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Role of the Human Herpesvirus 6 U69-Encoded Kinase in the Phosphorylation of Ganciclovir

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ABSTRACT

The human herpesvirus 6 (HHV-6) U69 gene product (pU69) is the presumed functional homolog of the human cytomegalovirus (HCMV) UL97-encoded kinase (pUL97), which converts ganciclovir to its monophosphate metabolite in HCMV-infected cells. It has been reported that insertion of U69 into baculovirus confers sensitivity to ganciclovir in insect cells (*J Virol* **73:**3284–3291, 1999). Our metabolic studies in HHV-6–infected human T-lymphoblast cells indicated that the efficiency of ganciclovir phosphorylation induced by HHV-6 was relatively poor. Recombinant vaccinia viruses (rVVs), expressing high levels of pU69 from two HHV-6 strains (representing the A and B variant), were constructed and used to compare the ganciclovir-phosphorylating capacity of pU69 and pUL97 in human cells. Metabolic studies with [8-^{3}H]ganciclovir showed that ganciclovir was phosphorylated in human cells infected with pU69-expressing rVVs, although the levels of phosphorylated ganciclovir metabolites were approximately 10-fold lower than those observed with pUL97. We also demonstrated that pU69, like pUL97, is expressed as a nuclear protein. Our results indicate that the limited phosphorylation of ganciclovir by pU69 may contribute to its modest antiviral activity against HHV-6 in certain cell systems.

HHV-6 is a lymphotropic and neurotropic β-herpesvirus that is closely related to human cytomegalovirus (HCMV) and human herpesvirus 7. It was first isolated in 1986 from the peripheral blood of patients with lymphoproliferative disorders (Salahuddin et al., 1986). HHV-6 exists as two distinct variants (designated A and B), that differ in antigenic properties (Ablashi et al., 1991) and in DNA sequence, with 90% nucleotide homology (Dominguez et al., 1999; Isegawa et al., 1999). Primary HHV-6 infection usually occurs before the age of 2 years and is associated with exanthema subitum (Yamanishi et al., 1988). Exanthema subitum is caused almost exclusively by the B variant; the pathogenic potential of HHV-6A in primary infection remains to be clarified. In adults, the overall seropositivity to HHV-6 is >90% (Dockrell and Paya, 2001). HHV-6 infection in the majority of cases results from the reactivation of latent virus during immunosuppression, as in transplant recipients and in persons infected with HIV. Apart from acting as a transactivator for other viruses in these patients, HHV-6 by itself has been implicated in a wide range of clinical manifestations such as fever, encephalitis, pneumonitis, hepatitis, graft failure, and bone marrow suppression (Singh and Carrigan, 1996; Emery, 2001; Mendez et al., 2001). Moreover, as a result of the complex interactions between β-herpesviruses, HHV-6 may act as a cofactor in cytomegalovirus disease (Dockrell and Paya, 2001). HHV-6 has also been associated with certain lymphomas, chronic fatigue syndrome, and multiple sclerosis, although a causal link with these syndromes is highly debated (Campadelli-Fiume et al., 1999).

The antiherpetic drugs active against HHV-6 are phosphonoformic acid (foscarnet), cidofovir (CDV), and the nucleoside analogs ganciclovir (GCV) and acyclovir, the latter showing only weak activity. Foscarnet has the best in vitro activity against HHV-6 but its long-term use in patients is hampered by serious side effects, mainly nephrotoxicity (MacGregor et al., 1991). Ganciclovir shows good and consistent activity against several HHV-6 clinical isolates in peripheral blood

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ABBREVIATIONS: HHV-6, human herpesvirus 6; pU69, human herpesvirus 6 U69 gene product; HCMV, human cytomegalovirus; pUL97, human cytomegalovirus UL97-encoded kinase; rVV, recombinant vaccinia virus; CBLC, cord blood lymphocyte; EGFP, enhanced green fluorescent protein; CDV, cidofovir; GCV, ganciclovir; FCS, fetal calf serum; PCR, polymerase chain reaction; RT, reverse transcriptase; VV, vaccinia virus; CC_{50}, compound concentration that causes 50% inhibition of cell growth as determined by cell counting; IC_{50}, the compound concentration that produced 50% inhibition of viral DNA replication; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; aa, amino acid; MCMV, murine cytomegalovirus; wtVV, wild-type vaccinia virus.

mononuclear cells (Manichanh et al., 2000), whereas in T-lymphoblastoid cell lines, a much less favorable antiviral activity and selectivity index is observed (Burns and Sandford, 1990; Reymen et al., 1995; Yoshida et al., 1998). Ganciclovir and acyclovir (or their recently approved prodrugs valganciclovir and valacyclovir) are the standard drugs for the treatment and prophylaxis of HCMV infections after transplantation. There is experimental evidence of similarities between HHV-6 and HCMV in their sensitivity to antiviral compounds; however, it is clear that the treatment of HHV-6 infections cannot merely be determined by the clinical experience obtained with HCMV. Clinical data on the efficacy of GCV against HHV-6 are limited to small-scale studies (Zerr et al., 2002).

The activity of ganciclovir is dependent on the conversion to its antivirally active triphosphate form by viral and cellular kinases. The HCMV-encoded kinase responsible for the first phosphorylation step is the protein kinase pUL97, encoded by the HCMV UL97 gene (Littler et al., 1992; Sullivan et al., 1992). In patients, the emergence of ganciclovir-resistant HCMV strains is the consequence of mutations in either the UL97 gene or the DNA polymerase UL54 gene (Baldanti et al., 1996; Smith et al., 1997; Chou et al., 2002). The HHV-6 U69–encoded protein (pU69) is homologous to pUL97 (Fig. 1) and has been reported to confer GCV sensitivity to baculovirus in insect cells (Ansari and Emery, 1999). Both pUL97 and pU69 show homology to other viral and cellular protein kinases (Chee et al., 1989) and to (auto)phosphorylate serine and threonine residues (He et al., 1997; Ansari and Emery, 1999). Recently, Manichanh and coworkers (2001) were the first to describe HHV-6 strains with reduced sensitivity to GCV. They identified a methionine-to-valine substitution at amino acid position 318 in pU69, which corresponds to the M460V/I mutation in pUL97 that is commonly detected in GCV-resistant HCMV strains and is situated in a conserved sequence (domain VIb) that is crucial for pUL97 kinase activity (Michel et al., 1999).

In the present study, we addressed the question of to which extent GCV is phosphorylated by pU69, both in HHV-6–infected cells and in a recombinant vaccinia virus system that was originally developed by Metzger and coworkers (1994) for the study of HCMV pUL97 (Zimmermann et al., 1997). This methodology, based on the expression of pU69 in mammalian cells, allowed us to determine the specific role of the pU69 kinase in the phosphorylation of GCV and to delineate a direct comparison with HCMV pUL97.

Materials and Methods

Antiviral Compounds. GCV (Cymevene) was purchased from Roche Pharmaceuticals (Basel, Switzerland), CDV (Vistide) from Gilead Sciences (Foster City, CA) and foscarnet (Foscavir) from AstraZeneca Pharmaceuticals (Brussels, Belgium). [8-^{3}H]GCV (10–20 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA).

Cells and Viruses. The human T-lymphoblastoid cell lines HSB-2 (American Type Culture Collection, Manassas, VA) and Molt-3 (Advanced Biotechnologies, Columbia, MD) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 0.1% sodium bicarbonate (all from Invitrogen, Carlsbad, CA). Human umbilical cord blood lymphocytes (CBLCs) were isolated by density-gradient centrifugation and cultured in growth medium supplemented with 10 μg/ml phytohemagglutinin-M (Roche Molecular Biochemicals, Mannheim, Germany) and 20 U/ml interleukin 2 (Roche Molecular Biochemicals). After 2 days of stimulation, cells were infected with HHV-6 and further cultured in medium containing 0.2 mg/ml phytohemagglutinin-M and 2 U/ml interleukin 2. Human cytosolic thymidine kinase-deficient osteosarcoma 143B cells and African green monkey CV-1 cells (both from American Type Culture Collection) were grown in Eagle's minimal essential medium containing 10% FCS, 2 mM L-glutamine, 0.75% sodium bicarbonate, and 1 mM sodium pyruvate (all from Invitrogen). The FCS content of the medium was reduced to 2% after cells were infected. All cell cultures were kept in a humidified, CO_2-controlled incubator at 37°C.

Strain GS of HHV-6A (kindly provided by Dr. R. C. Gallo when at the National Institutes of Health, Bethesda, MD) and strain Z29 of HHV-6B (Advanced Biotechnologies, Columbia, MD) were grown in HSB-2 and Molt-3 cells, respectively. Cell-free virus stocks were prepared as follows: HHV-6–infected HSB-2 or Molt-3 cells showing extensive cytopathic effect were concentrated 50-fold by centrifugation and disrupted by three freeze-thawing cycles. The cell debris were then removed by centrifugation (10 min at 350*g*), and the supernatant containing the released virus particles was ultracentrifuged for 2 h at 60,000*g*. The virus, now in pellet form, was finally resuspended in pure FCS, placed into aliquots, and frozen at −80°C.

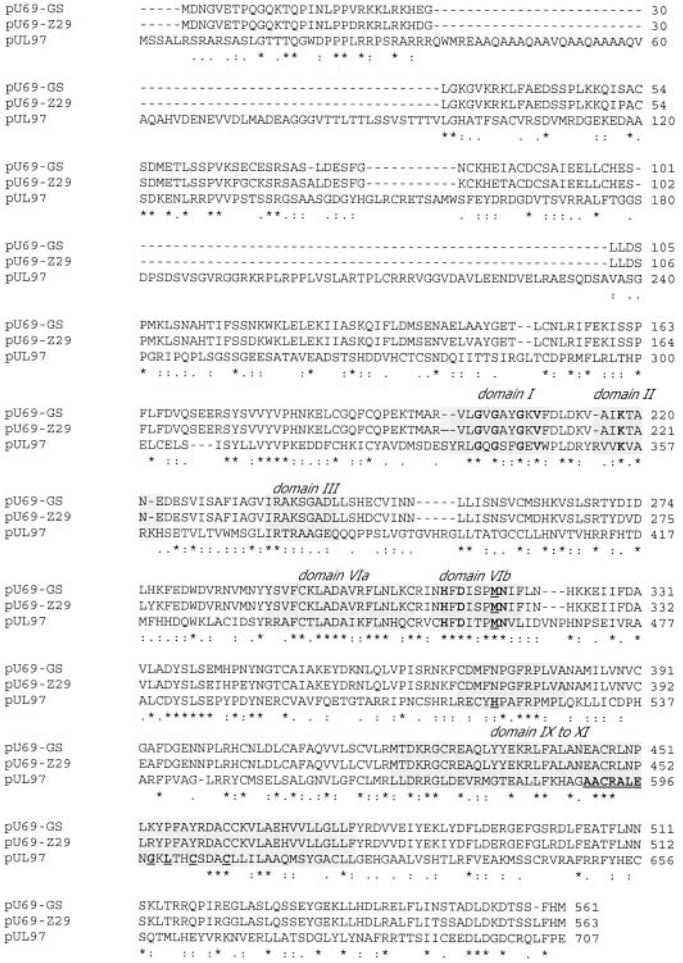

Fig. 1. ClustalW alignment of the HHV-6 pU69 and HCMV pUL97 protein sequences. The overall identity between HHV-6 GS and Z29 pU69 proteins is 94%. ⋆, identical residues; :, strong functional similarities; ., weak functional similarities. Published mutations conferring ganciclovir phenotypic resistance (Manichanh et al., 2001; Chou et al., 2002) are underlined. Conserved motifs are shaded. Amino acid residues that are highly conserved in protein kinases are in boldface type. The motif thus indicated in domains I and II is the putative nucleotide binding site.

Titers were determined by infecting T-lymphoblast cells with 5-fold dilutions of virus stocks. The 50% cell-culture infectious dose was determined according to the method used by Reed and Muench (1938).

Cytotoxicity Assays and Antiviral Studies with HHV-6. To determine cytotoxicity, uninfected cells were plated at a density of 0.8×10^6 cells/ml in the presence of 4- to 5-fold dilutions of compounds. HSB-2 and Molt-3 cultures were subcultivated every 3 to 4 days. The 50% inhibitory concentration (CC_{50}) was determined by cell counting after 12 days of culture.

Cells were infected with HHV-6 (50% cell-culture infectious dose = 100 per 10^6 cells) at a density of 5×10^6 cells/ml. After 90 min adsorption at 37°C, unadsorbed virus was removed by centrifugation, and cells were resuspended in culture medium at a density of 0.8×10^6 cells/ml. Cells were then plated in multiwell trays containing 4- to 5-fold dilutions of the antiviral compounds. HSB-2 and Molt-3 cultures were subcultivated every 3 to 4 days by 2-fold dilution with medium containing fresh compound. At days 10 to 13 postinfection (p.i.), when virus growth reached its maximum, the antiviral activity was determined either by scoring the cytopathic effect or by a DNA hybridization assay as described by Neyts et al. (2001). Antiviral activity was expressed as IC_{50} (the compound concentration that produced 50% inhibition of viral DNA replication).

Generation of Recombinant Vaccinia Viruses. DNA extracts from HHV-6A (GS)– or HHV-6B (Z29)–infected cells were prepared using the Qiamp DNA Blood mini kit (QIAGEN, Hilden, Germany). PCR amplification of the U69 ORF was performed in a reaction mixture containing 60 mM Tris-HCl, pH 8.5, 15 mM ammonium sulfate, 2.5 mM $MgCl_2$, 0.002% Triton X-100, 0.2 mM dNTPs (Invitrogen), 0.5 μM of each primer (Invitrogen), and 1 U/50 μl of proofreading DNA polymerase (ThermoZyme; Invitrogen). The following oligonucleotide primers were used: U69F/*Hin*dIII 5′-TCA AGC TTG AAT AAT TAT GGA CAA CGG TGT G-3′ (HHV-6A genomic positions 103858 to 103880) and U69R/*Eco*RI 5′-CCG GAA TTC TCC ATT ACT ATA TCA CAT ATG AAA G-3′ (positions 105566 to 105542). The amplification reaction consisted of 1 min of initial denaturation at 94°C, 35 thermal cycles of 30 s at 94°C, 45 s annealing at 63°C, and a 2 min elongation step at 72°C, followed by a final elongation at 72°C for 5 min. PCR products were size-separated on a 1% agarose gel and extracted using the QIAquick gel extraction kit (QIAGEN). The U69 amplicons of both strains were subcloned into a pCR4-TOPO T-vector (Invitrogen) and sequenced by automated fluorescence sequencing (ALFexpress; Amersham Biosciences Inc., Piscataway, NJ), using the Thermo Sequenase Cycle Sequencing kit (Amersham Biosciences Inc.) and 5′-Cy5 labeled primers from the same supplier. Selected clones were digested using *Hin*dIII and *Eco*RI restriction enzymes, and the resulting U69 fragments were cloned into a p7.5K131 vaccinia vector. The resulting recombinant vaccinia plasmids were again sequenced and used for the construction of recombinant vaccinia viruses (rVVs) as described previously (Metzger et al., 1994). Briefly, CV-1 cells were infected with a temperature-sensitive VV mutant to allow viral proteins to become expressed at 33°C. After 2 h, infected cells were transfected with the recombinant vaccinia virus plasmid and wild-type VV (strain Copenhagen) DNA. Recombinant vaccinia viruses, formed by homologous recombination, were propagated in 143B cells at 39.5°C under selective 5-bromo-2′-deoxyuridine (Sigma, St. Louis, MO) pressure. The rVV stocks, obtained by sonication of infected cultures, were then purified by two rounds of clonal selection. To this purpose, plaques were isolated from 143B cells, infected with serial dilutions of rVV stocks and maintained under selective pressure. The final rVV clones were again sequenced to ascertain correct insertion of the U69 genes and absence of mutations or deletions. The purified stocks were finally titrated on CV-1 cells.

Antiviral Studies with Recombinant Vaccinia Viruses. The activity of the antiviral compounds GCV and CDV against the recombinant vaccinia viruses was evaluated in CV-1 monolayer cells infected at an m.o.i. of 0.1 plaque-forming units per cell. After 2 h of virus adsorption, serial 4- to 5-fold dilutions (range, 10–500 μM) of the compounds were added to the cells. The cytopathic effect was scored at 24 h after infection and used to calculate IC_{50} value.

Production of Anti-pU69 Antiserum. Recombinant pU69 protein was prepared as follows: the HHV-6A (GS) U69 gene was PCR-amplified as described above using the following primers: U69F/*Eco*RI 5′-AAG AAT TCA TGG ACA ACG GTG TGG AGA-3 (genomic positions 103866 to 103884) and U69R/*Sal*I 5′-AAG TCG ACT CAC ATA TGA AAG AGA GAT GAT-3′ (positions 105554 to 105533). The PCR fragment was subcloned into a pGEM vector (Promega, Madison, WI), fused to the glutathione *S*-transferase tag of the bacterial expression vector pGEX-5X-3 (Amersham Pharmacia Biotech), and the resulting pGex-U69 (GS) construct was sequenced as described above. Expression and purification of the 88-kDa fusion protein were performed according to the manufacturer's recommendations, using BL21(DE3)pLysS cells (Promega). Although the protein was present predominantly in an insoluble form, yields were sufficient for immunization. Attempts to obtain pure pU69 protein by cleaving off the glutathione *S*-transferase moiety of the fusion protein using Factor Xa (Amersham Biosciences Inc.) were not successful, and the fusion protein was used for immunization. Two rabbits were immunized subcutaneously with 500 μg of either the native or denatured form of recombinant pU69, mixed with Freund's complete adjuvant (Sigma), and boosted after 4 weeks with 150 μg of protein in Freund's incomplete adjuvant (Sigma). The polyclonal antiserum was taken 2 weeks after the second immunization and subjected to protein G affinity chromatography (MAbTrap Kit; Amersham Biosciences Inc.). The isolated IgG fraction was subsequently purified on an affinity column (Amersham Biosciences Inc.), which was prepared according to the manufacturer's instructions, and contained the pU69 antigen coupled to a CNBr-activated sepharose resin. The anti-pU69 IgG containing eluate was desalted and concentrated on a Microcon 50-kDa cutoff filter (Millipore Corporation, Bedford, MA).

RT-PCR Analysis. Total RNA was extracted from 143B cells using the RNeasy kit (QIAGEN). DNA contamination was eliminated by digestion with 1 U/μl RNase-free DNase (Roche Molecular Biochemicals) at 37°C for 30 min, followed by 10 min of incubation at 65°C to inactivate residual DNase activity. First-strand cDNA synthesis from 1 μg of total RNA was carried out using 0.25 μg of oligo-$dT_{(15)}$ primer (Promega), 0.25 mM of each dNTP (Invitrogen), 60 U of porcine ribonuclease inhibitor (Amersham Biosciences Inc.), and 1.5 U RAV-2 reverse transcriptase (RT) (Amersham Biosciences Inc.) in a final reaction volume of 25 μl. Duplex PCR was done as described above using *Taq* polymerase (HT Biotechnologies, Cambridge, United Kingdom) and primers for both U69 [5′-TGC TTG CGA TTG TTC TGC GA-3′ (HHV-6A genomic positions 104123 to 104142) and 5′-CGG ATG ACA CCG GCT ATG AA-3′ (positions 104572 to 104553)] and human β-actin (5′-ATC CTC ACC CTG AAG TAC CCC A-3′ and 5′-GAA GGT CTC AAA CAT GAT CTG GGT-3′). To verify that the PCR signal was within linear range, an equal amount of sample was taken from the reaction tube after 25, 29, and 33 PCR cycles. The absence of contaminating DNA was certified by a control RT reaction (to which no RT was added), followed by PCR. PCR products were size-separated on a 2% agarose gel and visualized under UV illumination after ethidium bromide staining.

Western Blot Analysis. Total protein from approximately 5×10^6 cells was extracted with lysis buffer containing 0.02 M Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 25 mM β-glycerophosphate. After complete cell lysis, extracts were centrifuged at 350*g* for 10 min, and the clear supernatant was used for protein quantification using Bradford's reagent (Sigma). Samples were then denatured by 3-min boiling with 0.25 M Tris-HCl, pH 6.8, 10% SDS, 0.5% bromophenol blue, 0.25 M dithiothreitol, and 50% glycerol. Protein (50 μg) was loaded onto a 10% Tris-HCl Ready Gel (BioRad, Hercules, CA) and size-separated for 2 h at 100 V in a buffer consisting of 25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3. Electroblotting onto Hybond ECL nitrocellulose membrane (Amer-

sham Biosciences Inc.) was performed for 2 h at 0.8 mA/cm^2. After an overnight blocking in PBS containing 5% milk as a blocking reagent, the membrane was incubated for 30 min with rabbit polyclonal antiserum, raised against the denatured form of pU69 and diluted to a ratio of 1:500, then finally incubated with secondary anti-rabbit Ig horseradish peroxidase-conjugated antibody (1:5000) (DAKO, Glostrup, Denmark) for 45 min. Antibodies were diluted in 2% milk in PBS. All washes were performed in 0.1% Tween 20 in PBS. The U69 protein was finally visualized using the chemiluminescence ECL detection system (Amersham Biosciences Inc.) and exposure to Curix Blue HC-S Plus X-ray film (Agfa Gevaert, Mortsel, Belgium).

HPLC Analysis of Nucleoside Metabolism. Molt-3 cells (1×10^7 total) were infected with HHV-6B (Z29) at high m.o.i.. At days 3 to 5 p.i., a cytopathic effect was visible and 20 μCi of [8-^{3}H]GCV was added to a final concentration of 50 μM (specific activity, 400 mCi/mmol). 143B thymidine kinase (TK^-) cells were infected with recombinant vaccinia viruses at an m.o.i. of 1. Two hours p.i., 10 μCi of [8-^{3}H]GCV was added at the given concentration. After 12 or 24 h of incubation, cells were harvested, washed twice with ice-cold growth medium, and extracted with 66% methanol in water at 4°C. Cell extracts were centrifuged for 5 min at 23,000*g*, and supernatants were frozen at −20°C until analysis. Sample separation was performed on a Partisphere SAX anion exchange column (Whatman, Clifton, NJ) with an $NH_4H_2PO_4$ buffer system, pH 5.0, using gradient elution between 5 and 300 mM at a flow rate of 2 ml/min (Balzarini et al., 1998). One-minute fractions containing the phosphorylated metabolites of [8-^{3}H]GCV (mono-, di-, and triphosphates) were collected, and the radioactivity of each fraction was determined by liquid scintillation counting. Retention times for ganciclovir mono-, di-, and triphosphates were 12, 19, and 30 min, respectively, whereas (deoxy)guanosine phosphates eluted at 14 min (mono-), 22 min (di-), and 35 min (triphosphate).

Expression of EGFP-U69 Fusion Protein. The pEGFP-C2 vector (BD Biosciences Clontech, Palo Alto, CA) was used for the synthesis of a fusion construct between U69 (GS) and enhanced green fluorescent protein (EGFP). The U69 (GS) fragment was recovered from the pGex-U69 (GS) construct by *Eco*RI and *Sal*I digestion, thus allowing for in-frame insertion of U69 in pEGFP-C2 at the 3′ end of the EGFP gene. The resulting construct pEGFP/U69 was sequenced as described above and introduced into 143B cells using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol. Briefly, cells were seeded at a density of 5×10^4 per cm^2 in 6-well plates. After 24 h, a mixture of 3 μl of FuGENE 6 reagent and 1 μg of plasmid DNA was added directly to the cell-culture medium. Intracellular localization of EGFP and of the EGFP/pU69 fusion protein was evaluated 24 h after transfection on nonfixed cells with the use of standard fluorescence microscopy.

Statistics. The Wilcoxon two-sample test was used to compare ganciclovir phosphorylation between mock-infected and other samples. A p value of <0.05 was considered significant.

Results

Antiviral Activity of GCV Against HHV-6 in Human CBLCs and T-Lymphoblasts. The antiviral activity of ganciclovir against HHV-6A (GS) was evaluated in human CBLCs using a DNA hybridization assay and expressed as an IC_{50} value. Foscarnet and cidofovir were included as controls. The cytotoxicity of each compound was determined by cell counting and expressed as a CC_{50} value. IC_{50} values for ganciclovir and foscarnet, obtained in CBLCs, were in the same range (5.8 and 9.5 μM, respectively), whereas cidofovir was active at lower concentrations (0.56 μM) (Table 1). A comparable IC_{50} of 5.1 μM for ganciclovir has been reported for HCMV in MRC-5 cells (Talarico et al., 1999). Because of its relatively high cytotoxicity, GCV had a less favorable selectivity index of 17, compared with 182 for cidofovir and 68 for foscarnet. In the faster proliferating T-lymphoblastoma HSB-2 cells, a 10-fold decrease in the antiviral sensitivity and a significant increase (~3-fold) in cytotoxicity were observed for all compounds (except foscarnet). The IC_{50} values for ganciclovir, cidofovir and foscarnet were 32, 9.1, and 5.2 μM, respectively. As a result, the selectivity indices of the nucleoside analogs were much less favorable than those obtained in CBLCs (Table 1). Data obtained in HHV-6 (Z29)–infected Molt-3 cells were similar to those obtained for HHV-6 (GS) in HSB-2 cells (data not shown); in our experimental conditions, HHV-6 (Z29) grew relatively poorly in CBLCs, making difficult the assessment of the antiviral activity of ganciclovir in this cell system.

Metabolism of GCV in HHV-6 (Z29)–infected human T-Lymphoblasts. To investigate whether HHV-6 induces viral or cellular kinases capable of phosphorylating ganciclovir, uninfected and HHV-6B (Z29)–infected Molt-3 cells were incubated with [8-^{3}H]GCV, extracted, and analyzed by HPLC. In preliminary experiments, different parameters such as compound concentration, time of addition of radiolabeled compound, or duration of compound exposure were explored. A marked increase in phosphorylation was observed between 6 and 24 h of incubation, whereas only a small additional increase was seen between 24 and 48 h. At all incubation times, this increase was independent of [8-^{3}H]GCV concentration in a range of 2 to 50 μM. We therefore confined the experimental settings to one condition. In HHV-6 (Z29)–infected cells and uninfected cells (Fig. 2), the total concentration of phosphorylated ganciclovir was 29.04 ± 0.51 and 18.67 ± 0.23 pmol/10^7 cells, respectively, after 24 h of incubation with 50 μM GCV. This is a much lower value than that reported for HCMV-infected human fibroblast cells (Biron et al., 1985). The low level of ganciclovir phosphorylation could be caused by either a lower expression of the pU69 protein in HHV-6–infected T-lymphoblasts or by an intrinsic low phosphorylation capacity of pU69 for ganciclovir. To distinguish between these two possibilities, we turned to an rVV assay in which pU69 was expressed at high levels.

Expression of U69 by Recombinant Vaccinia Viruses. The U69 genes of HHV-6 strains GS and Z29 were cloned into the p7.5K131 vaccinia virus plasmid under the control of the p7.5K promoter and were used to generate recombinant vac-

TABLE 1
Antiviral activity in HHV-6A (GS)–infected cells
Antiviral activity and cytotoxic activity were determined 12 days p.i. Values are means ± S.D. for three or four experiments.

	IC_{50}	CC_{50}	SI
	μM		
CBLC			
Ganciclovir	5.8 ± 3.3	100 ± 6.2	17
Acyclovir	10 ± 5.3	277 ± 56	28
Cidofovir	0.56 ± 0.34	102 ± 13	182
Foscarnet	9.5 ± 4.8	647 ± 128	68
HSB-2			
Ganciclovir	32 ± 6.5	<50	<1.6
Acyclovir	181 ± 37	668 ± 77	3.7
Cidofovir	9.1 ± 1.5	<50	<5.5
Foscarnet	5.2 ± 1.3	1247 ± 57	240

HSB-2, human T-lymphoblastoma cell line; SI, selectivity index (ratio of CC_{50} to IC_{50}).

cinia viruses through homologous recombination between the termini of the VV TK gene in the wild-type VV and those flanking the multiple cloning site of the p7.5K131 plasmid. The resulting TK^- recombinant VVs (rVV-U69) were grown in 143B cells under selective pressure of the VV TK inhibitor 5-bromo-2′-deoxyuridine (Sigma). The HCMV pUL97 expressing rVV (rVV-UL97) (Metzger et al., 1994) and wild-type VV (wtVV) (strain Copenhagen) were included as controls. Expression levels of U69 mRNA in rVV-U69–infected 143B cells were assessed by RT-PCR (Fig. 3) and were found to be identical for both recombinants. To further investigate pU69 expression by the rVVs at the protein level, protein extracts of rVV-infected 143B cells at 24 h p.i. and of HHV-6– or HCMV-infected cells at 5 days p.i. were analyzed by Western blot using antiserum against recombinant pU69 (GS) (Fig. 4). pU69 was recognized as a clear band of 60 to 66 kDa, which correlates well with the predicted molecular weights of 63.6 and 63.9 for the rVV-expressed pU69 protein of the GS and Z29 strains, respectively. However, despite their almost identical molecular masses, the pU69 proteins from both HHV-6 strains had a different electrophoretic mobility, which we presume is a result of differences in their phosphorylation status. A similar observation was made previously by Ansari and Emery (1999). We observed an identical electrophoretic mobility in T-lymphoblasts infected with different HHV-6 strains (data not shown). No cross-reaction with HCMV pUL97 nor with cellular proteins of comparable size was observed.

Susceptibility of rVVs to Ganciclovir. Plaque reduction assays were performed in CV-1 cells to determine whether recombination with HHV-6 U69 would render VV susceptible to ganciclovir. Cidofovir was included as a control. Recombination with UL97 rendered VV sensitive to ganciclovir (IC_{50} = 75 μM); this was not the case for the U69-expressing rVVs, in which no significant antiviral effect of ganciclovir was obtained at concentrations lower than 500 μM. This suggests that although ganciclovir phosphorylation may occur in the rVV-infected cells, a low affinity of the VV DNA polymerase for ganciclovir triphosphate impedes the sensitivity of rVVs to GCV. For cidofovir, the IC_{50} remained unchanged after recombination with either viral kinase and was in the range of 35 to 75 μM for both wtVV and rVVs.

Metabolism of GCV in rVV-U69–Infected Cells. To investigate the capacity of pU69 to phosphorylate ganciclovir to its monophosphorylated metabolite, 143B TK^- human osteosarcoma cells were infected with rVVs expressing HHV-6 pU69 from both variants. The rVV-UL97 recombinant virus was used as a reference, and wtVV- and mock-infected 143B cells were included as negative controls. In a first series of experiments, rVV-Z29– and wtVV-infected cells were incubated with 5, 25, or 100 μM [8-^{3}H]GCV during 12 or 24 h, and the levels of phosphorylated metabolites were determined by cell extraction, HPLC analysis, and liquid scintillation counting (Table 2). The total level of [8-^{3}H]GCV phosphates increased with concentration on a linear basis. However, the difference between rVV-Z29– and wtVV-in-

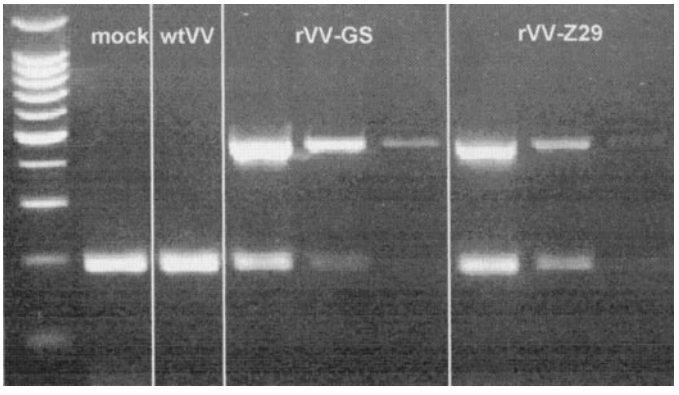

Fig. 3. Ethidium bromide–stained agarose gel showing the result of RT-PCR analysis for the U69 mRNA of the HHV-6 strains GS and Z29 expressed by rVVs on 1 μg of total RNA extracted from rVV-infected human 143B cells. A 449-base pair U69 fragment and a 186-base pair human β-actin fragment were coamplified in 33 PCR cycles. Equal amounts of sample were taken after 25, 29, and 33 PCR cycles to ensure that the signal was within linear range (left to right: 33, 29, and 25 cycles). Mock- and wild-type VV-infected cells were included as controls and subjected to 33 amplification rounds.

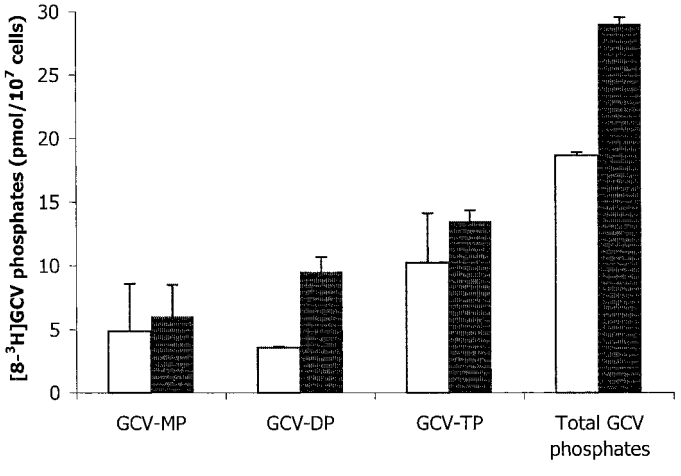

Fig. 2. Phosphorylation of GCV in uninfected and HHV-6 (Z29)–infected human Molt-3 T-lymphoblasts. Three to 5 days postinfection, 1×10^7 cells were pulsed for 24 h with 20 μCi of [8-^{3}H]GCV at a total concentration of 50 μM GCV. After methanol extraction, samples were analyzed by HPLC, and the radioactivity of the fractions containing the phosphorylated ganciclovir metabolites was determined by liquid scintillation counting. □, uninfected cells; ■, HHV-6B–infected cells; MP, monophosphate; DP, diphosphate; TP, triphosphate.

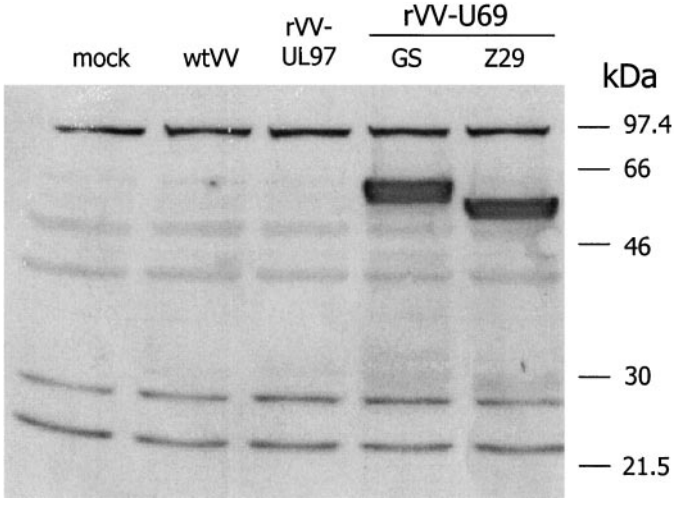

Fig. 4. Western blot analysis of pU69 of HHV-6 strains GS and Z29 expressed in 143B cells 24 h after infection with U69 recombinant vaccinia viruses. The pU69 protein was visualized by chemiluminescence after staining with anti-pU69 antiserum. Extracts from uninfected cells, wtVV-, and rVV-UL97–infected cells were included as controls.

fected cells was more pronounced after 24 h than after 12 h of incubation (ratio: 3-fold versus 2-fold difference) (Table 2), thus favoring 24 h of incubation for further experiments.

Next we compared the ganciclovir phosphorylation in cells infected with rVV-U69 and rVV-UL97. Total [8-^{3}H]GCV phosphate levels for rVV-U69–infected cells were 7.4 and 7.1 pmol/10^7 cells for the GS and Z29 variants, respectively, which is significantly higher than for mock-infected cells (1.8 pmol/10^7 cells) (Table 3). Most strikingly, the total [8-^{3}H]GCV phosphate level of rVV-U69–infected cells was approximately 10-fold lower than that of rVV-pUL97–infected cells (61 pmol/10^7 cells).

Intracellular Localization of pU69. The HCMV UL97 gene product is a nuclear protein (Michel et al., 1996), and a nuclear localization signal was mapped between amino acid (aa) 48 and 110 at the N-terminal region of the protein (Michel et al., 1998). Because the sequence homology between pU69 (563 aa) and pUL97 (707 aa) is most pronounced in the 400 C-terminal amino-acid residues, it seemed relevant to examine the intracellular expression pattern of pU69. The polyclonal antibody that was raised against HHV-6 pU69 showed a weak cross-reactivity with cellular cytoplasmic proteins. However, a marked accumulation of fluorescence was observed in the nucleus of rVV-U69–infected 143B cells. To provide unambiguous evidence for the nuclear localization of pU69, an EGFP/pU69 fusion construct was designed and transfected into 143B cells. Cells transfected with EGFP alone showed an overall green fluorescence of both the cytoplasm and nucleus (Fig. 5A), whereas introduction of the pEGFP/U69 fusion construct resulted in a strong fluorescent signal that was strictly confined to the nucleus (Fig. 5B). pU69 did not enter the nucleoli, which was also observed for pUL97 by Michel et al. (1996).

Discussion

Our in vitro data agree well with those from previous reports (Manichanh et al., 2000) and show a consistent activity of foscarnet against HHV-6. For ganciclovir, a major variable in the determination of the antiviral activity and cytotoxicity is the cell system that is being used to propagate the virus (i.e., established continuous cell lines versus freshly isolated blood lymphocytes) (Manichanh et al., 2000). The most probable explanation for the reduced anti–HHV-6 activity of ganciclovir in tumor-derived T-cell lines is that higher levels of endogenous nucleotides are present in these cells than in fresh lymphocytes, resulting in a higher competition at the viral DNA polymerase level. Furthermore, in enzyme assays, HHV-6 DNA polymerase was shown to be 4- and 6-fold less sensitive to inhibition by ganciclovir triphosphate as compared with the DNA polymerases of HSV-1 and HCMV, respectively (Bapat et al., 1989). To some extent, this explains the lesser antiviral potency of ganciclovir in cells infected with HHV-6 compared with HCMV and HSV-1. In this study, we focused on the HHV-6–dependent phosphor-

TABLE 2

Time and concentration dependence of [8-^{3}H]GCV phosphorylation in rVV (Z29)- versus wtVV-infected human 143B cells

Two hours postinfection, cells were pulsed with 5, 25, or 100 μ M [8-^{3}H]GCV for 12 or 24 h. Phosphorylated metabolites were measured by anion-exchange HPLC and liquid scintillation counting. Values are means ± S.D. for two independent experiments. Ratio column is ratio of total [8-^{3}H]GCV phosphate concentration for rVV-Z29 to that for wtVV.

Incubation Time	Concentration	Total [8-^{3}H]GCV phosphates		
		wtVV	rVV-Z29	Ratio
	μM	*pmol / 10^7 cells*		
12 h	5	1.6 ± 1.1	2.9 ± 1.4	1.8
	25	8.3 ± 3.7	15 ± 8.5	1.8
	100	29 ± 12	48 ± 29	1.7
24 h	5	4.1 ± 0.6	12 ± 1.0	2.9
	25	20 ± 3.5	56 ± 2.3	2.8
	100	82 ± 14	233 ± 19	2.8

TABLE 3

Phosphorylation of [8-^{3}H]GCV in rVV-infected human 143B cells

Two hours postinfection, cells were pulsed with [8-^{3}H]GCV (5 μ M) for 24 h. Phosphorylated metabolites were measured by anion-exchange HPLC and liquid scintillation counting. Values are means ± S.D. for three independent experiments.

	Phosphorylated metabolites of [8-^{3}H]GCV			
	MP	DP	TP	Total
	pmol / 10^7 cells			
rVV-GS	1.1 ± 0.5*	1.7 ± 0.33*	4.6 ± 4.5	7.4 ± 4.3*
rVV-Z29	1.7 ± 1.2*	1.5 ± 0.16*	4.0 ± 3.7	7.1 ± 4.8*
rVV-UL97	24 ± 22**	9.7 ± 3.1**	27 ± 20**	61 ± 41**
wtVV	0.44 ± 0.47	0.54 ± 0.32	2.0 ± 1.5	3.0 ± 2.0
Mock-infected	0.19 ± 0.21	0.47 ± 0.21	1.2 ± 0.70	1.8 ± 1.0

MP, monophosphate metabolites; DP, diphosphate metabolites; TP, triphosphate metabolites.

*$P < 0.05$; **$P < 0.01$; statistical significance compared with mock-infected condition (Wilcoxon two-sample test).

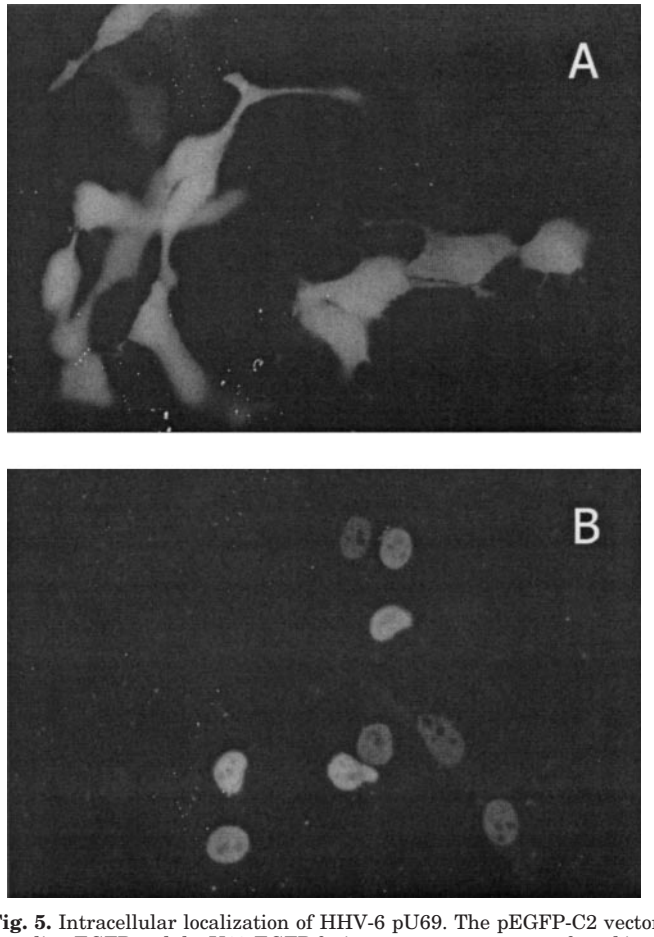

Fig. 5. Intracellular localization of HHV-6 pU69. The pEGFP-C2 vector encoding EGFP and the U69-EGFP fusion construct were transfected into human 143B cells. The fluorescence pattern was evaluated after 24 h using a fluorescein isothiocyanate filter-equipped fluorescence microscope. A, EGFP alone. B, pU69-EGFP fusion protein.

ylation of ganciclovir as an essential element in its antiviral activity against HHV-6.

We first investigated whether the phosphorylation of ganciclovir is altered in HHV-6–infected T-lymphocytes by the induction of either or both viral or cellular kinases. A moderate but significant increase in phosphorylation was observed in HHV-6–infected cells as compared with uninfected cells. Unlike the α-herpesviruses, HHV-6 does not produce thymidine kinase activity (Di Luca et al., 1990), but it may, like HCMV (Zimmermann et al., 1997), induce other enzymes capable of phosphorylating nucleoside analogs that account for the antiviral activity of ganciclovir against HHV-6. The enzyme that is most likely to be responsible for ganciclovir monophosphorylation is the protein encoded by the HHV-6 gene U69 (pU69), which is a presumed functional homolog of the HCMV-encoded pUL97 protein (Sullivan et al., 1992; Ansari and Emery, 1999). pUL97 has been shown to phosphorylate ganciclovir and, to a lower extent, acyclovir in both a recombinant vaccinia virus assay (Zimmermann et al., 1997) and an enzyme assay using purified pUL97 (Talarico et al., 1999).

To investigate the precise role of pU69 in HHV-6–induced GCV anabolism, we applied an rVV assay (developed earlier by Metzger et al.,1994) that guarantees high-level expression of pU69 in a mammalian cell system in the absence of other HHV-6 genes. By including the HCMV pUL97-expressing rVV in our experiments, we were able to make a direct comparison between the two viral kinases HHV-6 pU69 and HCMV pUL97. Expression of HHV-6 U69 at both the mRNA and protein level was shown to be constant in the vaccinia virus recombinants. In view of the important role that this protein may play in HHV-6 replication, the observed differences in electrophoretic mobility could account for variant-dependent variations in the biological properties of pU69. We therefore included U69 rVVs derived from the two different HHV-6 variants in all of our metabolism studies, but no major differences in their ability to phosphorylate ganciclovir were observed.

The HHV-6 U69 rVVs, unlike the rVV expressing HCMV pUL97, did not develop sensitivity to GCV in our experiments. Although these antiviral studies are compromised by the rather weak inhibitory effect of ganciclovir triphosphate on vaccinia virus DNA polymerase (St. Clair et al., 1980), they clearly indicated a lesser ability of HHV-6 pU69 to phosphorylate GCV compared with HCMV pUL97. This was clearly demonstrated in our metabolism studies in recombinant vaccinia virus–infected cells: pU69 of both HHV-6 isolates phosphorylated ganciclovir at approximately 10-fold lower levels than those of HCMV pUL97.

HHV-6 pU69, like HCMV pUL97, shares regions of homology with several cellular and viral protein kinases (Chee et al., 1989) encoded by herpes simplex virus, varicella-zoster virus, and obviously by other animal cytomegaloviruses (Michel et al., 1998). Although their overall sequences are quite divergent, several residues are highly conserved and are therefore considered to be essential for their kinase function. A homolog to the AACR motif (at aa position 590–593 in HCMV pUL97) that is crucial for ganciclovir phosphorylation (Sullivan et al., 1992) is encoded only by HHV-6 (ACR motif in the corresponding aa position 446–448). In their functional comparison between HCMV pUL97 and its murine homolog MCMV pM97, Wagner et al. (2000) found that despite the sensitivity of MCMV to ganciclovir, the level of total GCV phosphates was approximately 10-fold lower for pM97 than for pUL97. In addition, autophosphorylation by pM97 was hardly detectable, although pM97 (like pUL97) plays an important role in MCMV replication.

One striking observation was that both proteins showed a different cellular localization: pM97 is expressed in the cytoplasm, whereas pUL97 has been described previously as a nuclear protein (Michel et al., 1996). Together with the divergent optimal enzymatic reaction conditions described for pUL97 and HHV-6 pU69 (He et al., 1997; Ansari and Emery, 1999), this might indicate a relationship between cellular localization of these viral kinases and their (auto)phosphorylating capacity. We therefore investigated the intracellular localization of HHV-6 pU69 both in infected cells by immunofluorescence and in cells transfected with an EGFP/U69-fusion construct. Like HCMV pUL97, pU69 was expressed exclusively in the nucleus. This implies that major differences in their capacity of phosphorylating ganciclovir are caused by the intrinsic enzymatic properties of both kinases rather than by factors within the intracellular microenvironment. A more definite insight into the characterization of the enzymatic properties of pU69 could be obtained from phosphorylation studies using purified pU69 enzyme.

In conclusion, our studies demonstrate that the HHV-6 U69–encoded kinase, in contrast to HCMV pUL97, has a poor capacity to phosphorylate ganciclovir, explaining the appearance of relatively low levels of ganciclovir metabolites in HHV-6–infected cells and, hence, the relatively weak anti–HHV-6 activity of ganciclovir in some cell culture systems. Whether these data can be directly extrapolated to the in vivo situation is presently unclear. In HHV-6– or HCMV-infected patients, both the expression levels of HHV-6 pU69 and HCMV pUL97 and the competitive inhibition at the viral DNA polymerase level by the nucleoside triphosphates may differ from the in vitro situation, depending also on the tissue type in which HHV-6 and HCMV replicate. A definite conclusion with respect to the in vivo efficiency of ganciclovir should come from controlled clinical trials in patients undergoing HHV-6 reactivation.

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