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Human Cytomegalovirus UL97 Kinase Confers Ganciclovir Susceptibility to Recombinant Vaccinia Virus

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We analyzed whether the phosphotransferase encoded by the UL97 open reading frame of human cytomegalovirus (HCMV) alone is sufficient to confer ganciclovir (GCV) susceptibility to a foreign virus. Two vaccinia virus recombinants (T1 and A5) containing the UL97 open reading frames from a GCV-sensitive HCMV and from a GCV-resistant strain were constructed. T1 exhibited a GCV-sensitive phenotype in plaque reduction assays, whereas A5 did not. Moreover, T1-infected cell cultures showed a strongly increased incorporation of [^{14}C]GCV triphosphate into macromolecular DNA, compared with recombinant A5 or vaccinia virus controls, which could be inhibited by the addition of guanosine. This shows that UL97 kinase is the only additional gene product required to make vaccinia virus susceptible to GCV, and guanosine seems to be one natural substrate for the enzyme. The system described here should be very helpful for fast and detailed functional analyses of UL97 mutations found in GCV-resistant HCMV isolates.

Human cytomegalovirus (HCMV) is a major pathogen in recipients of bone marrow and solid-organ transplants and patients with AIDS (10, 12, 19). HCMV replication is inhibited by the nucleoside analog ganciclovir (GCV). GCV alone, or in combination with immunoglobulins, has shown benefits in transplant recipients (14, 15, 26, 33) and, together with foscarnet, is presently the drug of choice for the treatment of severe HCMV manifestations. The failure of GCV therapy of long-term-treated patients with underlying AIDS and also GCV resistance of HCMV strains in vitro have been reported (2, 11, 13, 28, 31).

The mechanism of GCV action depends on phosphorylation and formation of the nucleoside triphosphate (GCVTP), which results in the inhibition of the viral DNA polymerase and chain termination (15). Biron et al. described the metabolic activation of GCV in human diploid fibroblasts infected with HCMV (3). More recent evidence indicates that GCV is phosphorylated by an 80-kDa protein kinase homolog encoded by the HCMV UL97 open reading frame (ORF), and phosphorylation of GCV was shown after expression of the UL97 coding region in a bacterial system (17, 23). An altered UL97 ORF has been described by Sullivan and coworkers after selection of GCV-resistant HCMV in vitro (30). This largely GCV-resistant strain contained a 4-amino-acid deletion (amino acids 638 to 641; amino acid sequence AACR) within a region of the polypeptide conserved among protein kinases (16). In cotransfection and recombination experiments, the GCV resistance could be transferred to GCV-sensitive AD169 laboratory strains (24, 30). However, definite proof that GCV sensitivity can be due only to the UL97 kinase is still lacking and with these methods, the possible selection of preexisting GCV-resistant mutants from the AD169 population during the recombination or cotransfection procedures cannot be ruled out. Furthermore, production of HCMV recombinants is laborious, which limits the usefulness of this method for the examination of the UL97 kinase of GCV-resistant HCMV isolates. Recombination with AD169 may also result in chimeric viruses with altered replication efficiencies, making quantitative studies of drug resistance and other biological functions difficult. In addition, Sullivan et al. (29) and Lurain et al. (25) found their GCV-resistant HCMV strains to be double mutants with an additionally altered DNA polymerase gene. We analyzed HCMV UL97 functions after transfer of the coding region into naturally GCV-resistant vaccinia virus, which enabled us to investigate its effects in the absence of other HCMV gene products.

Cloning strategy. Firstly, we constructed a vaccinia virus recombinant expressing the UL97 kinase of the HCMV laboratory strain AD169. The UL97 coding sequence was inserted into the multiple cloning site of plasmid p7.5K131, a standard vaccinia virus expression vector, downstream from the potent 7.5K early/late promoter. Cloning of the whole UL97 coding region (Fig. 1A) was accomplished by the separate PCR amplification (30 s at 94°C, 45 s at 65°C, and 1 min at 72°C for 30 cycles) of three fragments, using the following oligonucleotide primers: pri.1-HindIII (5′-ATA AGC TTG GTA GCT AGT GCA GCC TTA G-3′) and pri.2-SalI (5′-GTT ATG CCG TCG ACA TGA GCG-3′) (1,024-bp N-terminal fragment), pri.3-SalI (5′-CGC TCA TGT CGA CGG CAT CAA-3′) and pri.4-KpnI (5′-CGG CGC GCG GTA CCC GTC TCC TG-3′) (518-bp middle fragment), and pri.5-KpnI (5′-CAG GAG ACG GGT ACC GCG CGC CG-3′) and pri.6-EcoRI (5′-AAC TGT TCC CCG AGT AAA TCG ATA GAA TTC CCG-3′) (618-bp C-terminal fragment). By careful design of the primers, several new restriction sites were introduced by silent point mutations which not only allowed the directional cloning of the fragments but also would later permit the exchange of corresponding sequences found in clinical HCMV isolates exhibiting impaired GCV susceptibility. Figure 1B shows the localization of the PCR primers in the UL97 ORF. The restriction enzyme recognition sites *Sal*I and *Kpn*I in the primers pri.2-SalI, pri.3-SalI, pri.4-KpnI, and pri.5-KpnI each introduce one silent mutation. The primer pri.1-HindIII contains an *Hin*dIII recognition site in addition to UL97 sequences, and pri.6-EcoRI contains an *Eco*RI tail. Both extra sequences are located outside of the UL97 coding region. The resulting clone (p7.5K-UL97) was used for recombination.

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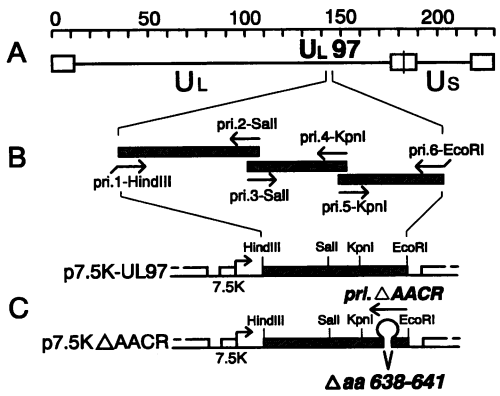

FIG. 1. Cloning strategy of HCMV UL97 coding region. (A) Schematic genomic organization of the HCMV genome with terminal and inverted repeats (open boxes) and the unique long (U_L) and unique short (U_S) regions. The scale is marked in kilobase pairs. (B) Overlapping fragments of the UL97 ORF were amplified by using primers with restriction endonuclease recognition sites for subsequent cloning into the vaccinia virus expression vector p7.5K131. (C) Expression vector p7.5K-UL97 containing the UL97 cistron driven by the 7.5K early/late promoter. p7.5KΔAACR containing the 12-bp deletion (deletion of amino acids [aa] 638 to 641) within the UL97 coding region generated by in vitro mutagenesis with the oligonucleotide primer pri.ΔAACR.

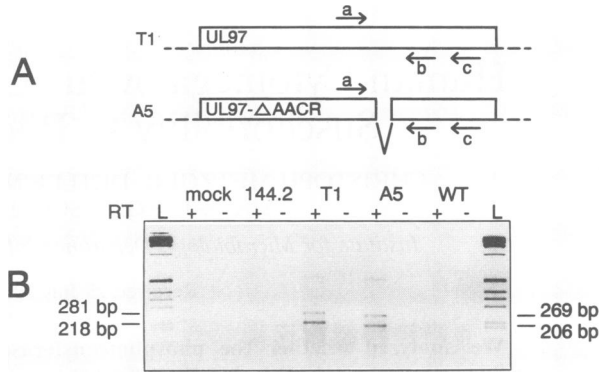

FIG. 2. RT-PCR amplification of 3′ fragments from UL97 mRNA. (A) Schematic map showing the positions of the oligonucleotide UL97-1903.minus (c) used for specific reverse transcription and the PCR primers UL97-1640.plus (a) and UL97-1840.minus (b) spanning the deleted region in the UL97 cistron. (B) Ethidium bromide-stained gel showing the 281-bp fragment in the sample T1+ (containing the UL97 coding region from wild-type AD169) which is produced by the amplification between UL97-1640.plus and the RT primer UL97-1903.minus (primers a and c). The 218-bp fragment is amplified by the two PCR primers (a and b). Amplification of cDNA from mutant A5 (containing the UL97 cistron with the 12-bp deletion) resulted in slightly smaller fragments of 206 and 269 bp, respectively. How the fragment with a length of about 500 bp was generated is unclear. mock, uninfected cells; 144.2, thymidine kinase-deficient vaccinia virus recombinant; WT, wild-type vaccinia virus (Copenhagen); +, with reverse transcription (RT) or −, without RT of extracted total RNA; L, DNA size marker (1 kb; Bethesda Research Laboratories).

For generation of the 12-bp deletion mutant (30), site-directed mutagenesis following a protocol published by Landt et al. was performed (22). This method requires one mutagenizing primer (12-bp deletion primer pri.ΔAACR [5′-GTG AGC TTA CCG TTC AAC GCG CCG GCG TGC TTA AAG AGC AAC GCC-3′]) and a pair of normal primers (pri.5-KpnI and pri.6-EcoRI). Briefly, a double-stranded DNA fragment obtained in a first PCR (amplified between pri.5-KpnI and pri.ΔAACR) was used together with the oligonucleotide pri.6-EcoRI to prime a second PCR. The resulting fragment that carries the 12-bp deletion was cut with *Eco*RI-*Kpn*I and recloned into p7.5K-UL97. Thereby, the C-terminal UL97 fragment of p7.5K-UL97 was replaced by the mutagenized cassette. In the resulting plasmid (p7.5KΔAACR), the complete sequence of the recloned fragment was determined to exclude the possibility of adventitious mutations. The vaccinia virus expression vectors thus generated were then used to produce vaccinia virus recombinants by insertion of the UL97 ORF into the thymidine kinase gene of vaccinia virus wild-type strain Copenhagen, according to standard protocols (8, 20). Recombinants obtained after the second round of selection were plaque purified, and insertions of UL97 sequences were confirmed by PCR analysis.

UL97 transcription in vaccinia virus-infected cells. After homologous recombination and subsequent selection, two recombinant vaccinia virus clones were isolated. Recombinant T1 contains the wild-type UL97 coding region, whereas recombinant A5 expresses the UL97 cistron with the 12-bp deletion. To verify that the foreign sequences were properly transcribed, a thymidine kinase-deficient human osteosarcoma cell line (143B) was infected with recombinants T1 and A5 as well as with wild-type vaccinia virus and another unrelated recombinant (144.2) which expresses a hepatitis B virus sequence. Since there are currently no antisera available to us which can be used to detect the UL97 protein, we analyzed RNA synthesis. Total RNA was isolated from infected and noninfected cells by guanidinium thiocyanate extraction (6). DNA contaminations were eliminated by DNase I treatment for 30 min at 37°C followed by phenol-chloroform extraction and ethanol precipitation before cDNA synthesis. To demonstrate expression of the UL97 coding region in T1- and A5-infected cells, cDNA was synthesized from the RNAs of T1-, A5-, 144.2-, wild-type-, and mock-infected cells with a specific antisense primer (UL97-1903.minus [5′-CAG CGT GTG CGA CAC CAG-3′]) by using Moloney murine leukemia virus reverse transcriptase (RT; Pharmacia). Subsequent RT PCR (30 cycles of 30 s at 94°C, 45 s at 58°C, and 50 s at 72°C) was performed with the primers UL97-1640.plus (5′-TTA TTG CAT GTC GGA GCT G-3′) and UL97-1840.minus (5′-AGG CGC CGT AGC TCA TTT G-3′), which amplify a 218-bp (wild-type UL97 cistron) fragment and a 206-bp (AACR deletion) fragment, respectively. To test for DNA contaminations, all RNA samples were also subjected to PCR amplification as described above without prior reverse transcription. Figure 2B shows that no amplified sequences were observed in the cDNA samples from noninfected cells or from cells infected with wild-type vaccinia virus or the unrelated recombinant 144.2. In samples from the T1- and A5-infected cells, three bands, which are due to RNA since they could only be observed after reverse transcription, were detected. The 281-bp fragment in the sample T1+ is due to an amplification between the 5′ PCR primer and the primer used for reverse transcription (UL97-1903.minus [Fig. 2, primer c]). The 218-bp fragment is amplified by the two PCR primers (UL97-1640.plus [primer a] and UL97-1840.minus [primer b]). In the case of the mutant A5, both fragments are slightly smaller than for the other samples. This was expected, since the mutant expresses the UL97 ORF with the 12-bp deletion. How the fragment with a length of about 500 bp was generated is unclear.

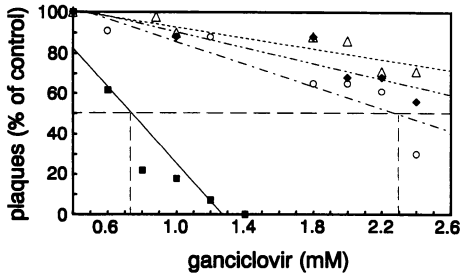

FIG. 3. Linear regression analysis of vaccinia virus plaque reduction assays. The IC_{50} (shown by long dashes) for the UL97 kinase-expressing recombinant T1 (0.7 mM) (■) is significantly lower than those for the other viruses. For the two recombinants A5 (△) and 144.2 (○) and wild-type vaccinia virus (◆), a slight reduction of plaque formation compared with untreated controls was observed. The required GCV concentrations in these cases were near the cytotoxic concentration. Therefore, the IC_{50} for recombinant A5 and wild-type vaccinia virus could not be determined (all values shown in the figure are the means of three independent experiments, each performed in duplicate).

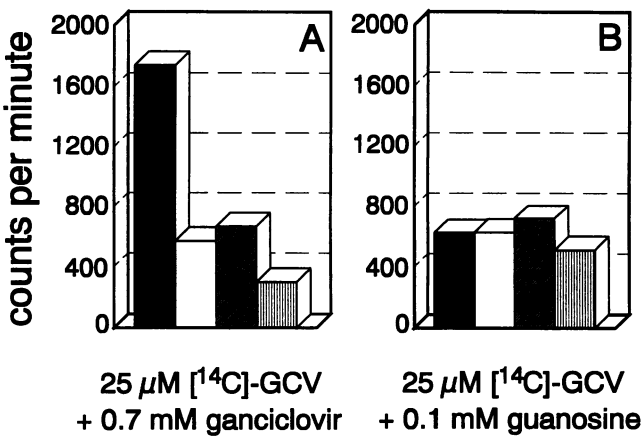

FIG. 4. Competition of [^{14}C]GCVTP incorporation into high-molecular-weight DNA. (A) Trichloroacetic acid-precipitable counts in the T1-infected culture (■), illustrating the incorporation of [^{14}C]GCVTP, were significantly higher than in A5- (▭), 144.2- (▨), and mock-infected (▩) cells. (B) Incorporation of [^{14}C]GCVTP in T1-infected cultures was inhibited by the addition of exogenous guanosine (all values shown in the figure are the means of two experiments).

Analysis of GCV susceptibility. To test whether introduction of the HCMV UL97 ORF resulted in a new vaccinia virus phenotype with respect to GCV sensitivity, plaque reduction assays were performed with 143B cells. The cells were grown in minimal essential medium (supplemented with 10% fetal bovine serum, L-glutamine [0.2 mM], penicillin [0.1 mg/ml], and streptomycin [0.1 mg/ml]) at 37°C in a humidified incubator with 5% CO_2. In pilot experiments, we had found that GCV has no quantifiable effect on the replication of wild-type vaccinia virus, except that plaque sizes tend to be slightly smaller. Moreover, the replication efficiencies of all vaccinia viruses used in this study were found to be equal (data not shown). For plaque reduction assays, the 143B cells were washed and one of the recombinants T1, A5, 144.2, or wild-type vaccinia virus (50 PFU per well) was added in a 0.4-ml total volume of complete minimal essential medium. After an adsorption period of 1 h, GCV-containing medium was added to give final GCV concentrations between 0.1 and 2.6 mM. Two days postinfection, plaques were counted and the 50% inhibitory concentrations (IC_{50}) were calculated by linear regression analysis in comparison with those of untreated controls (the range in all experiments was between 25 and 100 PFU per well). As is clearly demonstrated by the data presented in Fig. 3, UL97 expression had a drastic effect on the GCV susceptibility of the different viruses. The IC_{50} for the recombinant T1 was significantly lower (0.7 mM) than that for the other recombinants. For the two recombinants A5 and 144.2 and wild-type vaccinia virus, a slight reduction of plaque formation was observed, but even in the only case where the IC_{50} could be determined (recombinant 144.2) the GCV concentration required (2.3 mM) was already near the cytotoxic concentration. For this reason, the IC_{50} for recombinant A5 and wild-type vaccinia virus could not be determined. With this system, we were able to prove that the HCMV UL97 kinase is the only additional gene product necessary to confer GCV sensitivity to a virus. In particular, it shows that the HCMV DNA polymerase is not essential for GCV action but may make AD169 more sensitive to GCV (IC_{50} of 0.0025 to 0.035 mM) than are the UL97 vaccinia virus recombinants (29). Consequently, UL97 mutations may be the only cause of GCV resistance. In support of this scenario, we were able to confirm that the 12-bp deletion within a conserved sequence of the UL97 ORF (16, 30) abolishes its GCV phosphorylation activity. These findings do not, of course, exclude the possibility that mutations in other genes besides UL97 (e.g., HCMV DNA polymerase) can also cause GCV resistance, but to date, it can only be speculated to what extent mutations in other genes contribute to a GCV-resistant phenotype. It appears rather likely that the domain affected by the deletion corresponds either to the nucleoside binding site or to the phosphate transfer domain designated subdomain IX (16) or region VI (27) in other kinases. The active center of the herpes simplex virus thymidine kinase was proposed to consist of three functional regions, a nucleotide binding pocket, an ATP-binding site, and a thymidine binding site (7). In the UL97 kinase, such regions are not yet defined. However, there are some conserved residues which are shared with a large number of cellular and viral protein kinases as well as with some bacterial phosphotransferases (1, 4, 5, 9, 16, 18, 21, 32). The system described here should be very helpful to test the influence of other UL97 mutations on phosphorylation of GCV and other nucleoside analogs in further studies. For instance, Lurain and coworkers recently described a mutation in a conserved region of UL97, corresponding to region VI in protein kinases, which resulted in impaired GCV susceptibility in vitro. The mutation, which was found in three independently isolated GCV-resistant mutants of strain AD169, was a single G-to-T transversion at nucleotide 1380 in the UL97 coding sequence which converted an isoleucine into a methionine (4, 5, 16, 27). Finally, it might be of interest to have a GCV-sensitive vaccinia virus vector.

GCV phosphorylation and incorporation. In order to test whether there is a direct correlation between GCV sensitivity and the incorporation of GCVTP into high-molecular-weight DNA during infection, 143B cells were infected at a multiplicity of infection of 30 with the UL97 vaccinia virus recombinants T1 and A5 as well as with the unrelated TK^- recombinant 144.2 in six-well plates. The infected cultures were incubated for approximately one replication cycle (6 h) with 25 μM [^{14}C]GCV together with 0.7 mM (i.e., IC_{50} for T1; see Fig. 3) unlabelled GCV. The cultures were harvested and lysed with

phosphate-buffered saline–1% Triton X-100, and aliquots of the lysate were dotted onto Whatman 3MM paper. High-molecular-weight DNA was precipitated by incubation of the filters for 30 min with ice-cold 10% trichloroacetic acid. The filters were washed with ethanol, and bound radioactivity was determined in a beta counter. As is shown in Fig. 4A, there was a significantly increased incorporation of [^{14}C]GCVTP into high-molecular-weight DNA in cells infected with the T1 recombinant in comparison with incorporation in A5-, 144.2-, and mock-infected cells. This finding is in line with the assumption that the specific antiviral effect of GCV is due to a selective phosphorylation of GCV.

In a second series of experiments, we analyzed whether UL97 can also interact with a normal nucleoside. The 143Btk$^-$ cells were infected as described above, but instead of the nonradioactive GCV, 0.1 mM guanosine was added. As is indicated in Fig. 4B, [^{14}C]GCVTP incorporation in T1-infected cells was completely inhibited by guanosine. Since guanosine inhibited the incorporation of the radioactive GCV, we conclude that guanosine is one natural substrate for the UL97 kinase. Since other authors have shown a direct phosphorylating activity of the UL97 protein, we think that competition between guanosine and GCV is located at the level of initial phosphorylation (17, 23). Whether the UL97 kinase accepts all four nucleosides with the same efficiency is under investigation.

GCV is a fortuitous substrate of the UL97 kinase, and we have the first evidence that the enzyme also phosphorylates acyclovir, as suggested previously by Lurain and coworkers, but at significantly lower levels (24). Some preliminary data also suggest that there might be different catalytic domains for GCV and acyclovir. With this new system, it will be possible to examine substrate recognition for these and other nucleoside analogs in detail. Moreover, because vaccinia viruses replicate much more efficiently in tissue culture than HCMV, it should be extremely helpful to analyze the mechanisms of UL97 mutations and to screen and refine antiviral drugs.

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