ATP binding of G294A was 48.7 μM as compared with 30.0 μM of EBV wtTK. These results suggested that G294 participates in ATP binding and contributes to maintenance of structure. EBV TK mutants K297E, K297Q, and K297R lost their ATP-binding ability and enzyme activity. However, K297R was shown to have a preference for usage of GTP (K_m: 43.0 μM) instead of ATP (K_m: 87.6 μM) as the phosphate donor. This implies that, in addition to nucleotide binding, K297 was involved in the selection of phosphate donor. While EBV TK mutant T298S retained approximately 80% of wtTK enzyme activity, T298A lost its enzyme activity, suggesting that a hydroxyl group at this position is important for the enzyme activity. Interestingly, T298A retained its ATP-binding ability, suggesting a role of T298 in the catalytic process but not in the coordination of ATP. This study demonstrated that amino acid residues G294, K297, and T298 in the ATP-binding motif of EBV TK enzyme are essential for the enzymatic activity but are involved in different aspects of its action.

Thymidine kinase (TK)[1] plays a central role in the nucleotide salvage pathway. It catalyses the transfer of the γ-phosphoryl group of ATP to thymidine to produce thymidine monophosphate (TMP). TMP is phosphorylated further by cellular enzymes to TTP, which serves as a substrate for DNA polymerase during DNA replication. Herpesviral TKs have a broader substrate range than the cellular TK in terms of phosphorylation of nucleoside analogues (*1*). Epstein-Barr virus (EBV), a member of the gammaherpesvirinae, is associated closely with several types of malignancies, such as African Burkitt's lymphoma (*2, 3*), nasopharyngeal carcinoma (*4*), Hodgkin's disease (*5*), T cell lymphoma (*6*), gastric carcinoma (*7*), and post-transplantation lymphoproliferative disorder (*8*). The viral TK is encoded by BXLF1 open reading frame, which produces a 67 kD protein (*9*). EBV TK is expressed in the lytic cycle and is a potential target for specific antiviral and anticancer therapies (*10*−*14*).

EBV TK shares protein sequences and functional homology with herpes simplex virus type 1(HSV-1) TK but has an extended N-terminus. The function of this elongated amino terminus is unknown, but this hydrophilic region showed strong antigenicity. Specific TK antibodies detected in EBV-infected patients were almost all directed to the N-terminus (*15*). Biochemical studies revealed that EBV TK has K_m values of 22 μM for thymidine and 25 μM for ATP (*16*). Substrate specificity for the TK also has been determined (*16, 17*). However, the relationship between protein structure and function for the EBV TK has been addressed rarely. In our previous study, we showed that the N terminal 1−243 amino acids were dispensable for the TK activity, whereas the intact C terminal domain was essential (*18*). When 10 residues were deleted from the C-terminus, the TK activity was abolished completely. Apart from the N- and

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[1] Abbreviations: TK, thymidine kinase; EBV, Epstein-Barr virus; HSV, herpes simplex virus; VZV, varicella zoster virus; HHV-8, human herpes virus-8; GCV, ganciclovir; ACV, acyclovir; D4T, 3′-deoxy-2′,3′-didehydrothymidine; AZT, 3′-azido-3′-deoxythymidine; DDI, dideoxyinosine; IdU, iododeoxyuridine.

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Table 1: Primers Used in the Construction of Mutants with a Single-Amino Acid Change

mutants	sense primer (5 → 3)
G294A	AGGTGCCCCTG*CTG*TGGGAAAGA
G294V	AGGTGCCCCTG*TTG*TGGGAAAGA
K297E	TGGTGTGGGA*GAG*ACTACTATGC
K297Q	TGGTGTGGGA*CAG*ACTACTATGC
K297R	TGGTGTGGGA*CGG*ACTACTATGC
T298A	TGTGGGAAAG*GCT*ACTATGCTGA
T298S	TGTGGGAAAG*AGT*ACTATGCTGA

C-termini, little is known about the conserved regions of EBV TK. Recently, we identified the conserved regions in EBV TK for nucleoside binding and proved that they were important for activity, metal ion binding, and nucleoside substrate selection (*19*). Beside the nucleoside-binding site, EBV TK has another important conserved region, the ATP-binding site. Characterization of the ATP-binding site of EBV TK is necessary for better understanding of the complete picture of the catalytic mechanism.

ATP is a critical phosphate donor, supplying the phosphoryl group in the catalytic process of thymidine phosphorylation. Comparison of the protein sequences of many ATP-utilizing enzymes revealed that the ATP-binding regions were conserved in glycine-rich composition (*20*). On the basis of the crystal structure of adenylate kinase, this glycine-rich region likely bends to form a loop to bind ATP (*21*). From multiple alignments (*22*), six highly conserved sites were recognized in 12 herpesviral thymidine kinases. By mutational analyses, site 1 was suggested to have the function of binding ATP, while sites 3 and 4 were involved in nucleoside binding (*23*−*25*). When any one of the conserved glycines in site 1 was substituted with a valine, the HSV-1 TK became inactive (*23*). In another related study, replacement of one of the glycine residues of p21, a GTPase, in the conserved nucleotide-binding site increased the transforming ability of p21 and reduced the GTPase activity (*26*, *27*). For the adenylate kinase, mutation of the corresponding glycine residue in nucleotide-binding site does not produce similar biological effects (*28*). Disturbance of this glycine-rich region appears to produce different effects, and these results emphasize the importance of determining the functional role of the nucleotide-binding site. In this study, we attempted to identify the ATP-binding domain of EBV TK and to characterize biochemical properties of important residues in this region.

MATERIALS AND METHODS

Chemicals. Nucleoside analogues, thymidine, ATP, creatine phosphate, creatine phosphokinase, albumin, sodium fluoride, DTT, PMSF, and lysozyme were purchased from Sigma Corp. [H^3]-Thymidine and [γ-P^{32}]-ATP were obtained from NEN Corp.

Site-Directed Mutagenesis. Single-amino acid mutants were generated by recombinant PCR. The template plasmid, pET-TKB1B, encodes a full-length TK gene fused to a his-tag sequence contained in the vector (*29*). Primers were designed to modify specific amino acid codons (Table 1). Two PCR reactions were set up at the same time, one with a mutated antisense primer and a *Bam*HI recognition site-containing primer, the other with the mutated sense primer and a *Hind*III recognition site-containing primer. The two PCR products were gel purified, mixed at a 1:1 molar ratio in PCR reaction buffer, with one cycle of denaturation at 95 °C for 5 min, annealing at 52 °C for 5 min, and extension 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 pRsetA vector (Novagen) at the corresponding sites. DNA sequences around the mutated sites were confirmed by DNA sequencing (ABI PRISM dye terminator cycle sequencing kits).

Expression of the TK Protein. The recombinant TK plasmids obtained were used to transform *E. coli* strain BL21(DE3)pLysS. One colony was seeded into LB broth containing 200 μg/mL ampicillin and 25 μg/mL chloramphenicol and grown until the OD_{600} reached 0.4−0.6. Production of the recombinant TK was induced with IPTG (0.5 mM) for 2.5 h. The bacteria were collected and lysed in 1/50 volume of sample buffer (0.5 M NaCl, 20 mM Tris-HCl; pH 7.9, 10% NP-40), 1 mM PMSF, and 200 μg/mL lysozyme. After sonication (Ultrasonic, up400A), the lysate was assayed immediately for the TK activity and the remainder was stored at −70 °C for further use.

TK Activity Assay. The TK activity assay was performed as described previously (*15*). Ten microliters of the crude lysate was mixed with 75 μL of reaction mixture containing 0.16 M Tris-HCl (pH7.5), 0.14 mM albumin, 12.6 mM creatine phosphate, 11.2 U/mL creatine phosphokinase, 2.35 μM [H^3]-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. An aliquot of a 50-μL sample was spotted onto a Whatman DE-81 filter, washed twice with alcohol, and dried. The radioactivity on the membrane was measured in a liquid scintillation counter (LS-6000; Beckman). The relative specific activity was calculated by subtracting the H^3-reading from that of vector control, divided by the reading of wild-type TK, and then normalized with the corresponding TK amount quantitated from enhanced chemiluminescence (ECL) western blot.

ATP-Binding Assay. To determine the ability of wild-type and mutant enzymes to bind ATP, 10 μL of the crude lysate, which had already been assessed for the amount of protein expression using a TK monoclonal antibody, was mixed with 20 μL of the reaction mixture containing 0.16 M Tris-HCl (pH 7.5), 0.14 mM albumin, 12.6 mM creatine phosphate, 11.2 U/mL creatine phosphokinase, 1.9 mM DTT, 2.4 mM $MgCl_2$, 9 mM NaF, and 6 nM [γ-P^{32}]-ATP (6000 Ci/mmole). The mixture was irradiated with UV for 15 min (254-nm UV stratalinker 1800; Stratagene) to cross-link any TK−ATP complex. After UV-cross-linking, 10 μL Ni−NTA sepharose beads (Qiagen) and 190 μL binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl; pH 7.9, 10% NP-40) were added and the mixture was incubated for a further 30 min at room temperature to capture the wild-type and mutant proteins which had histidine tags at the N-terminus. The nickel beads in the reaction mixture were then pelleted by centrifugation at 13 000 rpm for 5 min. After washing twice with 400 μL washing buffer (50 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl; pH 7.9, 10% NP-40), the beads were resuspended in a 30-μL binding buffer and were boiled for 5 min and then separated on 10% SDS−PAGE. Dried gels were exposed to X-ray film for 1 day.

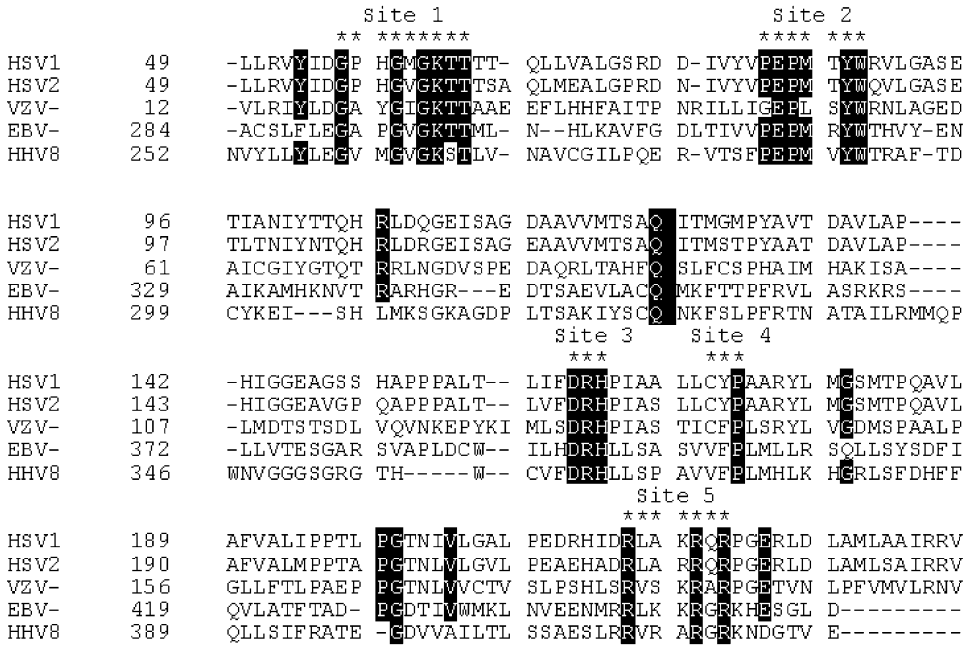

FIGURE 1: (a) Alignment of thymidine kinases from five human herpesviruses. Multiple alignment of the amino acid sequences of five human herpesviral thymidine kinases (including three alphaherpesviruses HSV-1, HSV-2, and VZV; and two gammaherpesviruses EBV and HHV-8) using Biology Workbench 3.0. Conserved residues (same amino acids present in at least four of the five herpesviral TKs) were marked black. Five conserved regions (sites 1−5 indicated by asterisks) were observed in the five human herpesviral thymidine kinases.

Determination of the Kinetics of the Wild-Type and Mutant TK Proteins for Different Phosphate Donors. Both the wild-type TK and mutants expressed from the pRsetA vector had histidine tags at the N-termini. The batch histidine beads purification was used for TK protein purification according to the manufacturer's instruction (Qiagen). The purification tube was prepared by loading 2 mL Ni−NTA agarose and washed with 10 mL loading buffer (0.5 M NaCl, 20 mM Tris-HCl; pH7.9, 10% NP-40) with rotation for 10 min. All subsequent steps were performed at 4 °C. Ten microliter crude lysate was clarified by centrifugation at 8000 rpm for 30 min, loaded to a purification tube. After overnight incubation, the tube was washed with two washing buffers (loading buffer plus 30 and 60 μM imidazole, respectively), each 10 mL for 10 min. The protein was obtained by incubating with three elution buffers (1 mL loading buffer plus 100, 200, and 300 μM imidazole, respectively) for 20 min. Purified proteins were assessed on Coomassie blue-stained gel, and its protein concentration was quantitated with BCA kit (BioRad, USA). The protein was stored at −70 °C for K_m determination.

To determine the kinetic factors of the wild-type and mutant EBV TKs for different phosphate donors, reaction conditions were similar to that for the TK activity assay described above except that concentrations of the phosphate donors varied from 1 to 500 μM. Lineweaver−Burk plots were used to determine the K_m and K_{cat} ($V_{max}/[E]$) values.

Analysis of Phosphate Donor Usage and Substrate Competitive Assay. Analysis of phosphate donor usage and substrate competitive assay were carried out using a similar procedure of the TK activity assay. Several NTPs, including ATP, TTP, CTP, and GTP, were tested for their utilization efficiency by the enzymes. The reaction buffer contains 0.16 M Tris-HCl (pH 7.5), 0.14 mM albumin, 12.6 mM creatine phosphate, 11.2 U/mL creatine phosphokinase, 7.5 mM NaF, 1.6 mM DTT, 1.5 mM $MgCl_2$, 2.35 μM [H^3]-thymidine (6.7 Ci/mmol), and 2mM NTP. The conditions for the substrate competitive assay were the same as those of the TK activity assay, except the various competitive nucleoside analogues were added and they were 50-fold greater in concentration than the radioactive thymidine.

Western Blot Analysis. The crude bacterial lysate was diluted 1:70 with loading buffer, fractionated by 10% SDS−PAGE, blotted on Immobilon-P (45 μm; Millipore), and blocked with 10 mM Tris-HCl (pH 7.4), 0.9% NaCl, and 4% skim milk (blocking buffer) for 1.5 h. Anti-TK monoclonal antibody 5F4-11, which was targeted to the N-terminus of EBV TK (*19*), was used as the primary antibody and was reacted for 1 h at room temperature. The blot was washed and incubated with horseradish peroxidase (HRP)-labeled goat antimouse antibody (Amersham) and was diluted 1:5000 in blocking buffer at room temperature for 1 h. After incubation, the blot was washed three times in washing buffer and then was developed with freshly prepared substrate for 1 min (ECL Western blotting; Amersham). The luminescence was detected by a short exposure to X-ray films. Relative amount of each protein was determined by a scanning densitometer (UltraScan XL; Pharmacia).

RESULTS

Alignment of Five Human Herpesvirus Thymidine Kinases. To gain further insights of the contribution of individual residues to ATP-binding activity, we aligned TK protein sequences of five human herpesviruses, including alpha-herpesviruses HSV-1, HSV-2, and VZV and gamma-herpesviruses EBV and HHV-8. Workbench 3.0 was used to identify conserved regions that might present functional domains. As shown in Figure 1, there are five conserved regions in these TK protein sequences. Among them, site 1, consisting of the motif -G X X G X G K T-, is rich in glycine residues. To identify a particular residue, a single amino acid and its position in the EBV TK was designated using

Protein		Residues	Sequence
EBV	TK	291–298	**G** A P **G** V **G** **K** **T**
HSV1	TK	56–63	**G** P H **G** M **G** **K** **T**
VV	TK	11–18	**G** P M F S **G** **K** **S**
E. coli	TK	9–16	S A M N A **G** **K** **S**
Human	TK	26–33	**G** P M F S **G** **K** **S**
Human	mtTK	57–64	**G** N I A S **G** **K** **T**
EF-Tu		14–21	**G** H V D S **G** **K** **S**
P21ras		10–17	**G** A G **G** V **G** **K** **S**
T4	DNK	8–15	**G** V K R S **G** **K** D
Yeast	UKY	9–16	**G** G P **G** A **G** **K** G
Yeast	GKY	8–15	**G** P S **G** T **G** **K** **S**
Yeast	AKY	10–17	**G** P P **G** A **G** **K** G
Human	AKH	15–22	**G** G P **G** S **G** **K** G
Bovine	AKB	15–22	**G** G P **G** S **G** **K** G
E. Coli	AKE	7–14	**G** A P **G** A **G** **K** G

FIGURE 2: Alignment of the sequences of the ATP- or GTP-binding motif from various enzymes. The ATP- or GTP-binding motifs from 13 ATP-binding proteins and 2 GTP-binding proteins were shown. The two GTP-binding proteins are elongation factor EF-Tu (*51*) and p21 ras (*52*). The 13 ATP-binding proteins include six thymidine kinases from EBV, HSV-1, vaccinia virus (VV) (*53*), *E. coli* (*54*), human cytosol (*55*), and mitochondria (*56*); four adenylate kinases from yeast (AKY) (*57*), human (AKH) (*58*), bovine (AKB) (*59*), and *E. coli* (AKE)(*60*); T4 phage deoxynucleotide kinase (DNK) (*61*); uridylate kinase (UKY) (*62*); and guanylate kinase (GKY) (*63*) from yeast. The conserved amino acids were marked in boldface.

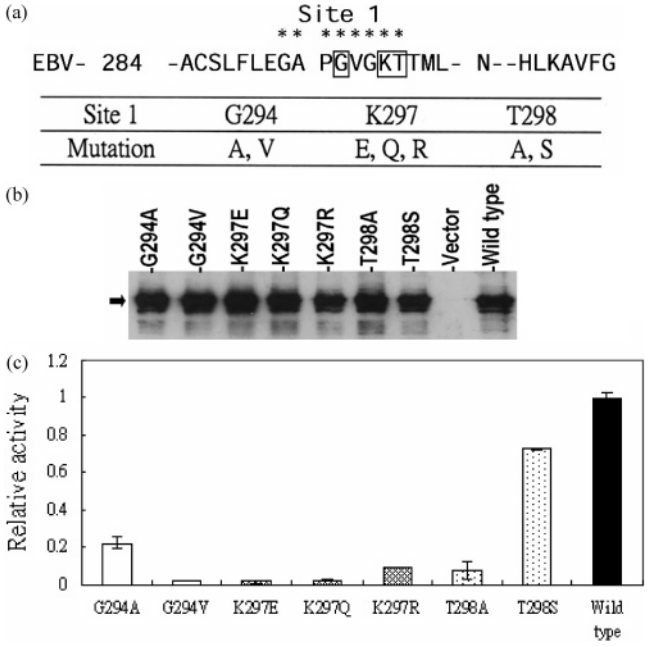

FIGURE 3: Expression of EBV TK mutants and enzyme activity assays. (a) Mutations created within conserved domain site 1 are indicated by asterisks and blocked. (b) Protein expression in *E. coli* BL21(DE3)pLysS transformed with plasmids G294A, G294V, K297E, K297Q, K297R, T298A, T298S, and wild-type EBV TK. Arrow indicates the expressed proteins of wild type and various mutants. (c) Relative TK activity was measured by using [^{3}H]-thymidine as the substrate. G294A and T298S retained 20−80% of the activity and the remaining mutants were almost inactive.

standard nomenclature, for example, G294 for glycine at residue 294. Three glycine residues (G291, G294, and G296 in EBV TK) are highly conserved in the five human herpesviral TKs, forming a so-called glycine loop or phosphate-binding motif (P loop), which is considered to be a characteristic of ATP-binding sites (*20, 30*). K297 and T298 are highly conserved in the five TKs as well. Furthermore, they were suggested to be involved in ATP binding through studies of crystal structure and mutational analysis of HSV-1 TK (*23*−*25*, *31*−*33*). In addition, the site 1 amino acid sequences of EBV TK and HSV-1 TK were compared with the corresponding sequences of various ATP-binding enzymes. As shown in Figure 2, all the ATP-binding domains of various viral TKs, human TKs, various adenylate kinases, and human cellular proteins are glycine-rich and have a lysine residue next to the last glycine of this particular region (*20, 34*−*41*). The lysine following the last glycine (K297 in EBV TK) is highly conserved in the various types of protein compared and is likely very important in catalytic function. After the lysine, residues are different, glycine for adenylate kinase and serine/threonine for the others (Figure 2). The role of this threonine at this site is also varied in different proteins (*34*). In this study, we chose the second glycine (G294), lysine (K297), and threonine (T298) in this putative ATP-binding region as the targets for mutation. A series of single-amino acid substitution mutants were generated to determine the respective functions and their biochemical roles.

TK Activities of the Site 1 Mutants. Three conserved residues, G294, K297, and T298, were chosen for functional analysis. The mutated residues are as listed in Figure 3a. Protein expression was examined by western blot analysis using EBV TK monoclonal antibody 5F4 (Figure 3b), and the relative activities of the mutants were determined as shown in Figure 3c.

Because glycine is the smallest amino acid and usually plays a crucial role in the maintenance of conformation, only alanine and valine of similar size were chosen to replace the glycine at position 294 (G294A and G294V). The results showed that substitution with alanine retained approximately 20% activity of the wild-type TK, but the mutant G294V was completely inactive (Figure 3c). This result indicated that the larger amino acid alanine could compensate partially for glycine but the much larger valine was not allowed.

Lysine 297 is a highly conserved residue in the herpesviral TKs and various other kinases. This residue was mutated to the positively charged amino acid, arginine, the negatively charged glutamate, and the neutral glutamine. Mutants K297E and K297Q did not have any activity and K297R maintained a residual activity (Figure 3c). The results indicated that the lysine is indispensable at this particular position.

Two substitution mutants for T298 were generated, T298S and T298A (Figure 3a). When T298 was substituted with alanine, a hydrophobic residue without a hydroxyl group, the enzyme activity was almost lost completely. However, the serine substitution did not inactivate the enzyme and about 80% activity was retained. These results imply that the hydroxyl group of threonine is crucial for activity.

ATP-Binding Analysis of the Site 1 Mutants. Because site 1 is a putative ATP-binding site, the ATP-binding ability was confirmed using an ATP-binding assay. With equivalent amounts of TK proteins determined by western analysis (Figure 4a), [γ-P^{32}]-ATP could be bound perfectly only by an intact ATP-binding pocket, whereas it could not be bound

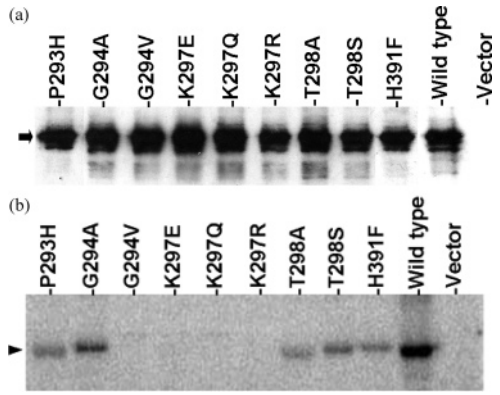

FIGURE 4: ATP-binding assay of EBV wtTK and its mutants. (a) The protein expression of all the mutants, which were applied to test ATP-binding ability. Arrow indicates the expressed proteins of wild type and various mutants. (b) [γ-P^{32}]-ATP was used as the phosphate donor to detect the binding ability of wild-type TK and site 1 mutants. P293H and H391F, which are the mutants of nonconserved residues, were used as controls. Arrowhead indicates the bound radioactive ATP by wild type and mutant proteins. The site 1 mutants of G294A, T298A, and T298S maintained the ability to bind ATP but not the G294V or any K297 mutants.

Table 2: Kinetic Parameters for Nucleotide Substrates of Wild-Type TK and Site 1 Mutants

		kinetic parameters		
EBV-TK proteins	nucleotide substrates	K_m (μM)	K_{cat} (10^{-3} s^{-1})	mean K_{cat}/K_m (10^{-3} s^{-1} μM^{-1})
TK	ATP	30.0 ± 7.15	28.10 ± 8.13	0.9369
	TTP	71.9 ± 9.40	10.47 ± 2.14	0.1456
	CTP	49.2 ± 1.34	18.67 ± 7.17	0.3796
	GTP	40.5 ± 2.34	21.27 ± 9.55	0.5250
G294A	ATP	48.7 ± 4.86	10.58 ± 0.88	0.2174
	TTP	ND[a]	ND	ND
	CTP	88.4 ± 4.40	8.82 ± 2.24	0.0998
	GTP	68.7 ± 5.10	7.62 ± 0.54	0.1109
K297R	ATP	87.6 ± 7.92	7.23 ± 4.14	0.0825
	TTP	ND	ND	ND
	CTP	107.5 ± 3.54	14.19 ± 9.43	0.1320
	GTP	43.0 ± 17.41	18.49 ± 4.86	0.4299
T298S	ATP	40.6 ± 6.22	24.05 ± 2.88	0.5923
	TTP	127.1 ± 7.64	7.37 ± 3.93	0.0580
	CTP	56.50 ± 15.98	13.48 ± 3.49	0.2385
	GTP	45.1 ± 2.97	17.03 ± 4.62	0.3775

[a] ND, not determined.

by a damaged pocket which exhibits loss of the binding ability. In this assay, we included two mutants as controls. P293H is a nonconserved substitution in the site 1 and H391F is a nonconserved substitution adjacent to site 3. Both mutants retained enzyme activity and have similar substrate preferences to wild-type TK (data not shown). As shown in Figure 4b, G294V and all K297 mutants did not have the ability to bind ATP, while the two mutants of the nonconserved residues, P293H and H391F, retained binding ability. G294A bound ATP with strong affinity, despite that its kinase activity was low. We determined the K_m values of mutants G294A and K297R for binding ATP. As shown in Table 2, the K_m's of G294A and K297R for ATP are 48.7 and 87.6 μM, respectively, and are higher than that of wild-type TK (30 μM). These data indicated that the capacities of these two mutants for ATP binding are reduced. The lower K_{cat} and K_{cat}/K_m values of G294A and K297R also showed the disruption of their catalytic efficiency. These results suggest that site 1 is involved in ATP binding, and G294 and K297 are important for both enzyme activity and ATP binding.

Of the T298 mutants, T298S retained both TK activity and ATP-binding ability. It was interesting to find that another mutant, T298A, which had the ability to bind ATP, lost most of the activity. This result implies that this threonine, although sitting at the ATP-binding site, may not participate directly in ATP binding but is very important for activity.

In addition, we determined the K_m's for thymidine of the K297R and T298S mutants. Both K_m values were similar to that of wild-type TK, suggesting that this region is not crucial for nucleoside binding (data not shown). We noticed that in the ATP-binding assay G294A and T298S bound significantly less ATP than did the wild type, while they have similar K_m values for ATP (Figure 4b and Table 2). We speculate that this difference may result from the different efficiency of UV cross-linking to ATP and the efficiency of the Ni-beads to capture the products.

Metal Ion Usage and Substrate Specificity of the Site 1 Mutants. In some nucleotide-binding proteins, the serine/threonine in the P loop plays an essential role in metal ion binding (*34*). We performed metal ion usage assays to see whether these residues also participate in metal ion binding. Three metal ions, Mg^{2+}, Mn^{2+}, and Zn^{2+}, were tested. H391F, a mutant in a nonconserved region of EBV TK, and D392E, a mutant involved in metal ion coordination (*19*), also were included. The results of wild-type TK were similar to those reported by Tung and Summers (*16*) in that Mg^{2+} is the preferred ion for EBV TK, Mn^{2+} is less favored, and Zn^{2+} could inhibit its activity, as shown in Figure 5a. The results revealed that the preferences for metal ion usage were similar in all the site 1 mutants.

Because TK is a potential target for antiviral drugs, the substrate specificity of these mutants was determined. Six well-known nucleoside analogues, ganciclovir (GCV), acyclovir (ACV), 3′-deoxy-2′,3′-didehydrothymidine (D4T), 3′-azido-3′-deoxythymidine (AZT), dideoxyinosine (DDI), and iododeoxyuridine (IdU), were tested and thymidine was included as the control. With the wild-type EBV TK, thymidine could not be competed by ganciclovir, acyclovir, and DDI, but was partially competed by D4T, and almost completely competed by AZT and IdU (Figure 5b). On the basis of assessment of competitive inhibition, the order of the various drugs for inhibition was IdU > AZT > D4T > DDI, GCV, and ACV. The results agreed with previously published studies which suggested that EBV TK seemed to favor pyrimidine nucleoside analogues but not purine nucleosides (*42*). As shown in Figure 5b, the mutants at site 1, G294A, K297R, and T298S, behaved almost the same as the wild-type TK in terms of substrate usage. These competition data suggested that the conserved residues in site 1 seemed not to play a crucial role in substrate specificity.

Analysis of Phosphate Donor Usage by the Site 1 Mutants. To investigate the preferential usage of phosphate donors, four phosphate donors (ATP, GTP, CTP, and TTP) were used in the TK activity assays. On the basis of the assessment of the wild-type TK, phosphate donor usage was ranked as ATP > GTP > CTP > TTP (Figure 6). No matter which phosphate donor was used, the mutants, other than G294A, K297R, and T298S, all remained inactive. G294A and T298S

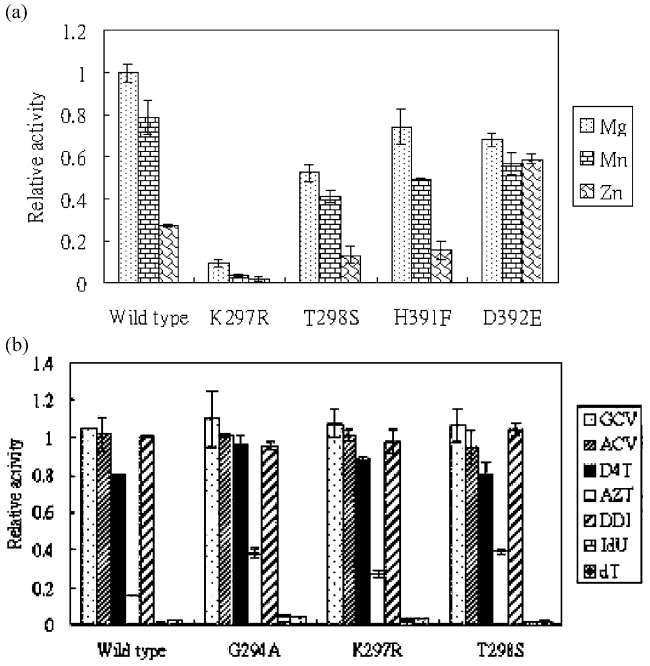

FIGURE 5: Metal ion usage and substrate specificity by the EBV wtTK and its mutants. (a) Three metal ions, magnesium, manganese, and zinc, were tested in the activity assays of EBV wtTK, K297R, and T298S. H391F, which was the mutant in the nonconserved region of EBV TK, and D392E, which was suggested to be mutant for metal ion binding, were tested as controls. Relative activity was determined by comparing the value with wild-type TK in the presence of magnesium. The bars represented the standard deviation from triplicate assays. (b) Ganciclovir (GCV), acyclovir (ACV), 3′-deoxy-2′,3′-didehydro-thymidine (D4T), 3′-azido-3′-deoxythymidine (AZT), dideoxyinosine (DDI), and iododeoxyuridine (IdU) were used to compete with [H^3]-thymidine for binding to wild-type TK, G294A, K297R, and T298S. Cold thymidine was used for the negative control. The bars represent the standard deviations from triplicate assays.

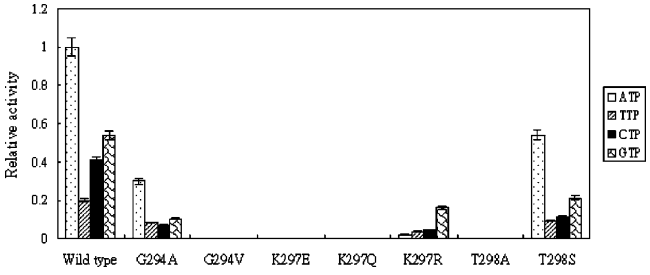

FIGURE 6: Phosphate donor analysis of EBV wtTK and its mutants. ATP, TTP, CTP, and GTP were used as phosphate donors to detect the activity of wild-type TK and site 1 mutants. The relative activity was determined by taking the activity obtained and divided by that of wild-type TK in the presence of ATP. The bars represent the standard deviations from triplicate assays.

revealed similar patterns in phosphate donor usage to the wild-type TK, with ATP as the most preferred donor. Surprisingly, K297R had a stronger preference for GTP as the phosphate donor. The K_m, K_{cat}, and K_{at}/K_m values for GTP also reflected this preference. The results implied that K297 plays a certain role in the choice of phosphate donor for EBV TK but not G294 or T298.

DISCUSSION

EBV TK is a potential target for the development of antiviral drugs and gene therapy (*10, 12*−*14*). Understanding the structure−function relationship of TK enzymes is valuable in elucidating the catalytic mechanism and application. Site-directed mutagenesis, in combination with crystal structure studies, has provided significant insights into HSV-1 TK. There is abundant information about the nucleotide-binding regions from other enzymes that bind nucleotides; however, less is known about the herpesviral TKs. In addition, the conserved residues do not always represent the same biological functions. In this study, we sought to determine the ATP-binding site and examine the roles of conserved amino acid residues in this region using site-directed mutagenesis, in the absence of any structural information on EBV TK.

The obvious reduction of TK activity in the mutants in site 1 (Figure 3c) indicated that this region is essential for activity. Loss of ATP-binding ability by the site 1 mutants (Figure 4b) suggested that this conserved region is, in fact, responsible for ATP binding. Our results also indicate that K297 at this site may play a certain role in phosphate donor utilization.

The consensus sequence for the P loop region is usually quoted in the literature as -G X X X X G K (T/S)- (*20*). However, herpesviral TKs and many other nucleotide-binding enzymes have a central glycine in this loop (Figure 2). Substitution of the three glycines with valine inactivated the HSV-1 TK activity (*23*), and replacement of these glycines with aspartic acid in pseudorabies TK also resulted in an inactive enzyme (*43*), indicating that these glycines are important for ATP binding. The 3D structures of HSV-1 TK and adenylate kinase indicate that these three glycines provide their flexible characteristic to form a loop and participate in ATP binding (*21, 32, 33*). Our results show that substitution with alanine could compensate partially for the contribution of G294, whereas it could not be replaced by valine. It is conceivable that alanine, the second smallest amino acid, could replace glycine to some extent although this may be more rigid than with glycine. The results presented suggest that this second glycine of the EBV TK (G294) is very important for the TK activity and contributes to maintain the conformation of the P-loop.

Mutants of K297 lost almost all their activity, suggesting that this lysine is essential. This result is consistent with those from other groups (*21, 44, 45*). The corresponding lysine has been mutated in both adenylate kinase and p21 (*28, 46*). In the latter, the K16N (p21) substitution dramatically reduced the affinity for guanine. The mutation K13Q in the *E. coli* adenylate kinase slightly reduces the affinity for substrates but drastically decreases its activity. Together with the results of these studies, it may be implied that a positive charge is required for stabilizing the transition state of the phosphoryl transfer reaction (*28*). From the structural data, in the ATP-binding site of adenylate kinase and HSV-1 TK, this lysine seems to neutralize the negative charge of the phosphate group in ATP (*32, 33*). The function of K297 in EBV TK may as well be the same.

The role of threonine at position 298 is an interesting issue. The T298A mutant had very low TK activity. However, the serine substitution maintained its activity. Similar results also were demonstrated in HSV-1 TK in that substitution by alanine inactivated the enzyme, whereas serine was able to replace this threonine (*23*). This clearly indicates that a hydroxyl group is important at this position. However, the actual role of this residue remains controversial. According

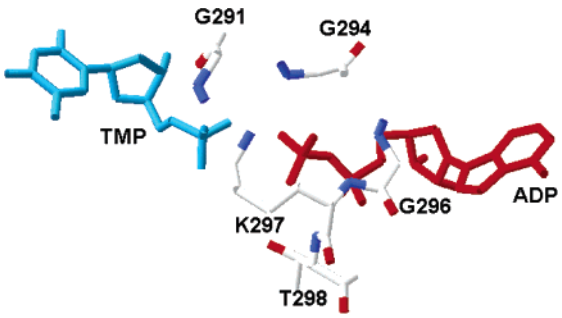

FIGURE 7: Hypothetical model of the protein structure of the conserved ATP-binding region in EBV TK. This model is based on the structure of the conserved site 1 of HSV-1 TK (1VTK). The position and geometry of TMP (blue color), ADP (red color), and conserved residues in the ATP-binding site are shown as labeled. The coordinates are indexed as 1VTK in the Protein Data Bank. Structural modeling exploration was conducted using the program Swiss-PdbViewer v.3.6b3.

to the results reported, there are three possible functions for this threonine at this position. The first possibility is based on the structural information from HSV-1 TK, which showed that this threonine precedes directly the glycine-rich loop (*33*, *47*), suggesting that the hydroxyl group is required to interact with ATP, perhaps by hydrogen bonding to the phosphoryl group. The second possibility is illustrated by a study in which the catalytic center of HSV-1 TK was predicted using the program GRID (*47*), where the data suggested that the corresponding threonine forms a hydrogen bond to coordinate the Mg^{2+}. Third, the hydroxyl group of this threonine has been postulated to form an ester intermediate to transfer the phosphoryl group (*23*). Our results of the ATP-binding analysis (Figure 4b) seem to support the third possibility, in that both T298A and T298S retained the ability to bind ATP, suggesting that the threonine of EBV TK may not be so important for direct binding of ATP and neither for metal ion coordination.

In an attempt to view the structure of the conserved ATP-binding site in EBV TK, we carried out protein modeling of site 1 on the basis of the structural information for HSV-1 TK (Figure 7). As in a typical P-loop structure, the polypeptides of EBV TK form a giant anion hole which may accommodate the phosphoryl group of ATP/ADP. The lysine of this fingerprint coordinates the phosphoryl group and its long side chain is speculated to participate in moving the phosphate group which is essential for phosphoryl transfer during catalysis, and T298 may also participate in the catalytic process. In addition to the conserved site 1, another region should participate in ATP binding. As in HSV-1 TK, the arginine in the conserved site 5 could contact the ATP and fix it, and another residue which is not in the conserved sites, such as Q331 (HSV-1 TK), could coordinate the adenine group of ATP (*33*). The combination of these interactions will support the stable binding of ATP with TK.

The results of substrate specificity analysis indicated that site 1 is involved in the binding of the nucleotide but not the nucleoside. Those mutants, G294A and T298S, which retained activity did not change their phosphate donor usage pattern in comparison with that of the wild-type TK (Figure 6, Table 2). Surprisingly, K297R changed its preferred usage of phosphate donor from ATP to GTP and was almost inactive in the presence of ATP but rather more active in the presence of GTP (Figure 6). This interesting result has not been reported for HSV-1 TK, implying that this lysine may be important for phosphate donor usage. Some enzymes, such as cAMP- and cGMP-dependent protein kinase, that have arginine instead of lysine, use ATP as the phosphate donor, but not GTP (*48*, *49*). Studies of the GTP-binding regions in GTP-binding proteins revealed two obvious characteristics different from that of the ATP-binding domain. First, more of the GTP-binding regions (corresponding to EBV TK T298) have serine instead of threonine in the ATP-binding domain (*20*). Second, while the P-loop was thought to participate in both GTP and ATP binding, two different additional domains were required for GTP and ATP binding (*50*). These results indicated that the choice of ATP or GTP is not a simple process determined only by a single residue or domain. We speculated that the relative position is very stringent and important, so the mutant K297R prefers to use GTP rather than ATP, although there is only a slight difference between ATP and GTP. The role of K297 in the choice of the phosphate donor in EBV TK is worthy of further study.

In addition to the results of our previous studies, which revealed the importance of the C-terminus of EBV TK [18] and characterized the conserved nucleoside-binding regions (*19*), this study identified the putative ATP-binding sites of EBV TK. Taken together, these results provide useful information to help in understanding the enzymatic activity of herpesviral TK and the extended study of the structure−function relationship of EBV TK. These results will be also useful when the enzyme is investigated as a potential target for antiviral drug development.

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