Conservative Mutations of Glutamine-125 in Herpes Simplex Virus Type 1 Thymidine Kinase Result in a Ganciclovir Kinase with Minimal Deoxypyrimidine Kinase Activities†

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ABSTRACT: The herpes simplex virus type 1 thymidine kinase (HSV-1 TK) is the major anti-herpes virus pharmacological target, and it is being utilized in combination with the prodrug ganciclovir as a toxin gene therapeutic for cancer. One active-site amino acid, glutamine-125 (Gln-125), has been shown to form hydrogen bonds with bound thymidine, thymidylate, and ganciclovir in multiple X-ray crystal structures. To examine the role of Gln-125 in HSV-1 TK activity, three site-specific mutations of this residue to an aspartic acid, an asparagine, or a glutamic acid were introduced. These three mutants and wild-type HSV-1 TK were expressed in *E. coli* and partially purified and their enzymatic properties compared. In comparison to the Gln-125 HSV-1 TK, thymidylate kinase activity of all three mutants was decreased by over 90%. For thymidine kinase activity relative to Gln-125 enzyme, the K_m of thymidine increased from 0.9 μM for the parent Gln-125 enzyme to 3 μM for the Glu-125 mutant, to 6000 μM for the Asp-125 mutant, and to 20 μM for the Asn-125 mutant. In contrast, the K_m of ganciclovir decreased from 69 μM for the parent Gln-125 enzyme to 50 μM for the Asn-125 mutant and increased to 473 μM for the Glu-125 mutant. The Asp-125 enzyme was able to poorly phosphorylate ganciclovir, but with nonlinear kinetics. Molecular simulations of the wild-type and mutant HSV-1 TK active sites predict that the observed activities are due to loss of hydrogen bonding between thymidine and the mutant amino acids, while the potential for hydrogen bonding remains intact for ganciclovir binding. When expressed in two mammalian cell lines, the Glu-125 mutant led to GCV-mediated killing of one cell line, while the Asn-125 mutant was equally as effective as wild-type HSV-1 TK in metabolizing GCV and causing cell death in both cell lines.

The herpes simplex virus thymidine kinases (HSV TKs)[1] are the pharmacological targets of most herpes virus treatments (*1, 2*), and more recently, HSV-1 TK has been utilized as a toxin gene therapeutic for cancer in combination with ganciclovir (*3, 4*). The basis for these uses is their ability to specifically phosphorylate anti-herpes virus nucleoside drugs such as acyclovir (ACV), ganciclovir (GCV), and 5-bromovinyldeoxyuridine (BVDU) (*1, 2, 5*). This viral targeting is based primarily on the differences in substrate specificity of HSV TK compared to the host cellular TKs. Besides thymidine and ATP, the HSV-1 TK utilizes a much broader range of substrates that include most deoxypyrimidine nucleosides, many guanosine derivatives (e.g., ACV or GCV), and most purine and pyrimidine nucleoside triphosphates (*6*−*9*). HSV TK also possesses a thymidylate kinase (TMPK) activity, but this activity is restricted to only deoxypyrimidine monophosphate substrates (*7*−*9*).

Using a photoactive TMP analog, [^{32}P]5N$_3$dUMP, proteolytic mapping studies of HSV-1 TK identified the site of photoincorporation as a region of the thymine base binding site inclusive in the peptide Ile^{112}-Tyr^{132} (*10*). This report, and others (*7, 8, 11, 12*), concluded that the thymine base of TMP and thymidine bind in one shared site. This was subsequently confirmed in comparisons of two X-ray crystal structures of HSV-1 TK with bound thymidine or TMP (*13, 14*). Two initial X-ray crystal structures of HSV-1 TK have been published (*13, 14*), one with bound thymidine or ganciclovir (*13*) and the other with thymidine, 5-iododeoxyuridine monophosphate, or a complex with TMP and ADP (*14*). Additional structures have been reported with bound acyclovir, penciclovir, and other nucleoside drug substrates and inhibitors (*15, 16*). Within the pyrimidine base binding site, all structures have indicated that hydrogen bonding between Gln-125 of HSV-1 TK and the N3 and O4 atoms

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[1] Abbreviations: BVDU, 5-bromovinyl-2′-deoxyuridine; ACV, acyclovir; GCV, ganciclovir; HSV-1 TK, herpes simplex virus type 1 thymidine kinase; TMPK, thymidylate kinase; 5N$_3$dUMP, 5-azido-2′-deoxyuridine-5′-monophosphate; 5N$_3$dU, 5-azido-2′-deoxyuridine; IPTG, isopropyl-β-D-thiogalactopyranoside; AZT, 3′-azido-2′,3′-dideoxythymidine; dC, deoxycytidine; d4T; 2′,3′-didehydro-2′,3′-dideoxythymidine; TCA, trichloroacetic acid; SA, specific activity; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; BSA, bovine serum albumin;. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide.

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of the pyrimidine base was evident (*13*−*15*). In the complex with ganciclovir or acyclovir, Gln-125 was shown to form hydrogen bonds with the N1 and O6 atoms of the guanine base of GCV (*13*, *15*, *16*).

It has been reported that site-directed mutagenesis of Gln-125 to Glu, Leu, or Asn can modulate the substrate affinities for thymidine and ACV in the context of HSV-1 TK in antiviral drug resistance (*17*). To examine the role of Gln-125 in HSV-1 TK activity in the context of gene therapeutic applications, we made three separate site-specific mutations of this residue to an Asp, an Asn, or a Glu residue. These three mutants and wild-type HSV-1 TK were expressed in *E. coli*, partially purified, and then compared for their ability to phosphorylate deoxypyrimidine and acyclic purine substrates. For each mutation, the ability to phosphorylate deoxypyrimidine substrates was greatly modified, while activity for the acyclic purines was variable. Kinetic constants for thymidine and GCV were also determined. The molecular basis for the obtained results was evaluated using Flexidock molecular modeling simulations of the different enzyme active sites. The cDNAs for each mutant HSV-1 TK were incorporated into a retroviral plasmid for expression in two mammalian cell lines and evaluation of sensitivity to GCV killing. The potential uses of these mutants in gene therapy applications and in the design of new HSV-1 TK proteins with different activities is discussed.

MATERIALS AND METHODS

Materials. All reagents and nucleotides were purchased from Sigma Chemical Co. unless otherwise indicated. [*methyl*-^{3}H]Thymidine (71 Ci/mmol), [*methyl*-^{3}H]AZT (19 Ci/mmol), [8-^{3}H]ganciclovir (17 Ci/mmol), and [8-^{3}H]-acyclovir (15 Ci/mmol) were purchased from Moravek Biochemicals. [5-^{3}H]Deoxycytidine (20 Ci/mmol) was from American Radiolabeled Chemicals. [γ-^{32}P]ATP was from ICN Radiochemicals. Whatman DE81 filter paper disks and DE-52 resin were purchased from Fisher. Automated DNA sequencing of plasmids was done by the UAMS Molecular Resource Laboratory using a model 377 DNA sequencer from Applied Biosystems. Oligonucleotide primers were synthesized in the UAMS Molecular Biology Core Facility. Restriction endonucleases, Vent polymerase, and T4 DNA ligase were from New England Biolabs.

Mutagenesis and Expression Vector Constructions. Starting with a previously prepared pET-9a (Novagen) HSV-1 thymidine kinase construct pET-TK1 (*12*), a 1.5 kb *Sal*I−*Bam*HI fragment was excised and subcloned into a pUC118 vector (pUC118-TK). Single-stranded pUC118-TK DNA was purified from cultures of JM107 after infection with helper phage M13K07 as previously described (*18*). Three mutagenic oligonucleotide primers were prepared to replace Gln-125 with Asp-125, Asn-125, or Glu-125 as follows: ACAAGCGCCGACATAACAATG for Asp, ACAAGCGCCAACATAACAATG for Asn, and ACAAGCGCCGAAATAACAATG for Glu. The three primers were used with an Amersham Sculptor in vitro mutagenesis kit as per the manufacturer's directions as adapted from Sayers *et al.* (*19*). Plasmids isolated from the resulting colonies were sequenced by automated sequencing to confirm the presence of the mutation and its identity. After confirmation of each mutation, the DNA pieces were released by *Sal*I−*Bam*HI restriction digests and religated back into the original pET-TK1 expression plasmid for transformation. The resulting colonies were again sequenced to confirm the presence of the mutated residues.

Enzyme Purification. E. coli BL21SY211 cells, in which T7 RNA polymerase is under the control of the IPTG-inducible lacUV5 promoter (*20*), were transformed with each pET-TK plasmid. These cells were grown to $A_{600} = 0.6$, induced by 1 mM IPTG for 2.5 h, and lysed in buffer A (20 mM Tris buffer, pH 8.1, 10% glycerol, 1 mM DTT, 40 mM KCl, 1 mM EDTA, 1 mM PMSF, and aprotinin (1 μg/mL)) by sonication for 3 min. The homogenate was then centrifuged at 10000*g* for 30 min to separate insoluble material. The soluble lysate was loaded onto a column of DE-52 in tandem sequence with a heparin agarose column (both 15 × 2 cm) and washed extensively with buffer A. The heparin agarose column was uncoupled from the DE-52 column, and bound proteins were eluted in 50 mL of buffer A plus 300 mM NaCl. The resulting fraction with thymidine kinase activity was concentrated to approximately 10 mL in an Amicon filtration apparatus, diluted with three volumes of buffer A (minus NaCl), and reconcentrated. This approach has reproducibly led to greater than 80% purified HSV TK as determined by Coomassie blue staining with cumulative yields ranging from 0.5 to 2 mg of total protein. The stabilities of the mutant enzyme preparations relative to wild-type preparations were identical when the preparations were stored at −20 °C for up to 6 weeks (data not shown).

Enzyme Assays. The activity of the purified HSV-1 TK was determined using the following standard reaction mixture for thymidine as a substrate (*12*): 3 μg of protein, 4 μM [*methyl*-^{3}H]thymidine (0.1 μCi/mmol), 20 mM potassium phosphate, pH 7.6, 1 mM DTT, 5 mM ATP, 5 mM $MgCl_2$, 25 mM NaF, 40 mM KCl, and 0.5 mg/mL BSA in a total volume of 25 μL for 10 min at 37 °C. To identify phosphorylated products, 20 μL of the reaction mixture was loaded onto a DE-81 filter and dried, washed once in 1 mM ammonium formate and twice more in 95% ethanol, and counted for radioactivity (*12*). Radioactivity was determined using an LKB 1214 Rackbeta liquid scintillation counter and corrected for background using controls with enzyme incubated in the absence of ATP. For TMPK activity, 20 μM [*methyl*-^{3}H]TMP was substituted for thymidine in the above assay mixture, and [^{3}H]TDP product was determined by separation from [^{3}H]TMP on thin-layer PEI-cellulose plates developed in 0.35 M LiCl (*21*). The amount of product converted to TDP was quantitated by scintillation counting of the excised TMP and TDP spots. The filter binding assay was used to quantitate the phosphorylation of [^{3}H]dC (200 μM) and [^{3}H]AZT (200 μM) with the four enzymes. To compare relative enzymatic activities for [^{3}H]GCV (50 μM) and [^{3}H]ACV (50 μM), reactions were loaded onto small DE-52 columns packed in Pasteur pipets as previously described (*22*). Unreacted nucleoside substrates do not bind to this resin, and phosphorylated products were eluted in 100 mM ammonium bicarbonate and quantified for radioactivity.

For determination of K_m values of thymidine and GCV for the four enzymes, the filter binding assay was utilized. The basic reaction components were as above, and the following enzyme concentrations, substrate ranges, and times were used under linear initial velocity conditions: For [^{3}H]-thymidine (parent Gln-125TK) 0.1 μg of TK, 0.1−4 μM,

10 min; (Asn-125TK) 0.2 μg of TK, 0.25−10 μM, 15 min; (Asp-125TK) 4 μg of TK, 0.1−2.5 mM, 30 min; (Glu-125TK) 0.5 μg of TK, 0.5−2.5 μM, 15 min. For [^{3}H]GCV (parent Gln-125TK) 2 μg of TK, 5−100 μM, 15 min; (Asn-125TK) 0.6 μg of TK, 0.5−15 μM, 15 min; (Asp-125TK) not determined; (Glu-125TK) 4 μg of TK, 50−1000 μM, 25 min. Each reaction was done in triplicate. For calculation of kinetic constants, nonlinear regression Michaelis−Menten analyses were done using PSI-PLOT version 5.0 (Poly Software International).

Photaffinity Labeling of HSV-1 TK. The TMP photoaffinity analog [^{32}P]5N$_3$dUMP was synthesized enzymatically using HSV-1 TK, 5-azido-deoxyuridine, and [γ-^{32}P]ATP as previously described (*10*, *12*). For photolabeling studies, 10 μg of the DE-52/heparin agarose purified HSV TKs was incubated with 10 μM [^{32}P]5N$_3$dUMP for 10 s. The sample was then irradiated for 90 s with a hand-held UV lamp (254 nm UVP-11, Ultraviolet Products, Inc.) at a distance of 3 cm. Reactions were terminated by addition of an equal volume of 10% TCA, incubated on ice for 10 min, and pelleted by centrifugation at 13000*g* for 5 min. The protein was resuspended in a solubilization mixture (*23*) and separated on 10% SDS−polyacrylamide gels. Dried gels were exposed to film for 2 days.

Molecular Modeling of Substrate Interactions with HSV-1 TK. The active-site region of wild-type HSV-1 TK with bound thymidine or GCV was excised from the coordinates for the X-ray crystal structure of HSV-1 TK (kindly provided by Dr. Mark Sanderson (*14*)) and loaded into the Flexidock module of the molecular modeling program SYBIL 6.3. Because the molecular interactions between bound thymidine and GCV were similar in the crystal structures except for rotation of Gln-125, this residue was defined as movable in the Flexidock program. As a control for this assumption, thymidine was placed in the Gln-125 HSV-1 TK active site and the lowest energy conformation determined. The lowest energy conformation was identical to that reported for thymidine in the crystal structure (*14*). Thus, lowest energy conformations for bound thymidine and GCV were done for the Gln-125, Glu-125, Asp-125, and Asn-125 active sites.

Expression and Characterization of HSV-1 TKs in Cell Lines. A Moloney murine leukemia virus derived plasmid for the expression of HSV TK, termed pLENTK, has been previously constructed (*24*). A unique *Bsp*EI−*Mlu*I restriction fragment within the HSV TK sequence of pLENTK contains the Gln-125 mutation site. This fragment was removed from wild-type plasmid and replaced with the analogous fragments encoding each mutant. The new pLEN-mutant-TK constructs were sequenced to confirm the presence of the mutation. Along with wild-type HSV-1 TK plasmid, each mutant-TK plasmid was transfected individually into the murine fibroblast cell line NIH 3T3 and the human colon tumor cell line HCT-116 using lipofectin reagent (GIBCO/BRL) (2 μg of plasmid, 14 μL lipid/1 $\times$ 10^6 cells). Cells were maintained in RPMI 1640 media and selected with G418 (200 μg/mL for 2 weeks) as previously described (*24*). At least eight individual G418 resistant cell clones were picked and grown up for further characterization. Each clone was screened initially for growth inhibition by 25 μM GCV. Those clones that were sensitive were further analyzed for HSV TK protein expression by Western blot analysis with a polyclonal, rabbit anti-HSV TK antibody (a gift from Dr. Margaret Black). For each clone, cell numbers were normalized to 1 $\times$ 10^6, and equal protein loading was confirmed for each sample by gel staining. Blotted HSV-1 TK protein bands were visualized on film using ECL chromophore reagents (Amersham). For analysis of GCV sensitivity of different clones, NIH3T3 and HCT-116 cell sets were seeded in 96 well plates in 0.1 mL of media (15 000 cells/well). The next day, a dose range of GCV (0.005−5 μM, n = 3) was added in 0.1 mL of media. After 4 days, MTT (50 μg/well) was added for 1.5 h, followed by DMSO solubilization of the cells and absorbance reading at 540 nm (*25*).

RESULTS

Mutagenesis and Expression of Gln-125 Mutants of HSV-1 TK. Expression plasmids derived from wild-type pET-TK1 (*12*) encoding the Asp, Asn, or Gln changes were individually transformed into BL21 *E. coli*, grown, and induced with IPTG. After cell pelleting and sonication, the resulting mutant HSV TKs, along with wild-type HSV TK and pET9a control preparations, were partially purified over tandem DE-52 and heparin agarose columns. This is a modification of our previously described purification method for HSV-1 TK (*12*), in that we have changed the pH of the lysis buffer from 7.6 to 8.1. This change allows the bulk of expressed HSV-1 TK to flow through the DE-52 column, instead of weakly absorbing as in the previous procedure (*12*), followed by elution of bound HSV TK from the heparin agarose column. Under the conditions utilized, no TK or TMPK activities in the *E. coli* pET9a extracts were detected (data not shown).

Enzymatic Activities of the Gln-125 HSV-1 TK Mutants. As an initial screen for activity, each expressed HSV-1 TK enzyme was assayed for phosphorylation of the following substrates: thymidine (4 μM), TMP (20 μM), ACV (50 μM), GCV (50 μM), deoxycytidine (dC; 200 μM), and 3′-azido-2′,3′-dideoxythymidine (AZT; 200 μM). Enzymatic conditions for optimal wild-type HSV-1 TK activities were utilized; thus, results presented in Table 1 for each substrate were normalized to 100% values for comparative purposes. As expected, the wild-type Gln-125 HSV-1 TK efficiently phosphorylated each of these substrates. For each mutant, there was a striking decrease in their ability to phosphorylate pyrimidine nucleosides, and minimal TMPK activity for the TMP substrate. For metabolism of GCV and ACV, the Asn-125TK retained most of these phosphorylation activities, while activities for the Asp-125TK and Glu-125TK were decreased to 14% and 7% for ACV and 0.7% and 5% for GCV, respectively.

Due to these observed differences in purine versus pyrimidine metabolism, and because of the use of HSV-1 TK in cancer gene therapy applications with GCV (*3*, *4*), the K_m and $k_{cat(app)}$ for thymidine and GCV were determined for each of the four HSV-1 TKs and are listed in Table 2. Unlike the comparative substrate screening assays presented in Table 1 that used wild-type HSV-1 TK conditions, linear velocity conditions for each enzyme and substrate were established prior to K_m determinations (see the Materials and Methods). For thymidine, the K_m values increased relative to that of the wild-type enzyme approximately 3-fold for the Glu-125 enzyme, 20-fold for the Asn-125 enzyme, and 6000-fold for the Asp-125 enzyme. The k_{cat} doubled for the Asp-

Table 1: Analysis of Reaction Products for Wild-Type and Mutant HSV-1 TKs[a]

TK enzyme	product formation (% of wild-type Gln-125)					
	TMP	TDP	GCVMP	ACVMP	dCMP	AZTMP
Gln-125 (WT)	100	100	100	100	100	100
Glu-125	43(6)	7(0.8)	5(0.6)	7(1)	0.2(0.1)	48(5)
Asn-125	22(4)	6(0.7)	85(6)	92(5)	0.2(0.1)	9(1)
Asp-125	0.5(0.1)	4(0.5)	0.7(0.1)	14(3)	>0.1(>0.1)	8(0.9)

[a] Purified HSV TKs (3 μg) were incubated for 10 min with each indicated [^{3}H]substrate at the following concentrations: 1 μM thymidine; 20 μM TMP; 50 μM GCV or ACV; 200 μM dC; 200 μM AZT in triplicate. Filter binding assays were used to quantitate T, dC, and AZT metabolites (*12*). TMP to TDP conversion was quantitated by separation of product on PEI-cellulose TLC plates (*21*). ACVMP and GCVMP were isolated on mini-DEAE-cellulose columns (*22*). The amounts of product determined for wild-type HSV-1 TK reactions were normalized to 100% values. The estimated standard deviations (percent) for each reaction are included in parentheses.

Table 2: Kinetic Constants of HSV-1 TK Gln-125 Mutants for Thymidine and Ganciclovir[a]

HSV-1 TK	thymidine			ganciclovir		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Gln-125 WT	0.9	0.06	6.7×10^4	69	0.47	6.8×10^3
Asn-125	20	0.13	6.5×10^3	50	0.08	1.7×10^3
Glu-125	3	0.003	844	473	0.04	82
Asp-125	6000	0.11	18	ND	ND	ND

[a] ND = not determined.

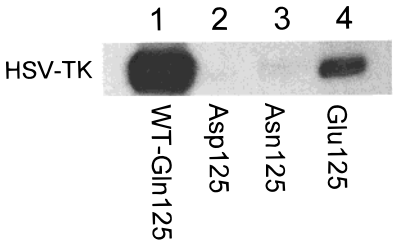

FIGURE 1: Autoradiograph of photolabeled wild-type and mutant HSV-1 TKs. Partially purified extracts from each HSV-1 TK were photolabeled with the TMP photoaffinity analog [^{32}P]5-azido-dUMP as described in the Materials and Methods (*5, 9*). Photolabeled proteins were separated on 10% SDS−polyacrylamide gels followed by autoradiography of the dried gel.

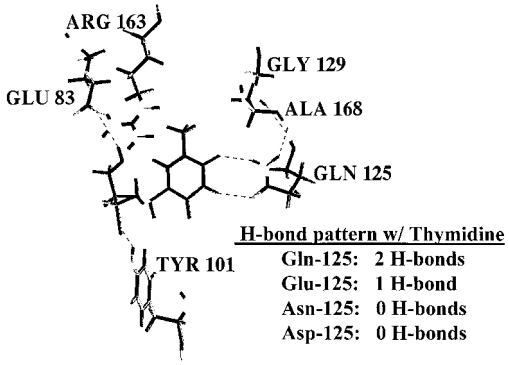

FIGURE 2: Flexidock modeling of hydrogen bonding between thymine and amino acid 125. The Flexidock module of SYBIL 6.3 was used to generate the lowest energy conformations of bound thymidine with wild-type Gln-125 HSV TK and the three mutant HSV TKs as described in the Materials and Methods. The predicted number of hydrogen bonds formed with each residue at position 125 is listed on the lower right side of the figure.

125 and Asn-125 enzymes, while a 20-fold decrease in k_{cat} was determined for the Glu-125TK. For GCV, the K_m decreased from 69 μM for the wild-type enzyme to 50 μM for the Asn-125TK enzyme, while the K_m increased to 473 μM for the Glu-125 enzyme. These mutations also caused a 6-fold and 12-fold decrease, respectively, in the k_{cat} compared to that of the wild-type enzyme. Interestingly, no linear velocity conditions could be established for the Asp-125TK with GCV. As was shown in Table 1, this enzyme will phosphorylate a small amount of GCV; however, it does not generate product in an initial velocity-dependent manner. The basis for this lack of activity was not further evaluated.

Photoaffinity Labeling of the Gln-125 HSV-1 TK Mutants. The thymidine and TMP photoaffinity analog [^{32}P]5-azido-dUMP has proven useful as an active-site cross-linking reagent for studying HSV-1 TK (*10, 12*). This analog was used for photo-cross-linking of the four HSV-1 TKs as described in the Materials and Methods. As shown in the autoradiograph in Figure 1, [^{32}P]5-azido-dUMP was photoincorporated efficiently into the wild-type Gln-125 enzyme. This photoincorporation was decreased by over 90% (by densitometry comparison) for the Glu-125 mutant, and only trace photoincorporation was detected for the Asp and Asn mutants. These results further demonstrate the diminished binding affinities of the three mutant HSV-1 TKs for pyrimidine substrates.

Molecular Modeling Comparisons of Thymidine and Ganciclovir in the Gln-125 and Mutant HSV-1 TK Active Sites. The Flexidock component of the molecular modeling program SYBIL 6.3 (see the Materials and Methods) was used with the coordinates of the wild-type Gln-125 HSV-1 TK crystal structure (*14*) to dock thymidine or GCV in the active site of each of the four HSV-1 TK enzymes. As shown in Figure 2, loss of hydrogen bonding between the N3 and O4 of the thymine base and Asn-125 or Asp-125 may be a molecular contributor to the decreased pyrimidine substrate phosphorylation activities of these enzymes. This analysis predicts that the Glu-125 enzyme may still form one hydrogen bond, thus retaining its thymidine phosphorylation activity. Analysis of GCV in the active site predicts that it is still able to maintain hydrogen bonding with the Asp, Asn, or Glu residues (data not shown), and thus could contribute to the retention of this phosphorylation activity with the Asn-125 mutant. Introduction of the negatively charged Asp and

Glu residues clearly attenuated the GCV phosphorylation activities relative to the Asn mutation, and it is thus likely that this charge difference also contributes to the observed changes in enzymatic properties.

Cellular Expression of HSV-1 TK and Sensitivity to GCV Killing. The cDNA for each mutant was incorporated into a Moloney murine leukemia virus derived expression plasmid (*24*). The plasmids for wild-type HSV-1 TK and each mutant were individually transfected into either NIH3T3 cells or the human colon tumor cell line HCT-116. Following drug selection in G418, individual cell clones were evaluated for sensitivity to GCV and relative levels of HSV-1 TK expression. Using an HSV-1 TK antibody and extracts normalized by protein and cell number, the relative expression levels of HSV-1 TK in each cell clone were determined. Cell sets having equivalent expression of an HSV-1 TK and roughly equivalent cell growth rates were identified and selected for comparative study. As shown in Figure 3, these NIH3T3 and HCT-116 cell sets were plated in 96-well plates and evaluated for dose-dependent cell killing by GCV for 4 days. Increasing GCV concentrations led to proportionally more cell killing in the wild-type or Asn-125TK clones tested, and apparently both enzymes function similarly with regard to intracellular GCV metabolism and effects on cell viabilities. For the Glu-125TK mutants, minimal cell killing was observed in the NIH3T3 cells. However, the Glu-125TK expressed in the HCT-116 cells led to minor decreases in cell viability at lower GCV concentrations, but at the highest concentration, 5 μM, cell viabilities dropped precipitously. For the Asp-125TK-expressing HCT-116 cell lines, GCV had little effect on cell viability, although expression of HSV-1 TK protein was detected by an antibody (data not shown). Similar negative results were found with the Asp-125TK expressed in the NIH-3T3 cell line (data not shown). As expected, GCV had little effect on the cell viabilities of the non-HSV-1 TK-expressing parental cells. In a separate study, it was demonstrated by metabolic labeling with [^{3}H]-GCV that the levels of intracellular GCV metabolites detected correlated with the cell killing sensitivities presented in Figure 3 (*26*).

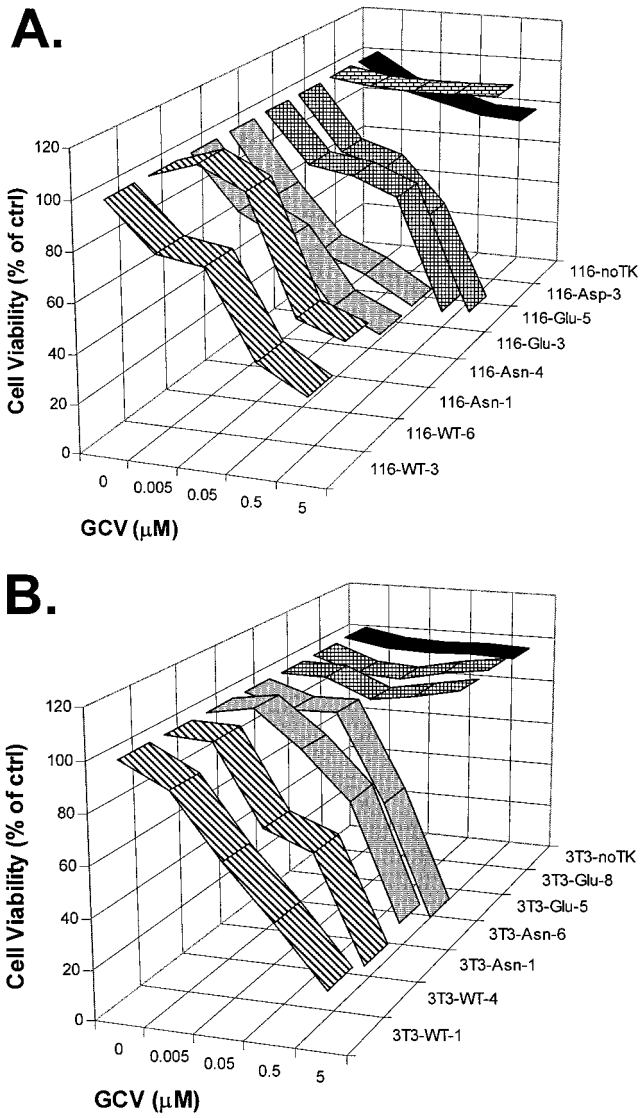

FIGURE 3: Sensitivity to GCV killing of wild-type and mutant HSV-1 TK expressing HCT-116 and NIH3T3 cell lines. HCT-116 and NIH3T3 cell lines stably expressing wild-type or mutant HSV-1 TKs under control of a Moloney murine leukemia virus promoter were prepared as described in the Materials and Methods. Cells were plated in triplicate and exposed to 0, 0.05, 0.5, or 5 μM GCV for four days. Cell viabilities were determined using MTT dye as described in the Materials and Methods. Results are presented as the percent of untreated cell controls for each cell line. (A) NIH3T3 cell lines; (B) HCT-116 cell lines; parental cells (no TK, dark bands); Asp-125TK (Asp-*n*, brick band); Glu-125TK (Glu-*n*, cross-hatch bands); Asn-125TK (Asn-*n*, gray bands); wild-type HSV-1 TK (WT-*n*, striped bands).

DISCUSSION

Multiple X-ray crystal structures of HSV-1 TK have highlighted the importance of Gln-125 in forming hydrogen bonds with pyrimidine and purine substrates such as GCV (*13*−*17*). We have demonstrated that fairly conservative mutations of Gln-125 to Glu, Asp, or Asn in HSV-1 TK can significantly alter the substrate specificity and overall enzyme activity. Cumulatively for the three mutant enzymes, the enzymatic and photoaffinity data indicated a decrease in binding and utilization of TMP, which is the second substrate for the TMP kinase activity of wild-type HSV TK. The decreased photoaffinity labeling of the Glu mutant and trace photoincorporation into the Asp and Asn mutants with the active-site-directed TMP photoaffinity analog [^{32}P]5-azido-dUMP further demonstrate the poor interactions with TMP. We had previously determined that following activation 5-azido-dUMP covalently cross-links to an amino acid in the peptide comprising residues 112−132 (*10*). The lack of photolabeling of the mutant enzymes suggests that the site of cross-linking is at or near the vicinity of Gln-125. Molecular modeling of the three mutations using a Flexidock program has predicted that loss of hydrogen bonding between thymidine and the Asp-125 or Asn-125 mutants contributes to the altered activities, while hydrogen bonding with each mutant enzyme and GCV is still retained. Clearly, the introduction of a negative charge (Glu, Asp) in the active site versus the more conserved Asn residue is another contributing factor to the altered activities.

As presented in Table 2, the Asn-125 mutation led to a 22-fold increase in the K_m for thymidine and 10-fold decrease in the k_{cat}/K_m value compared to those of wild-type HSV-1 TK. For GCV, the K_m decreased slightly by 1.4-fold to 50 μM. However, a 4-fold decrease of the k_{cat}/K_m value was determined for this enzyme. As shown in Figure 3, cellular

expression of the Asn-125TK enzyme plus GCV was just as effective at killing HCT-116 and NIH-3T3 cells as wild-type HSV-1 TK. In a separate study that further characterized the cellular effects of the Asn-125TK (*26*), it was determined that it more efficiently metabolized GCV better than wild-type HSV-1 TK, yet it did not efficiently phosphorylate thymidine or deoxycytidine intracellularly (*26*). In Figure 3A, a surprising result was the ability of the Glu-125TK to effectively kill the HCT-116 cells following GCV addition. The Glu-125 had a 79-fold and 82-fold lower k_{cat}/K_m value compared to wild-type HSV-1 TK for thymidine and GCV, respectively (Table 2). This mutant has proven useful in demonstrating that it does not require a large amount of phosphorylated GCV metabolites to cause cell death in some cell lines (*26*, Figure 3). Also, subsequent analysis of the mode of cell death in the Glu-125TK-expressing cells has indicated a nonapoptotic cell death mechanism distinct from that of wild-type- or Asn-125TK-expressing cells (*26*). The Asp-125 HSV-1 TK had no detectable GCV killing when expressed in either cell line, consistent with its poor utilization of GCV in the kinetic assays. This mutant could prove useful for further advanced enzymatic kinetic/mechanistic studies due to its demonstrated ability to phosphorylate GCV and lack of nonlinear initial velocity kinetics.

A previous study evaluated the effect that single amino acid substitutions at Gln-125 (Asn, Asp, Glu, or Leu) had on thymidine and ACV phosphorylation (*17*). In this study, it was reported that their Glu-125 mutant had no detectable activities, and the Asn-125 mutant had a 50-fold and 3-fold increase in the K_m values of thymidine and ACV, respectively (*17*). These mutant HSV-1 TKs were not expressed in mammalian cell lines, and cumulatively these results differ significantly from the data presented herein for our HSV-1 TK mutants. Other than the expression of their HSV-1 TK mutant enzymes as glutathione-*S*-transferase fusion constructs (*17*), the reasons for the discrepancies in the results for the same mutants is not clear. In other previous reports, a series of random insertional oligonucleotide mutagenesis studies on HSV-1 TK have demonstrated the catalytic role of the amino acids spanning residues 159−172 (*27*−*29*), and many mutants have been identified which have altered or improved substrate specificities for GCV and ACV (*28*, *29*). As a goal toward improving HSV-1 TK gene therapy strategies, some of the mutant HSV-1 TKs generated by random insertional mutagenesis were tested for cell killing efficacy in mammalian cells (*29*). One of these mutants, which had four changed residues (Ile-160 to Leu; Phe-161 to Leu; Ala-168 to Val; Leu-169 to Met), was shown to have 43-fold and 20-fold greater sensitivities to cell killing with GCV and ACV, respectively (*29*). The K_m of GCV for this mutant was 5-fold lower than that of wild-type HSV-1 TK, and the k_{cat} of the mutant enzyme for thymidine, ACV, and GCV remained the same as that of wild-type HSV-1 TK (*29*). Because these amino acid changes occur in a distinct catalytic region of HSV-1 TK and the Gln-125 appears to be only involved in nucleoside base binding (*13*−*17*), it should be possible to construct hybrid HSV-1 TKs comprising mutations from both sites to generate an enzyme with minimal TK/TMPK activities and maximal acyclic purine nucleoside phosphorylation activities.

In other cell culture studies, it has been demonstrated that the more HSV-1 TK protein is expressed in a cell, the more efficient GCV metabolism and cell killing are (*30*−*33*). Whether by improving expression or catalytic efficiencies, these cumulative results for HSV-1 TK indicate that any method of increasing GCV metabolism could result in increased therapeutic benefits. Because the K_m for thymidine is over 70−100 times lower than that of GCV for wild-type HSV-TK (*29*), we hypothesized that in a cellular environment the Asn-125 mutant would act primarily as a GCV kinase, particularly as thymidine and its metabolites will compete less with GCV for binding in the active site. This appears to be the case in both cell lines tested for the Asn-125 mutant, and even in the HCT-116 cells expressing the Glu-125 mutant. The GCV metabolism properties and mechanistic aspects of GCV cell killing by the Asn-125 and Glu-125 mutants in the HCT-116 cells have been further evaluated in a separate study (*26*). These types of HSV-1 TK mutants described herein will allow the evaluation of whether the TK/TKMP activities of HSV-1 TK cause any cellular problems related to altered nucleotide metabolism and pool sizes. This could be especially important in the cancer gene therapy trials for myeloma (*34*) and leukemia (*35*) that administer HSV-1 TK-expressing T-lymphocytes to patients for immune protection and surveillance following bone marrow transplants. HSV-1 TK acts as a safety gene in these studies to allow termination of the treatment via GCV if graph-versus-host disease develops; thus, use of an HSV-1 TK that is predominantly a GCV kinase could prove to be safer and more effective. Efforts to characterize the expression and metabolism of GCV in T-lymphocytes expressing Glu-125 and Asn-125 HSV-1 TKs are currently in progress.

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