Differential Properties of Cytomegalovirus pUL97 Kinase Isoforms Affect Viral Replication and Maribavir Susceptibility

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Running title: Functional properties of HCMV pUL97 isoforms
The human cytomegalovirus (HCMV)-encoded kinase pUL97 is required for efficient viral replication. Previous studies described two isoforms of pUL97, full length (M1) and a smaller isoform likely resulting from translation initiation at codon 74 (M74). Here, we report the detection of a third pUL97 isoform during viral infection resulting from translation initiation at codon 157 (isoform M157). The consistent expression of isoform M157 as a minor component of pUL97 during infection with clinical and laboratory-adapted HCMV strains was suppressed when codon 157 was mutagenized. Viral mutants expressing specific isoforms were generated to compare their growth and drug susceptibility phenotypes, as well as pUL97 intracellular localization patterns and kinase activity. The exclusive expression of isoform M157 resulted in substantially reduced viral growth and resistance to the pUL97 inhibitor maribavir while retaining susceptibility to ganciclovir. Confocal imaging demonstrated reduced nuclear import of amino-terminal deletion isoforms compared to isoform M1. Isoform M157 showed reduced efficiency of various substrate protein interactions and autophosphorylation whereas Rb phosphorylation was preserved. These results reveal differential properties of pUL97 isoforms that affect viral replication, with implications for the antiviral efficacy of maribavir.
IMPORTANCE

The HCMV UL97 kinase performs important functions in viral replication that are targeted by the antiviral drug maribavir. Here, we describe a naturally occurring short isoform of the kinase that when expressed by itself in a recombinant virus results in altered intracellular localization, impaired growth and high level resistance to maribavir when compared to the predominant full length counterpart. This is another factor to consider in explaining why maribavir appears to have variable antiviral activity in cell culture and in vivo.
INTRODUCTION

The product of the HCMV UL97 gene (pUL97) is a serine/threonine protein kinase that phosphorylates itself and multiple viral and host proteins (1). It is expressed early in infection (2, 3), localizes predominantly to the nucleus using amino-terminal nuclear localization signals (4, 5), and is also observed in the cytoplasm later in the infection cycle (2, 5, 6). It is also present in the HCMV virion (7). Genetic deletion of UL97 results in a severe (10-1000 fold) replication defect in vitro (8-10), as does mutation of the K355 lysine residue critical for kinase activity (6). UL97 is therefore an attractive target for antiviral drug development (1, 11-14) as an alternative to the UL54 DNA polymerase target of all currently licensed HCMV antivirals including ganciclovir (GCV).

Maribavir (MBV) is a benzimidazole riboside pUL97 inhibitor that inhibits HCMV replication potently (15) but variably depending on cell culture conditions (16). While MBV showed promise in early clinical trials, two recently-published phase III trials demonstrated that MBV was no more effective than placebo at preventing HCMV viremia after allogeneic stem cell transplantation or liver transplantation (17, 18). Since resistance to MBV was not observed in these trials (19), potential explanations for their outcome may include an insufficient drug dosage or understanding of how in vivo host cell conditions affect the function of pUL97 and consequences of its inhibition.

It is difficult to determine which of the multiple functions of pUL97 are most important in facilitating viral replication. Early in the infection cycle it may activate the major immediate-early promoter (20), phosphorylate retinoblastoma (Rb) protein (21) to influence the cell cycle as a CDK ortholog (22-24), interact with cyclins (25), and...
phosphorylate the viral polymerase accessory protein pUL44 (26, 27) and the nuclear mRNA export factor pUL69 (28). Later in the infection cycle, pUL97 may facilitate nuclear egress by phosphorylating the viral tegument protein pp65 (29), nuclear lamins (30, 31), and may also function through kinase-independent mechanisms (32).

The UL97 open reading frame (ORF) contains several potential alternative in-frame ATG translation initiation sites downstream of the first ATG codon (M1) and upstream of K355, located at codons 38, 74, 111, 157, and 330. Previous studies documented two pUL97 isoforms, and results using pUL97 expressed by transient transfection implicated M74 as the start site for the second isoform (4, 27, 33). Both isoforms exhibited autophosphorylation activity and were incorporated in HCMV virions (4). Moreover, two nuclear localization sequences, NLS1 and NLS2, were identified in the N-terminus of pUL97 indicating a differential, isoform-specific efficiency of nuclear import (5).

The present objective was to investigate the use of alternate translation initiation sites in the UL97 ORF in the context of HCMV replication. We found that during normal HCMV infection, a majority component of full-length pUL97 is consistently accompanied by a shorter isoform translated beginning with M157. Recombinant phenotyping of HCMV strains showed significant differences in substrate phosphorylation, subcellular localization, and growth and drug susceptibility profiles of the smaller isoform that may affect the antiviral efficacy of MBV.
MATERIALS AND METHODS

Cells. Human foreskin fibroblast (HFF) and human embryonic lung (HEL) fibroblast cell lines were maintained at 37°C in a 5% CO₂ atmosphere in Eagle’s minimal essential media supplemented with 7.5 - 10% fetal bovine serum (FBS, Hyclone) during the growth phase and 3% FBS after reaching confluence. HFFs and HELs were maintained by passaging (app. 1:3) once or twice per week. Human embryonic kidney 293T cells were cultivated in Dulbecco’s modified Eagle media supplemented with 10% FBS. For HCMV infections, cells were selectively seeded in 6-well, 12-well, or 24-well plates and infected at MOIs between 0.01 to 3 under standard conditions (12, 34).

Viral clones and strains. The recombinant viruses UL97-HA (expressing wild type UL97 tagged with HA epitope), UL97(Mx4)-F (expressing Flag tagged UL97 containing the mutations M38A/M74L/M111L/M157A) and UL97(M1L)-HA were constructed using traceless bacterial artificial chromosome (BAC) mutagenesis (35). To generate the internal kanamycin resistance cassette bounded by a repeated UL97 segment, the oligonucleotides 5-aphAI/UL97-PstI and 3-aphAI-PstI (Table 1) were used to amplify a fragment containing PstI restriction sites (unique in UL97) using the vector pEP-S/aphAI (kindly provided by B. K. Tischer, Free Univ. Berlin, Germany (35)). This PCR product was then inserted into the cloning constructs for UL97-HA, UL97(Mx4)-F and UL97(M1L)-HA, and tagged UL97 mutant versions were generated. Recombination was achieved by the use of these transfer vectors, inserted at homologous sites into the previously constructed AD169-derived BAC pHB15-ΔUL97 using primers 5-ΔUL97 and 3-ΔUL97 (Table 1). After cleavage at a specific I-Scel site, the kanamycin resistance marker was removed by a second recombination step. The resulting recombinant BACs
were verified via restriction fragment length polymorphism, PCR amplification of the inserted gene region and nucleotide sequencing. For virus reconstitution, BAC DNA was transfected into HFF cells, before infectious virus released to the supernatants could be passaged and grown to viral stocks.

Additional strains were generated for secreted alkaline phosphatase (SEAP) reporter-based analysis of replication and drug susceptibility. These are based on the BAC BA1 that was cloned from the AD169-derived wild type HCMV strain T2211 and contains a SEAP reporter gene under control of an ectopic HCMV major immediate early (MIE) promoter adjacent to gene US3 (36, 37). The BAC BA9 was derived from BA1 by replacing codons 134-654 of UL97 with a galK selection marker (36) and transfected into HFF to yield HCMV strain T3110 (ΔUL97). Strains expressing kinase-inactive pUL97 (K355M and ΔK355) have been previously described (38). Additional HCMV recombinant strains harboring the desired UL97 mutation(s) (Table 2) were created by recombination of BAC BA9 with a transfer vector containing the UL97 mutation (generated by PCR mutagenesis) in addition to an Frt-flanked kanamycin resistance marker, followed by removal of the kanamycin marker with Flp recombinase, as previously described (36). BAC DNA was then transfected into HFF cultures to reconstitute live HCMV. The absence of parental BAC galK sequences in the recombinant virus was confirmed by PCR, and the entire UL97 sequence of each recombinant virus was assessed by standard automated fluorescent dideoxy sequencing to ensure the presence of the desired mutation(s) and the absence of unintended ones.

Historical clinical HCMV isolates C1-C3 and C076 (39) were obtained from transplant recipients not receiving anti-CMV therapy. A series of additional clinical
isolates collected between 1990 and 2012 was obtained from the diagnostic repositories for clinical specimens of the virological institutes at Erlangen (11 isolates) and Sydney (31 isolates). The UL97 gene region of primary or low-passage virus isolates was amplified using a nested-PCR reaction. First round amplification was performed with primers UL97.140304T and UL97.142644B, and second round with primers UL97.140341T and UL97.142418B (Table 1). All PCR products were analyzed by nucleotide sequencing.

**Comparative growth of UL97 mutant strains.** SEAP activity in the supernatants of 24-well cultures of HEL or HFF cells was assayed using a chemiluminescent substrate as relative light units (RLU) at days 1, 4, 5, 6, 7, and 8 after the inoculation of a set of comparison strains at a multiplicity of infection (MOI) of 0.01 to 0.02 (36, 37). The mean SEAP activities and standard deviations from 4 replicate wells were used to construct the growth curves. Similar SEAP activities at 24h were used to calibrate an equivalent MOI.

Viral genome equivalents were measured by quantitative real-time PCR (qPCR, 7500 RealTime PCR System and SDS Software, Applied Biosystems) of supernatants collected from HFFs infected with recombinant HCMVs (MOI 0.1) at days 0, 2, 4, 6, 8, 10 and 12 post-infection. Parental AD169 was taken as a control for normal viral replication in vitro. Following digestion of supernatant samples with proteinase K, an IE1 gene region within exon 4 was amplified using primers CMV 3' and CMV 5' together with the TaqMan probe CMV MIE FAM/TAMRA (Table 1). Control plasmid containing IE1 cDNA served as an internal amplification standard. All samples were analyzed in triplicate and the standard deviation was calculated.
Phenotypic assay of antiviral susceptibility. SEAP yield reduction assays for GCV and MBV susceptibility of HCMV strains were performed as previously described (37, 40). Briefly, cell-free virus stock was inoculated onto 6-day-old HEL (MBV) or HFF (GCV) monolayers in 24-well culture plates at an MOI of 0.01 to 0.02 and cultured under a range of drug concentrations (5 per assay) and a no-drug control. The drug concentration required to reduce the supernatant SEAP activity by 50% (EC50) was determined by curve fitting. A minimum of seven assay replicates performed on at least four separate dates were used to calculate the mean EC50 and standard deviation for each strain. Susceptible and resistant control strains were included for quality control and to enable comparison with previous publications.

Western blot analysis. HFF or HEL cells infected with HCMV recombinant strains were washed in ice-cold phosphate-buffered saline and lysed using 2% sodium dodecyl sulfate (SDS), standard lysis buffer (50mM Tris/HCl (pH 8.0), 150mM NaCl, 5mM EDTA, 0.5% NP-40, 1mM PMSF, 2 μg/ml aprotinin, leupeptin and pepstatin) or NSRB (62.5 mM Tris-HCl pH 6.8, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 2% SDS, 10% Glycerol, 5% beta-mercaptoethanol). Lysate was separated on SDS-polyacrylamide gels followed by transfer to a polyvinylidene difluoride (PVDF) membrane (Millipore). Immunoblot analysis was performed for pUL97 using a carboxy-terminal mouse monoclonal antibody (Alabama) (6), a central region monoclonal antibody (Rijeka) (41) or rabbit polyclonal antibodies (2, 42). Antibodies against pp65 (Virusys), UL44 (Virusys), actin (Sigma AC-15 and 2066), and Rb (4H1; Cell Signaling Technology) were used according to manufacturers’ instructions. Horseradish peroxidase-conjugated goat anti-mouse (Santa Cruz Biotechnology; sc-2005) or anti-rabbit (Santa Cruz
IgG secondary antibody, and luminol were used according to manufacturers’ instructions (Sigma-Aldrich; A8511). Digital images were acquired using LAS-1000 (Fujifilm Europe GmbH), ChemiDoc XRS (BioRad), or 4000 MM (Kodak) luminescence imagers.

Confocal laser-scanning microscopy. Immunofluorescence analysis and confocal microscopy was performed as described elsewhere (43). In brief, HCMV-infected cells (MOI 0.1) were grown on coverslips, before fixation and immunostaining was performed by the use of 4% paraformaldehyde and a polyclonal antibody against pUL97 (42).

Immunoprecipitation and in vitro kinase assays. pUL97 immunoprecipitation and in vitro kinase assays using anti-pUL97 rabbit polyclonal antibody was performed as previously described (29).

RESULTS

Isoforms of pUL97 result from translation initiation at M1 and M157 during HCMV infection. The presence of two pUL97 isoforms during infection of human HFFs with HCMV strain AD169 has been described (4, 5, 27, 33). Potential translation start sites for UL97 coding sequence include M1 for full-length pUL97 and others at M38, M74, M111, M157, and M330. Prior work indicated that M74 may serve as an initiation site when pUL97 is expressed by transient transfection (4). We therefore focused on M74, but also evaluated a role for M111 or M157 during viral infection. Immunoblot analysis was performed on protein extracts from HFFs infected with parental HCMV (AD169) or recombinant strains containing stop codons at positions 74, 111 or 157.

Using a rabbit polyclonal antibody (Ulm) to pUL97 (Fig. 1A), infection with wt HCMV resulted in a prominent pUL97 isoform migrating at ~ 100kDa (band 1) and a shorter,
less prominent isoform at ~80 kD (band 3). Mutation of M1 to a leucine residue (M1L) resulted in loss of band 1, confirming loss of the full-length isoform and appearance of an intermediate-size isoform (band 2) likely resulting from translation initiation at M74. The isoform represented by band 3 was also observed during infection with strains containing stop codons at 74 and 111 but not 157, indicating that it may result from initiation at codon M157.

To confirm these results, a mouse monoclonal antibody was used to detect pUL97 during infection with the strains described above. Also included were strains lacking intact pUL97, including one with stop codons replacing both M74 and M330, and another with codons 134-654 deleted (HCMVΔUL97). Two isoforms were observed during infection with BAC-cloned strain AD169 derivatives (wt HCMV, Figure 1B and C) and an intermediate-size isoform (M74) was again detected during infection with HCMV UL97(M1L) (band 2). The smallest isoform (band 3) was not detected in strains with stop codons at 157 or 330. No signal was observed during infection with strains lacking intact pUL97, demonstrating the specificity of the pUL97 monoclonal antibody. These results are consistent with isoforms of pUL97 translated from M1 and M157 during HCMV infection.

In order to verify M157 as a site of isoform initiation, and to control for the possibility that this isoform is simply a degradation product of full-length pUL97, a recombinant virus with M157 replaced by leucine (HCMV UL97(M157L)) was generated. The recombinant viruses HCMV UL97(M74L) and HCMV UL97(M74L/M157L) were made to address the previously mentioned role for M74 in pUL97 isoform translation (4) and for the finding that translation appears to initiate at M74 when M1 is mutated (Figures 1A
and B). To assess whether smaller isoforms of pUL97 resulted from autophosphorylation, a recombinant virus lacking the critical kinase residue (HCMV UL97(ΔK355)) was utilized. Finally, a virus in which amino acids 1-156 were deleted was made to provide a size marker for the pUL97 polypeptide comprising amino acids 157-707. Two isoforms were clearly detected during infection with wt HCMV (Figure 1C, arrows), with the smaller isoform again being present in a minority amount relative to full-length pUL97 and co-migrating with pUL97(157-707). Mutation of M157, but not M74, markedly reduced the intensity of the smaller isoform despite equal amounts of full-length pUL97, leaving a faint background signal that was observed in this blot but not consistently in others (Fig 2A). The presence of the smaller isoform was not affected by the absence of pUL97 kinase activity (HCMV UL97(ΔK355)).

Taken together, these results indicate that multiple isoforms of pUL97 are made during viral infection, with full-length pUL97 (isoform M1) predominant over the isoform originating at M157 (isoform M157).

**Translation of the M157 isoform occurs during infection with clinical isolates of HCMV.** To determine whether translation of the M157 isoform occurred during infection with clinical isolates, lysate was prepared from HFFs infected with one of four clinical isolates of HCMV (CS1-3, CO76) and analyzed by immunoblot using anti-pUL97 mAb. Lysate from cells infected with HCMV UL97(M74L/M157L) and HCMV UL97(157-707) served as negative and positive controls, respectively, for detection of isoform M157. Both isoforms M1 and M157 were observed during infection with CS1, 2, and 3 (Figure 2A, arrows). For CS076, the M157 isoform band was barely visible probably because of the reduced total amount of pUL97 in this sample.
Additional clinical isolates, R3 and R4 (12, 44) were analyzed for the presence of pUL97 isoforms using anti-pUL97 polyclonal antibody (Ulm). Three polypeptides were detected, consistent with the presence of isoforms (Fig. 2B), but in different relative amounts than findings using the anti-pUL97 monoclonal antibody (Fig 1B and C and Fig. 2A). To determine whether this difference in detection depended on the pUL97 antibody reagents, the same lysates from cells infected with strain AD169 or the clinical isolates R3 and R4 were subjected to immunoblot analysis using anti-pUL97 polyclonal antibody (Ulm) as in Fig 2B, and two anti-pUL97 monoclonal antibodies – “Alabama” (as used in Figs 1B and C, 2A), and “Rijeka”. The relative detection of isoforms varied among the strains and pUL97 antibody reagents (Figure 2C). In contrast to findings with the polyclonal antibody, both monoclonal antibodies showed the smaller isoforms to be always present in lesser quantities compared to the full-length isoform.

Thus, the translation of multiple pUL97 isoforms in HCMV-infected cultured cells could be demonstrated for several strains of HCMV and may therefore also have relevance during infection in vivo. Indeed, the amino terminal portion of UL97 is known to be well conserved among >60 published clinical isolate sequences, including codons M1, M38, M74, M111 and M157 (45, 46). Sequence analysis of 5 laboratory HCMV strains and an additional 42 clinical isolates also showed complete conservation of these ATG codons (Table 2). Therefore it is anticipated that all clinical isolates have the potential to express multiple pUL97 isoforms.

Isoform M1 but not M157 is sufficient for normal viral replication. The role of each pUL97 isoform in HCMV replication was assessed by multi-step growth curve analysis of HCMV recombinants. Replication of HCMV UL97(M1L) and HCMV UL97(M74L)
in HEL cells was similar to that of a simultaneously inoculated control, parental AD169-derived (wt) strain (Figure 3A). Additional multi-step growth curve analysis after infection of HEL cells indicated that while HCMV UL97(M74L/M157L) grew with wild-type kinetics, HCMV UL97(157-707) was growth attenuated but not to the degree of a recombinant strain in which the kinase activity of pUL97 was genetically abrogated by deletion of the critical catalytic residue K355 (HCMV UL97(ΔK355) (Figure 3B). HCMV UL97(157-707) also demonstrated growth impairment in HFFs (Figure 3C), although to a lesser extent than in HELs. An alternate growth comparison of this mutant in HFF culture against wild type AD169 by DNA qPCR showed a modest reduction of viral DNA yield over time, while strains producing the other isoforms appeared to grow as well as AD169 (Fig. 3D). Therefore, it appears that no single isoform, including full-length pUL97 (isoform M1), is required for efficient viral replication.

An HCMV mutant lacking isoform M1 results in altered subcellular localization of pUL97. NLS1 is located between codons 6-35 and is therefore missing in smaller isoforms, whereas NLS2, located between codons 190-213, is retained (4, 5). To assess for changes in localization of pUL97 resulting from loss of NLS1 during infection, confocal microscopy experiments were performed after infection of HFFs (MOI=0.01) with recombinant viruses representing full length (UL97(Mx4)-F) or short isoforms (UL97(M1L)-HA) (Fig. 4). Virus expressing only full length pUL97 (UL97(Mx4)-F, panels 10-18) showed dominant nuclear localization as expected, with concentration of signals at the periphery of viral replication compartment (panel 17b). In contrast, during infection with HCMV UL97(M1L)-HA, pUL97 demonstrated reduced nuclear localization, and the pUL97 that was imported into the nucleus was concentrated in
replication compartments (panels 25-27). Thus, the absence of NLS1 impairs but does not abrogate nuclear import of pUL97 during infection.

Isoform 3 exhibits a reduced level of substrate protein interaction. pUL97 has been shown to directly interact with several proteins, including the HCMV-encoded UL83 (pp65) and UL44 proteins (27, 42). To determine the roles of the various isoforms in protein-protein interaction, pUL97 from cells infected with wt HCMV, HCMV UL97(Mx4)-F, HCMV UL97(M1L), and HCMV UL97(157-707) was immunoprecipitated using rabbit polyclonal anti-pUL97 antibody. The relative amounts of input pUL97 isoforms and pUL44 were comparable across infections, whereas input pp65 was slightly more variable but was similar in HCMV AD169 and UL97(157-707) (Figure 5, bottom panels). The pUL97 polypeptides immunoprecipitated to a similar degree with the exception of the isoforms made during infection with HCMV UL97(M1L)-HA, which were present in greater abundance (Figure 5, top panel). Both pp65 and pUL44 co-immunoprecipitated with pUL97 during infection with recombinant viruses expressing all pUL97 isoforms (AD169 and UL97-HA) as well as solely isoform M1 (UL97(Mx4)-F). The efficiency of pUL44 co-immunoprecipitation was slightly reduced based on the amount relative to immunoprecipitated pUL97 during infection with HCMV UL97(M1L)-HA, whereas pp65 co-immunoprecipitated proportionately.

The interaction of pUL97 isoform M157 (HCMV UL97(157-707)) with both pUL44 and pp65 appeared less robust, with both being detectable only after prolonged exposure. Similar results pertaining to the interaction of pUL97(157-707) with pp65 were observed after immunoprecipitation of pUL97 from HCMV-infected cells using the mouse monoclonal antibody (data not shown). These results indicate that the amino-terminus of
pUL97 is necessary for normal pUL97 protein-protein interactions during infection.

**Phosphorylation of pUL97 substrates by the M157 isoform.** pUL97 autophosphorylation is often used as a surrogate assay of biologically relevant kinase activity in its wild type and mutant forms (1, 41, 47). To determine isoform-specific properties of autophosphorylation, an *in vitro* kinase assay was performed using HCMV-infected HFFs (MOI of 0.1). Five dpi, cell lysates were prepared in CoIP buffer (Fig. 6A) or RIPA buffer (Fig. 6B), and pUL97 was immunoprecipitated and analyzed in an *in vitro* kinase assay (29). Analysis of a recombinant virus expressing only isoform M1 (UL97(Mx4)-F) demonstrated autophosphorylation comparable to wild-type controls (AD169, UL97-HA) (Figure 6A, top panel). During infection with HCMV UL97(M1L)-HA, autophosphorylation was readily detectable, whereas when expressed in isolation, isoform M157 exhibited relatively reduced autophosphorylation. The arrow indicates a phosphorylated product that likely represents pp65. While phosphorylation of pp65 by pUL97(157-707) appeared to be relatively reduced, the interaction of pUL97(157-707) with pp65 was, as previously observed (Figure 5), less robust, thus pp65 phosphorylation appears to be limited at the level of substrate protein binding. To exclude the presence of additional co-immunoprecipitating kinases, we routinely performed control reactions in which we applied the pUL97 inhibitor Gö6976 so that pUL97 specificity of the phosphorylation signals could be assured (data not shown). The reduced phosphorylation capacity of isoform M157 was also reflected by a limited degree of standard substrate phosphorylation, i.e. exogenously added recombinant human histones 1-4 (Fig. 6B).

Based on these results, it appears that isoform M157 is capable of autophosphorylation but to a lesser degree than isoform M1, and substrate phosphorylation may be affected as.
well, possibly due to loss of direct interaction.

Phosphorylation of Rb during HCMV infection is dependent on pUL97 kinase activity (21). pUL97 contains three putative Rb binding domains, of which one located between codons 149-153 is absent from isoform M157 (1). To assess whether infection with HCMV UL97(157-707) resulted in pUL97-kinase dependent Rb phosphorylation, HFFs that were cultured in conditions of serum starvation were mock-infected or infected with wt HCMV or HCMV UL97(157-707) at an MOI of ~3 under various concentrations of MBV and lysate was prepared at 24 hpi. The phosphorylation status of Rb was determined by assessing the electrophoretic mobility shift observed after immunoblot analysis of total Rb (21). Infection with HCMV UL97(157-707) resulted in Rb phosphorylation comparable to infection with AD169-derived parental wt HCMV, and this was effectively inhibited by MBV (Figure 6C). Therefore, normal phosphorylation of Rb occurs during infection in a pUL97-kinase dependent manner when isoform M157 alone is expressed.

Isolated expression of isoform M157 is associated with MBV resistance. In order to assess whether pUL97 isoforms have a role in determining susceptibility to the antiviral agents GCV and MBV, EC50 values for each drug against recombinant strains were determined (Table 3). While HCMV UL97(M157L) was susceptible to both GCV and MBV, HCMV UL97(157-707) was significantly resistant to MBV but susceptible to GCV. Therefore, isoform M157 is dispensable for the antiviral efficacy of GCV and MBV, but the exclusive expression of this pUL97 isoform results in resistance to MBV and not to GCV.
Expanding on previous reports of pUL97 isoforms, we used specific viral mutants to demonstrate that an additional isoform results from translation initiation at codon M157 in HCMV-infected cell cultures. Phenotypic characterization of a HCMV recombinant strain expressing only isoform M157 indicated that the amino terminus is required for the normal nuclear localization of pUL97, efficient HCMV replication, and maribavir susceptibility.

The alternate ATG start sites for translation of pUL97 are all highly conserved among clinical isolates on the basis of published (45, 46) and new data (Table 2), supporting the presumption that isoform M157 of pUL97 is routinely expressed during HCMV infection in vivo in similar relative quantities and kinetics as observed in the laboratory and clinical strains examined here.

Not all publications have detected multiple pUL97 isoforms, instead reporting just one isoform that corresponds in size to full-length pUL97 (48). Indeed, at early times during infection, or when DNA synthesis was inhibited with fosfomycin (data not shown), this isoform was not readily observed, likely due to the low overall amount of total pUL97. However, as total pUL97 accumulated at late times during infection, we consistently detected the isoform translated from M157 and confirmed its identity by mutagenizing that residue, although it represented a minor component relative to full-length pUL97.

The isoform of pUL97 described in transient transfection systems as resulting from translation initiation at M74 (4, 5, 33) was not readily detected in virus-infected cells in this study using a monoclonal antibody (Fig. 1 and 2). These discordant results...
may reflect the differences in the expression of pUL97 during infection versus transient transfection, as well as the performance characteristics of different anti-pUL97 antibodies (Fig 2C).

The exclusive expression of isoform M157 of pUL97 by an in-frame deletion mutant resulted in a growth defect that was more apparent in HEL fibroblasts than HFFs, consistent with the known greater sensitivity of HCMV growth in HEL cells to loss of pUL97 kinase activity (16). However, in contrast to UL97 knockout mutants, infection with HCMV UL97(157-707) did not show the typical UL97-defective cytopathic appearance (49, 50). In addition, isoform M157 maintained sufficient kinase activity to result in full ganciclovir susceptibility, which requires initial pUL97-mediated phosphorylation, (51, 52). Rb phosphorylation was also maintained, despite loss of a Rb-binding LxCxE motif in pUL97 centered on C151 (53). Isoform M157 also exhibited partial autophosphorylation activity, adding to prior data showing that a wide range of pUL97 residues affect autophosphorylation in vitro (41, 54). In this case, decreased autophosphorylation may result from the absence of known serine and threonine targets that are clustered in the missing amino terminal part of pUL97, such as S3 (54). However, it is unclear if autophosphorylation is required for the normal function of pUL97.

The partial growth defect of HCMV UL97(157-707) may result from loss of pUL97 function due to the observed disruption of normal intracellular localization and/or protein-protein interactions. Isoform M157 exhibited reduced interaction with at least two known pUL97 interacting partners, pp65 and pUL44. While the pp65 interaction domain has not yet been defined, the pUL44 interaction domain has been mapped to codons 366-
Since this domain is present in isoform M157, the impaired interaction with pUL44 most likely results from abnormal subcellular localization during infection (Figure 4). As pp65 is nonessential for efficient HCMV replication in HFFs (55), it is unlikely that loss of phosphorylation of this substrate explains the observed growth defect. It remains to be determined whether phosphorylation of pUL44 (26) and other pUL97 substrates such as pUL69 (28) are affected.

MBV resistance has emerged in HCMV isolates from treated individuals, involving mutations mapping to the ATP binding region of pUL97 (codons 409 and 411) (56, 57), consistent with the action of MBV as an ATP-competitive kinase inhibitor (58). Additional mutations in this vicinity that confer MBV resistance have been observed in cell culture (15, 19, 38, 59). All of these mutations confer significant MBV resistance while retaining near-normal viral growth and kinase activity sufficient for GCV phosphorylation and susceptibility. Deletion of the amino terminal residues of pUL97 would not appear to involve the ATP-competitive MBV binding site. The suppression of Rb phosphorylation by MBV during infection with HCMV UL97(157-707) at concentrations well below the EC50 for this virus, and comparable to the EC50 for a strain expressing full length pUL97 (Figures 6C), provides indirect evidence that MBV inhibits the kinase activity of isoform M157. Rather, it is likely that isoform M157 lacks some critical function of full-length pUL97 that results in MBV resistance because a greater fraction of the remaining viral growth would then be independent of the pUL97 kinase activity that is inhibited by MBV. This isoform could have nonkinase biological activities as previously described for pUL97 (32).

The finding of multiple pUL97 isoforms with differential kinase and growth
properties adds to the biological complexity of this antiviral drug target. A variable
degree of HCMV growth that can occur without normal pUL97 kinase activity may have
influenced the results of the two Phase III MBV prophylaxis trials. As additional Phase II
trials of MBV are ongoing (ClinicalTrials.gov Identifier NCT01611974 and EudraCT
number 2010-024247-32), a more detailed understanding of the pUL97 isoform-specific
interactions, substrates, and functional overlap with cellular enzymes and pathways is
needed to guide the development of antiviral compounds targeting pUL97.

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FIGURE LEGENDS

FIG 1. pUL97 isoform translation during HCMV infection.
HFFs were infected with wt AD169-derived HCMV or recombinant strains in which the indicated ATG codons were replaced with ones encoding leucine (L) or a stop codon (*). pUL97 isoforms were detected by immunoblots of infected cell lysates using (A) a rabbit polyclonal antibody (pAb-Ulm) or (B and C) a pUL97 mouse monoclonal antibody (mAb) (Alabama). Arrows 1, 2 and 3 indicate bands corresponding to isoforms M1, M74 and M157.

FIG 2. pUL97 isoform translation during infection with clinical isolates.
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(A and B) HEL cells or (C) HFFs were infected with wt or recombinant HCMV strains with indicated genotypes. All infections were at calibrated MOI of 0.01-0.02. Supernatant SEAP activity at days 1 and 4-7 or 8 post-infection was used to assess relative growth.
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**FIG 5.** Interaction of pUL97 isoforms with pUL44 and pp65. HFFs were infected with HFFs were infected with AD169 or recombinant HCMVs expressing individual pUL97 isoforms. Using anti-pUL97 polyclonal antiserum (pAb-Boston), pUL97 was precipitated from cell lysates followed by immunoblot analysis for pUL97 (pAb-Ulm), pUL44, and pp65. Expression controls consisted of immunoblots of cell lysates before immunoprecipitation.

**FIG 6.** Phosphorylation of pUL97 substrates in vitro and during viral infection. (A) An in vitro kinase assay on proteins immunoprecipitated from infected cell lysates by pUL97 antibody. Autophosphorylation of pUL97 (arrows) and phosphorylation of co-immunoprecipitated pp65 was assessed by autoradiography (upper panel). Expression
and precipitation control immunoblot panels show pUL97 and pp65 before and after immunoprecipitation.

(B) In vitro phosphorylation of human histone mixture H1–H4 (Roche) by pUL97 immunoprecipitated from HCMV-infected cells, as shown by autoradiography.

(C) HFFs were cultured in media containing 0.1% FBS for 24 hours prior to infection with HCMV strains (MOI of 1-3) expressing wt pUL97 or pUL97(157-707) in the presence or absence of MBV at the indicated concentrations. After infection, cells were maintained under serum starvation until protein lysates were obtained at 24 hpi. Rb phosphorylation was assessed by differential mobility on SDS-PAGE.
Table 1. Oligonucleotide sequences

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV 3’</td>
<td>GAGCAGACTCTCAGAGGATCGG</td>
</tr>
<tr>
<td>CMV 5’</td>
<td>AAGCGGCCTCTGTATAACCAAG</td>
</tr>
<tr>
<td>CMV MIE FAM/TAMRA</td>
<td>CATGCAGATCTCCTCAATGCGGCG</td>
</tr>
<tr>
<td>5-aphAI/UL97-PstI</td>
<td>TAGCTGCAGAAGCTGTCACTGTGAGCCCGACGGCGCGGTTTCCCCC</td>
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<td></td>
<td>GTAGCGCCGGCTACGTAGGGATAACGGGTAATCGATT</td>
</tr>
<tr>
<td>3-aphAI-PstI</td>
<td>TAGCTGCAGGCAGTGTATACCAAGGACCTGCTGCGG</td>
</tr>
<tr>
<td>5-ΔUL97</td>
<td>CGGTGTGATAGCTAGCTGAGCCTTGAACGGGAGACTGTCG</td>
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<tr>
<td></td>
<td>CCACTTACGGATAACGGGTAATCGATT</td>
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<tr>
<td>3-ΔUL97</td>
<td>ACCTTCTCTGTGGCCCTTTCCCTCAGCAACCAGTGCTCCGCGTGTC</td>
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<td>CCGGAGTGGCGACAGTGCTTCCCTGCTAGGGCTGACATGCTA</td>
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<td></td>
<td>CCACACCGAGCCAGTGGATAACCAACCCAGGTAATCGATT</td>
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<tr>
<td>UL97.140304T</td>
<td>TGTTGGAGACCTGGTTTCCCGG</td>
</tr>
<tr>
<td>UL97.142644B</td>
<td>CCTTTGCCCTCAGCAACCGTC</td>
</tr>
<tr>
<td>UL97.140341T</td>
<td>CTGGAGGTGACGACGCGTGTC</td>
</tr>
<tr>
<td>UL97.142418B</td>
<td>GGGCACACGGAGCATCTTGG</td>
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Table 2. Analysis of the N-terminal sequence of UL97 from clinical HCMV isolates

<table>
<thead>
<tr>
<th>HCMV source</th>
<th>Specimen isolation year</th>
<th>ATG (M1/38/74/111/157) conservation</th>
<th>NLS1 / NLS2 conservation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD169</td>
<td>-</td>
<td>✓</td>
<td>✓ / ✓</td>
</tr>
<tr>
<td>Merlin</td>
<td>-</td>
<td>✓</td>
<td>✓ / ✓</td>
</tr>
<tr>
<td>TB40E</td>
<td>-</td>
<td>✓</td>
<td>✓ / ✓</td>
</tr>
<tr>
<td>Towne</td>
<td>-</td>
<td>✓</td>
<td>Q19E / ✓</td>
</tr>
<tr>
<td>FIX</td>
<td>-</td>
<td>✓</td>
<td>✓ / ✓</td>
</tr>
<tr>
<td>Clinical isolates from Australia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27 isolates</td>
<td>1991-1996</td>
<td>✓</td>
<td>✓ / ✓</td>
</tr>
<tr>
<td>4 isolates</td>
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<td>Q19E / ✓</td>
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<tr>
<td>Clinical isolates from Europe</td>
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<tr>
<td>10 isolates</td>
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<td>✓</td>
<td>✓ / ✓</td>
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<tr>
<td>1 isolate</td>
<td>2012</td>
<td>✓</td>
<td>Q19E / ✓</td>
</tr>
</tbody>
</table>

*Amino acid substitution indicated where noted, otherwise no changes*
Table 3. Genotypes and Phenotypes of Recombinant Viruses

<table>
<thead>
<tr>
<th>BAC</th>
<th>Virus</th>
<th>UL97 Genotype</th>
<th>Ganciclovir</th>
<th>Maribavir</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>EC50^3</td>
<td>SD^4</td>
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<tr>
<td><strong>Controls</strong></td>
<td></td>
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<td></td>
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<tr>
<td>BA29</td>
<td>T3261</td>
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<td>BA27</td>
<td>T3259</td>
<td>C592G</td>
<td>3.07</td>
<td>0.49</td>
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<td>T3679</td>
<td>V353A</td>
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<tr>
<td>BA109</td>
<td>T3418</td>
<td>ΔK355</td>
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<td>7.80</td>
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<tr>
<td><strong>UL97 isoform mutants</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>BA235</td>
<td>T3820</td>
<td>M1L</td>
<td>1.26</td>
<td>0.34</td>
</tr>
<tr>
<td>BA234</td>
<td>T3819</td>
<td>M74L</td>
<td>1.17</td>
<td>0.29</td>
</tr>
<tr>
<td>BA270</td>
<td>T3947</td>
<td>M157L</td>
<td>1.42</td>
<td>0.43</td>
</tr>
<tr>
<td>BA299</td>
<td>T4045</td>
<td>157-707</td>
<td>0.88</td>
<td>0.23</td>
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<tr>
<td>BA296</td>
<td>T4047</td>
<td>M74L/M157L</td>
<td>1.62</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Table footnotes:
**bold** Items associated with decreased drug susceptibility

- **BAC**: HCMV strain AD169-derived bacterial artificial chromosome clone name
- **1**: Recombinant HCMV strain derived from BAC clone (serial number)
- **2**: Amino acid substitution or codon range in BAC and recombinant virus
- **3**: Mean drug concentration (µM) required to reduce SEAP growth by 50%
- **4**: Standard deviation of the EC50 values
- **5**: Number of assays (performed over at least 4 separate dates)
- **6**: Ratio of EC50 to baseline strain
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