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Expression of the Human Cytomegalovirus *UL97* Gene in a Chimeric Guinea Pig Cytomegalovirus (GPCMV) Results in Viable Virus with Increased Susceptibility to Ganciclovir and Maribavir

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Abstract

In lieu of a licensed vaccine, antivirals are being considered as an intervention to prevent congenital human cytomegalovirus (HCMV) infection. Ideally, antiviral therapies should undergo pre-clinical evaluation in an animal model prior to human use. Guinea pig cytomegalovirus (GPCMV) is the only small animal model for congenital CMV. However, GPCMV is not susceptible to the most commonly used HCMV antiviral, ganciclovir (GCV), rendering *in vivo* study of this agent problematic in the guinea pig model. Human cytomegalovirus (HCMV) susceptibility to GCV is linked to the *UL97* gene. We hypothesized that GPCMV susceptibility to GCV could be improved by inserting the HCMV (Towne) *UL97* gene into the GPCMV genome in place of the homolog, *GP97*. A chimeric GPCMV (GPCMV::*UL97*) expressed *UL97* protein, and replicated efficiently in cell culture, with kinetics similar to wild-type GPCMV. In contrast, deletion of *GP97* resulted in a virus (GPCMVΔ*GP97*) that grew poorly in culture. GPCMV::*UL97* had substantially improved susceptibility to the inhibitory effects of GCV in comparison to wild-type GPCMV. Additionally, GPCMV::*UL97* exhibited improved susceptibility to another antiviral undergoing clinical trials, maribavir (MBV; benzimidazole riboside 1263W94), which also acts through *UL97*.

Keywords

Guinea pig cytomegalovirus; ganciclovir; maribavir; congenital CMV infection; antiviral therapy; bacterial artificial chromosome

1. Introduction

Congenital infection with human cytomegalovirus (HCMV) is a major public health problem in the developed world. Congenital HCMV infection occurs in up to 2% of all deliveries, and

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is responsible for a variety of neurodevelopmental sequelae, including sensorineural hearing loss (SNHL) (Demmler, 1995). Although there is no licensed vaccine to HCMV, antiviral agents are available. A recent study of antiviral treatment of newborns with symptomatic congenital HCMV infection involving the central nervous system (CNS) indicated that ganciclovir therapy resulted in improved hearing outcomes (Kimberlin et al., 2003). Another recent study of passive immunotherapy, using high-titer HCMV antibody, suggested that treatment of HCMV infection in pregnancy resulted in improved neurodevelopmental outcomes in congenitally infected infants (Nigro et al., 2005). Thus, there is considerable interest in evaluating antivirals for use in treatment of the fetus and newborn infant, toward the goal of improving pregnancy outcomes.

Ideally, antiviral therapies would undergo pre-clinical evaluation in an animal model prior to licensure for human use. However, the species specificity of HCMV precludes study of this virus in animals, and necessitates the study of species-specific CMVs in their respective animal hosts. Among the small animal models, the guinea pig cytomegalovirus (GPCMV) model has unique advantages over other rodent models. These advantages include a disease pathogenesis similar to that observed in humans, including neurological injury and SNHL (Bia et al., 1983; Schleiss and Lacayo, 2006). Additionally, GPCMV is transmitted *in utero* producing congenital infection and disease in the newborn pup (Schleiss and Lacayo, 2006). This feature makes the GPCMV model particularly relevant to studies of antiviral therapy in pregnancy. However, there are disadvantages to the GPCMV model for the study of antivirals. GPCMV is resistant to medically relevant doses of the most commonly used antiviral for HCMV, ganciclovir (Matthews and Boehme, 1988; Williams et al., 2003), and attempts to study this agent in the guinea pig model have met with limited success (Fong et al., 1987; Woolf et al., 1988). A newer agent being explored in clinical trials, the benzimidazole riboside 1263W94, (Maribavir [MBV]; Biron et al., 2002) is similarly inactive against GPCMV (Williams et al., 2003). Other antivirals, such as cyclic cidofovir (HPMPC) and BAY 38-4766, are active against GPCMV *in vitro* and *in vivo*, but these agents are not yet licensed for use in HCMV infection (Bourne et al., 2000; Bravo et al., 2006; Schleiss et al., 2006; White et al., 2006; Kern, 2006).

The molecular basis for the decreased susceptibility of GPCMV to GCV and MBV, compared to HCMV, is unknown. To become active, GCV requires phosphorylation by the *UL97* kinase protein prior to conversion into the active triphosphate form (Sullivan et al., 1992). GCV-resistant viral strains of HCMV can be generated in tissue culture under GCV selection or through prolonged drug therapy in patients, and resistant strains are characterized by mutations to genes *UL54* or *UL97* (Baldanti et al., 2004). MBV-resistant strains of HCMV also have mutations in the *UL97* gene, indicating a common target for both drugs, although the mode of action by MBV is different from that of GCV (Biron et al., 2002). All animal CMVs, including GPCMV, appear to encode a homolog to the HCMV *UL97* gene (Fox and Schleiss, 1997; Michel and Mertens, 2004; Romaker et al., 2006). A comparison of the *UL97* ORF of wild-type and GCV resistant HCMV strains with *GP97* demonstrated divergence at critical key amino acids that were hypothesized play a role in resistance to GCV (Fox and Schleiss, 1997; Michel and Mertens, 2004). Therefore, we evaluated whether GPCMV could be rendered sensitive to GCV if the virus were engineered to encode the HCMV *UL97* gene in the place *GP97*. An infectious bacterial artificial chromosome (BAC) of the GPCMV genome in *E. coli* was used to generate an intertypic, chimeric virus encoding the HCMV *UL97* gene (GPCMV::*UL97*), and the antiviral susceptibility of this virus to GCV was compared to that of wild-type GPCMV. Additionally, a *GP97* knockout (GPCMVd*GP97*) was generated to explore the role of the *GP97* protein in the virus life cycle.

2. Materials and methods

2.1. Cells, viruses and oligonucleotides

GPCMV (strain 22122, ATCC VR682), GPCMV BAC derived virus, and vAM403, an enhanced green fluorescent protein (eGFP)-tagged wild-type GPCMV (McGregor and Schleiss, 2001) were propagated on guinea pig fibroblast lung cells (GPL; ATCC CCL 158) in F-12 medium supplemented with 10% fetal calf serum (FCS; Gibco-BRL), 10,000 IU of penicillin/liter, 10 mg of streptomycin/liter (Gibco-BRL), and 7.5% NaHCO₃ (Gibco-BRL). HCMV (strains Towne and Toledo) were propagated on human foreskin fibroblast (HFF) cells in DMEM media with 10% FCS. Virus titrations were carried out on six-well plates overlaid with 1.5% (wt/vol) methylcellulose. Plaques were stained with 10% Giemsa stain or visualized by fluorescence microscopy. All oligonucleotides were synthesized by Sigma-Genosys (The Woodlands, TX) and are listed in Table 1.

2.2. Recombinant shuttle vectors

A partial clone of the GPCMV *UL97* homolog, *GP97*, was cloned in the PCR vector, pCR 2.1 (Invitrogen) following amplification of a 2124 bp fragment using primers *GP97F1* and *GP97R1* (Table 1). This fragment contained the *GP97* promoter and start codon, but was truncated 55 codons upstream of the native stop codon. The *GP97* insert from this plasmid, pKTS 596, was removed as a *EcoR*I insert and inserted into pUC19, yielding pKTS 597, which was then truncated by *Bgl*II digestion, yielding pKTS 598. This *Bgl*II collapse retained 593 bp of upstream *GP97* sequence (including the amino-terminal 39 codons), and 308 bp of downstream *GP97* sequence (spanning codons residues E₄₄₇ through F₅₄₄). Next, the HCMV *UL97* gene (Towne strain) was cloned by PCR amplification, using primers *UL97F1* and *UL97R1*. This downstream primer was a degenerate primer in which novel *EcoR*V and *Bam*H I sites were introduced 3' of the native stop codon. The resulting clone, pKTS 612, contained the *UL97* cassette cloned as a ~2.1 kb *Bam*H I insert. This insert was cloned into the unique *Bgl*II site of pKTS 598, resulting in a chimeric, *GP97/UL97* hybrid plasmid. This clone (pKTS 613) was modified by insertion of a kanamycin resistance cassette, amplified by PCR from the plasmid pACYC177 using primers Km1 RV and Km2 RV (Table 1). The resultant clone, pKTS 615, was used in generation of recombinant BAC virus (see below). To produce a construct for generation of *GP97* null virus, plasmid pKTS 598 (the *GP97* deletion construct) was modified by insertion of a kanamycin resistance cassette, using primers Km1 Bam and Km2 Bam (Table 1). This cassette was cloned as a *Bam*H I fragment into the *Bgl*II site of pKTS 598, yielding plasmid pKTS 622.

For generation of a rescue of the *GP97* deletion virus (GPCMVd*GP97*) a PCR product spanning the *GP96* and *GP97* genes (approximately 2.7 kb) was generated using wild-type GPCMV DNA template and primers res*GP97F4* and res*GP97R4* (Table 1). This introduced unique *Hind*III sites onto the 5' and 3' ends of the PCR product. The product was gel-purified, digested with *Hind*III and cloned into pUC19. Positive clones were verified by sequence analysis. The rescue plasmid was designated pGP9697.

2.3. EGFP-*GP97* expression plasmids

The entire *GP97* coding sequence (Fox and Schleiss, 1997) was PCR amplified from viral DNA using the primers Hd*GP97F2* and Hd*GP97R2* (Table 1), which amplified the *GP97* coding sequence (1803 bp) and incorporated *Hind*III sites at the 5' and 3' ends, to enable in-frame cloning as a *Hind*III fragment into the 3' end of the eGFP expression vector pEGFP-C1 (Clontech). This facilitated expression of *GP97* under HCMV IE promoter control and as a fusion with the C-terminal domain of the eGFP protein. The construct was designated pEGFP*GP97* and encoded a fusion of the eGFP ORF (249 aa) and the *GP97* ORF (605 aa). Subsequently, convenient restriction enzyme sites were used to make progressive truncations

to the 3' end of the *GP97* coding sequence: *EcoR* I digestion and re-ligation resulted in the generation of pEGFPGP97Ec (encoding 544 aa of *GP97* ORF); *Pst* I digestion created pEGFPGP97Pst (encoding 424 aa of *GP97* ORF); *Kpn* I digestion created pEGFPGP97Kpn (encoding 255 aa of *GP97* ORF); and *Bam*H I digestion created pEGFPGP97Bm (encoding 154 aa of *GP97* ORF). The pEGFPC-1 empty vector and pEGFPpp65, an eGFP pp65 fusion construct (McGregor et al., 2004b), were used as controls. The pEGFPpp65 plasmid (HCMV *UL83* fused to the C-terminal domain of eGFP) was used as a positive control, with the eGFPpp65 fusion protein exhibiting a nuclear fluorescent pattern. Transfected cells were counterstained with DAPI for localization of the nucleus (McGregor et al., 2004a).

2.4. Generation of recombinant BACmids and viruses and growth curve analyses

An inducible recombination system (ET system) was introduced into DH10B bacterial cells containing the GPCMV BAC plasmid using a protocol previously described (McGregor et al., 2004a) to generate recombination-positive electrocompetent cells. *GP97* targeting shuttle vectors (pKTS 615 and pKTS 622) were linearized with *EcoR* I, bands purified using GeneClean® II (Qbiogene), suspended to a concentration of 1 µg/µl, and electroporated into GPCMV BAC-containing cells. Recombinant colonies were isolated by chloramphenicol (12.5 µg/µl) and kanamycin (20 µg/µl) selection and DNA analyzed by *EcoR* I and *Hind* III restriction digestion to verify the accuracy of the predicted genome configuration (McGregor and Schleiss, 2001). Insertion of the *UL97* and kanamycin cassettes into the *GP97* wild-type locus was confirmed by PCR using primers flanking the modified *GP97* locus (diagGP97F3 and diagGP97R3, Table 1).

For generation of recombinant viruses, large-scale GPCMV BAC DNA was purified from *E. coli* and used to transfect GPL cells using Lipofectamine 2000 (Invitrogen). Virus stocks were generated as previously described, and one-step growth curves were carried out using an eGFP-tagged GPCMV, vAM403 (McGregor and Schleiss, 2001) and mutant (GPCMV::*UL97*, GPCMVd*GP97*) and rescue viruses (GPCMVR*GP97*) as described elsewhere (McGregor and Schleiss 2001).

2.5. Rescue of *GP97* deletion virus

The GPCMVd*GP97* was rescued to verify the mutant virus phenotype. Rescue virus was generated as previously described for the rescue of an essential gene knockout (McGregor et al., 2004a; Schleiss et al., 2006). Briefly, 1 µg of *GP97* deletion mutant GPCMV BAC DNA was co-transfected with 20 µg of rescue plasmid (pGP9697) onto GPL cells. Rescued viral plaques were picked and subject to further rounds of plaque purification by limiting dilution. Viral DNA was extracted from virus-infected cells as previously described (McGregor and Schleiss, 2001). Rescue of *GP97* was confirmed by PCR and by restriction profile analysis of viral DNA.

2.6. RT-PCR analysis of *UL97* and *GP97* mRNAs in infected cells

Chimeric virus (GPCMV::*UL97*) or wild-type GPCMV (vAM403, McGregor and Schleiss, 2001) were used to infect GPL cells at a multiplicity of infection (MOI) of 0.5 pfu/cell. At 1 hr post-adsorption the monolayers were washed and overlaid with fresh F-12 media. Samples were obtained at 0, 2, 4, 6, 8, 12 and 24 hr post-infection. RNA was extracted using a Qiagen RNA isolation kit and samples treated with RQ1 DNase (Promega) following the manufacturer's protocols. All RNA levels were normalized by OD (260 nm) prior to assay. Samples were assayed for *GP97* expression using primers RT-*GP97*F5 and RT-*GP97*R5 or for *UL97* expression with primers RT-*UL97*F2 and RT *UL97*R2 (Table 1). All RNA samples were also evaluated for cellular GAPDH expression with primers, RT-GAPDHF1 and RT-GAPDHR1 (Table 1). RT-PCR was conducted using the OneStep RT-PCR Kit (Qiagen) according to the manufacturer's specifications: reverse transcription (50 °C for 30 minutes),

95 °C for 10 minutes, PCR for 35 cycles (94 °C 15 sec, 57 °C 15 sec, 72 °C 20 sec), followed by a final extension at 72 °C for 10 minutes. Products were analyzed by agarose gel electrophoresis. Controls were water (no template) and PCR only (no RT stage).

2.7. Western blot and immunofluorescence assays

For Western blot, wild-type and recombinant GPCMV_s were used to inoculate GPL cells at a MOI of 1 and monolayers harvested at 96 hours post-infection. An exception to this was the *GP97* deletion virus, which was inoculated at a MOI of 0.5; cells were harvested once complete cytopathic effect (CPE) was obtained. Additionally, HCMV (Toledo strain) was used to inoculate HFF cells at a MOI of 3 with harvest at 96 hours post-infection. Mock-infected cells were used as controls. Lysates were subjected to SDS-PAGE (10% gel) and Western blot assay as previously described (Schleiss et al., 1999) using polyclonal anti-HCMV rabbit *UL97* antiserum (the generous gift of D. Coen, Harvard University; 1/500 dilution). The secondary antibody employed was anti-rabbit IgG conjugated to horseradish peroxidase (Accurate Chemical and Scientific Co.) at a 1:20,000 dilution followed by detection with chemiluminescence using the manufacturer's specifications (SuperSignal West Pico; Pierce, Rockford, Ill.).

For immunofluorescence assay, GPL cells were inoculated with wild-type and recombinant GPCMV_s at a MOI of 0.1. Once complete CPE was noted, cells were washed with PBS and then fixed with 100% methanol at -20 °C for 72 hr. The cells were then processed with primary antibody, anti-GPCMV gB mouse monoclonal antibody 29:29, at a dilution of 1/500, and a secondary antibody (rabbit anti-mouse IgG conjugated to FITC [Sigma]) used at 1/500 dilution. Cellular nuclei were stained with DAPI using mounting fluid (Vector labs) following the manufacturer's specifications.

2.8. Antiviral susceptibility assays

Antiviral susceptibilities of wild type and recombinant GPCMV_s were determined by plaque reduction assay, as previously described (Schleiss et al., 2005). Confluent monolayers were inoculated with 50–100 PFU per well of eGFP-expressing GPCMV BAC derived wild-type virus (McGregor and Schleiss, 2001), chimeric virus (GPCMV::*UL97*), and, for GCV, a HCMV (Towne) control assay was carried out on HFF cells. After adsorption (1.5 hr) cells were washed, and a 0.5% methylcellulose-media overlay containing serial dilutions of antiviral (GCV or MBV) was added and plaques enumerated after 96 hr of incubation. The drug concentration required to reduce 50% of virus plaque count, compared to the no-drug control, was defined as the IC₅₀ value (Table 2). IC₅₀ values were established by linear regression analysis of dose-response curves using InStat 3.0 (GraphPad software, San Diego, CA). Results indicated (Table 2) represent mean ±SD for 4 independently conducted experiments (with each individual experiment containing a minimum of 3 replicate wells) examining GCV, and 3 experiments examining MBV, for determination of the IC₅₀s for wild-type and GPCMV::*UL97* chimeric viruses, respectively. Wild-type and chimeric *UL97* viruses were tested in parallel for each independent experiment, using identical batches of low-passage GPL cells for each individual experiment, to help account for any batch-to-batch variation in the physiology of the cells.

3. Results

3.1. Transient expression of *GP97* in GPL cells

The HCMV *UL97* protein targets the cell nucleus, but in contrast the murine cytomegalovirus (MCMV) homolog, *M97*, is cytoplasmic; moreover, although MCMV is sensitive to GCV, this antiviral effect is not dependent on *M97* (Wagner et al., 2000). Hence, prior to any in-depth studies with *GP97*, we sought to determine if this protein targeted the nucleus. The full-length

GP97 ORF (605 aa) was cloned into the C-terminal domain of an eGFP expression vector, allowing visualization of the cellular localization of the resultant fusion protein with a predicted size of 854-amino acids (Table 2). C-terminal truncations of the *GP97* ORF were generated using convenient restriction enzyme sites and eGFP patterns of protein localization within the cell were visualized (Table 2). Cells transfected with either the full-length eGFP-*GP97* construct or C-terminal collapsed versions exhibited eGFP expression in the nucleus (summarized in Table 2), confirming that *GP97* is a nuclear targeting protein. Since the eGFP-*GP97* fusion encoded by pEGFP*GP97*Bm, encoding the N-terminal 154 aa of *GP97*, targeted the cell nucleus, we presume that *GP97* encodes a N-terminal NLS similar to *UL97*, supporting the hypothesis that *GP97* is a functional homolog of *UL97*.

3.2. Generation of *UL97* Chimera, *GP97* Deletion, and *GP97* rescue viruses

Two different recombinant GPCMV s were generated that targeted the knockout of *GP97*. First, a *GP97* deletion virus (GPCMVd*GP97*) was constructed to determine if the loss of *GP97* had an effect on the ability of the virus to replicate in tissue culture. Second, a chimeric GPCMV (GPCMV::*UL97*) was constructed that substituted the HCMV *UL97* gene in place of the *GP97*, both to determine if *UL97* could functionally complement the loss of *GP97* as well as test whether this chimeric virus exhibited improved susceptibility to GCV and MBV. Previous studies using GPCMV cloned as a BACmid in *E. coli* demonstrated the amenability of manipulating the genome by an ET recombination strategy (McGregor et al. 2004a,b; Schleiss et al., 2006). This system was therefore used to generate a deletion of nearly the entire native *GP97* coding sequence (pGPCMVd*GP97*), deleting codons L₃₉ to E₄₄₇ of the predicted 605 amino acid protein (Fig. 1). Similarly, ET recombination was performed using the shuttle vector pKTS 615 (Fig. 1) to generate a *UL97*:*GP97* chimera (pGPCMV::*UL97*) containing an in-frame fusion of the N-terminus of *GP97* ORF (codons 1-38) with HCMV (Towne strain) *UL97* (codons 1-707) at a *Bgl* II restriction site. The shuttle vector introduced the *UL97* stop codon and a kanamycin selection marker into the GPCMV BAC in place of *GP97*.

Initially, to confirm the genomic structure of the recombinant BACs, DNA was analyzed by *Eco*R I restriction, which provides the best overall profile of the integrity of the GPCMV genome following recombination induction (McGregor and Schleiss, 2001). Secondly, diagnostic PCR was performed to confirm targeted insertion or deletion in the *GP97* locus (Fig. 1A). PCR confirmed the predicted modifications, with amplification of the *GP97* deletion generating a ~0.9 kb PCR product, and amplification of the chimeric locus generating a ~3 kb PCR product (Fig. 1B). In contrast, amplification of the wild-type *GP97* locus generated a PCR product of ~1.2 kb (Fig. 1B). The modifications were further confirmed by sequence and Southern blot analyses (data not shown).

Recombinant BAC DNAs were next transfected onto GPL cells to generate virus. By day 18 post-transfection the chimeric virus generated from pGPCMV::*UL97* BAC DNA had spread across the cell monolayer in a manner similar to wild-type virus, in contrast to *GP97* deletion virus, which demonstrated dramatically fewer plaques and had reduced spread across the monolayer (data not shown). Replication kinetics of wild-type and recombinant viruses were assessed in one-step growth assays. The results from the growth curve assay (Fig. 2) indicated that the GPCMV::*UL97* virus was only slightly impaired in tissue culture growth, compared to wild-type GPCMV. This sharply contrasted with GPCMVd*GP97*, which was severely impaired. To confirm that the *GP97* deletion was responsible for the growth impaired phenotype of the GPCMVd*GP97* mutant virus, a rescue of *GP97* was performed, using plasmid pGP96*GP97*. PCR (primers diag*GP97*F3 and R3, Fig. 1C) confirmed the wild-type genome configuration of rescue virus. Rescue of GPCMVd*GP97* resulted in a virus that grew with kinetics similar to wild-type virus (Fig. 2). These results suggested that *UL97* is functional in

a chimeric GPCMV, and that it successfully complements the loss of *GP97*. Although *GP97* is not an essential gene, its loss severely impairs virus growth.

3.3 Distribution of gB in wild type and mutant virus infected cells

Studies on a HCMV deletion mutant indicated that the loss of the *UL97* affected virus maturation (Wolf et al., 2001; Prichard et al., 2005) and this was recently characterized by a redistribution of the location of the viral antigen gB during late stages of HCMV infection (Azzeh et al., 2005). We examined if expression of *UL97* could functionally complement any alteration in GPCMV gB distribution conferred by deletion of *GP97*. At late stages of infection, wild type and recombinant viruses were assessed for gB distribution using an anti-GPCMV gB monoclonal antibody (Fig. 3). This experiment demonstrated that loss of *GP97* in GPCMV::d*GP97* resulted in retention of the gB in a perinuclear location at late stages of virus infection (Fig. 3B, 3C), as opposed to a general cytoplasmic distribution found with both wild-type GPCMV (Fig. 3A) and chimeric virus (Fig. 3D). The chimeric virus demonstrated near-normal distribution of gB in infected cells, implying functional substitution of function conferred by *UL97* in the context of the GPCMV genome.

3.4 *UL97* expression in chimera-infected cells

Expression of *UL97* was confirmed in GPCMV::*UL97* virus infected cells by both RT-PCR and Western blot assays. RT-PCR assay indicated that *UL97* mRNA could be detected as early as 4 hours post-infection, similar to the expression of *GP97* in wild-type GPCMV (Fig. 4A; Fox and Schleiss, 1997). Since *UL97* in the chimeric virus is under *GP97* promoter control this was the expected result. The insertion of the kanamycin cassette 3' to *UL97* was not expected to affect expression of the upstream *GP96* or *GP95* based on published data (Fox and Schleiss, 1997). *UL97* protein expression in GPCMV::*UL97* infected cells was confirmed by Western blot of cell lysate using *UL97* specific antisera (Fig. 4B). The predicted M_r of *UL97* is ~80 kDa (Michel et al., 1996). In GPCMV::*UL97* infected lysate, a band of 80 kDa was detected, similar to that observed in lysates from HCMV (Toledo) infected HFFs (Fig. 4B). No bands were detected in the wild-type GPCMV infected lysates, lysates from *GP97* deletion virus (GPCMVd*GP97*) or mock-infected cell controls (Fig. 4B).

3.5 Susceptibility of chimeric viruses to ganciclovir and maribavir

Since *UL97* appeared functional in the chimeric GPCMV we wanted to determine if this modified GPCMV susceptibility to GCV. Plaque reduction assays were carried out as described in materials and methods in the presence or absence of increasing concentrations of GCV. Specific GCV 50% growth inhibitory values (IC_{50} values) were calculated for both wild-type GPCMV and chimeric GPCMV. The results summarized in Table 3 demonstrate the GCV IC_{50} value of the chimeric virus was drastically improved ($15.2 \pm 3.0 \mu M$) compared to a wild-type GPCMV, which exhibited an IC_{50} value of $174.7 \pm 16.0 \mu M$ (mean $\pm$ SD of four replicate experiments). The HCMV Towne control demonstrated full GCV susceptibility in this assay, with an IC_{50} of $3.0 \pm 0.6 \mu M$.

The susceptibilities of the chimeric virus and wild-type GPCMV for the antiviral MBV were also compared by growth reduction assay in the presence or absence of serial dilutions of drug. These analyses revealed that wild-type GPCMV had a MBV IC_{50} of $62.05 \pm 4.5 \mu M$. Table 3 demonstrates that the chimeric virus had increased susceptibility to MBV compared to wild-type GPCMV, with an IC_{50} of $14.05 \pm 0.8 \mu M$ (mean $\pm$ SD of three replicates). Presumably, the modified antiviral sensitivities to both GCV and MBV of the chimeric GPCMV can be attributed to the functional expression of *UL97* in chimeric virus-infected cells.

4. Discussion

Although antiviral therapy requires further study as an interventional strategy for congenital HCMV infection, this virus cannot be effectively studied in animal models. The guinea pig is unique as the only small animal model for congenital CMV infection, likely due to the striking similarities in placenta structure and biology between guinea pigs and humans (Kaufmann, 2004; Carter, 2007; Mess, 2007). Since GPCMV congenital infection has a similar pathogenesis in guinea pigs as occurs in humans (Griffith et al., 1985; Kumar and Nankervis, 1978; Schleiss, 2002), this model is well-suited to study antivirals aimed at preventing congenital CMV infection.

We hypothesized that GPCMV could be made sensitive to the antiviral GCV if the virus encoded a wild type *UL97* gene in place of the homolog *GP97* gene. This strategy has been applied to other viruses with mixed levels of success. A chimeric *tk*-negative herpes simplex virus carrying *UL97* in place of *UL13* was reported to partially complement the loss of *UL13*, and to have modified sensitivity to GCV (Ng et al., 1996). In contrast, substitution of *UL97* for the *M97* homolog (Rawlinson et al., 1997) failed to complement for the loss of the *M97* gene (Wagner et al., 2000). This chimeric virus grew poorly, with kinetics similar to a *M97* knockout, and deletion or substitution of *M97* did not affect viral sensitivity to GCV, which implied that MCMV sensitivity to GCV could not be directly attributed to the *M97* gene (Wagner et al., 2000; Prichard et al., 1999).

Potentially, the difference in function between *UL97* and *M97* proteins may relate to cellular location of these proteins. The *M97* protein is located predominantly in the cytoplasm, whereas the *UL97* protein targets the nucleus (Wagner et al., 2000; Michel et al., 1996). Because of these differences, it was important to determine the cellular location of *GP97* protein. Transient expression studies of a series of eGFP-*GP97* fusion constructs determined that the *GP97* protein, in the absence of other viral proteins, appeared to be a nuclear targeting protein. The *GP97* protein encodes a putative N-terminal nuclear localization signal (NLS), as is found in the *UL97* protein (Michel et al., 1996). Analysis of the predicted coding sequence revealed the presence of an arginine-rich region, spanning codons 8-12 (KRRRD), similar to a sequence identified in *UL97* (RARRRQ). An arginine-rich NLS of KRRRH has also been identified for the HCMV pp65 tegument protein (Schmolke et al., 1995). Interestingly, studies by Prichard et al. (2005) suggest that HCMV pp65 and *UL97* interact within the nucleus, and that this interaction may be linked to tegument assembly. Since the homolog to pp65 in GPCMV has been identified, *GP83*, (Schleiss et al., 1999) and the protein functions in a similar manner to pp65 (Schleiss et al., 1999; McGregor et al., 2004), it will be worth investigating potential *GP83/GP97* interactions in future studies. These studies could be extended into HCMV by the generation of chimeras encoding *GP83* and/or *GP97* in place of their HCMV homologs to compare protein-protein interactions in HCMV mutant (*UL97/UL83*) and intertypic chimeric mutant virus (*GP97/GP83*) infected cells.

Two *GP97* knockout GPCMV mutants were generated via GPCMV BAC mutagenesis, a *GP97* deletion virus (GPCMVd*GP97*) and a *UL97* chimeric GPCMV (GPCMV::*UL97*). The *GP97* deletion GPCMV BAC regenerated viable virus in tissue culture, but the virus was severely impaired for growth. The phenotype of the *GP97* deletion virus is similar to the phenotype described for a HCMV (AD169 stain) *UL97* knockout virus (Prichard et al., 1999; Wolf, et al. 2001; Prichard et al., 2005) and illustrated by a modified distribution of gB antigen (Azzeh et al., 2006). Expression of *UL97* protein in cells infected with the GPCMV::*UL97* chimera restored a normal profile of gB cellular distribution. This result, coupled with the near wild-type growth kinetics of the chimeric virus, suggests that *UL97* functionally complements the loss of *GP97*.

The chimeric virus had increased susceptibility to GCV and MBV as reflected in improved IC₅₀ values (Table 3). This result strongly suggests that the *UL97* is functionally active in chimeric virus-infected cells. Interestingly, the chimeric GPCMV has a more favorable IC₅₀ value for GCV than that published for rat CMV, and similar to that reported for rhesus monkey CMV (Williams et al., 2003). The improved susceptibility of chimeric GPCMV to GCV and MBV creates an opportunity for future GCV antiviral studies to be performed in the guinea pig model at clinically relevant doses of these agents. It is interesting to note that the published GCV IC₅₀ value for wild type GPCMV has ranged from 71 μ M to 191 μ M (Fong et al., 1987; Williams et al., 2003); our results, using an eGFP-tagged virus to enable detection of plaques, revealed a IC₅₀ of ~175 μ M (Table 3) for wild-type virus, although the differences among assay techniques preclude definitive comparisons. In contrast, the chimeric virus demonstrated IC₅₀s of ~15 μ M. Improved susceptibility of the chimeric GPCMV to MBV was also noted. Wild-type GPCMV exhibited an IC₅₀ of ~60 μ M this finding was comparable to that described by Williams and colleagues (Williams et al., 2003). In contrast, MBV susceptibility was reduced >4-fold, to ~14.5 μ M, in the chimeric virus, a level of susceptibility similar to that described for HCMV. This result was of particular interest in light of the fact that MBV, which is currently in clinical trials (Biron et al., 2002), is active against HCMV strains resistant to other CMV antivirals (Drew et al., 2006). Study of this and other antiviral agents that target the *UL97* kinase should prove to be of increased relevance in GPCMV model using this chimeric virus; moreover, the potential antagonistic properties of MBV against GCV activity (Chou and Marousek, 2006) could be determined in this more relevant, “humanized” guinea pig model. Creation of other chimeric GPCMV's encoding HCMV genes using BAC technology could allow study for the novel study of immunogenic HCMV proteins, such as gB, in the context of an animal model, which may in turn aid development of successful vaccines.

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Fig. 1. Analysis of modified GPCMV BAC mutants

(A) Schematic representation of the *GP97* loci on wild type, chimeric and deletion GPCMV BACs (pGPCMV::UL97 and pGPCMVdGP97). Common primers flanking the mutated *GP97* locus (diagGP97F3 and R3, Table 1) were used to PCR-amplify the *GP97* loci of the wild type and mutant GPCMV BACs. The predicted sizes of the PCR product for each *GP97* locus is shown: wild type GPCMV (1.2 kb); chimera pGPCMV::UL97 (3 kb); deletion pGPCMVdGP97 (0.9 kb). (B) Analysis of *GP97* loci via PCR assay. PCR reactions were performed on wild-type and mutant GPCMV BACs using primers diagGP97F3 and R3 (Table 1) that flank the *Bgl* II sites at the 5' and 3' ends of the *GP97* gene. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Lanes: (1) chimera pGPCMV::UL97 (3 kb); (2) deletion pGPCMVdGP97 (0.9 kb); (3) wt GPCMV (1.2 kb); Invitrogen kb marker. Arrows (left) indicate PCR product and (right) kb band ladder size. (C) Rescue of *GP97* deletion GPCMV back to wild type status. GPCMVdGP97 was rescued back to wild type virus by co-transfection of BAC pGPCMVdGP97 with rescue plasmid pGP9697, as described in materials and methods. Viral DNA of plaque-purified rescue virus was analyzed to verify rescue of the *GP97* locus by diagnostic PCR assay using common *GP97* primers flanking the modified region. PCR products were analysed as described in (B). Lanes: (1) Invitrogen kb ladder; (2) wt GPCMV (1.2 kb); (3) GPCMVdGP97 (0.9 kb); (4) Rescue virus, GPCMVdGP97 (1.2 kb). Arrows (left) indicate kb ladder and (right) PCR products.

Fig. 2. One-step growth curves of GPCMV mutants vs. wild type GPCMV
 eGFP-tagged wild type GPCMV (vAM403), chimeric *UL97* GPCMV (GPCMV::*UL97*), *GP97* deletion GPCMV (GPCMVd*GP97*) and *GP97* deletion rescue virus (GPCMVVR*GP97*) were used to inoculate GPL cells in separate wells of six-well dishes as described in materials and methods. Viruses: vAM403 (circle); GPCMV::*UL97* (square); GPCMVd*GP97* (triangle); GPCMVVR*GP97* (open triangle). Cells were infected at a MOI of 0.5 pfu/cell.

Fig. 3. Immunofluorescence assay for gB distribution within wild type GPCMV and mutant viruses in infected GPL cells

IFL assay was carried out as described in Materials and Methods at late stage of virus infection, using mouse anti-GPCMV gB monoclonal antibody and secondary anti-mouse IgG conjugated to FITC (pictures A, B, C and D). Cells were also counterstained with DAPI to locate the nucleus (pictures E, F, G and H). Infected monolayers: wild type GPCMV (A and E); GPCMVdGP97 (B, C, F and G); GPCMV::UL97 (D and H).

Fig. 4. Expression of *UL97* in chimeric GPCMV-infected cells

(A) Comparison of the kinetics of *GP97* and *UL97* gene expression by chimeric (GPCMV::*UL97*) and wild type GPCMV using RT-PCR assay. GPL cells in separate wells of six well dishes were infected with viruses (1 pfu/cell; either wt GPCMV or GPCMV::*UL97*) and at various time points post-infection (0, 2, 4, 6, 8, 12, 24 hr) RNA was isolated from infected cells and subjected to RT-PCR as described in materials and methods. (i) RT-PCR of *UL97* mRNA from chimeric virus infected cells at time points 0 to 24 hr post infection. (ii) RT-PCR of *GP97* mRNA from wt GPCMV infected cells at time points 0 to 24 hr post infection. (iii) RT-PCR of endogenous cellular mRNA (GAPDH) from GPCMV::*UL97* infected GPL cells (0 to 24 hr post infection). Controls (a and b lanes) include no RT step (a) and no template (b) assays. (B) Western blot of *UL97* protein expressed by GPCMV::*UL97* infected cells. Infected or uninfected cell lysates were separated by SDS-PAGE (10%) and transferred to a nytran membrane for immunoblot assay using *UL97*-specific antisera. Lanes: (1) mock-infected GPL cells; (2) wt GPCMV; (3) and (4) GPCMV::*UL97*; (5) GPCMVd*GP97*; (6) mock-infected HFF cells; (7) HCMV (Toledo) infected HFF cells.

Table 1

List of oligonucleotides used in PCR and RT-PCR experiments (see Materials and Methods).

Primer Names	Sequences (5' to 3' direction)
GP97F1	GGCTCCGATCGGAGATTTCCT
GP97R1	CTCTATACATTCGTGAATTCCTG
UL97F1	CTATGTCTCCGCACTTCGGTCTCGG
UL97R1	GATGGCGGATCCTCGGTATTTCCCGCCGGTGGGTTGATATCCTTCT
Km1 RV	GATAGAGATATCGATTTATTCAACAAAGCCACG
Km2 RV	TAGATAGATATCGCCAGTGTACAACCAATTAACC
Km1 Bam	GATAGAGGATCCGATTTATTCAACAAAGCCACG
Km2 Bam	AGAGACGGATCCGCCAGTGTACAACCAATTAACC
Hd GP97F2	TGATACAAGCTTCGACGTCCAATGTTTTAAAG
HdGP97R2	TAGATAAAGCTTCCTATCGGTAGTCCGCTTGAAAC
diaGP97F3	CTTTCTAGACGTCTTGCTAG
diagGP97R3	CAAGGTCCTCATGAGACAG
resGP97F4	AGATGAAAGCTTTAAAGAGACTATGCGCCACAGACTGGAGGAGAAAC
resGP97R4	TAGATAAAGCTTCCTATCGGTAGTCCGCTTGAAAC
RT-GP97F5	GATGTAGTGCTTCAGGCACGGC
RT-GP97R5	GACGACAGAAGTTCCGAGCCG
RT-UL97F2	CTTCGAGCACCAGTCCACG
RT-UL97R2	CAGGTCGCCCAGGCTCACGTC
RT-GAPDHF1	GGGCAAGGTCATCCCAGAG
RT-GAPDHR1	TGGAAGAATGGCTGTCACTGTT

Table 2
Cellular location of full length and C-terminal truncated versions of GP97

GP97 was PCR amplified as a *Hind* III fragment and cloned in-frame into the C-terminal domain of eGFP in the unique *Hind* III site of pEGFP-C1 (Clontech). Using convenient restriction sites within the *GP97* ORF a series of 3' truncated *GP97* ORFs were generated. The series of eGFP-*GP97* expression plasmids were transfected separately onto GPL cells in six well dishes and eGFP tagged protein transiently expressed for 24 hr as described in materials and methods. The cellular location of full length and truncated eGFP-*GP97* fusion proteins is summarized. Plasmids pEGFPpp65 (McGregor et al., 2004a) and pEGFP-C1 empty vector were used as controls for nuclear targeting and general cell fluorescence respectively. GPL cells were also counterstained with DAPI to locate the nucleus.

GFP Plasmid	GP97 aa content	eGFP cellular location
pEGFPGP97	1-605	nucleus
pEGFPGP97Ec	1-544	nucleus
pEGFPGP97Pst	1-424	nucleus
pEGFPGP97Kpn	1-255	nucleus
pEGFPGP97Bm	1-154	nucleus
pEGFP-C1	0	cytoplasm + nucleus
pEGFPpp65	0	nucleus

Table 3

Antiviral susceptibilities of wild-type and recombinant CMVs to GCV and MBV. Results represent mean $\pm$ SD for 4 experiments examining GCV, and 3 experiments examining MBV, for determination of IC₅₀s for wild-type and GPCMV::UL97 chimeric viruses, respectively (see Materials and Methods for details).

Ganciclovir Susceptibility	
Wild-Type GPCMV	174.7 $\pm$ 16 μ M
GPCMV::UL97	15.2 $\pm$ 3 μ M
HCMV (Towne)	3.0 $\pm$ 0.6 μ M
Maribavir Susceptibility	
Wild-Type GPCMV	62.05 $\pm$ 4.5 μ M
GPCMV::UL97	14.5 $\pm$ 0.8 μ M