Engineering of a Chimeric RB69 DNA Polymerase Sensitive to Drugs Targeting the Cytomegalovirus Enzyme*

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Detailed structural and biochemical studies with the human cytomegalovirus (HCMV UL54) DNA polymerase are hampered by difficulties to obtain this enzyme in large quantities. The crystal structure of the related RB69 DNA polymerase (gp43) is often used as a model system to explain mechanisms of inhibition of DNA synthesis and drug resistance. However, here we demonstrate that gp43 is ~400-fold less sensitive to the pyrophosphate analog foscarnet, when compared with UL54. The RB69 enzyme is also able to discriminate against the nucleotide analog inhibitor acyclovir. In contrast, the HCMV polymerase is able to incorporate this compound with similar efficiency as observed with its natural counterpart. In an attempt to identify major determinants for drug activity, we replaced critical regions of the nucleotide-binding site of gp43 with equivalent regions of the HCMV enzyme. We show that chimeric gp43-UL54 enzymes that contain residues of helix N and helix P of UL54 are resensitized against foscarnet and acyclovir. Changing a region of three amino acids of helix N showed the strongest effects, and changes of two segments of three amino acids in helix P further contributed to the reversal of the phenotype. The engineered chimeric enzyme can be produced in large quantities and may therefore be a valuable surrogate system in drug development efforts. This system may likewise be used for detailed structural and biochemical studies on mechanisms associated with drug action and resistance.

Infection with the human cytomegalovirus (HCMV), which belongs to the Herpesviridae, remains an important health problem in immunocompromised persons (1–7). Several drugs that target the viral DNA polymerase (UL54) have been developed to treat the infection (8–12). Cidofovir (CDV), ganciclovir (GCV), or its prodrug valganciclovir are nucleotide or nucleoside analog inhibitors, respectively, that are intracellularly phosphorylated to their triphosphate form and compete with natural nucleotide pools for incorporation (13–20). These compounds are characterized by an acyclic sugar moiety with the equivalent of a 3’-hydroxyl group that is required for the next nucleotide incorporation event (21). Thus, once incorporated, these compounds interfere with DNA synthesis at various positions downstream (18, 22, 23). In contrast, compounds that lack the 3’-hydroxyl group, such as the antitherpetic drug acyclovir (ACV) (Fig. 1), act as chain terminators (24, 25). Although active against HCMV, ACV is not approved for treatment of HCMV infection, and its efficacy is inferior to GCV or CDV (8, 26, 27). The pyrophosphate analog foscarnet (phosphonoformic acid, PFA) is the third approved anti-HCMV drug that inhibits UL54 (Fig. 1) (28, 29). However, toxicity, problems with oral bioavailability, and the rapid development of resistance can limit the clinical utility of each of the approved drugs.

PFA is a broad spectrum antiviral agent that was shown to inhibit various polymerases, including enzymes encoded by herpes simplex virus (HSV), human herpesvirus, HCMV, and the reverse transcriptase (RT) of the human immunodeficiency virus type 1 (HIV-1) (28, 29). Progress has been made in elucidating the mechanism of inhibition of HIV-1 RT (30, 31). Site-specific footprinting experiments revealed that the enzyme can oscillate between two conformations, referred to as pre- and post-translocation (32). The 3’ end of the primer still occupies the nucleotide-binding site in the pre-translocated complex (33, 34). Binding of the next nucleotide requires translocation of the enzyme relative to its nucleic acid substrate (35). The dNTP substrate can bind to and is incorporated in the post-translocated complex. In contrast, PFA traps the pre-translocational complex, which provides a plausible mechanism for inhibition (30, 32). The mechanism of action might be similar with the HCMV enzyme; however, the limited solubility of UL54 makes it difficult to produce the purified enzyme in sufficient amounts required for detailed biochemical and structural studies (36, 37). Combined in vitro transcription/translation systems and the baculovirus expression system have proven successful for the expression of UL54 and the related HSV polymerase (UL30) (38–43). The UL30 apoenzyme has been crystallized (44); however, crystallographic data for UL54 are not available (45).

Like the related phage RB69 DNA polymerase (gp43), UL54 and UL30 belong to the polymerase α family (46). The RB69 polymerase can be expressed in its soluble form in Escherichia coli as a recombinant protein with a 6×His tag (47). The RB69 sequence was fused with the nucleotide-binding domain of the human cytomegalovirus DNA polymerase UL54. This hybrid enzyme is active and incorporates dNTPs efficiently to form DNA (48). For the purpose of this study, we chose to use the nucleotide-binding domain of UL54 because its structure is well characterized, and because the domain is derived from a different herpesvirus.

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The UL54 coding sequence was cloned into pCITE4b (Novagen) by use of the EcoRI and HindIII sites to generate pCITE4b/UL54. We also generated a 3′–5′-exonuclease negative construct that contains the D542A substitution. The RB69 DNA polymerase (gp43) coding sequence was kindly provided by Dr. Sylvie Doublée (University of Vermont) and Dr. Jim Karam (Tulane University). The gp43 coding sequence was cloned into pPR-IBA1 (IBA) using the BsaI site to generate pPR-IBA1/gp43. This construct facilitates protein purification through Strep-tag affinity chromatography (IBA). D222A and D327A substitutions were introduced to remove the 3′–5′-exonuclease activity. Constructs for the production of mutant enzymes were generated by site-directed mutagenesis. The amino acid substitutions were introduced with PfuUltra DNA polymerase (Stratagene) according to the manufacturer’s recommendations.

**Protein Expression**—The HCMV polymerase UL54 was expressed in rabbit reticulocyte lysate with a coupled in vitro transcription-translation system (Promega). Reactions were conducted essentially as described previously (41, 42). The RB69 DNA polymerase and chimeric RB69/HCMV enzymes were expressed as described previously (47). All enzymes were purified using Strep-tag affinity chromatography (IBA) according to the manufacturer’s recommendations. Heterodimeric reverse transcriptase p66/p51 was expressed and purified as described (54).

**Nucleic Acids and Chemicals**—Oligodeoxynucleotides used in this study were chemically synthesized and purchased from Invitrogen. The following sequences were chemically synthesized and purchased from Fermentas Life Sciences, and PFA was purchased from Sigma.

**Synthesis of ACV-TP**—ACV (1.5 mmol) was dissolved in 200 μl of dry 1,3-dimethyl-2-oxohexahydropyrimidine, N,N′-dimethylpropylene urea with 12–15 molecular sieves under nitrogen and stirred for 24 h. The mixture was chilled with an ice-water bath and stirred for 1 h, followed by slow addition of phosphorus oxychloride (3 eq) and stirred for an additional 25 min. A solution of tributylammonium pyrophosphate (4 eq) in 200 μl of N,N′-dimethylpropylene and tributyl amine (15 eq) was simultaneously added to the reaction. After 45 min the reaction was quenched with ice-cold water. The reaction was washed with chloroform, and the aqueous layer was collected and co-evaporated with deionized water three times. The residue was redissolved in 100 μl of deionized water and purified on ion-exchange column by high performance liquid chromatography. The final product was co-evaporated with water five times, giving a total yield of ACV-TP (NH₄)₄ of 18% with purity ≥95%. The molecular weight of the ACV-TP was confirmed by liquid chromatography-mass spectrometry/ tandem mass spectrometry m/z (M + 1) 466 → 152 (55).
**Enzyme Kinetics**—100 nM DNA/DNA primer-template hybrid T1/P1 (100 nM) was preincubated for 5–10 min at 37 °C with a given DNA polymerase in a buffer containing 25 mM Tris-HCl (pH 8), 50 mM NaCl, 0.5 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, and 5% glycerol. To compare different enzymes in single nucleotide incorporation assays, we adjusted the enzyme concentration and the time point of the reaction such that the values were in the middle of the chosen concentration range of the dNTP substrate and/or inhibitor was chosen such that the region between 807 and 815 in helix P (556–564 in gp43) plays an important role in this regard (7, 42). Some of the amino acids of this segment can interact with the bound nucleotide, whereas others appear to be involved in interhelical interaction with residues 779–784 (477–482 in gp43) of helix N (49, 50) (Fig. 2B). In an attempt to closely mimic the structure of UL54, we replaced amino acids that differ at equivalent positions in gp43 according to the sequence alignment shown in Fig. 2A. These changes involve the block of three amino acids between the conserved residues Lys-477 and Gln-481/Lys-482 of helix N, referred to as block A, and two blocks of three amino acids between conserved residues Gln-556 and Asn-564 of helix P (556–564 in gp43) (49) using the chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (59) The sequence alignment output was graphically prepared with ESPRIPT software (60). Conserved residues are highlighted in black, and similar residues are boxed (61).

**RESULTS**

Experimental Design—The goal of this study was to engineer and to characterize a chimeric RB69/UL54 enzyme that facilitates the study of PFA-mediated inhibition of DNA synthesis and drug resistance. We focused on segments of helix N and helix P that are located in close proximity to the phosphates of the bound dNTP substrate in the ternary complex with gp43 (Table 1 and Fig. 2) (49). Several amino acids in these regions are likewise implicated in binding of PFA by UL54. Phenotypic susceptibility assays, and our previous biochemical data suggest that the region between 807 and 815 in helix P (556–564 in gp43) plays an important role in this regard (7, 42). Some of the amino acids of this segment can interact with the bound nucleotide, whereas others appear to be involved in interhelical interaction with residues 779–784 (477–482 in gp43) of helix N (49, 50) (Fig. 2B). In an attempt to closely mimic the structure of UL54, we replaced amino acids that differ at equivalent positions in gp43 according to the sequence alignment shown in Fig. 2A. These changes involve the block of three amino acids between the conserved residues Lys-477 and Gln-481/Lys-482 of helix N, referred to as block A, and two blocks of three amino acids between conserved residues Gln-556 and Asn-564 of helix P, referred to as block B and block C, respectively. Of note, the alignment also points to several amino acid changes in equiva-

### TABLE 1

<table>
<thead>
<tr>
<th>Nomenclature for chimera (block)</th>
<th>Region</th>
<th>RB69 residues</th>
<th>HCMV residues</th>
<th>Comments</th>
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<tr>
<td>A</td>
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<td>Val-478</td>
<td>Trp-780</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Phe-479</td>
<td>Val-781</td>
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<tr>
<td></td>
<td></td>
<td>Asn-480</td>
<td>Ser-782</td>
<td></td>
</tr>
<tr>
<td>A/V781I</td>
<td>Helix</td>
<td>Chimera A V4791</td>
<td>Chimera A V7811</td>
<td>Foscarnet resistance conferring mutation*</td>
</tr>
<tr>
<td>B</td>
<td>Helix P</td>
<td>Ile-557</td>
<td>Met-808</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asn-558</td>
<td>Ala-809</td>
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<td></td>
<td>Arg-559</td>
<td>Leu-810</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Helix P</td>
<td>Leu-601</td>
<td>Val-812</td>
<td>None</td>
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<td></td>
<td></td>
<td>Leu-602</td>
<td>Thr-813</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ile-603</td>
<td>Cys-814</td>
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<tr>
<td>ABC/R784A</td>
<td>Helix N</td>
<td>Chimera A</td>
<td>Chimera A</td>
<td>Conserved residue</td>
</tr>
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<td></td>
<td></td>
<td>R784A</td>
<td>R784A</td>
<td></td>
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<td></td>
<td>Helix P</td>
<td>Chimera BC</td>
<td>Chimera BC</td>
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<tr>
<td>ABC/Q807A</td>
<td>Helix N</td>
<td>Chimera A</td>
<td>Chimera A</td>
<td>Conserved residue conferring resistance to foscarnet*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chimera BC</td>
<td>Chimera BC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Helix P</td>
<td>Chimera BC</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>Q807A</td>
<td>Q807A</td>
<td></td>
</tr>
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</table>

* A 5.2-fold increase in foscarnet resistance is indicated (62).
* A 6-fold increase in foscarnet resistance is shown (42).
We generated chimeric gp43-based enzymes in which block A of helix N and blocks B/C of helix P have been replaced by equivalent regions of UL54. Several conserved amino acids within these regions interact with the phosphates of the nucleotide (Fig. 2B). Arg-482 of helix N as well as Gln-556 of helix P appear to contact the γ-phosphate of the bound nucleotide. The same region is also implicated in PFA binding. The Q807A mutation in helix P in UL54 increases the IC₅₀ value for PFA (42). However, a potential role of helix N in PFA resistance remains to be defined. Of note, Phe-479 of helix N in gp43 is equivalent to Val-781 in UL54, and the V781I substitution in the HCMV enzyme shows decreased phenotypic susceptibility to PFA (62). This residue is likewise located in the vicinity of the γ-phosphate, and this region appears to be important for PFA binding. Thus, to test how close the chimeric enzyme may mimic the natural HCMV polymerase, we introduced V781I and alanine substitutions at conserved residues Arg-784 (helix N) and Gln-807 (helix P), respectively, against the background of the chimeras.

Efficiency of Nucleotide Incorporation—We initially determined steady-state kinetic parameters for single nucleotide incorporation events, and we compared several chimeric enzymes with WT gp43 (Table 2). Throughout this study, we used 3′–5′-exonuclease negative mutants to prevent potentially confounding effects through the editing activity. Replacing block A (helix N) with the equivalent UL54 region caused 2–3-fold reductions in the efficiency of single nucleotide incorporation events. Introducing the V781I mutation against this background caused similar reductions in kinetic parameters. Chimeric enzymes containing block A (helix N) as well as block B/C (helix P) of UL54 showed 10-fold reductions in the same assay. Alanine mutations at conserved positions Arg-784 and Gln-807 that were introduced against this mutational background did not cause further significant reductions in the efficiency of nucleotide incorporation. Overall, these findings show that replacing critical elements of helix N and helix P with their UL54 counterparts can cause reductions in enzymatic activity. Because of the lack of a corresponding expression/purification protocol for UL54, it is at this point difficult to assess whether the activity of the HCMV enzyme is intrinsically reduced or whether the chimeric nature of the enzyme may cause such deficits. However, the enzymatic activities are sufficiently high to measure potential changes in drug sensitivity, provided that the concentrations of each of the enzymes to be compared are appropriately adjusted as outlined under “Experimental Procedures”.

Sensitivity to PFA—The ability to inhibit single nucleotide incorporation events with PFA was expressed in both IC₅₀ and Kᵦ values (Table 3). Fig. 3 shows an example of the assay used to evaluate the extent of inhibition. We compared gp43 with UL54 and HIV-1 RT as another control. UL54 and HIV-1 RT are sensitive to inhibition with PFA and have IC₅₀ values between 0.6 and 0.9 μM (Table 3). In contrast, gp43 has an IC₅₀ value greater than 300 μM, which renders the enzyme ~400-fold less susceptible to PFA when compared with UL54. Thus, despite the evidence for structural and functional links between gp43 and UL54, these data point to important differences between the two enzymes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>kₐ₀⁻¹</th>
<th>Kᵦ</th>
<th>kₐ₀/Kᵦ</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp43</td>
<td>0.61</td>
<td>0.49</td>
<td>1.25</td>
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<tr>
<td>A</td>
<td>0.39</td>
<td>0.92</td>
<td>0.42</td>
</tr>
<tr>
<td>A/V781I</td>
<td>0.51</td>
<td>0.92</td>
<td>0.55</td>
</tr>
<tr>
<td>ABC</td>
<td>0.46</td>
<td>2.7</td>
<td>0.17</td>
</tr>
<tr>
<td>ABC/R784A</td>
<td>0.39</td>
<td>3.3</td>
<td>0.12</td>
</tr>
<tr>
<td>ABC/Q807A</td>
<td>0.50</td>
<td>5.6</td>
<td>0.14</td>
</tr>
</tbody>
</table>

* kₐ₀ is the enzyme turnover number that is calculated by normalizing the maximum velocity of the reaction to the enzyme concentration.

** Kᵦ is the substrate concentration at half-maximum velocity of the reaction.

* The previously reported gp43 values for dCTP kₐ₀ and Kᵦ are 1.0 s⁻¹ and 0.57 μM, respectively (51).

* Standard deviations were determined on the basis of at least three independent experiments and represent a maximum of 12% of the reported value.
Reactions were monitored in the presence of constant concentrations of dNTPs and increasing concentrations of PFA. The reactions conditions were chosen such that the maximum of the available primer-template substrate was used in the absence of PFA. PFA-mediated inhibition of DNA synthesis is illustrated by the reduction of the intensity of the signal corresponding to the migration pattern of a full-length product (t) that is indicated by the corresponding arrow. The migration pattern of the 5’ end radioactively labeled primer (s) and the product of the terminal transferase activity (tt) is illustrated by corresponding arrows. The asterisk points to the exogenous background exonuclease activity present in samples with HCMV polymerase UL54 expressed in vitro transcription/translation system.

**TABLE 3**

Kinetic constants for inhibition of nucleotide incorporation by PFA

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC$_{50}$ $\mu$M</th>
<th>Fold$^d$ change</th>
<th>$K_i$ $\mu$M</th>
<th>Fold change</th>
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<tr>
<td>UL54$^e$</td>
<td>0.87$^f$</td>
<td>Reference$^g$</td>
<td>0.076</td>
<td>Reference</td>
</tr>
<tr>
<td>RT</td>
<td>0.60</td>
<td>1.4$^e$</td>
<td>1.1</td>
<td>11$^e$</td>
</tr>
<tr>
<td>gp43</td>
<td>333</td>
<td>380$^h$</td>
<td>NA$^i$</td>
<td>NA</td>
</tr>
<tr>
<td>gp43</td>
<td>333</td>
<td>Reference</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>A</td>
<td>7.5</td>
<td>44$^k$</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>ABC</td>
<td>3.3</td>
<td>100$^l$</td>
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<tr>
<td>A/V781I</td>
<td>7.5</td>
<td>Reference</td>
<td>0.99</td>
<td>Reference</td>
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<tr>
<td>ABC</td>
<td>3.3</td>
<td>Reference</td>
<td>0.55</td>
<td>Reference</td>
</tr>
<tr>
<td>ABC/R784A</td>
<td>37</td>
<td>11$^m$</td>
<td>5.9</td>
<td>11$^m$</td>
</tr>
<tr>
<td>ABC/Q807A</td>
<td>27</td>
<td>8.2$^n$</td>
<td>6.1</td>
<td>11$^n$</td>
</tr>
</tbody>
</table>

$^a$ IC$_{50}$ is the inhibitory concentration of PFA that reduced the nucleotide incorporation activity of enzyme by 50%. Values were calculated by fitting at least 10 data points to a sigmoidal dose-response (variable slope) equation using GraphPad Prism (version 5.0).

$^b$ Fold change is calculated as the ratio of the IC$_{50}$ or $K_i$ values of the reference and the query enzyme in the subgroup such that the ratio is more than 1.

$^c$ $K_i$ is the inhibitor dissociation constant. Values were calculated by globally fitting the 42-data point $K_i$ experiment to the general mixed model of inhibition using GraphPad Prism (version 5.0).

$^d$ The HCMV polymerase UL54 was expressed in the in vitro transcription/translation system.

$^e$ Standard deviations were determined on the basis of at least three independent experiments and represent a maximum of 25% of the reported value.

$^f$ Reference defines the enzyme whose inhibitory constants were used as a reference value in the subgroup. Subgroups of enzymes are separated by an extra row.

$^g$ Reference values of the enzyme whose inhibitory constants were used as a reference for the enzymes in the subgroup such that the ratio is more than 1.

$^h$ NA means not available.

IC$_{50}$ and $K_i$ measurements show that helix N is a critical structural determinant that mediates PFA sensitivity (Table 3). The chimeric enzyme containing block A (helix N) from UL54 shows ≈50-fold reductions in IC$_{50}$ values when compared with WT gp43. This value is 3-fold increased when the V781I mutation is introduced against this mutational background. A similar trend is observed for the $K_i$ values. The enzyme with blocks A (helix N) and B/C (helix P) of UL54 showed further subtle increases in sensitivity to PFA. Overall, we measured a 100-fold increase as compared with WT gp43. Moreover, alanine changes at conserved residues Gln-807 and Arg-784 confer 8–11-fold resistance in the context of our biochemical measurements. Together, these findings identify helix N as an important determinant for PFA sensitivity, whereas helix P, in relation to helix N, appears to be a minor contributor. However, the resistance data unambiguously indicated that changes in either one of the two helices can affect PFA susceptibility.

To confirm these phenotypes, we devised a gel-based assay that allowed us to monitor the effects of PFA over multiple template positions (Fig. 4). We focused on the chimeric enzyme with blocks A and B/C of UL54, referred to as gp43-UL54ABC, that showed the lowest IC$_{50}$ values. In agreement with the aforementioned data, we demonstrated that both HIV-1 RT and UL54 were sensitive to PFA. Concentrations as low as 1 $\mu$M had significant levels of inhibition as judged by full-length product formation, although gp43 is not inhibited at concentrations of 10 $\mu$M.
RB69-HCMV Chimeric DNA Polymerases

In the presence of increasing concentrations of dGTP or ACV-TP.

FIGURE 5. Efficiency of nucleotide incorporation for ACV-TP and dGTP. Graphical representation of single nucleotide incorporation events monitored in the presence of increasing concentrations of dGTP or ACV-TP. Values were calculated by fitting the data points to Michaelis-Menten function using GraphPad Prism (version 5.0).

**DISCUSSION**

In light of the problems associated with the development of an expression system for the HCMV DNA polymerase (UL54) (37), we engineered and characterized chimeric enzymes derived from the related RB69 polymerase (gp43) with critical elements of its UL54 counterpart. We replaced regions of helix N and helix P that are located in close proximity to the triphosphate moiety of an incoming nucleotide. Such effects may be exacerbated with acyclic nucleotide analog inhibitors that rely even more on specific contacts through their phosphates. To test this hypothesis, we asked whether gp43, UL54, and gp43-UL54ABC showed differences in efficiency of incorporation of ACV-TP.

To assess the ability of viral enzymes to use ACV-TP as a substrate for DNA synthesis, we determined the steady-state parameters for incorporation of ACV-TP and its natural counterpart dGMP (Fig. 5 and Table 4). The ratio of $V_{max}/K_m$ is indicative of the efficiency of nucleotide incorporation, and the ratio of $V_{max}/K_m$ (dGTP) and $V_{max}/K_m$ (ACV-TP) defines the selectivity for the inhibitor. Among the three enzymes, the RB69 enzyme demonstrated the highest efficiency for incorporation of dGMP but the lowest for ACV-CP. The gp43-UL54ABC showed significantly reduced rates of incorporation of dGMP as compared with UL54. The selective advantage of incorporation of the natural nucleotide over the inhibitor was >300. Of significance, the gp43-UL54ABC mutant enzyme does not show such a selective advantage. The mutant enzyme behaved almost exactly like UL54. The selectivity values for UL54 and gp43-UL54ABC were close to 1, suggesting that these enzymes accept ACV-TP and dGTP as substrates for DNA synthesis with similar efficiencies.

**TABLE 4**

Kinetic constants for dGTP and ACV-TP incorporation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$V_{max}$ $^a$ (nM/min)</th>
<th>$K_m$ $^b$ (μM)</th>
<th>$V_{max}/K_m$</th>
<th>$V_{max}$ $^a$ (nM/min)</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}/K_m$</th>
<th>SEL$^c$</th>
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<tr>
<td>UL54$^d$</td>
<td>2.2 ± 0.086$^e$</td>
<td>0.041 ± 0.0067</td>
<td>54</td>
<td>12.2 ± 0.13</td>
<td>0.039 ± 0.023</td>
<td>31</td>
<td>1.7</td>
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<tr>
<td>gp43$^f$</td>
<td>9.2 ± 0.12</td>
<td>0.079 ± 0.053</td>
<td>117</td>
<td>8.6 ± 0.45</td>
<td>24 ± 2.8</td>
<td>0.36</td>
<td>325</td>
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<tr>
<td>ABC</td>
<td>5.1 ± 0.23</td>
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<td>5.1 ± 0.14</td>
<td>0.41 ± 0.059</td>
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$^a$ $V_{max}$ is the maximum velocity of the reaction.

$^b$ $K_m$ is the substrate concentration at half-maximum velocity of the reaction.

$^c$ SEL means selectivity, which is calculated as a ratio of $K_m$ for dGTP over $K_m$ for ACV-TP.

$^d$ UL54, the HCMV polymerase, was expressed in the in vitro transcription/translation system. The previously reported UL54 values for dGTP $K_m$ is 0.11 μM (42).

$^e$ Standard deviations were determined on the basis of at least two independent experiments.

$^f$ The previously reported gp43 value for dGTP $K_m$ is 0.17 μM (51).

In general, mutations that affect susceptibility to antiviral drugs often involve subtle structural changes that do not include conserved amino acids, which makes it difficult to model such effects if only the structures of related enzymes are available. The chimeric enzymes designed in this study may help to tackle this problem.

Replacing the critical regions of helix N of gp43 with the equivalent region of UL54 caused marked increases in sensitivity to PFA (Table 3). The introduction of naturally occurring, resistance-conferring mutations against this mutational background translated this phenotype in biochemical terms. Thus, a limited number of amino acids can be changed to produce a chimeric gp43-UL54 enzyme that shows a similar behavior to an antiviral drug as observed with WT UL54. The substitution of the three amino acids of helix N showed the strongest PFA resensitization effects, although the contribution of changes in helix P are minor. However, helix P contains various amino acids that are involved in HCMV resistance to PFA, CDV, and GCV (7). Changes at these amino acids may directly affect binding of these inhibitors or indirectly through intermolecular interactions between helix N and helix P. Thus, future studies.
aimed at elucidating mechanisms of resistance to the approved anti-HCMV enzymes may be performed with the gp43-UL54ABC enzyme in which important regions of both helices are replaced.

A structural model of T7 DNA polymerase bound to a primer-template and nucleotide substrate provided a better understanding of the biochemical consequences of specific ACV resistance conferring mutations in the HSV-1 polymerase (UL30) (63); however, the detailed role played by helix N and helix P in drug susceptibility remained elusive. Modeling studies based on the crystal structure of UL30 in its apo form suggest an equivalent role for the two helices gp43 (44), although a comparison of the structures of the apo form of gp43 and the ternary complex revealed significant conformational changes upon nucleotide binding (52). We have superimposed the structures of UL30 (apo form) and of gp43 (ternary complex) and arrive to the same conclusion (Fig. 2B). Moreover, there are also several amino acid changes in the relevant regions of the two helices of UL30 and UL54 that can affect susceptibility to antiviral drugs. The resistance profile of the two enzymes is not identical in this region (64). Thus, the approach to engineer gp43-based chimeric enzymes may likewise provide a valuable tool to study differences in drug susceptibility among the various polymersases that belong to the Herpesviridae.

We have further demonstrated that changes of residues that are located in close proximity to the γ-phosphate of a bound nucleotide, either as part of helix N or as part of helix P, diminish the inhibitory effects of PFA. These findings are consistent with our previous mutational studies on UL54, and point to this region as a possible binding site for PFA (42). Binding of PFA to the pre-translocated complex of HIV-1 RT depends on the presence of Mg2+ ions, which suggests that the inhibitor binds in close proximity to the location that occupies the β- and γ-phosphates of the bound nucleotide in the post-translocated complex (30, 32, 35). Like the nucleotide, the presence of PFA stabilizes the complex, presumably as a result of a conformational change that traps the ligand. The structures of binary and ternary complexes of the RB69 enzyme show a similar conformational change that involves helix N and helix P (49, 50). Thus, the differences between gp43 and UL54 in sensitivity to PFA may also be linked to potential differences in the ability of the two enzymes to trap the inhibitor.

The incorporation of nucleotides and nucleotide analogs follow the sequence of binding, conformational change, and catalysis (65–68). Sensitivity to a certain nucleotide analog can likewise be affected by these parameters (21, 34, 69–72). Our previous mutational studies on UL54, and point to this region as a possible binding site for PFA (42). Binding of PFA to the pre-translocated complex of HIV-1 RT depends on the presence of Mg2+ ions, which suggests that the inhibitor binds in close proximity to the location that occupies the β- and γ-phosphates of the bound nucleotide in the post-translocated complex (30, 32, 35). Like the nucleotide, the presence of PFA stabilizes the complex, presumably as a result of a conformational change that traps the ligand. The structures of binary and ternary complexes of the RB69 enzyme show a similar conformational change that involves helix N and helix P (49, 50). Thus, the differences between gp43 and UL54 in sensitivity to PFA may also be linked to potential differences in the ability of the two enzymes to trap the inhibitor.

Taken together, these findings/results indicate that the chimeric gp43-UL54 enzyme can provide a valuable tool to study the mechanisms of action and resistance to PFA and to nucleotide analog inhibitors. Although various other regions of the enzyme, including its 3′–5′-exonuclease activity can also contribute to differences in drug susceptibility, our findings reveal that helix N and helix P play an important role in this context (7, 40). gp43-UL54ABC and the various resistant mutant enzymes can be expressed and purified at high yields, which facilitates detailed structural and biochemical studies.

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