**Engineering of a Single Conserved Amino Acid Residue of Herpes Simplex Virus Type 1 Thymidine Kinase Allows a Predominant Shift from Pyrimidine to Purine Nucleoside Phosphorylation**

Received for publication, January 17, 2006, and in revised form, May 15, 2006

Published, JBC Papers in Press, May 15, 2006, DOI 10.1074/jbc.M600414200

Jan Balzarini 1,2, Sandra Liekens 1, Nicola Solaroli 2, Kamel El Omari 2, David K. Stammers 2, and Anna Karlsson 2

From the 1 Rega Institute for Medical Research, K. U. Leuven, B-3000 Leuven, Belgium, 2 Karolinska Institute, Huddinge University Hospital, S-141 86 Huddinge/Stockholm, Sweden, and 3 The Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, United Kingdom

Studies of herpes simplex virus type 1 (HSV-1) thymidine (dThd) kinase (TK) crystal structures show that purine and pyrimidine bases occupy distinct positions in the active site but approximately the same geometric plane. The presence of a bulky side chain, such as tyrosine at position 167, would not be sterically favorable for pyrimidine or pyrimidine nucleoside analogue binding, whereas purine nucleoside analogues would be less affected because they are located further away from the phenylalanine side chain. Site-directed mutagenesis of the conserved Ala-167 and Ala-168 residues in HSV-1 TK resulted in a wide variety of differential affinities and catalytic activities in the presence of the natural substrate dThd and the purine nucleoside analogue drug ganciclovir (GCV), depending on the nature of the amino acid mutation. A168H- and A167F-mutated HSV-1 TK enzymes turned out to have a virtually complete knock-out of dThd kinase activity (at least 4–5 orders of magnitude lower) presumably due to a steric clash between the mutated amino acid and the dThd ring. In contrast, a full preservation of the GCV (and other purine nucleoside analogues) kinase activity was achieved for A168H TK. The enzyme mutants also markedly lost their binding capacity for dThd and showed a substantially diminished feedback inhibition by thymidine 5′-triphosphate. The side chain size at position 168 seems to play a less important role regarding GCV or dThd selectivity than at position 167. Instead, the nitrogen-containing side chains from A168H and A168K seem necessary for efficient ligand discrimination. This explains why A168H-mutated HSV-1 TK fully preserves its GCV kinase activity (Vmax/Km 4-fold higher than wild-type HSV-1 TK), although still showing a severely compromised dThd kinase activity (Vmax/Km 3–4 orders of magnitude lower than wild-type HSV-1 TK).

Herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) recognizes, besides thymidine and 2′-deoxyuridine, also 2′-deoxyoxycytidine and 2′-deoxythymidylate (dTMP) as natural substrates. In contrast with its cytosolic TK-1 counterpart, HSV-1 TK can also recognize a variety of dUrd analogues with modifications in the base moiety (i.e. 5′-substituted dUrd derivatives such as (E)-5′-(2-bromovinyl)-2′-deoxyuridin (BVUD, brivudin)) and/or in the sugar part, such as 1β,3β-arabinofuranosylthymine (araT) or BVaraU (sorivudine) (see Fig. 1). Interestingly, HSV-1 TK can also recognize a variety of guanine nucleoside analogues containing an acyclic or cyclic analogue of the 2′-deoxyribose moiety (i.e. ganciclovir (GCV), acyclovir (ACV), penciclovir (PCV), etc.) (Fig. 1). Specific recognition of these purine nucleoside analogues as a substrate for phosphorylation is required to express a potent antiviral activity of the test compounds.

Several attempts have been made to increase the catalytic activity of HSV-1 TK for GCV and ACV by mutagenesis of the active site of the enzyme. Random sequence mutagenesis at a segment of the putative nucleoside binding site created new HSV-1 TKs with preferential phosphorylation of GCV and/or ACV. In these studies, six codons (encoding Leu-159, Ile-160, Phe-161, Ala-168, Leu-169, and Leu-170) were targeted (1–3). Thymidine and GCV phosphorylation could not be improved, but ACV phosphorylation could be enhanced for the TK enzymes containing Leu-160 + Leu-161 + Val-168 + Met-169 (4.3-fold) or Leu-161 + Ser-168 + Tyr-169 + Cys-170 (2.0-fold). In a number of cases, the ratios of GCV/dThd or ACV/dThd phosphorylation by the mutant enzymes could be increased by 20–30-fold (1). The creation of a second generation semi-randomized mutant TK library further optimized the enzyme for GCV/ACV substrate sensitivity. These HSV-1 TK mutants contained, besides other mutations in the substrate active site, predominantly 168F/Y (4). The ratio of catalytic capacity of the mutant TKs with GCV or ACV versus dThd increased up to 11–83-fold or 70–567-fold, respectively (4). One mutant HSV-1 TK (I160F + F161A + A168F) (designated

---

* This work was supported by Project GOA-2005/19 from the Flemish “Geconcerteerde Onderzoeksacties” (to J. B.), the Belgian “Federatie tegen Kanker” (to J. B.), and the European Commission (Project QLRT-2000-01004) (to J. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Rega Institute for Medical Research, K. U. Leuven, Minderbroedersstr. 10, B-3000 Leuven, Belgium. Tel.: 32-16-337352; Fax: 32-16-337340; E-mail: jan.balzarini@rega.kuleuven.be.

2 The abbreviations used are: HSV-1, herpes simplex virus type 1; TK, thymidine kinase; GCV, ganciclovir; BVUD, bromovinyldeoxyuridin; dUrd, 2′-deoxyuridine; dTMP, 2′-deoxythymidylate; araT, 1β,3β-arabinofuranosylthymine; BVaraU, bromovinylarabinofuranosyluracil; ACV, acyclovir; PCV, penciclovir; dTTP, 2′-deoxythymidine-5′-triphosphate; GST, glutathione S-transferase; WT, wild-type.
Mutant Ala-167 and Ala-168 HSV-1 TK

SR-39 (L159I + I160F + F161L + A168F + L169H) showed a 124-fold decrease in $K_m$ for GCV compared with wild-type TK. The SR-26 mutant HSV-1 TK showed a 124-fold decrease in $K_m$ for dThd, GCV, and ACV, and the SR-26 mutant HSV-1 TK showed the highest ratio of GCV kinase/dThd kinase activity. In particular, A168H HSV-1 TK proved the most interesting enzyme mutant with fully preserved GCV catalytic substrate activity and heavily compromised dThd kinase activity both at the same time. Such a discriminative activity has never previously been observed by one single amino acid point mutation in HSV-1 TK. The insights obtained have resulted in a new predictive structural modeling approach for generating substrate-specific HSV-1 TKs and are instrumental in optimizing HSV-1 TK enzyme constructs for use in combined gene/chemotherapy of cancer or other diseases.

**EXPERIMENTAL PROCEDURES**

Construction, Expression, and Purification of Wild-Type and Mutant HSV-1 TK—Mutant HSV-1 TK enzymes were derived from the TK sequence cloned in pGEX-5X-1 (Amersham Biosciences AB, Uppsala, Sweden) (7). Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene; Westburg, Leusden, The Netherlands) as described previously (9). The two complementary oligonucleotide primers (Invitrogen) that were used contained the desired mutation at amino acid position 167 or 168 of HSV-1 TK (Table 1). The mutant DNA was transformed into competent Escherichia coli XL-1 blue. Plasmid preparations from ampicillin-resistant colonies were checked by sequencing of the TK gene on an ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA) using the ABI Prism Big Dye Terminator cycle-sequencing ready reaction kit (Applied Biosystems) and transfected in E. coli BL21(DE3)pLysS. Transfected bacteria were grown overnight in 2YT (yeast/tryptone) medium containing ampicillin (100 μg/ml) and chloramphenicol (40 μg/ml) and then diluted in fresh medium. After further growth of the bacteria at 27 °C (for 5 h), isopropyl-$β$-D-thiogalactopyranoside (Sigma) was added to a final concentration of 0.1 mM to induce the production of the GST-TK fusion protein. After 16 h of further growth at 27 °C, the cells were pelleted and resuspended in lysis buffer (50 mM Tris, pH 7.5, 1 mM dithiothreitol, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and 0.15 mg/ml lysozyme). Bacterial suspensions were passed through a SLM Aminco French pressure cell dThd- and GCV-phosphorylating capacity of the mutated HSV-1 TK enzymes and to the design and construction of mutant HSV-1 TKs with superior GCV/dThd catalytic ratios than the existing mutant HSV-1 TKs. In particular, we aimed to fully preserve the catalytic activity for GCV and related analogues and, at the same time, to abolish as much as possible the dThd catalytic activity. It was found that A167F- and A168H-mutated HSV-1 TKs showed the highest ratio of GCV kinase/dThd kinase activity. In particular, A168H HSV-1 TK proved the most interesting enzyme mutant with fully preserved GCV catalytic substrate activity and heavily compromised dThd kinase activity both at the same time. Such a discriminative activity has never previously been observed by one single amino acid point mutation in HSV-1 TK. The insights obtained have resulted in a new predictive structural modeling approach for generating substrate-specific HSV-1 TKs and are instrumental in optimizing HSV-1 TK enzyme constructs for use in combined gene/chemotherapy of cancer or other diseases.

**EXPERIMENTAL PROCEDURES**

Construction, Expression, and Purification of Wild-Type and Mutant HSV-1 TK—Mutant HSV-1 TK enzymes were derived from the TK sequence cloned in pGEX-5X-1 (Amersham Biosciences AB, Uppsala, Sweden) (7). Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene; Westburg, Leusden, The Netherlands) as described previously (9). The two complementary oligonucleotide primers (Invitrogen) that were used contained the desired mutation at amino acid position 167 or 168 of HSV-1 TK (Table 1). The mutant DNA was transformed into competent Escherichia coli XL-1 blue. Plasmid preparations from ampicillin-resistant colonies were checked by sequencing of the TK gene on an ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA) using the ABI Prism Big Dye Terminator cycle-sequencing ready reaction kit (Applied Biosystems) and transfected in E. coli BL21(DE3)pLysS. Transfected bacteria were grown overnight in 2YT (yeast/tryptone) medium containing ampicillin (100 μg/ml) and chloramphenicol (40 μg/ml) and then diluted in fresh medium. After further growth of the bacteria at 27 °C (for 5 h), isopropyl-$β$-D-thiogalactopyranoside (Sigma) was added to a final concentration of 0.1 mM to induce the production of the GST-TK fusion protein. After 16 h of further growth at 27 °C, the cells were pelleted and resuspended in lysis buffer (50 mM Tris, pH 7.5, 1 mM dithiothreitol, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and 0.15 mg/ml lysozyme). Bacterial suspensions were passed through a SLM Aminco French pressure cell
press (Beun de Ronde, La Abcoude, The Netherlands) and ultracentrifuged (11,000 rounds/min, 4 °C, 30 min). GST-TK was purified from the supernatant using glutathione-Sepharose 4B (Amersham Biosciences) as described by the supplier. The protein content of the purified fractions was assessed with the Bradford reagent (Sigma) using bovine serum as the concentration standard.

Radiolabeled Compounds—[3H]Ganciclovir (12.4 Ci/mmol), [3H]lobucavir (26 Ci/mmol), and [3H]penciclovir (16 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). [3H]dThd (50 Ci/mmol) was from Amersham Biosciences.

dThd and GCV Kinase Assays—The ability of the purified HSV-1 TK WT and mutant HSV-1 A167 and A168 TK preparations to phosphorylate [3H]dThd and [3H]GCV was determined as follows. The standard reaction mixture contained 2.5 mM MgCl₂, 10 mM dithiothreitol, 1 mg/ml bovine serum albumin, 2.5 mM ATP, 10 mM NaF, a variety of [3H]dThd and [3H]GCV concentrations, and WT HSV-1 TK and mutant Ala-167 and -168 HSV-1 TK preparation in a total reaction mixture of 50 μl of 50 mM Tris-HCl, pH 8.0. The reaction mixture was incubated at 37 °C for 30 min. At this time point, it was ascertained that the reaction still proceeded linearly for all HSV-1 TK enzyme mutants evaluated in the presence of dThd and GCV concentrations that were approximately equal to the respective Km values and five times the K₅ values. (i.e. For dThd, 0.15 and 0.75 μM for WT TK; 50 and 250 μM for A168Y TK; 750 and 3750 μM for A168H TK; 200 and 1000 μM for A168W TK; 5 and 25 μM for A168I TK; 50 and 250 μM for A168E TK; 200 and 1000 μM for A168K TK; 10 and 50 μM for A168N TK; 5 and 25 μM for A168C TK; 5 and 25 μM for A168F TK; 1000 and 5000 μM for A167C TK; 100 and 500 μM for A167N TK; 5 and 25 μM for A167I TK; 2 and 10 μM for A167T TK; 5 and 25 μM for A167E TK; 100 and 500 μM for A167F TK; 150 and 750 μM for A167Y TK; 200 and 1000 μM for A167H TK; 500 and 2500 μM for A167W TK; 250 and 1250 μM for A167K TK; 10 and 50 μM for A167N TK; 100 and 500 μM for A167C TK; 50 and 250 μM for A167F TK; 15 and 75 μM for A168S TK; and 15 and 75 μM for A168F TK; 150 and 750 μM for A167Y TK; 200 and 1000 μM for A167H TK; 500 and 2500 μM for A167W TK; 250 and 1250 μM for A167K TK; 10 and 50 μM for A167N TK; 100 and 500 μM for A167C TK; 50 and 250 μM for A168S TK; and 15 and 75 μM for A168F TK.) The reactions were terminated by spotting an aliquot of 45 μl onto DE-81 disks (Whatman; Maidstone, UK) that were instantly immersed and thoroughly washed three times in 1 mM HCOONa and one time in ethanol (70%). Finally, the disks were dried and assayed for radioactivity in a scintillant. The influence of different concentrations of dThd, BVDU, and dTTP on the phosphorylation of [8-3H]GCV was determined by incubating 50-μl reaction mixtures at 37 °C for 30 min in the presence of 1.6 μM [8-3H]GCV (1 μCi), a variety of compound concentrations (i.e., 8, 40, 200 and 1000 μM), and WT HSV-1 TK, mutant A167F, or A168H HSV-1 TK.

The phosphorylation of different concentrations of [3H]GCV, [3H]PCV, and [3H]ACV (i.e., 400, 200, 150, 125, 100, 67, 40, 20, 15, 12.5, 10, 6.7, and 4 μM) in the presence of wild-type HSV-1 TK and mutant A168H HSV-1 TK was carried out as described above. The reaction mixtures were incubated for 30 min at 37 °C and then subjected to high performance liquid chromatography analysis using a Parti-sphere-SAX column. A linear gradient of 5 mM (NH₄)₂HPO₄, pH 5.0 (buffer A), to 0.3 M (NH₄)H₂PO₄, pH 5.0 (buffer B), was used to separate the metabolites as follows: 5 min of 100% buffer A, 15 min of a linear gradient to 100% buffer B, 20 min of 100% buffer B, 5 min of a linear gradient to 100% buffer A, and 5 min of equilibration with buffer A. The flow rate was 2 ml/min.

Modeling Studies—The Protein Data Bank-coded HSV-1 TK structures 1E2J (10) and 1K12 (11) complexed, respectively, with dThd and GCV were overlaid using the COOT model-building tool for molecular graphics (12). The same software was used to generate the different enzyme mutants, the carbon-α chain was kept identical to the model structures, and the side chains were fitted by hand in the active site. The pictures were drawn by PYMOL (DeLano Scientific, San Carlos, CA).

**RESULTS**

Eleven different amino acid mutations have been introduced in the catalytic substrate-active site of HSV-1 TK at positions 167 and 168, and the mutated enzymes were evaluated for their potential to phosphorylate dThd and GCV. The kinetic Km and Vmax constants were determined for each of the mutant enzymes and depicted in Tables 2 and 3. Figs. 2 and 3 depict the phosphorylating capacity (Vmax/Km) of the individual mutant Ala-167 and Ala-168 TK enzymes for dThd and GCV. Among the TKs mutated at codon 167, replacing alanine by other amino acids with relatively small functional groups, such as serine, cysteine, or threonine, did not markedly affect the kinetic properties of the enzyme with respect to dThd and GCV phosphorylation. In contrast, bulky side-chains at position 167, such as the aromatic amino acids Tyr, Phe, His, and Trp dramatically compromised the dThd kinase activity, yet retained pronounced GCV-phosphorylating capacity. The mutant A167F TK had the highest GCV-phosphorylating capacity (~8-fold higher than the previously reported A167Y TK) (9) and a deleted dThd kinase activity. The higher Vmax/Km value of A167F TK was mainly because of a substantially lower Km for

---

**TABLE 1**

| Mutation | Sense primer sequence |
|----------|-----------------------|
| A167Y    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A167H    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A167W    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A167I    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A167K    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A167N    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A167C    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A167F    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A167E    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A167Y    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A167S    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A167T    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A168Y    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A168H    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A168W    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A168I    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A168K    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A168N    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A168C    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A168F    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A168E    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A168Y    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A168S    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A168T    | 5'-CCACCCCAACTTGTCTGAC-3' |
| A168F    | 5'-CCACCCCAACTTGTCTGAC-3' |

**JULY 14, 2006 • VOLUME 281 • NUMBER 28 • JOURNAL OF BIOLOGICAL CHEMISTRY 19275**
Mutant Ala-167 and Ala-168 HSV-1 TK

TABLE 2
Kinetic values of HSV-1 TK mutated at amino acid position 167

Data are the mean ± S.D. for at least two independent experiments. The data obtained for WT and A167/H HSV-1 TK represent the mean ± SD of four independent experiments.

| Kinetic parameter | WT | A167Y | A167H | A167V | A167I | A167E | A167K | A167C | A167T | A167S | A167F |
|-------------------|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Substrate: dThd   |    |       |       |       |       |       |       |       |       |       |       |
| $V_{\text{max}}$  | 323 ± 76 | NA     | NA    | NA    | 5.2 ± 0.21 | NA    | 35 ± 13 | 1035 ± 230 | 388 ± 144 | 882 ± 54 | NA     |
| $K_{\text{m}}$    | 0.23 ± 0.13 | NA     | NA    | NA    | 909 ± 0   | 0.006  | 1042 ± 295 | 1.7 ± 0.64 | 2.5 ± 0.72 | 0.36 ± 0.084 | NA     |
| $V_{\text{max}}/K_{\text{m}}$ | 1404 |       |       |       |       |       |       |       |       |       |       |
| Substrate: GCV    |    |       |       |       |       |       |       |       |       |       |       |
| $V_{\text{max}}$  | 109 ± 35 | 96 ± 3.2 | 29 ± 3.2 | 19 ± 11 | NA    | NA    | 29 ± 13 | 41 ± 2.1 | 220 ± 40 | 102 ± 57 | 391 ± 146 | 80 ± 0.71 |
| $K_{\text{m}}$    | 18 ± 6.0 | 136 ± 18 | 266 ± 145 | 877 ± 13 | NA    | NA    | 190 ± 85 | 11 ± 2.3 | 61 ± 21 | 28 ± 11 | 9.5 ± 3.6 | 15 ± 0.71 |
| $V_{\text{max}}/K_{\text{m}}$ | 6.1 | 0.71 | 0.11 | 0.022 | 0.150 | 3.7 | 0.35 | 3.6 | 3.6 | 0.41 | 5.3 |

ATPase activity was not measurable, NA, not active.

$\text{NA} = $ not active.

$V_{\text{max}}/K_{\text{m}}$ expressed as $\mu$M.

$V_{\text{max}}$ expressed as pmol/100 ng of protein/30 min.

$K_{\text{m}}$ expressed as $\mu$M.

GCV compared with the A167Y TK mutant enzyme (Table 2). Also the charged mutants A167E and A167K TK, and surprisingly also the mutant A167L TK, had neither measurable dThd kinase nor GCV kinase activity. When the ratio of the GCV/ dThd-phosphorylating potential of all 167 mutated TK enzymes were compared, A167F-mutated TK proved to be the most successful enzyme (Table 2, Fig. 2).

Similar studies were performed for a broad variety of Ala-168 mutant TK enzymes (Table 3). Similar to the Ala-167 mutants, the amino acid changes with less-bulky side chains showed virtually no compromised GCV kinase activity but a more pronounced attenuated dThd kinase activity. The aromatic amino acids resulted in an even better GCV-phosphorylating activity than the corresponding Ala-167-mutated TKs, except for A168W. Mutant A168C TK had pronounced GCV kinase but a ~40-fold compromised dThd kinase activity. The charged amino acid substitutions resulted in a better GCV phosphorylation than the corresponding Ala-167 mutants, but in both (A168E and A168K) TK mutants, dThd kinase was virtually abolished (Table 3). Interestingly, the mutant HSV-1 A168H

![FIGURE 2. Phosphorylating capacity of mutant Ala-167 HSV-1 TK enzymes in the presence of thymidine (dThd) or ganciclovir (GCV).](image)

![FIGURE 3. Phosphorylating capacity of HSV-1 mutant Ala-168 TK enzymes in the presence of thymidine (dThd) or ganciclovir (GCV).](image)
TK proved to be endowed with the highest discriminating capacity between GCV and dThd phosphorylation. Its $K_m$ for GCV was identical to the $K_m$ of wild-type HSV-1 TK for GCV, and its $V_{max}$ was ~4-fold higher than wild-type TK, whereas its dThd kinase-phosphorylating potential was $>3$–$4$ orders of magnitude lower than wild-type TK (Table 3, Fig. 3). Whereas several mutant Ala-167 HSV-1 TKs were completely devoid of dThd kinase activity (below the limit of detection) (Table 1), the mutant Ala-168 HSV-1 TKs usually had detectable dThd kinase activities, although in a number of cases (i.e. A168H, A168E, A168K), this residual TK activity proved at least $3$–$4$ orders of magnitude lower than wild-type HSV-1 TK activity (Table 3).

The $K_m$ and $V_{max}$ values for each mutant enzyme were obtained by measuring the direct conversion of GCV or dThd to their corresponding phosphate derivatives. To reveal whether the mutant TK enzymes that show the highest ratio of GCV versus dThd phosphorylating potential (i.e. A168H TK and A167F TK) still keep the potential of non-functionally binding dThd, we have assessed the competition of dThd and the dThd analogue BVDU at a range of concentrations for the mutant HSV-1 TKs in the presence of radiolabeled GCV. Clearly, dThd (and BVDU) had substantially lost the capacity to bind to the mutant A168H and A167F HSV-1 TK enzymes, because they poorly inhibit mutant TK-catalyzed GCV phosphorylation (Fig. 4). The feedback inhibition of GCV phosphorylation by dTTP was also much less pronounced than with wild-type TK (Fig. 4).

Given the differences in GCV and ACV phosphorylation found for previously reported HSV-1 TK mutants by Black et al. (4), we performed phosphorylation studies with GCV but also with ACV and PCV and found identical kinetic properties for wild type and the mutant A168H enzyme for each individual purine nucleoside analogue (data not shown). These data confirm that introduction of the A168H mutation into HSV-1 TK does not affect phosphorylation of any of the guanine nucleoside analogues but results in heavily compromised dThd kinase activity.

**DISCUSSION**

The $K_m$ value of wild-type HSV-1 TK for GCV, but not for dThd, proved 2.5–3.5-fold lower under our experimental conditions than previously reported by other investigators. Field et al. (13) report a $K_m = 66 \mu M$ for GCV against native HSV-1 (Patton strain) TK from HSV-1-infected HeLa cell cultures. Kokoris and Black (5) found 47.6 $\mu M$ for recombinant HSV-1 TK (derived from the pET23d; HSV TK-Dummy vector) purified on a 3-aminothymidine affinity column, and Bohman et al. (14) report a $K_m = 45 \mu M$ for GCV against purified native HSV-1 (F strain) TK derived from HSV-1 TK gene-transfected mammary carcinoma FM3A TK−/HSV-1 TK− cell cultures. The somewhat lower $K_m$ of 18 $\mu M$ for GCV against purified recombinant HSV-1 (Lyons strain) TK found in our study could not be explained by the presence of GST covalently linked to the HSV-1 TK, because it was ascertained that removal of GST from the wild-type and A168H HSV-1 TK fusion protein did not change the $K_m$ value for GCV (a $K_m$ of 18 and 13 $\mu M$ for the wild-type and A168H GST-HSV-1 TK fusion protein versus a $K_m$ of 21 and 18 $\mu M$ for the wild-type and A168H HSV-1 TK).

However, differences in $K_m$ for GCV may perhaps be explained by the different virus strains from which the TKs were derived. A wide variety of catalytic activities and affinities could be observed for the mutant enzymes in the presence of different amino acids at positions 167 and 168. Most of the increased or decreased catalytic activities for dThd and GCV could be explained in a structural context. Mutations at amino acid residue 167 show an abolishment of the dThd kinase activity in the presence of bulky side chains (Fig. 5). Indeed, the 5-methyl group of dThd or any other substituents at this position would
clash with large amino acid side chain residues. It is noticeable that the GCV activity varies among the bulky residues. The A167F mutation in TK presents the highest GCV activity. A167Y TK activity is lower than the latter, despite the side chains being of similar bulk. We suggest that tyrosine at 167 makes a hydrogen bond via its hydroxyl side chain to the purine ring and freezes GCV in a conformation not optimal for the phosphorylation. In conclusion, hydrophobic and bulky side chains at position 167 seem to be the best residues to select for GCV activity, apart from residues such as tryptophan that are probably too bulky to fit in the binding pocket. Adding any charged or polar residues does not improve GCV binding.

Mutations at residue 168 have a lower discriminating power, regarding GCV and dThd activities as substrates, compared with mutations at residue 167. Indeed, mutated residue side chains would be oriented in a way that is less disruptive for GCV and dThd binding (Fig. 6). Most of the mutations seem to affect the $K_{m}$ for dThd (from 3- to 4000-fold) but not the $V_{max}$ (Table 2); therefore, only the apparent binding affinity of dThd is affected rather than its rate of phosphorylation. Additionally, the same putative active site modifications are situated around dThd and not GCV. Mutations Lys, His, and Trp significantly increase the dThd $K_{m}$ at least 1000–4000-fold. In the case of Lys and His, it is interesting to note that both residues have nitrogen on their side chain, which appears somehow to be involved in interference with dThd binding and not GCV binding presumably because the latter has no oxygen to make a bond with the mutated residue.

Profound structural insights in the substrate active site of HSV-1 TK are required to enable rational development of novel enzyme constructs that recognize new nucleoside analogues as a substrate or improve the substrate affinity and/or catalytic activity of existing substrates. Because combined gene/chemotherapy of cancer makes use of the HSV-1 TK gene as a suicide gene to be used in combination with anti-herpetic drugs such as GCV or ACV, our findings may have a direct application in this field. Indeed, mutant HSV-1 TKs with substantially high $K_{m}$ values for the natural substrate dThd can have a distinct advantage in preferentially phosphorylating purine nucleoside analogues such as GCV, ACV, or PCV because of reduced competition with dThd for the active site and a less pronounced feedback inhibition by dTTP, which also binds to the dThd site. Because GCV and ACV have markedly higher $K_{m}$ values for wild-type HSV-1 TK than dThd (−100- and 1,000-fold, respectively), knocking out of the pyrimidine nucleoside phosphorylation site may become crucial to guaranteeing an efficient phosphorylation of the purine nucleosides in the in vivo (tumor) environment. In fact, our preliminary investigations reveal that uncloned human osteosarcoma (OST TK) tumor cell cultures transduced by the mutant A168H HSV-1 TK gene construct became markedly more sensitive to the cytostatic activity of GCV, PCV, and ACV than the parent cell cultures. Moreover, the addition of 50 µM thymidine or BVDU did not reverse the cytostatic activity of these test compounds against the OST TK−/HSV-1 A168H TK tumor cells under conditions where GCV, PCV, and ACV toxicity could be reversed by dThd in osteosarcoma tumor cell cultures transduced by the wild-type HSV-1 TK gene (data not shown). These preliminary findings validate the beneficial kinetic properties of the mutant A168H HSV-1 TK gene construct. The novel mutant enzyme constructs reported in this study may also prove their value in imaging reporter gene expression in vivo through non-invasive positron emission tomography (PET) scan detection of fluorinated (18F) nucleoside analogues (in particular F-GCV and F-PCV). Our findings that the fluorinated PCV is equally efficiently converted to its monophosphate derivative as the parent
unsubstituted PCV against A168H TK opens interesting perspectives for the mutated A168H (or A167F) HSV-1 TK construct to be used for such an application. Also, other indications, including the prevention of graft versus host disease using a suicide gene expressed in T-lymphocytes (15, 16), may make use of engineered enzyme constructs as described above.

Acknowledgments—We thank Kristien Minner, Ria Van Berwaer, and Lizette van Berckelaer for excellent technical assistance and Christiane Callebaut for dedicated editorial help.

REFERENCES
1. Black, M. E., Newcomb, T. G., Wilson, H.-M. P., and Loeb, L. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3525–3529
2. Kokoris, M. S., Sabo, P., Adam, E. T., and Black, M. E. (1999) Gene Ther. 6, 1415–1426
3. Kokoris, M. S., Sabo, P., and Black, M. E. (2000) Anticancer Res. 20, 959–964
4. Black, M. E., Kokoris, M. S., and Sabo, P. (2001) Cancer Res. 61, 3022–3026
5. Kokoris, M. S., and Black, M. E. (2002) Protein Sci. 11, 2267–2272

3 J. Balzarini and G. Bormans, unpublished data.