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# A Point Mutation in the Human Cytomegalovirus DNA Polymerase Gene Confers Resistance to Ganciclovir and Phosphonylmethoxyalkyl Derivatives

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**Ganciclovir-resistant mutant 759[r]D100 derived from human cytomegalovirus strain AD169 contains two resistance mutations, one of which is in the *UL97* gene and results in decreased ganciclovir phosphorylation in infected cells [V. Sullivan, C. L. Talarico, S. C. Stanat, M. Davis, D. M. Coen, and K. K. Biron, Nature (London) 358:162–164, 1992]. In the present study, we mapped the second mutation to a 4.1-kb DNA fragment containing the DNA polymerase gene and showed that it confers ganciclovir resistance without impairing phosphorylation. Sequence analysis of the 4.1-kb region revealed a single nucleotide change that resulted in a glycine-to-alanine substitution at position 987 within conserved region V of the DNA polymerase. Recombinant viruses constructed to contain the DNA polymerase mutation but not the phosphorylation defect displayed intermediate resistance (4- to 6-fold) to ganciclovir relative to the original mutant 759[r]D100 (22-fold); the recombinant viruses also displayed resistance to ganciclovir cyclic phosphate (7-fold), 1-(dihydroxy-2-propoxymethyl)-cytosine (12-fold), and the phosphonylmethoxyalkyl derivatives (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)adenine and (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (8- to 10-fold). However, the recombinant viruses remained susceptible to certain related compounds. These results imply that the human cytomegalovirus DNA polymerase is a selective target for the antiviral activities of ganciclovir, certain of its derivatives and phosphonomethoxyalkyl derivatives; support a role for region V in substrate recognition; and suggest the possibility of clinical resistance of human cytomegalovirus to these compounds because of polymerase mutations.**

Human cytomegalovirus (HCMV) is a serious and often life-threatening pathogen of newborn and immunocompromised individuals including transplant recipients and patients with AIDS (47, 56). Only a few drugs are available to treat or suppress HCMV infections; these include the nucleoside analogs ganciclovir {[9-(1,3-dihydroxy-2-propoxymethyl)-guanine]; DHPG} and, at high doses, acyclovir, and the $PP_i$ analog foscarnet. These drugs have limited efficacies, they cause toxic side effects, there are difficulties in drug delivery and distribution associated with these drugs, and there is the potential (3, 49) or actual emergence of resistant virus (16, 25). These limitations highlight the need both to develop alternative therapies for HCMV infections and to understand the mechanisms of anti-HCMV drug resistance.

We have previously described a DHPG-resistant HCMV mutant, 759[r]D100, derived from strain AD169, which was susceptible to foscarnet (3) and recently showed that mutant 759[r]D100 contains two DHPG resistance mutations (51). One of these lies in the *UL97* gene and controls DHPG phosphorylation (51). In the study described here we mapped the second DHPG resistance mutation to a conserved region of the HCMV DNA polymerase (*pol*) gene and showed that it confers resistance not only to DHPG but also to several other promising antiherpesvirus drugs. The results have implications for drug mechanisms and polymerase function and indicate the potential for drug resistance in the clinical setting.

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## MATERIALS AND METHODS

**Cells and viruses.** Human foreskin fibroblasts (HFFs) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 0.03% glutamine, 0.075% $NaHCO_3$, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (DME-10). Human embryonic lung fibroblast cells (MRC-5 cells) were obtained from the American Type Culture Collection and were maintained as described previously (4). Wild-type HCMV strain AD169 was originally provided by J. Nelson. Stocks of HCMV strain AD169, mutant 759[r]D100 (3), and recombinants GDG[r]H5 and GDG[r]P53 (this study) were prepared in HFFs as described previously (49) and were stored in liquid nitrogen.

**Antiviral drugs.** DHPG (synthesized at Burroughs Wellcome by L. Beauchamp) was prepared in equimolar NaOH, filter sterilized, and stored at −20°C. 1-[(1,3-Dihydroxy-2-propoxy)methyl]cytosine (DHPC) was synthesized at Burroughs Wellcome by L. Beauchamp and was prepared as described previously (2). Ganciclovir cyclic phosphate {DHPG-cyclic phosphate; 9-[(2-hydroxy-1,3,2-dioxaphosphorinan-5-yl)oxymethyl]guanine phosphate-oxide, also known as 2′ nor-cyclic GMP} was kindly provided by R. L. Tolman (Merck Sharp & Dohme Laboratories, Rahway, N.J.) and was prepared in water, filter sterilized, and stored at −20°C. DHPG homophosphonate (17), which was kindly provided by E. Reist (Stanford Research Institute), and (*S*) - 1 - (3 - hydroxy - 2 - phosphonylmethoxypropyl)adenine (HPMPA) and (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC), which were kindly provided by J.

Martin (Bristol-Meyers, Wallingford, Conn.) were prepared in water, filter sterilized, and stored at −20°C.

**Drug susceptibility assays.** Plaque reduction assays were carried out on HFFs or MRC-5 cell monolayers as described previously (10, 49), and viral plaques were visualized by staining cell monolayers with crystal violet 10 to 14 days postinfection. The criteria, including statistical analysis, used to determine whether viruses differed significantly in their susceptibilities to drugs have been described previously (7).

**Anabolism of DHPG.** MRC-5 cell monolayers in 60-mm-diameter dishes were infected at a multiplicity of infection (MOI) of 0.1 to 0.5. Three to four days postinfection the cells were pulse-labeled with 50 μM $^{14}$C-labeled DHPG (specific activity, 52 mCi/mmol) which had been purified by high-pressure liquid chromatography to remove guanine contaminants. Cells were extracted with perchloric acid, and DHPG anabolites were measured with a cation-exchange column as described previously (24).

**Marker transfer experiments.** To prepare infectious AD169 DNA (5 to 50 PFU/μg), HFFs were infected with HCMV strain AD169 at an MOI of 0.01. Once a generalized cytopathic effect was observed, the cells were scraped into the medium and were pelleted by centrifugation at low speed for 15 min at 4°C. The supernatant (containing extracellular virus) was stored on ice. The cells were resuspended in 0.01 M Tris-HCl–0.01 M NaCl–0.003 M $MgCl_2$ (pH 7.4), incubated on ice for 10 min, and then disrupted by Dounce homogenization; the nuclei were removed by low-speed centrifugation for 10 min at 4°C. The supernatant containing the cytoplasmic fraction was combined with the medium containing the extracellular virus, and the virions were pelleted by centrifugation at 12,000 × *g* for 1 h at 4°C. Viral DNA was prepared by resuspension of the virions in 10 mM Tris-HCl (pH 8)–0.1 mM EDTA; this was followed by the addition of 1% sodium dodecyl sulfate and incubation with 100 μg of proteinase K per ml for 5 h at 37°C. The DNA was extracted once with chloroform, twice with phenol, and once again with chloroform and was then dialyzed for 48 h against 10 mM Tris (pH 8)–0.1 mM EDTA, yielding approximately 2 μg of AD169 DNA per $10^6$ infected cells. Transfections were carried out by calcium phosphate precipitation as described previously (7), with the following modifications. HFFs between passages 5 and 10 were used throughout the procedure; the transfection mixture contained 0.2 μg of AD169 infectious DNA and at least a fivefold molar excess of 759$^r$D100 DNA fragments relative to homologous sequences found in AD169, which were prepared as previously described (9). At 3 to 6 h after transfection, cells were treated with 20% glycerol in DME-10 for 2 min. HCMV plaques could be detected after 7 to 21 days. The cells were harvested after a generalized cytopathic effect was obtained, and the titers of the progeny virus were determined in the absence of drug and the presence of the indicated concentrations of DHPG.

**Cloning and sequencing.** Plasmids pHF54 and pHF4 consist of the *Hin*dIII F fragments of GDG$^r$H5 and 759$^r$D100, respectively, cloned into the *Hin*dIII site of pUC18 (58). The *Hin*dIII F fragment of GDG$^r$H5 was isolated from GDG$^r$H5 viral DNA. The *Hin*dIII F fragment of 759$^r$D100 was subcloned from cosmid vector pC7S37. pC7S37 consists of a partial *Sau*3A fragment of 759$^r$D100 viral DNA, which includes the *Hin*dIII F, M, and Z fragments cloned into the *Bam*HI site of cosmid vector pCV108 (12). The entire DNA polymerase open reading frame plus flanking sequences 7 bp upstream and 253 bp downstream were isolated as a 4.1-kb *Rsr*II to *Sst*I fragment as follows. pHF54 was digested with *Rsr*II plus *Sst*I, treated with T4 DNA polymerase and the Klenow fragment to convert 3′ and 5′ overhangs to blunt ends, and ligated into the *Sma*I site of pGEM7Zf(+) (Promega Corp.) to generate pC54POL. pHF4 was digested with *Rsr*II, treated with the Klenow fragment to fill in the 5′ overhangs, and then digested with *Sst*I and the 4.1-kb fragment ligated into the *Sma*I and *Sst*I sites of pGEM7Zf(+) to generate pC7POL. The 4.1-kb fragment was subsequently excised from pC7POL with *Eco*RI plus *Sst*I, blunt-ended as described above, and ligated into the *Sma*I site of pGEM7Zf(+) to generate pPOL71.

A series of plasmids containing 3′ deletions in the 759$^r$D100 DNA polymerase gene were generated by exonuclease III treatment of *Asu*II plus *Sst*I-digested pPOL71. This procedure was carried out with a commercial kit (Erase-a base; Promega Corp.). The pPOL71-derived plasmids were sequenced as double-stranded DNA by using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical). pUC/M13 reverse and universal primers were used, and when necessary, oligonucleotide primers were designed by using the wild-type AD169 sequence (34). The following synthetic oligonucleotides (Oligos Etc. Inc.) were used: VSPOL1, 5′-TCAGGAAGACTATGTAGTGG-3′ (positions 3260 to 3279), and VSPOL2, 5′-ACTTCATCGAGT GAGAGGC-3′ (complementary to positions 3709 to 3727), 5′-CAGCAGATCCGTATCT-3′ (positions 2391 to 2406), 5′-ACTGGACCATGGCCAGA-3′ (positions 1303 to 1319), and 5′-TTGATGGCACGGACGA-3′ (positions 4051 to 4066).

For direct polymerase chain reaction (PCR) sequencing of GDG$^r$P53 and AD169 DNAs, a 150-cm$^2$ flask of HFFs was infected with each virus. When generalized cytopathic effects were observed, the cells were scraped into the medium, freeze-thawed three times, and sonicated for 10 to 30 s. The cellular debris was removed by low-speed centrifugation, and virus was pelleted from the supernatant by microcentrifugation for 1 h at 4°C. The virus pellet was resuspended in 10 mM Tris-HCl–1 mM EDTA (pH 7), chloroform extracted once, phenol extracted twice, chloroform extracted once more, and ethanol precipitated. The DNA was resuspended in water and amplified by a standard PCR protocol (8) by using primers VSPOL1 and VSPOL2. The PCR products were then prepared for DNA sequencing as described previously (31) and were sequenced by using Sequenase according to the manufacturer's specifications (United States Biochemical).

## RESULTS

**Mapping of DHPG resistance to the *pol* gene.** We previously identified two nonoverlapping cosmids from DHPG-resistant mutant 759$^r$D100 that could transfer DHPG resistance in marker transfer experiments. The resistance mutation in one of these cosmids was shown to lie within the *UL97* gene that controls ganciclovir phosphorylation, while the other cosmid, pC7S37, includes the viral DNA polymerase (*pol*) gene (51). Since the antiviral action of DHPG involves inhibition of viral DNA synthesis (37, 42, 44) and its triphosphate form has been shown to be an inhibitor of HCMV DNA polymerase in vitro (4, 23, 38), we tested the possibility that the ability of pC7S37 to transfer DHPG resistance was due to a *pol* mutation.

The *pol* gene of 759$^r$D100 was isolated from pC7S37 as a 4.1-kb *Rsr*II to *Sst*I fragment inserted in pGEM7Zf(+). The resulting plasmid, pPOL71, was tested in marker transfer experiments. While progeny virus from HFFs transfected

TABLE 1. Susceptibilities of HCMV strains AD169, GDG$^r$P53, and 759$^r$D100 to various drugs

| Drug | $ED_{50}$ (μM) of[a]: | | |
|---|---|---|---|
| | AD169 | GDG$^r$P53 | 759$^r$D100 |
| DHPG | 2.5 | 10 | 55 |
| DHPC | 14 | 170 | 97 |
| DHPG cyclic phosphate | 16 | 110 | 100 |
| DHPG homophosphonate | 6.0 | 7.0 | 6.6 |
| Acyclovir | 70 | 100 | 130 |
| HPMPA | 0.84 | 6.8 | 7.2 |
| HPMPC | 0.52 | 5.8 | 3.4 |

[a] $ED_{50}$s represent typical results from plaque reduction assays. Similar fold differences in susceptibilities among the three viruses were observed in at least one other assay for each drug.

with AD169 DNA alone contained no detectable progeny that was resistant to 20 μM DHPG (≤0.017%), 5% of the progeny from cells cotransfected with plasmid pPOL71 and AD169 DNAs formed plaques at this drug concentration. These results demonstrated efficient transfer of DHPG resistance by pPOL71.

Recombinant virus GDG$^r$P53 was isolated from progeny virus derived from infectious pPOL71 and AD169 DNAs after plating in 20 μM DHPG and was further plaque purified in the absence of DHPG. Its susceptibility to DHPG was investigated by a plaque reduction assay (Table 1). GDG$^r$P53 was resistant to DHPG, exhibiting a 50% effective dose ($ED_{50}$) of 10 μM, which was fourfold greater than that of AD169 ($ED_{50}$, 2.5 μM). This was an intermediate level of resistance relative to that of the parental mutant 759$^r$D100, for which the $ED_{50}$ was 55 μM in this assay. Other recombinants derived from the same marker transfer behaved similarly (50). These results are consistent with our earlier findings (51) that 759$^r$D100 contains more than one mutation that affects resistance to DHPG and that recombinants containing the *UL97* mutation also exhibit intermediate DHPG resistance. Thus, like the *UL97* gene, the *pol* gene of 759$^r$D100 contains a mutation that accounts for part of this mutant's resistance.

**Phosphorylation of DHPG in recombinant GDG$^r$P53-infected cells.** Since the DHPG resistance of mutant 759$^r$D100 was associated previously with its inability to induce phosphorylation of DHPG in infected cells (3), we investigated whether recombinant GDG$^r$P53 is also altered in its ability to induce conversion of DHPG to its phosphorylated derivatives. MRC-5 cells were mock infected or infected with AD169, GDG$^r$P53, or 759$^r$D100 at an MOI of 0.4. Four days after infection the cells were labeled with $^{14}$C-labeled DHPG for the indicated times. Cells were then harvested and the levels of DHPG anabolites were determined (Fig. 1A). As reported previously (3), 759$^r$D100 is impaired in its ability to induce DHPG phosphorylation. However, DHPG-resistant recombinant GDG$^r$P53 was able to induce phosphorylation of DHPG in infected cells as efficiently as wild-type AD169. Thus, the DHPG resistance mutation in the *pol* gene is unrelated to phosphorylation of the drug.

**An independent recombinant containing a *pol* mutation for DHPG resistance.** In a separate marker transfer experiment, unseparated *Hin*dIII fragments of 759$^r$D100 DNA were shown to transfer DHPG resistance efficiently to AD169 (50). No progeny arose in cells transfected with the digested mutant DNA alone. One of the plaques formed in 50 μM DHPG by progeny virus from cells cotransfected with 759$^r$D100 *Hin*dIII fragments was picked, plaque purified

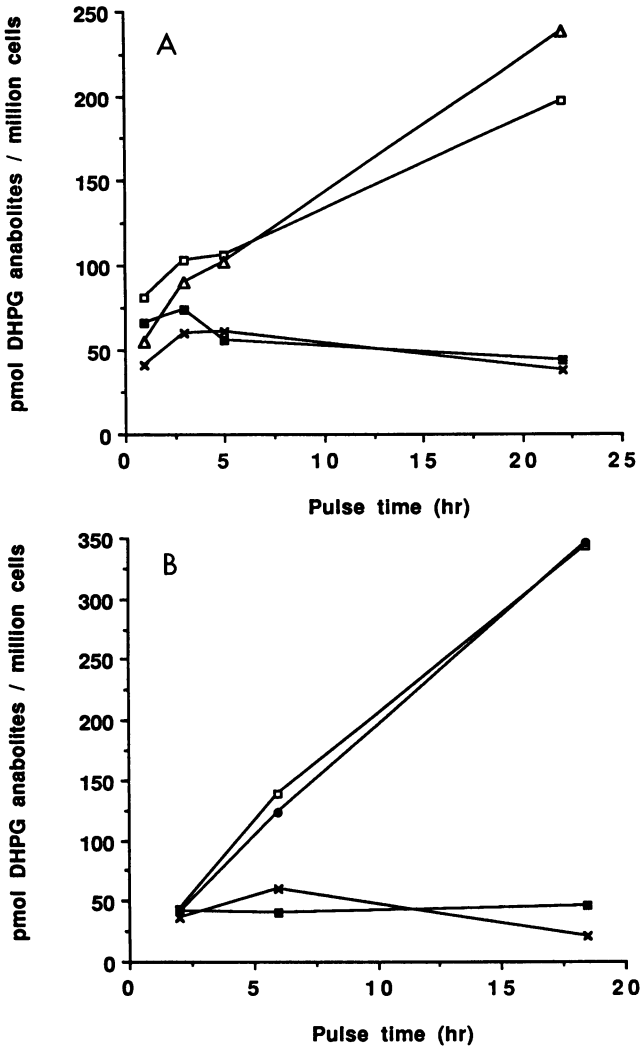


FIG. 1. Accumulation of $^{14}$C-labeled DHPG anabolites. (A) MRC-5 cells were infected with AD169 (□), 759$^r$D100 (■), or GDG$^r$P53 (△) at an MOI of 0.4 or were mock infected (×). Three days postinfection the cells were labeled with $^{14}$C-labeled DHPG for 3, 5, and 22 h. The cells were harvested and the levels of DHPG anabolites (in picomoles per $10^6$ cells) were determined (see text). (B) MRC-5 cells were infected with AD169 (□), 759$^r$D100 (■), or GDG$^r$H5 (●) at an MOI of 0.25 or were mock infected (×). Four days postinfection the cells were labeled for 2, 6, and 18.5 h with $^{14}$C-labeled DHPG. The levels of DHPG anabolites (in picomoles per $10^6$ cells) are shown.

twice in the absence of DHPG, and designated GDG$^r$H5. Like GDG$^r$P53, GDG$^r$H5 exhibited intermediate resistance to DHPG ($ED_{50}$, 14 μM, which was sixfold greater than that of AD169) and was able to induce phosphorylation of DHPG (Fig. 1B). The *pol* gene from GDG$^r$H5 was cloned (plasmid pC54POL), and like that of 759$^r$D100, it efficiently transferred DHPG resistance to wild-type AD169 in marker transfer experiments (plating efficiency in 50 μM DHPG, 4.1% for progeny derived from pC54POL plus AD169 DNA versus <0.017% for AD169 DNA alone). These data provide independent confirmation that the *pol* gene of 759$^r$D100 contains a mutation that confers resistance to DHPG but that is unrelated to effects on DHPG phosphorylation.

**Region V mutation.** To identify the precise mutation in

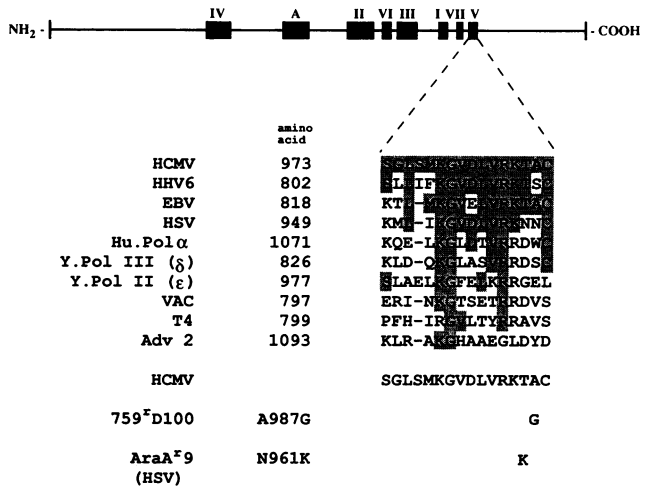


FIG. 2. Amino acid sequence of conserved region V from various DNA polymerases. The line at the top represents the HCMV DNA polymerase. The solid boxes represent regions of sequence conserved between DNA polymerases (26, 32, 57); region I contains amino acids 905 to 920, region II contains amino acids 696 to 742, region III contains amino acids 804 to 846, region IV contains amino acids 379 to 421, region V contains amino acids 973 to 988, region VI contains amino acids 771 to 789, and region A contains amino acids 538 to 598. Below is shown the amino acid sequence of region V from a number of DNA polymerases including HCMV (34); human herpesvirus 6 (HHV6) (52); Epstein-Barr virus (EBV) (1); HSV (27); human polymerase α (Hu. Pol α) (57); yeast polymerase III (Y. Pol III), the homolog of human DNA polymerase δ (5); yeast polymerase II (Y. Pol II), the homolog of human DNA polymerase ε (41); vaccinia virus (VAC) (18), T4 (46), and adenovirus type 2 (Adv 2) (28). Amino acids identical to those in the HCMV sequence are shaded. The amino acid substitutions in HSV mutant AraA$^r$9 (26) and HCMV mutant 759$^r$D100 discussed in this report are given at the bottom.

759$^r$D100 that confers DHPG resistance, the 4.1-kb *Rsr*II plus *Sst*I fragment containing the 759$^r$D100 DNA polymerase gene was sequenced. When compared with the wild-type AD169 sequence (34), the only nucleotide change observed in the entire *pol* gene plus flanking sequences was a C-to-G change at position 3619. Sequence analysis of pC54POL with oligonucleotide primers VSPOL1 and VSPOL2 demonstrated that this mutation is also present in the *pol* gene of GDG$^r$H5, and direct PCR sequencing of GDG$^r$P53 DNA with the same primers also demonstrated the presence of this mutation. The presence of a C at position 3619 in the infectious AD169 DNA used for marker transfer experiments was also confirmed by direct PCR sequencing, thus eliminating the possibility that virus polymorphism might have given rise to a G at this position in our AD169 stock.

The C3619G mutation results in a substitution of alanine by glycine at amino acid position 987 in the predicted protein (Fig. 2). This amino acid change lies within the conserved region V of the DNA polymerase (57). An alanine is also found at this position in region V of Epstein-Barr virus (1); however, the alanine is not conserved in herpes simplex virus (HSV) or varicella-zoster virus (asparagine) (13, 27, 33, 43, 55); human herpesvirus 6 (serine) (52); or other human (57), yeast (5, 41), or viral polymerases (18, 28, 46) that have sequences homologous to region V. A glycine was not found at this position in any of these polymerases.

**Susceptibility and resistance to other antiviral drugs.** To characterize the nature of the *pol* mutation further, we performed plaque reduction assays, comparing the susceptibilities of 759$^r$D100, GDG$^r$P53, and the parental wild-type strain AD169 to various compounds which have been shown to inhibit HCMV. The $ED_{50}$s of the various drugs for these viruses are summarized in Table 1. In several cases (see below) we obtained similar results with the independent recombinant GDG$^r$H5 (50), which confirmed that the phenotypes observed with GDG$^r$P53 were due to the *pol* mutation.

We first compared drugs with acyclic sugar-like moieties similar to those of DHPG. 759$^r$D100 and GDG$^r$P53 exhibited substantial resistance ($ED_{50}$s, 7- to 12-fold greater than that of AD169; Table 1) to DHPC, which consists of the acyclic moiety of DHPG linked to a cytosine base. 759$^r$D100 and GDG$^r$P53 were also six- to sevenfold resistant to DHPG cyclic phosphate (Table 1). Comparable results were obtained with GDG$^r$H5 in dot blot hybridization assays of drug resistance (50). This magnitude of resistance was similar to that displayed by GDG$^r$P53 and GDG$^r$H5 to DHPG itself (Table 1). However, 759$^r$D100 and GDG$^r$P53 exhibited little or no resistance to the related compound DHPG homophosphonate (Table 1).

Several compounds with acyclic sugar-like moieties similar to that of acyclovir were also tested. For GDG$^r$P53 (Table 1) and GDG$^r$H5 (52), the $ED_{50}$s of acyclovir were not meaningfully different from that of AD169. 759$^r$D100 exhibited a slightly higher $ED_{50}$, as reported previously (3). In contrast, as shown in Table 1, GDG$^r$P53 and 759$^r$D100 exhibited substantial resistance (8- to 10-fold) to HPMPA and HPMPC, which contain the acyclic moieties of acyclovir, with the 5′-like $CH_2OH$ replaced by a phosphonate. Similar results were obtained with GDG$^r$H5 and an additional recombinant derived from the cloned 759$^r$D100 *pol* gene (50). Thus, the *pol* mutation of 759$^r$D100 transferred into AD169 conferred resistance not only to DHPG but also to DHPC, DHPG cyclic phosphate, HPMPA, and HPMPC, but it displayed little or no resistance to certain closely related compounds.

## DISCUSSION

In the study described here we used marker transfer and sequencing analyses to map a DHPG resistance mutation unrelated to DHPG phosphorylation to a highly conserved region of the viral DNA polymerase. We then examined the effect of this mutation on virus susceptibility to a variety of antiviral drugs. The results have implications for the mechanisms of action of DHPG and several other drugs, DNA polymerase function, and HCMV drug resistance in clinical settings.

**Implications for mechanisms of DHPG.** DHPG is known to inhibit HCMV viral DNA synthesis (37, 42, 44), and it has been shown that DHPG triphosphate can inhibit the viral DNA polymerase in vitro (4, 23, 38). However, there has been no direct evidence that inhibition of the viral DNA polymerase is responsible for the antiviral action of this compound. Indeed, the inhibition of HSV *pol* mutants in culture by DHPG was not found to correspond to the susceptibilities of the viral polymerases to DHPG triphosphate in vitro, raising the possibility that virus replication is inhibited by mechanisms other than inhibition of DNA polymerase (22). The results presented here by mapping a DHPG resistance mutation to the *pol* gene demonstrate that for HCMV the viral DNA polymerase is a selective target for the antiviral action of DHPG. There have been reports of

DHPG resistance associated with DNA polymerase mutants of HSV (11, 35). However, this phenotype was not definitively mapped to the DNA polymerase in one case (35) and was unable to be confirmed in the other (10).

We have previously mapped a DHPG resistance mutation that impairs DHPG phosphorylation to another gene, *UL97*, whose role in HCMV replication is unknown (51). If *UL97* is an essential gene, then it would be conceivable that DHPG inhibits HCMV replication solely by inhibition of UL97, with DHPG phosphorylation merely being a by-product. However, the demonstration of a DHPG resistance mutation in the HCMV *pol* gene argues strongly against this concept, since there is considerable evidence that DHPG must be phosphorylated to its triphosphate form to interact with herpesvirus DNA polymerases (4, 17, 25, 38).

**Implications for DHPG cyclic phosphate, HPMPA, and HPMPC.** Like DHPG, DHPG cyclic phosphate (also known as 2′ nor-cyclic GMP), HPMPA, and HPMPC are potent inhibitors of herpesvirus replication in cell culture and in vivo (14, 15, 17, 20, 25, 45, 53). The mechanism of inhibition of herpes simplex virus by these drugs is independent of phosphorylation by the HSV thymidine kinase (14, 20, 25, 30, 53). Similarly, we found no difference in susceptibility to these drugs between 759$^r$D100, which is impaired for DHPG phosphorylation because of a *UL97* mutation, and GDG$^r$ P53, which is not impaired for DHPG phosphorylation. Thus, the DHPG kinase activity of *UL97* does not appear to be required for the anti-HCMV activities of these drugs.

DHPG cyclic phosphate is converted to DHPG triphosphate in cells; however, the activity of the cyclic phosphate, which is at best a poor inhibitor of HSV DNA polymerase, cannot be ascribed to this conversion (25). The present data showing that an HCMV *pol* mutation confers resistance to DHPG cyclic phosphate demonstrates that HCMV DNA polymerase is a selective target for the action of this drug. The resistance of GDG$^r$P53 and GDG$^r$H5 to the cyclic phosphate was similar in magnitude to their resistance to DHPG (Table 1) (50). The simplest explanation of this observation is that inhibition of HCMV by DHPG cyclic phosphate involves conversion of the drug to DHPG triphosphate. This would appear to differ from its reported mechanism against HSV (25). However, it is possible that the cyclic phosphate or some metabolite other than DHPG triphosphate interacts with the HCMV DNA polymerase in a manner so like that of DHPG triphosphate that the *pol* mutation confers resistance to it in a similar manner.

HPMPC has been shown to inhibit HCMV DNA synthesis (42), presumably by inhibiting the DNA polymerase. Although it has been shown that phosphorylated derivatives of HPMPA can inhibit the HSV DNA polymerase in vitro (21, 30, 40) and that mutants of HSV and HCMV resistant to polymerase inhibitors are hypersensitive to HPMPA (21, 49), direct evidence that the anti-HCMV actions of HPMPC and HPMPA involve inhibition of the HCMV DNA polymerase has been lacking. Indeed, for HSV, the possibility that the target of HPMPA is one other than the viral polymerase has been raised (6, 21). Our finding that a HCMV *pol* mutation confers resistance to HPMPA and HPMPC demonstrates that the HCMV DNA polymerase is a target for these drugs.

**Implications for polymerase function.** The 759$^r$D100 DNA polymerase mutation causes a single amino acid substitution of alanine by glycine within conserved region V of the protein. Only one other DNA polymerase mutation has been mapped to region V: HSV mutant AraA$^r$9, which displays hypersensitivity to DHPG (10), contains an asparagine to lysine substitution (26) in the amino acid corresponding to that adjacent to the alanine-glycine mutation described here (Fig. 2). It is interesting that mutations at adjacent positions give rise to resistance in one case and hypersensitivity in the other. Single amino acid substitutions in the HSV DNA polymerase that map three or four residues apart and that similarly give rise to opposite aphidicolin and phosphonoacetic acid phenotypes have been observed previously (26, 29). In these cases, perhaps the resistance mutation alters the charge, polarity, or size of a residue involved in substrate recognition to decrease the affinity of DNA polymerase for the drug while the hypersensitivity mutation alters a neighboring residue to increase the affinity of DNA polymerase.

As is true for a number of HSV *pol* mutations (10), substantial differences in resistance to different nucleoside analogs were conferred by this HCMV mutation. Fairly subtle changes, such as removal of the 3′ moiety of DHPG to yield acyclovir or conversion of the 5′-$CH_2OH$ of DHPG to the phosphonate to yield DHPG homophosphonate, greatly decreased the resistance of the mutant relative to that of the wild-type parent. On the other hand, substitution of cytosine for the guanine of DHPG to yield DHPC led to even greater resistance. Thus, this particular mutation very specifically alters the recognition properties of the polymerase, particularly in terms of its specificity for the sugar-like moieties of antiviral agents.

Drug resistance mutations that affect HSV DNA polymerase alter conserved regions I, II, III, V, VII, and A of the protein (26, 29, 32, 33, 36, 39, 54). This implies that there is a role for these conserved regions in deoxynucleoside triphosphate and $PP_i$ binding, since these drugs are mainly analogs of deoxynucleoside triphosphates and $PP_i$ and compete with these natural substrates for enzyme binding sites. The mapping of a second drug resistance mutation within region V supports the involvement of this region in substrate recognition and suggests that such a functional role is conserved in the HSV and HCMV enzymes. It has not been possible to separate the deoxynucleoside triphosphate and $PP_i$ binding sites. The majority of HSV polymerase mutants display altered specificities for both nucleoside and $PP_i$ analogs or map in the immediate vicinity of alterations which affect both (26, 29, 36, 39). It is therefore interesting that neither of the mutations within region V confer more than marginal resistance or hypersusceptibility to $PP_i$ analogs (3, 10); however, more mutations within this region must be analyzed to determine whether this observation is of any significance.

**Implications for clinical drug resistance.** DHPG is one of the few antiviral drugs used to treat patients with HCMV infections, and virus resistant to this drug has been isolated from patients on long-term therapy with DHPG (16, 19, 48). Resistance of HCMV has mainly been associated with impaired DHPG phosphorylation (3, 48); however, the isolation of a DNA polymerase mutant of HCMV that confers resistance to DHPG and several other drugs suggests that such mutants may arise in a clinical setting. This particular mutant remains susceptible to foscarnet (3); whether other DHPG-resistant HCMV *pol* mutants behave similarly remains to be seen.

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