Murine cytomegalovirus resistant to antivirals has genetic correlates with human cytomegalovirus

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Human cytomegalovirus (HCMV) resistance to antivirals is a significant clinical problem. Murine cytomegalovirus (MCMV) infection of mice is a well-described animal model for in vivo studies of CMV pathogenesis, although the mechanisms of MCMV antiviral susceptibility need elucidation. Mutants resistant to nucleoside analogues aciclovir, adefovir, cidofovir, ganciclovir, penciclovir and valaciclovir, and the pyrophosphate analogue foscarnet were generated by in vitro passage of MCMV (Smith) in increasing concentrations of antiviral. All MCMV antiviral resistant mutants contained DNA polymerase mutations identical or similar to HCMV DNA polymerase mutations known to confer antiviral resistance. Mapping of the mutations onto an MCMV DNA polymerase three-dimensional model generated using the Thermococcus gorgonarius Tgo polymerase crystal structure showed that the DNA polymerase mutations potentially confer resistance through changes in regions surrounding a catalytic aspartate triad. The ganciclovir-, penciclovir- and valaciclovir-resistant isolates also contained mutations within MCMV M97 identical or similar to recognized GCV-resistant mutations of HCMV UL97 protein kinase, and demonstrated cross-resistance to antivirals of the same class. This strongly suggests that MCMV M97 has a similar role to HCMV UL97 in the phosphorylation of nucleoside analogue antivirals. All MCMV mutants demonstrated replication-impaired phenotypes, with the lowest titre and plaque size observed for isolates containing mutations in both DNA polymerase and M97. These findings indicate DNA polymerase and protein kinase regions of potential importance for antiviral susceptibility and replication. The similarities between MCMV and HCMV mutations that arise under antiviral selective pressure increase the utility of MCMV as a model for in vivo studies of CMV antiviral resistance.

INTRODUCTION

Human cytomegalovirus (HCMV) is an important pathogen of immunocompromised individuals, and the development of antiviral-resistant HCMV strains is an increasing hindrance to the successful treatment and prevention of CMV-related illness. Animal models are necessary for in vivo studies of CMV antiviral susceptibility and resistance due to the species-specificity of HCMV that restricts permissive replication to humans and human cell lines. Murine cytomegalovirus (MCMV) infection in mice is an excellent model that has been extensively studied with respect to pathology and immunology (Hudson, 1979; Koffron et al., 1998; Lagenaur et al., 1994; Yuhasz et al., 1994). The entire MCMV genome has also been sequenced, and shares many regions of similarity with HCMV (Rawlinson et al., 1993, 1996).

HCMV and MCMV are inhibited by antivirals that target the viral DNA polymerase, including nucleoside analogues aciclovir (ACV), adefovir (ADV), cidofovir (CDV), ganciclovir (GCV), penciclovir (PCV), the ACV pro-drug valaciclovir (VCV) and the pyrophosphate analogue foscarnet (FPA) (De Clercq, 2001; Rawlinson, 2001; Smee et al., 1995). HCMV and MCMV are inhibited by similar concentrations of these antivirals in vitro, except MCMV exhibits increased sensitivity to ACV (Boyd et al., 1993; Chrisp & Clissold, 1991; Cole & Balfour, 1987; Rawlinson et al., 1997; Smee et al., 1995).

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences reported in this paper are AY529137–AY529146, AY529127–AY529133 and AY529134–AY529136.
et al., 1995; Xiong et al., 1997a, b). The nucleoside analogues ACV, ADV, CDV, GCV and PCV require intracellular phosphorylation prior to incorporation into replicating viral DNA by the HCMV DNA polymerase (Dé Clercq, 2001). In HCMV-infected cells, ACV, GCV and PCV are initially monophosphorylated by the HCMV UL97 protein kinase, and further phosphorylated to the triphosphate form by cellular enzymes (Biron et al., 1985; Littler et al., 1992; Sullivan et al., 1992; Talarico et al., 1999; Zimmerman et al., 1997). ADV and CDV are monophosphorylated and do not require activation by viral proteins prior to cellular conversion to their triphosphate forms (Xiong et al., 1997a, b).

HCMV strains resistant to ADV, CDV, PFA or GCV can develop through mutations of the DNA polymerase gene (encoded by UL54), which can confer multi-drug resistance depending on the mutation position (Chou, 1999; Chou et al., 2003; Erice, 1999). HCMV UL97 protein kinase mutations are most often described as responsible for the development of GCV-resistance in HCMV isolates, although earlier studies omitted assessment of DNA polymerase mutations (Chou, 1999; Erice, 1999). Although currently not documented in clinical isolates, mutations of HCMV UL97 protein kinase can confer resistance to ACV and PCV (Talarico et al., 1999; Zimmerman et al., 1997), with potential implications for the use of VCV prophylaxis (Feinberg et al., 1997; Lowance et al., 1999).

MCMV M54 and M97 are positional, sequence and primary structure homologues of HCMV DNA polymerase and UL97 protein kinase (Elliott et al., 1991; Rawlinson et al., 1993, 1996). The MCMV homologues retain DNA polymerase and protein kinase domain regions important for protein function (Elliott et al., 1991; Rawlinson et al., 1997). No other protein kinase homologues are known to be encoded by MCMV (Rawlinson et al., 1996). Phosphorylation assays have failed to show detectable levels of ACV- and GCV-phosphorylation either in MCMV-infected cells or by recombinant M97 protein, despite MCMV susceptibility to ACV and GCV in vitro (Burns et al., 1981; Ochiai et al., 1992; Wagner et al., 2000). However, while HCMV UL97 substituted for MCMV M97 enhances GCV phosphorylation by recombinant MCMV, HCMV UL97 does not complement the function of MCMV M97 with respect to virus replication (Wagner et al., 2000). Additional correlative information is therefore necessary to determine the complementarities of HCMV and MCMV antiviral susceptibility and virus replication.

In order to further analyse CMV replication and antiviral susceptibility, we have generated a series of MCMV mutants resistant to ACV, ADV, CDV, GCV, PCV, PFA and VCV and characterized the genetic mutations associated with the resultant changes in phenotype. Regions of MCMV DNA polymerase and the putative protein kinase (pM97) important for MCMV antiviral susceptibility and virus replication have been identified. The mutations of these MCMV antiviral-resistant strains demonstrate remarkable similarity to mutations known to confer antiviral resistance to HCMV isolates defined from infected patients, and correlate with DNA polymerase and protein kinase regions of functional importance.

**METHODS**

**Antivirals.** Reagent grade ACV and VCV were kindly provided by GlaxoWellcome (UK), ADV and CDV by Gilead Sciences (USA), GCV by Roche Pharmaceuticals (Australia), PCV by SmithKline Beecham Pharmaceuticals (UK) and PFA by Astra Pharmaceuticals (Australia). Antivirals were resuspended in pyrogen-free water (Baxter) to a final concentration of 10 μM (except PFA, which was resuspended to a final concentration of 50 μM), filter-sterilized and stored in aliquots at −20 °C until use.

**MCMV strains.** MCMV laboratory strain Smith was obtained from ATCC. The virulent laboratory strain K181 and nine wild-type MCMV isolates (G3A, G4, K17A, K17G, N1, W2, W5, W8 and W9) were kindly provided by Professor Geoffrey Shellam from the Department of Microbiology, University of Western Australia, Perth, Australia. Viral titres were quantified in primary mouse embryo fibroblasts (MEFs) by standard plaque assays, as described previously (Scott et al., 2000).

**Generation of antiviral-resistant MCMV isolates.** MCMV antiviral resistant mutants (ACVres, ADVres, CDVres, GCVres, PFAres and VCVres) were generated by continuous passaging of MCMV laboratory strain Smith in MEFs in increasing concentrations of each antiviral. Mutants were selected against both ACV and the ACV pro-drug VCV, to compare the resistance mutations generated by antiviral agents that differed in bioavailability. Initially, Smith strain was cultured in 25 cm² flasks containing minimal essential media (MEM) + 2 % fetal bovine serum (FBS) and 0.1 μM of antiviral. Virus was passaged to new MEF cultures after 100 % cytopathic effect (CPE) was observed, or one week of culture where CPE was less than 100 %, and antiviral concentration increased two-fold at each passage. At higher antiviral concentrations, cultures were carried out in six-well plates, where plates were centrifuged at 600 g for 30 min to enhance virus infectivity. Passaging of virus continued to the highest concentration of antiviral where virus continued to be isolated without cellular toxicity (Table 1).

Each antiviral-resistant mutant was plaque purified a total of five times by culturing serial 1 : 10 dilutions of virus and sterile selection following inoculation of virus. The concentration range for each antiviral was as follows: 0.825–480 μM ACV (increased to 1.825–480 μM for analysis of the VCV-resistant mutant), 0.93–240 μM ADV, 0.0625–16 μM CDV, 0.625–160 μM GCV, 1.25–320 μM PCV, 6.25–1800 μM PFA and 1.825–480 μM VCV. The 50 and 90 % inhibitory concentrations (IC50 and IC90) were calculated by linear regression from plots of percentage reduction in plaque numbers at each antiviral concentration against log drug concentrations.

**Plaque reduction assays (PRA).** PRA was carried out in triplicate using 30 p.f.u. per well of virus as described previously (Rawlinson et al., 1997), except that plates were centrifuged at 600 g for 30 min following inoculation of virus. The concentration range for each antiviral was as follows: 0.47–120 μM ACV (increased to 1.825–480 μM for analysis of the VCV-resistant mutant), 0.93–240 μM ADV, 0.0625–16 μM CDV, 0.625–160 μM GCV, 1.25–320 μM PCV, 6.25–1800 μM PFA and 1.825–480 μM VCV. The 50 and 90 % inhibitory concentrations (IC50 and IC90) were calculated by linear regression from plots of percentage reduction in plaque numbers at each antiviral concentration against log drug concentrations.

Resistance was defined as a greater than twofold increase in IC50 and IC90 values compared with parent (Smith) strain. At the final round of plaque purification, mutant virus was cultured in the presence of media without antiviral and cell-associated virus harvested and stored in MEM + 10 % FBS at −80 °C.

**M54 and M97 DNA PCR.** M54 and M97 PCR was carried out on DNA extracted from MCMV-infected MEFs as described previously (Scott et al., 2002). The entire M54 gene was amplified in four
overlapping segments (M54A, M54B, M54C and M54D) of approximately 800 to 900 bp. M54A was amplified using primers M54.T8 (5'-ATCATCAGATAAGGAGGG-3') and M54.B7 (5'-TGGAGGCCCTTCGGCGAAAGT-3'). M54B using primers M54.T6 (5'-CATAGGGAAACGAAGCTATT-3') and M54.B5 (5'-CGAG-GAACAGGAAATGGCATG-3'), M54C using primers M54.T4 (5'-GTTGGCAAGATCTAGTGGC-3') and M54.B3 (5'-AGAG-CAAGAACATGGCGGT-3'), and M54D using primers M54.T2 (5'-AAGGACAGGAAACGTTAGAAGG-3') and M54.B1 (5'-CTCC-GATTTCGAGTACTGAG-3'). The entire M97 gene was amplified by PCR in three overlapping segments (M97A, M97B and M97C) of approximately 800 to 900 bp, using primers M97.T1 (5'-TCCGATCATCCTGCTGGT-3') and M97.B7 (5'-AGGCGCGCC-GTACAGGAAGG-3') for M97A, M97.T3 (5'-ATTCCCCGCTGCCTGCGCCTGTGTTGACAGGACGGGCCG-3') and M97.B4 (5'-GGGCGGGCCGCGCTGTGACAGGACGGCCG-3') for M97B, and M97.T5 (5'-AGGCCGCTGCTAATGGCAACATCCT-3') and M97.B1 (5'-GACCGTCTGCATTACATGCAG-3') for M97C. Each reaction consisted of 50 mM KCl, 10 mM Tris/HCl pH 9.0, 0.1 mM Triton-X-100, 5 mM MgCl2, 0.25 mM dNTP, 0.4 mM forward and reverse primer, 1 U Taq DNA polymerase (Promega) and 2 µl DNA template in a total reaction volume of 50 µl. Reaction conditions consisted of an initial denaturation step at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min.

M54 and M97 sequencing. PCR products were purified as described previously using polyethylene glycol (PEG) 8000 (Scott et al., 2002), and sequenced using ABI Prism BigDye terminator chemistry (Applied Biosystems). Forward and reverse PCR primers were used for sequencing, as well as internal forward and reverse primers specific for each PCR product. The entire M54 and M97 gene sequences were assembled by comparison to Smith consensus sequences (Rawlinson et al., 1996), translated using Alttrans and whole protein sequences for each gene aligned using CLUSTALW (Thompson et al., 1997). This alignment included the bacteriophage RB69 DNA polymerase that has previously been used as a model for the analysis of herpes simplex virus and CMV DNA polymerase resistance mutations (Huang et al., 1999; Chou et al., 2003). An MCMV DNA polymerase model was built using the structure of the closest matched template of known three-dimensional structure, the Tgo polymerase from Thermococcus gorgonarius (PDB code 1TGO) (Hopfner et al., 1999) with the help of COBASER as contained in Sybyl6.92 (Tripos). The model was subjected to energy minimization to optimize the geometry and remove steric overlap in the structure and Verify3D (Luthy et al., 1992) was used to evaluate the quality of the model.

Growth kinetic assays. Growth kinetic assays comparing the wild-type parent strain (Smith) to each of the antiviral-resistant mutants were carried out in triplicate. Virus was inoculated at an m.o.i. of 0.01 p.f.u. per cell onto MEFs with centrifugal enhancement and cultured for 4 days. Cell-free (media) and cell-associated virus titres were quantified by calculation of tissue culture infective dose (TCID) in 96-well plates containing confluent MEFs. The number of infected cells per plaque for each MCMV strain was also counted after 4 days of culture to determine focus expansion and plaque size measured following photography. These analyses were performed as a blind trial.

Table 1. Antiviral susceptibilities of wild-type MCMV parent strain (Smith) and corresponding MCMV antiviral-resistant mutants

<table>
<thead>
<tr>
<th>Antiviral</th>
<th>Mutant selection* (µM)</th>
<th>Parent IC₅₀† (µM)</th>
<th>Mutant IC₅₀‡ (µM)</th>
<th>Increase‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACV</td>
<td>60</td>
<td>1.2 ± 0.1</td>
<td>26.0 ± 2.5</td>
<td>21.7</td>
</tr>
<tr>
<td>ADV</td>
<td>60</td>
<td>0.5 ± 0.1</td>
<td>135.1 ± 70.1</td>
<td>270.2</td>
</tr>
<tr>
<td>CDV</td>
<td>8</td>
<td>0.2 ± 0.2</td>
<td>7.0 ± 3.9</td>
<td>35</td>
</tr>
<tr>
<td>GCV</td>
<td>80</td>
<td>6.5 ± 2.0</td>
<td>51.8 ± 18.4</td>
<td>6.7</td>
</tr>
<tr>
<td>PCV</td>
<td>160</td>
<td>8.1 ± 1.7</td>
<td>22.2 ± 4.8</td>
<td>2.7</td>
</tr>
<tr>
<td>PFA</td>
<td>800</td>
<td>80.9 ± 14.7</td>
<td>439.8 ± 65.1</td>
<td>5.4</td>
</tr>
<tr>
<td>VCV</td>
<td>240</td>
<td>2.3 ± 0.6</td>
<td>279.1 ± 140.2</td>
<td>121.3</td>
</tr>
</tbody>
</table>

*Final antiviral concentration for isolation of the antiviral resistant mutant.  †IC₅₀ 50 % inhibition concentration (µM) of antiviral.  ‡Indicates fold increase of mutant IC₅₀ from that of the parent strain (Smith).

RESULTS

Antiviral susceptibilities of the MCMV antiviral-resistant mutants

All seven MCMV antiviral-resistant mutants (ACVres, ADVres, CDVres, GCVres, PCVres, PFAres and VCVres) were isolated in antiviral concentrations that exceeded the IC₅₀ and IC₉₀ values of MCMV-sensitive strains (Rawlinson et al., 1997; Smee et al., 1995). PRA confirmed the MCMV mutants had significant increases in IC₅₀ and IC₉₀ values compared with those of the parent (Smith) strain (Table 1). The increases in antiviral inhibitory concentrations ranged from 2.7-fold for the PCVres mutant to 270.2-fold for the ADVres mutant compared with the parent (Smith) strain (Table 1).

Mutants generated against nucleoside analogues that require phosphorylation (ACV, GCV, PCV and VCV) also demonstrated cross-resistance to other antivirals within this group.
Fig. 1. MCMV mutants resistant to ACV (ACVres), GCV (GCVres), PCV (PCVres) and VCV (VCVres) are cross-resistant to other nucleoside analogue antivirals. The IC_{50} of ACV, GCV and PCV are shown with error bars for parent (Smith) strain (Fig. 1), and 12- and 15-fold increases in IC_{50} values, respectively (results not shown). As expected, resistance to ACV was also observed for the MCMV mutant generated against VCV (Fig. 1). The level of resistance that VCVres demonstrated to ACV was very high, requiring inhibitory concentrations of ACV that were 200-fold that of the parent (Smith) strain, and 10-fold that of the ACVres mutant (Fig. 1). Cross-resistance to GCV was only observed with the PCVres mutant, and not ACVres or VCVres. All mutants tested (ACVres, GCVres and VCVres) demonstrated cross-resistance to PCV (Fig. 1).

MCMV DNA polymerase mutations associated with resistant phenotype

All seven antiviral-resistant mutants contained mutations of MCMV DNA polymerase (Table 2 and Fig. 2). These mutations occurred in regions of MCMV DNA polymerase that were invariant in nine sequenced wild-type (antiviral sensitive) strains (AY529137–AY529146) and mutations were verified by repeat amplification and sequencing reactions using a second set of different primers. No MCMV DNA polymerase mutations were found outside of codons 360–870 corresponding to the DNA polymerase domain region (Fig. 2), identified from alignment with HCMV sequences (Chou et al., 1999). Half of the MCMV DNA polymerase mutations (4/8) occurred within or in close proximity to DNA polymerase domain III (Table 2 and Fig. 2).

The ACVres, ADVres and VCVres mutants contained identical amino acid mutations (P826R) between DNA polymerase functional domains I and VII (Table 2 and Fig. 2).

Table 2. MCMV M54 (DNA polymerase) and M97 (protein kinase) mutations associated with antiviral resistance correlate with HCMV resistance mutations

<table>
<thead>
<tr>
<th>MCMV mutant</th>
<th>DNA polymerase resistance mutations</th>
<th>Protein kinase resistance mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACVres</td>
<td>L678V</td>
<td>V781I (PFA, GCV)</td>
</tr>
<tr>
<td>ADVres</td>
<td>P826R</td>
<td>V955I (VCV)</td>
</tr>
<tr>
<td>GCVres</td>
<td>L467I</td>
<td>C-L501I (CDV, GCV)</td>
</tr>
<tr>
<td>PCVres</td>
<td>T753A</td>
<td>M395V (GCV), M460V (GCV)</td>
</tr>
<tr>
<td>PFAres</td>
<td>ND</td>
<td>A679V (PFA, GCV)</td>
</tr>
</tbody>
</table>

*HCMV resistance mutations homologous or occurring in similar regions to the MCMV mutations (1) Scott et al., 2004; (2) Cihlar et al., 1998a; (3) Cihlar et al., 1998b; (4) Cihlar et al., 1998c; (5) Cihlar et al., 1998d. MCMV resistance mutations with identical HCMV mutations are indicated in bold.

ND, Not done.
corresponding to DNA polymerase sequencing. The CDVres mutant contained one mutation of the mutant from original stocks and repeat and the ADVres mutation verified by repeat plaque purification (Fig. 2). These mutants were isolated at different time points, except T753A of PCVres, also correlated with regions of HCMV DNA polymerase associated with antiviral resistance (Cihlar et al., 1998a, b; Scott et al., 2004). This included the third mutation of CDVres (D709N), located one codon upstream of the HCMV mutation K805Q associated with CDV resistance and the DNA polymerase domain III N terminus (Cihlar et al., 1998a).

**MCMV DNA polymerase three-dimensional structure**

An MCMV DNA polymerase model generated using the *T. gorgonarius* Tgo polymerase crystal structure had good Verify3D scores for the majority of the protein (Fig. 3a). *T. gorgonarius* Tgo polymerase demonstrated greater similarity to HCMV and MCMV DNA polymerase than bacteriophage RB69 DNA polymerase, which has previously been used as a model for the study of herpes simplex virus and CMV antiviral resistance mutations (Huang et al., 1999; Chou et al., 2003). The catalytic domain regions of MCMV DNA polymerase exhibited the best Verify3D scores, and the N-terminal region of the protein model had the lowest score (Fig. 3a). A small number of insertions in the MCMV sequence relative to the template protein were not amenable to modelling and were removed from the model during alignment. These regions were predominantly within the N-terminal or extreme C-terminal region of MCMV DNA polymerase.

The MCMV DNA polymerase mutations associated with antiviral resistance in this study were analysed using the model (Fig. 3b). Only subtle perturbations in the MCMV DNA polymerase model were observed on alteration of the residues associated with antiviral resistance. However, most of the resistance mutations were located in the model surrounding an MCMV aspartate triad (D624, D791 and D910) homologous to the catalytic aspartate triad (D717, D912) of *T. gorgonarius* Tgo polymerase, a motif important for nucleotidyl transfer and metal binding (Rodriguez et al., 2000).

**M97 protein mutations associated with resistant phenotype**

Mutants resistant to antivirals that require phosphorylation (PCVres, GCVres and VCVres) contained mutations of pM97 not present in MCMV antiviral-sensitive strains, in addition to the DNA polymerase mutations described above (Rawlinson et al., 1997) (Table 2 and Fig. 4). Mutations of pM97 were not detected in the ACVres mutant, suggesting the P826R M54 (DNAPol) mutation described above is solely responsible for the antiviral resistance of this isolate (Table 2 and Fig. 4). No pM97 mutations were detected in PFAres, demonstrating non-specific M97 mutations did not result from passaging of virus.

Regions of pM97 corresponding to protein kinase functional domains were defined by alignment with HCMV UL97 protein kinase, herpes simplex virus UL13 and other protein kinase sequences (Chee et al., 1989; Hanks & Quinn,
1991). The pM97 mutations of the PCVres and VCVres mutants (M395V and T393M, respectively) occurred within regions corresponding to the protein kinase domain VIb conserved motif (DxxxxN) (Table 2 and Fig. 4). The M97 mutation of GCVres (P479L) was located between consensus motifs for protein kinase domains VIII (APE) and IX (DxxxxG) (Table 2 and Fig. 4). Alignment of the MCMV pM97 sequences with UL97 protein kinase sequences from HCMV antiviral-resistant isolates indicated the pM97 domain VIb mutation of PCVres was homologous to a mutation (M460V) that confers HCMV resistance to GCV, with the pM97 mutation of VCVres only two codons upstream of this site (Fig. 4) (Chou et al., 1995).

**Replication impairment of the MCMV antiviral-resistant mutants**

All MCMV antiviral-resistant mutants were impaired in replication ability, producing smaller plaque sizes and 3- to 36-fold less cell-associated and cell-free virus titres than the parent (Smith) strain (Fig. 5). The most reduction in replication ability was observed with the VCVres mutant that contained single DNA polymerase and pM97 mutations (Fig. 5). The replication impairment of VCVres was greater than that observed for the ACVres and ADVres mutants (all containing identical DNA polymerase mutations), indicating the additional pM97 mutation of VCVres contributed to the overall decrease in replication ability of this isolate (Fig. 5 and Table 2). More than one mutation in MCMV DNA polymerase (the CDVres and GCVres mutants) or mutations in both DNA polymerase and pM97 (the GCVres, PCVres and VCVres mutants) tended to have a greater impact on replication ability than single DNA polymerase mutations. The PFAres mutant that contained a single DNA polymerase mutation corresponding to domain VI was the least replication-impaired mutant (three- to fivefold reduction in TCID$_{50}$) (Fig. 5). Only a moderate reduction in plaque size was observed for the PFAres mutant at day 4, and
by day 7 the PFAres plaque sizes were equivalent to those observed for parent Smith strain (Fig. 5).

**DISCUSSION**

The antiviral-resistant and replication-impaired phenotypes of the seven MCMV mutants were associated with M54 DNA polymerase and the M97 putative protein kinase mutations that developed in vitro under selective pressure from antivirals. The involvement of the MCMV mutations in reduced antiviral susceptibility and replication ability is further supported by their correlation with HCMV mutations associated with antiviral resistance and their detection within protein regions of functional importance. In turn, the MCMV mutations associated with antiviral resistance and replication impairment have suggested important regions of MCMV DNA polymerase and pM97 that indicate functional elements of the HCMV and cellular homologues of these proteins.

The MCMV DNA polymerase mutation P826R between domains I and VII of the ACVres, ADVres and VCVres mutants was identical in position but different in substitution to a mutation (P826A) found in an ACV-resistant MCMV mutant generated in a separate study (Minematsu et al., 2001). Furthermore, this MCMV mutation correlates with an HCMV DNA polymerase mutation (V955I) we have recently identified in a patient who developed an HCMV-related illness despite receiving VCV prophylaxis (Scott et al., 1995). Domain I is the most conserved functional domain of DNA polymerases and is of major importance in substrate binding, polymerase activity and virus replication (Huang et al., 1999; Ye & Huang, 1993). Domain VII is involved in polymerase activity and recognition of ACV (Hwang et al., 1992; Ye & Huang, 1993). These two important domains are maintained as β-sheets in a herpesvirus DNA polymerase model (Huang et al., 1999), and β-sheet turns are predominantly associated with proline (P) residues (Stryer, 1988). This suggests the antiviral resistance and replication impairment observed for ACVres, ADVres and VCVres might have resulted from altered interaction of DNA polymerase domains I and VII with nucleoside analogues and natural substrate.

The similarity observed between MCMV and HCMV mutations associated with antiviral resistance suggests MCMV antiviral-resistance and replication-impairment is potentially conferred via similar mechanisms as HCMV. Two mutations of CDVres (the δ-region C mutation L467I and domain III mutation V717L) were identical in location and amino acid substitution to HCMV DNA polymerase mutations that confer resistance to CDV and GCV (Cihlar et al., 1998a, b; Lurain et al., 1992). The L467I mutation is identical in position to a δ-region C mutation of HCMV DNA polymerase (L501F) that produces enhanced exonuclease activity (Kariya et al., 2000), and presumably more efficient removal of incorporated inhibitor. The third CDVres mutation (D709N) aligned adjacent to the N-terminal codon of HCMV DNA polymerase domain III (K805), which when substituted with glutamine (Q) confers resistance to CDV (Cihlar et al., 1998a). The D709N mutation is also congruent with domain III mutations of
HCMV DNA polymerase that interfere with DNA template binding (Ye & Huang, 1993). The P734S mutation of the GCVres mutant is in close proximity to an HCMV DNA polymerase mutation we have detected in an HCMV strain isolated from a patient demonstrating clinical resistance to GCV, PFA and CDV (Scott et al., 2004). The T753A mutation of the PCVres mutant did not correlate with antiviral-resistant mutations of HCMV, but was in close proximity to ACV-resistant mutations of herpes simplex virus and varicella-zoster virus that result in decreased substrate binding (Huang et al., 1999; Schmit & Boivin, 1999; Visse et al., 1999). The A679V mutation of DNA polymerase domain VI was the only genetic alteration identified in the PFAres mutant, and corresponds in location with mutations of HCMV and other herpesvirus DNA polymerases associated with PFA-resistance (Cihlar et al., 1998a; Schmit & Boivin, 1999). DNA polymerase domains II and VI are the only two functional domains of HCMV DNA polymerase associated with PFA-resistance (Cihlar et al., 1998a). The GCVres mutant also contained a mutation (L678V) within MCMV DNA polymerase domain VI, and an association between this domain and HCMV GCV resistance has been suggested but not proven (Jabs et al., 2001).

The conservative nature of almost all the resistance mutations identified in MCMV polymerase suggests that the mutations alter antiviral susceptibility of the polymerase via subtle perturbations of the protein. This is supported by analysis of the MCMV antiviral resistance mutations using the backbone of T. gorgonarius Tgo polymerase crystal structure (Hopfner et al., 1999). Most of the resistance mutations in MCMV CDVres, GCVres, PCVres and PFAres are clustered in domains III and VI and surround a catalytic triad formed by conserved aspartic acid residues D624 (domain II), D791 and D793 (both in domain I). Analyses of Thermococcus sp. polymerases have demonstrated the importance of this catalytic aspartate triad for nucleotidyl transfer and Mg$^{2+}$ binding (Rodriguez et al., 2000). Therefore, the MCMV mutations potentially alter the local structure and hence orientation of the three aspartates in the triad, reducing the polymerase efficiency. This resulting reduction in polymerase efficiency would give the exonuclease domain an improved chance to remove any defective DNA strands containing antiviral nucleoside analogues, allowing the viral polymerase to complete replication successfully. A further potential mechanism of resistance involving disruption of an Mn$^{2+}$- and Zn$^{2+}$-binding site known to associate with the catalytic aspartate triad (Hopfner et al., 1999) is also suggested by the P826R mutation present in MCMV ACVres, ADVres and VCVres given this residue’s proximity to this metal-binding region. The position of L4671 at the end of a helix in the nuclease domain suggests that this mutation may alter the processivity of the nuclease activity and potentially have a concomitant effect on the incorporation of inhibitors. Detailed study of the enzymology of the MCMV DNA polymerase will shed further light on the mechanism of action of these antiviral resistance mutations.

Interestingly, the two MCMV mutants generated against ACV (one against the ACV pro-drug VCV) produced identical DNA polymerase mutations (P826R), but the VCVres mutant also contained an additional mutation in M97. It is not known whether MEFs contain the specific dipeptide transporters responsible for the increased bioavailability of VCV in intestinal cells (Han et al., 1998; Landowski et al., 2003), nor VCV hydrolase required for cleavage of L-valine from VCV to produce ACV (Burnette et al., 1995). It is therefore unclear whether these mechanisms contributed to the additional M97 mutation observed in the VCVres mutant, and no evidence exists suggesting the frequency of additional resistance mutations is related to exposure to higher antiviral concentrations. However, similar to results observed here with MCMV VCVres, HCMV isolates containing mutations in both DNA polymerase and UL97 have increased resistance to GCV compared with isolates containing single UL97 mutations (Smith et al., 1997). In HCMV isolates, high-level resistance related to the length of exposure to antiviral (Smith et al., 1997), whereas ACVres and VCVres were exposed to antiviral for similar lengths of time. The differences observed here for ACVres and VCVres were therefore most likely the result of random selection, enhanced by the process of plaque purification for each mutant.

The detection of three separate pM97 mutations in MCMV mutants resistant to ACV, GCV and PCV suggest the involvement of the putative M97-protein kinase in MCMV susceptibility to nucleoside analogues that require phosphorylation by virally encoded enzymes. Two of these mutations (T393M and M395V of the MCMV VCVres and PCVres mutants, respectively) reside within a region of pM97 homologous to the protein kinase domain Vlb consensus motif (DxxxxN) important for phosphotransfer and catalytic activity (Chee et al., 1989; Hanks & Hunter, 1995). The M395V mutation of the PCVres mutant is identical in position and amino acid substitution to the UL97 protein kinase mutation (M460V) frequently detected in GCV-resistant HCMV isolates (reviewed by Chou, 1999; Erice, 1999). The MCMV GCVres mutation (P479L) did not correlate with HCMV UL97 protein kinase mutations associated with antiviral resistance, and cannot be directly attributed to the observed phenotypic changes of this mutant due to the presence of two additional DNA polymerase mutations. However, the GCVres P479L mutation is located midway between protein kinase domain VIII (APE) involved in peptide recognition, and the peptide substrate binding domain IX (DxxGxG), and is therefore positionally central to the protein kinase catalytic core (Hanks & Hunter, 1995). No mutations were detected in the pM97 sequence (KTCDAL) homologous with the UL97 protein kinase AACRAL motif that has a strong association with antiviral resistance in HCMV GCV-resistant strains (Chou, 1999; Erice, 1999; Sullivan et al., 1993). Furthermore, the domain Vlb mutation of MCMV VCVres did not confer cross-resistance to GCV despite being homologous to an HCMV UL97 protein kinase mutation that is common in
HCMV GCV-resistant isolates (Chou, 1999; Erice, 1999). This difference may account for the low levels of GCV phosphorylation by MCMV pM97 compared with HCMV UL97 (Wagner et al., 2000). However, the low levels of phosphorylated GCV produced in MCMV-infected cells are sufficient for inhibition of MCMV replication, as shown in this study and others (Rawlinson et al., 1997; Smee et al., 1995; Wagner et al., 2000), suggesting MCMV DNA polymerase has a high affinity for trace levels of phosphorylated nucleoside analogues produced by the M97 putative protein kinase (Wagner et al., 2000).

MCMV and HCMV are similar in the DNA polymerase and protein kinase mutations that develop under antiviral selective pressure, the evidence suggesting potential involvement of the respective protein kinase homologues in antiviral susceptibility to nucleoside analogues, and the DNA polymerase and protein kinase regions that appear to be of functional importance. These similarities indicate a close relationship between MCMV and HCMV antiviral resistance and demonstrate the utility of MCMV as an animal model for CMV antiviral studies.

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