Cytomegalovirus UL97 Kinase Catalytic Domain Mutations That Confer Multidrug Resistance

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Human cytomegalovirus UL97 kinase mutations that commonly confer ganciclovir resistance cluster in different parts of the gene than those conferring resistance to maribavir, an experimental UL97 kinase inhibitor. The drug resistance, growth, and autophosphorylation phenotypes of several unusual UL97 mutations in the kinase catalytic domain were characterized. Mutations V466G and P521L, described in clinical specimens from ganciclovir-treated subjects, conferred a UL97 kinase knockout phenotype with no autophosphorylation, a severe growth defect, and high-level ganciclovir, cyclopropavir, and maribavir resistance, similar to mutations at the catalytic lysine residue K355. Mutations F342S and V356G, observed after propagation under cyclopropavir in vitro, showed much less growth attenuation and moderate- to high-level resistance to all three drugs while maintaining UL97 autophosphorylation competence and normal cytopathic effect in cell culture, a novel phenotype. F342S is located in the ATP-binding P-loop and is homologous to a c-Abl kinase mutation conferring resistance to imatinib. UL97 mutants with relatively preserved growth fitness and multidrug resistance are of greater concern in antiviral therapy than the severely growth-impaired UL97 knockout mutants. Current diagnostic genotyping assays are unlikely to detect F342S and V356G, and the frequency of their appearance in clinical specimens remains undefined.

Ganciclovir (GCV) and its oral prodrug valganciclovir are currently used as first-line antiviral therapy for human cytomegalovirus (CMV) infection (1). Maribavir (MBV) remains experimental after phase III clinical trials showed low toxicity but inadequate antiviral efficacy at the dose tested (2,3). Additionally, methyleneacyclophosphate nucleoside analogs, such as cycloprocavir (CPV), are being investigated for clinical use (4,5). The CMV UL97 kinase (pUL97) has important roles in the initial phosphorylation of GCV and CPV that is essential for the antiviral activity of these drugs and as a direct antiviral target for the kinase inhibitor MBV, since UL97 shutoff results in severe viral growth impairment in vitro (6).

Genetic mechanisms of resistance to these drugs have been investigated with particular attention to cross-resistance, as it affects combined or sequential antiviral drug usage in cases of treatment failure. So far, UL97 mutations preferentially selected under GCV and MBV in vitro and in vivo are distinct and confer no cross-resistance (7,8), while partial GCV-CPV cross-resistance exists (9,10). Seven canonical UL97 mutations (M460V/I, H520Q, C592G, A594V, L595S, and G603W) are involved in >80% of reported GCV-resistant clinical isolates, of which M460I and H520Q confer considerable CPV cross-resistance (9,10). Less-common GCV resistance mutations occur at codons 590 to 607, and rare ones outside this range have been reported in pediatric clinical specimens, such as V466G (11), which conferred a slow-growth phenotype and GCV resistance (12), or P521L, which was suspected but not proven to cause GCV resistance (13). MBV resistance mutations that have no major impact on viral growth have been identified at codons 337, 353, 397, 409, and 411 in the kinase ATP-binding domain (7,14,15).

The objective of this study was to characterize the drug resistance, growth, and autophosphorylation phenotypes of the following noncanonical UL97 mutations encountered in different settings: a novel UL97 mutation F342S, located in the conserved kinase ATP binding P-loop and selected in vitro under CPV, mutation V356G, reported as a minority subpopulation after in vitro exposure to CPV (16), and mutations V466G and P521L, reported in clinical CMV sequences (11–13). Autophosphorylation assays were used to assess biological UL97 kinase activity, which affects viral growth competence (6,17–19).

MATERIALS AND METHODS

Antiviral compounds. CPV and MBV were supplied by Microbiotix and ViroPharma, respectively, and dissolved as 10 mM stock solutions in dimethyl sulfoxide, from which aqueous final dilutions were made. GCV sodium salt (Cytovene, Roche) was used as aqueous solutions from licensed pharmaceutical material.

Viral strains, clones, and mutants. CMV strains were derived from laboratory strain AD169 modified with a secreted alkaline phosphatase (SEAP) reporter gene at US3 (strain T2211) and subsequently cloned as a bacterial artificial chromosome (BAC) (20). Derivative BAC-cloned strains include T3261 (a baseline strain containing an inert 34-bp Frt motif upstream from UL97) (20). The BAC clone BA9 (derived from BA1 by replacing a large part of UL97 with a bacterial gIIIk cassette) was used to derive additional UL97 mutant BACs by recombineering, as described previously (20), with the desired DNA sequence contained in a transfer vector which also carried a kanamycin resistance selection marker that was subsequently removed by induction of Flp recombinase. To facilitate in vitro analyses of the mutant UL97 kinases, additional BACs were constructed that contained a short sequence (5’-TACCCATATGACGTCCC TGATTACC-GTATCCCATATGACGTCCCTGATTACC-GTGCTCA TTAGGCTCCCTGATTACGGC-3’) encoding 3 tandem copies of the influenza hemagglutinin epitope tag (3XHA) in frame with the UL97 gene sequence.

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sequence between codons 2 and 3. Strains with mutations at codon 355 that render the expressed kinase inactive (K355M or K355del, where the entire codon was deleted in frame) (6) were used as controls for growth and autophosphorylation studies.

Newly constructed BACs were examined for correct HindIII restriction digest patterns at each stage. BACs were transfected into human foreskin fibroblast (HFF) cultures to reconstitute live CMV, which was subsequently propagated in HFFs under standard conditions (20). New recombinant viruses were sequenced throughout UL97 to verify their intended genotype. In vitro propagation of the error-prone exonuclease mutant T2294 (containing UL54 pol mutation D413A) under CPV to accelerate the discovery of new resistance mutations was performed as described previously (9).

**Viral growth and drug susceptibility assays.** Cell culture supernatant SEAP activity assayed with a chemiluminescent substrate was used to assay viral growth and drug susceptibility (20). A low-multiplicity inoculum, calibrated by assaying the SEAP activity at 24 h postinfection, was used in multicycle growth curve comparisons at days 4 to 8 in HFF and human embryonic lung (HEL) fibroblast cultures. The drug concentration required to reduce supernatant SEAP activity by 50% (50% effective concentration [EC50]) was used to assess viral susceptibility (20). A low-multiplicity inoculum, calibrated by assaying the SEAP activity at 24 h postinfection, was used in multicycle growth curve comparisons at days 4 to 8 in HFF and HEL fibroblast cultures. The drug concentration required to reduce supernatant SEAP activity by 50% (50% effective concentration [EC50]) was used to assess viral susceptibility (20).

**UL97 kinase autophosphorylation assays.** pUL97 autophosphorylation was assayed in vitro after immunoprecipitation from infected-cell extracts (17–19, 22). UL97 epitope-tagged (3 × HA) strains were used to infect HFF cultures at high multiplicity where feasible and extracted at 5 to 7 days postinfection. Strains with the UL97 knockout phenotype were passaged as infected cells until the characteristic cytopathic effect (CPE) appeared normal and moderate viral growth under CPV to generate live mutant CMV strains. BAC mutants representing different genotypes were constructed and transfected into HFF to allow for weekly passaging of the virus by transfer of ~30% of cells to a confluent uninfected HFF monolayer.

**RESULTS**

**Selection of UL97 mutation F342S under CPV.** We reported the preferential selection of UL97 mutations M460I and H520Q under CPV previously (9). Under the same conditions, a single selection experiment (M110) resulted in the selection by passage 8 (under 4 μM CPV) of a subpopulation of M460I, but by passage 10 (under 8 μM CPV), it had been replaced by an estimated 40% sequence subpopulation of F342S and became the only UL97 genotype detected at passages 15 (under 20 μM CPV), 18, and 19 (under 25 μM CPV). During this time, the viral cytopathic effect (CPE) appeared normal and moderate viral growth under CPV allowed for weekly passaging of the virus by transfer of ~30% of cells to a confluent uninfected HFF monolayer.

**Construction of recombinant viruses.** To compare the phenotypes conferred by specific mutations, corresponding recombinant BAC clones were constructed and transfected into HFF to generate live mutant CMV strains. BAC mutants representing V356G and F342S yielded progeny virus with normal CPE and culture characteristics, whereas mutants V466G and P521L displayed the abnormal type 2 CPE typical of UL97-deficient strains, such as the K355M and K355del mutants (7, 23). Cell-free stocks were made and used for reporter-based growth and drug susceptibility assays.

**Growth characteristics.** Multicycle reporter-based growth assays (Fig. 1) showed the F342S mutant to have growth similar to that of the matching wild-type strain in HFF cultures and to be more sensitive in HEL fibroblasts but ultimately approach the same level of growth as the wild type. The differences in the HFF and HEL growth curves illustrate the greater sensitivity of HEL cells in showing a growth impact of decreased UL97 kinase function (24). The V356G mutant was more growth-attenuated than the F342S mutant but grew better than the V466G and P521L.
TABLE 1 Genotypes and phenotypes of recombinant viruses

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<tr>
<th>BAC clone</th>
<th>Virusb</th>
<th>UL97 mutationc</th>
<th>Ganciclovir</th>
<th>入学号</th>
<th>SD</th>
<th>No. of assays</th>
<th>Ratio</th>
<th>Cytochrome</th>
<th>No. of assays</th>
<th>Ratio</th>
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UL97 mutants

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<th>No. of assays</th>
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a Data in boldface are associated with decreased drug susceptibility.

b Serial number of recombinant live CMV strain derived by transfection of BAC into fibroblasts.

c Mutation introduced into BAC and recombinant virus.

d Mean drug concentration (μM) required to reduce SEAP growth by 50% at 6 to 7 days postinfection.

e Standard deviation of the EC50 values.

f Assays were performed over at least 3 separate dates.

g Ratio of EC50 to that of baseline strain (3261).

h Independent BAC clone and derived virus containing the same P521L mutation as BA24.

DISCUSSION

The CMV UL97 P-loop F342S mutant, selected under CPV, showed significant multidrug resistance while maintaining relatively normal viral growth and autophosphorylation activity, in contrast to other multidrug-resistant mutants, such as those with the K355M/del, V466G, and P521L mutations, which severely impaired UL97 kinase autophosphorylation and viral growth. Another mutation, V356G, reported as a minor sequence subpopulation with and without exposure to CPV, also conferred multidrug resistance with autophosphorylation competence and moderate growth retardation. The newly characterized mutations F342S and V356G are distinctive in their location next to critical functional pUL97 residues. Their unexpected finding in vitro raises concern that they may occasionally emerge in clinical isolates and escape detection by routine genotyping assays. The levels of drug resistance conferred by these mutations are equivalent to those resulting from mutations commonly associated with the respective drugs (8, 9, 15).

FIG 2 In vitro autophosphorylation assay. In vitro kinase assays used [γ-32P]ATP and immunoprecipitated pUL97 from control and mutant UL97 strains. (A) Phosphorimages of radiolabeled proteins. (B) Corresponding HA-immunoprecipitated pUL97 in the same blot as the phosphorimage. The HA-tagged full-length pUL97 corresponds to the upper band in the phosphorimage lanes. The lower, non-HA-tagged radioactive band likely represents matrix protein pp65 that coimmunoprecipitated with and was phosphorylated by pUL97. The position of the 82-kDa molecular mass marker is shown. Lanes: 1, K355M mutant; 2, V466G mutant; 3, P521L mutant; 4, F342S mutant; 5, V356G mutant; 6, wild type; 7, wild type plus 5 μM MBV.
The mutation F342S is adjacent to one of the 3 glycine residues (338, 340, and 343) that together define the conserved motif of the kinase ATP-binding P-loop (Hanks subdomain I) (25). F342S is positionally homologous (Fig. 3) to the c-Abi kinase mutation Y253F/H that arises frequently in vivo after imatinib exposure to confer cross-resistance to multiple related kinase inhibitors (26, 27). The mutation is postulated to modify the flexibility of the P-loop and the conformation required for inhibitor binding (27). A similar effect of F342S would be plausible for resistance to maribavir, which is an ATP-competitive pUL97 inhibitor (28). The significant CPV and GCV resistance of the F342S mutant suggest that the kinase P-loop can also be involved in the binding of these drugs, thus offering a mechanism of viral cross-resistance to substrates, as well as inhibitors of pUL97, that has little impact on viral growth.

The mutation V356G is adjacent to the conserved catalytic K355 residue that is conventionally disrupted to create inactive kinase control mutants (6, 17). Despite this proximity, the V356G mutant retained substantial kinase activity and a normal cytopathic appearance yet conferred multidrug resistance. It was, however, more growth impaired than the F342S mutant. The V356G mutation has been detected only as a subpopulation of less than 10% by deep sequencing both in baseline strain AD169 cultures and in virus passaged under CPV (16). Its prospective role in CMV drug resistance remains unclear for this reason.

Isolated reports of V466G and P521L in clinical specimens (11, 13) are surprising given their UL97 knockout phenotype. Extensive diagnostic genotyping literature reveals no other case reports of these mutations (8, 29, 30). Although such mutants are inherently resistant to all antivirals requiring or inhibiting the action of pUL97, their selection is unexpected since comparable levels of drug resistance are conferred by mutations with far less damaging effects on viral growth (7). While it may be argued that the UL97 knockouts could be less attenuated in vivo than in vitro, their detection may also be a technical artifact of the genotyping procedure. V466G was reported as a mixed mutant−wild-type sequence in one of two specimens from the same subject that otherwise contained the same well-known UL97 resistance mutations (11). Similarly, P521L was detected in only one specimen of a series from the same individual, without information about admixture with the wild type (13). In neither case was the atypical mutation described as confirmed on independent reanalysis of the original or serial specimens, nor was it found in a matching culture isolate. Considering other recent cases of clinical specimens reported as containing UL54 DNA polymerase mutations that confer a non-viable phenotype (31, 32), more-stringent validation is needed of the authenticity of unusual mutations found during diagnostic genotyping.

Clinical implications of such mutations as F342S and V356G arise from the possibility of their underdetection, along with associated multidrug resistance not previously suspected. These are now the furthest-upstream UL97 mutations known to confer definite GCV resistance with relatively preserved growth competence. Current diagnostic genotyping, including methods used in randomized valganciclovir clinical trials, seldom covers UL97 codons below positions 360 to 400 (11, 30, 33), thus leaving the above-described mutations undetected if present. Expanded UL97 sequencing of specimens from subjects receiving prolonged antiviral therapy is advisable.

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REFERENCES


