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Phosphorylation of aciclovir, ganciclovir, penciclovir and S2242 by the cytomegalovirus UL97 protein: a quantitative analysis using recombinant vaccinia viruses

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Abstract

We used recombinant vaccinia viruses (rVV) containing the UL97 open reading frame (ORF) of the human cytomegalovirus (HCMV) to investigate the UL97-dependent phosphorylation of different nucleoside analogs. The rVV T1 expressed the wild-type UL97 protein whereas rVV A5 contained a 12 bp deletion in the UL97 which had been known to be responsible for resistance of HCMV to ganciclovir (GCV). The rVV T1opal was generated which contained a stop codon at position 1089 of the UL97 ORF and which expressed a truncated UL97 protein. We quantitatively analyzed the capability of these rVVs to phosphorylate GCV, penciclovir (PCV), aciclovir (ACV) and 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl] purine (S2242) as well as the natural nucleosides deoxycytidine and deoxythymidine. Moreover, we compared their phosphorylating capability with that of herpes simplex virus type 1 strains. In thymidine kinase (TK)-deficient 143B cells infected with rVV T1, the three compounds GCV, ACV and PCV were phosphorylated with different efficiency whereas in cells infected with the rVV A5 a markedly reduced but not completely abolished phosphorylation of these compounds was observed. In rVV T1opal-infected cells no specific phosphorylation of the compounds was detectable at all. Neither S2242 nor the natural substrates of TKs were phosphorylated by any of the vaccinia recombinants. The rVVs proved to be a suitable tool for analysis of UL97-dependent phosphorylation of nucleoside analogs and also allowed to quantitatively study the influence of UL97 mutations on drug phosphorylation. © 1997 Elsevier Science B.V.

Keywords: Phosphorylation UL97; Recombinant vaccinia viruses; Nucleoside analogs

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1. Introduction

Nucleoside analogs are widely used inhibitors of human herpesvirus replication. The antiviral activity of these substances depends on metabolic activation by phosphorylation to their respective mono-, di- and triphosphates. The preferential initial phosphorylation to the nucleoside monophosphate by viral enzymes is one mechanism of selective antiviral activity. Herpes simplex virus (HSV), varicella zoster virus (VZV) and Epstein–Barr virus (EBV) encode thymidine kinases (TK) capable to phosphorylate antiviral compounds. The biochemical and genetic characteristics of these enzymes have been well established (Darby et al., 1986; Koyano et al., 1996; Littler and Arrand, 1988; Reusser, 1996; Tung and Summers, 1994). Furthermore, mutants with impaired TK activity as well as TK-negative HSV strains have been described (Darby and Field, 1981; Schnipper and Crumpacker, 1980).

Human cytomegalovirus (HCMV) encodes for an 80 kD protein by the UL97 open reading frame (ORF) which has been shown to be necessary for phosphorylation of ganciclovir (GCV) in infected cells (Littler et al., 1992; Sullivan et al., 1992). Several mutations in UL97 have been described to confer GCV resistance and their responsibility for resistance has been proven by homologous recombination into the HCMV laboratory strain AD169 (Baldanti et al., 1995; Chou et al., 1995; Hanson et al., 1995; Lurain et al., 1994; Stanat et al., 1991; Sullivan et al., 1992). However, the procedure of HCMV recombination and purification of recombinant viruses is laborious and time consuming. Additionally, in contrast to HSV TK, quantitative determination of UL97-dependent phosphorylation of GCV, aciclovir (ACV) and penciclovir (PCV) has not been shown due to a quite low specific phosphorylating activity of the UL97 protein in HCMV-infected cells (Lurain et al., 1992). We have reported the construction of recombinant vaccinia viruses (rVV) containing the UL97 ORF allowing the analysis of UL97 gene functions in the absence of other HCMV gene products (Metzger et al., 1994). In order to study the UL97-dependent phosphorylation of the nucleoside analogs GCV, ACV, PCV and 2-amino-7[(1,3-dihydroxy-2-propoxy)methyl] purine (S2242), rVVs were used containing either the entire UL97 ORF derived from AD169 or mutated UL97 ORFs.

2. Materials and methods

2.1. Cells and viruses

143B TK-deficient cells were used for infection with rVV and HSV1 strains. Experiments with HCMV strain AD169 were performed using human foreskin fibroblasts (HFF) as described previously (Michel et al., 1996). HSV1 strains were grown and titrated on Vero cells. K1 and K1r HSV1 strains were biologically characterized as GCV/ACV-sensitive and -resistant, respectively (Hampl, unpublished data). Construction of rVV T1 and A5 containing the UL97 ORF has been described previously (Metzger et al., 1994), recombinant vaccinia T1opal was selected from T1 by 17 cell culture passages in the presence of 50 μM GCV and subsequent plaque purification.

2.2. Antiviral compounds

Ganciclovir (Cymeven®) was purchased from Syntex, Aachen, Germany and aciclovir (Zovirax®) from Wellcome, Burgwedel, Germany. Penciclovir was a gift from SmithKline Beecham, Philadelphia, PA, and S2242 was kindly provided by Dr. Winkler (Hoechst, Frankfurt/Main, Germany).

2.3. Analysis of nucleoside anabolism

[8-^{3}H]ganciclovir (12.4 Ci/mmol), [8-^{3}H]penciclovir (18.6 Ci/mmol), [8-^{3}H]aciclovir (12.7 Ci/mmol) (Moravek, Brea, USA), [methyl-^{3}H]thymidine (61 Ci/mmol) and [5-^{3}H]deoxycytidine (26 Ci/mmol) (ICN, Costa Mesa, CA, USA) were used for analysis of nucleoside anabolism as described previously (Michel et al., 1996). [^{14}C]S2242 (44 mCi/mmol) was kindly provided by Drs Winkler and Lötzsch (Hoechst, Frankfurt/Main, Germany). 143B cells were infected with rVVs or HSV strains at a MOI

of 2. One hour post infection (h.p.i.) 5 μCi of HPLC-purified radioactive-labelled compound was added at a specific radioactivity of 100 mCi/mmol (44 mCi/mmol for S2242). After 20 h of incubation, cells were harvested and washed three times with phosphate buffered saline (PBS). The cells were resuspended in PBS and extracted with 0.5 M perchloric acid. The extracts were neutralized to pH 6.8 with 2.5 M KOH in 1.5 M KH_2PO_4, centrifuged for 5 min at $14\,000 \times g$ and the supernatant was used for HPLC. HPLC was performed on a Sephasil C_{18} reversed phase column (250 by 4.6 mm, Pharmacia, Uppsala, Sweden) with 20 mM KH_2PO_4, pH 6.0, 7.5% methanole (or 15% methanole for PCV) as the mobile phase performing an isocratic elution at a flow rate of 1.0 ml/min. Fractions containing the GCV-phosphates (mono-, di- and triphosphates) and the unphosphorylated compound were collected according to their retention times as determined by external standard runs. The radioactivity of each fraction was determined by liquid scintillation counting. The amount of phosphorylated compound was normalized for 10^5 cells using the amount of unphosphorylated compound as an internal standard. HFF were infected with HCMV at a MOI of 1. At 24 h.p.i. 10 μCi of radioactive-labelled substance was added and analyzed as described above after further 48 h of incubation.

2.4. DNA-sequencing

Sequence analysis was performed by the dideoxynucleotide chain termination method (Sambrook et al., 1989) using the T7-sequencing-kit (Pharmacia, Uppsala, Sweden) and the Deaza G/A T7-sequencing-mixes (Pharmacia, Uppsala, Sweden).

2.5. Western blot analysis

Total cell lysates were extracted according to Sambrook et al. (1989) from cell monolayers infected with the indicated rVV as described above. Following 20 h.p.i., proteins were separated on a 12% sodium dodecyl sulfate polyacrylamide gel and semi-dry transferred to nitrocellulose membranes. The membranes were probed with anti-UL97 polyclonal rabbit serum (Michel et al., 1996) at a dilution of 1:1000 and with horseradish peroxidase-conjugated anti-rabbit IgG at a dilution of 1:5000 (Amersham, Braunschweig, Germany). For detection, the ECL western blot detection system (Amersham, Braunschweig, Germany) was used.

2.6. Analysis of drug susceptibility

The sensitivity of the HSV1 strains to inhibition by antiviral drugs was tested in a quantitative microtiter cytopathic effect (CPE) reduction assay and was expressed as an inhibitory concentration value (IC_{50}) exactly as described by Kruppenbacher et al. (1994). Susceptibility of HCMV strain AD169 was characterized by focus reduction assay. Staining of HCMV-infected cells was performed according to Gleaves et al. (1987). In brief, HCMV-infected HFF were quantified by immunoperoxidase staining 20 h.p.i. As primary antibody we used a mouse monoclonal antibody to delayed-early p52 HCMV protein (Dako A/S, Glostrup, Denmark) diluted 1:30 (all dilutions of antibodies were made in PBS/1% BSA). After incubation for 30 min at room temperature, wells were washed three times with PBS and a secondary antibody (horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins, Dako) was added using a dilution of 1:100. After additional incubation for 30 min, infected cells were visualized by addition of a substrate (3,3′-diamino-benzidine-tetrahydrochloride dihydrate, Fluka, Neu-Ulm, Germany). The number of HCMV-infected cells was determined (1 infected cell = 1 early antigen producing unit, EAU) by using a light microscope. For determination of 50% inhibitory concentrations of the nucleoside analogs, immediately after quantification of infected cells 10–20 EAU per well of 96-well microtiter plates were mixed with fresh HFF and antiviral compounds were added to reach final concentrations of 0.4–400 μM in 2-fold dilution steps. Control wells containing virus without antiviral substances were included in each assay. After incubation at 37°C and 5% CO_2 for 96 h, focus formation was determined (1 focus = at

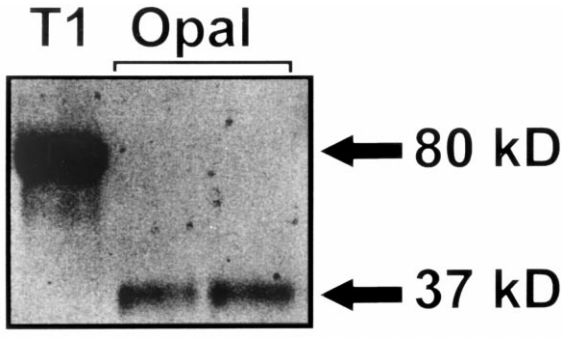

Fig. 1. Western blot analysis of UL97 protein expressed by rVV T1 (lane 1) and two rVV T1opal (lanes 2 and 3).

least 5 HCMV-infected adjacent cells) utilizing immunoperoxidase staining as described above, but using a mouse monoclonal antibody against the lower matrix HCMV protein pp65 (Dako) as primary antibody. The effective dose for each antiviral compound was expressed as IC_{50} representing an inhibitory concentration of drug (μM) which reduced the number of viral foci by 50%.

3. Results

3.1. Phosphorylation of antiviral compounds

In order to quantitatively study the phosphorylating capacity of the UL97 protein for different antiviral compounds, rVV T1 carrying the wild-type UL97 ORF and rVV A5 were used. A5 carried a UL97 ORF with a 12 bp deletion which results in a 4-amino-acid deletion of residues 590–593 in the UL97 protein and which had been found in a GCV-resistant HCMV isolated by selection from strain AD169 under drug pressure in vitro (Biron et al., 1985; Sullivan et al., 1992). Additionally, we used a new rVV which carried a point mutation at position 1089 introducing an opal stop codon into the UL97 ORF. It was confirmed by western blot analysis that this variant T1opal produced a truncated UL97 protein of about 37 kDa (Fig. 1). We analyzed the expression of UL97 after infection with different MOI and found the same level of UL97 expression if the MOI was 1 or higher, as used in the experiments (data not shown). We have shown recently that the HCMV-encoded UL97 protein expressed by rVV provides no detectable nucleoside kinase activity although an UL97-dependent GCV phosphorylation was clearly detectable (Michel et al., 1996). In order to quantitatively measure the intracellular phosphorylation of the different antiviral compounds, 143B cells were infected with the UL97-expressing rVVs. For comparison, 143B cells were infected with HSV1 strains and HFF were infected with HCMV. Results are summarized in Table 1. Phosphorylation of GCV, ACV and PCV could be easily detected by using the

Table 1
Phosphorylation of antiviral compounds or natural nucleosides

Virus	pmol of nucleoside phosphates/10^5 cells[a]					
	GCV	ACV	PCV	dT	dC	S2242
rVV T1	84.7 ± 10.2	9.5 ± 1.5	34.9 ± 8.9	5.0 ± 0.3	4.1 ± 0.3	9.5 ± 1.5
rVV A5	17.1 ± 5.5	6.3 ± 1.0	15.4 ± 1.1	4.8 ± 0.3	3.6 ± 0.4	9.0 ± 0.9
rVV T1opal	5.9 ± 0.6	4.6 ± 0.4	6.3 ± 1.6	4.8 ± 0.3	3.6 ± 0.3	9.1 ± 1.0
VV wild-type	5.1 ± 0.6	5.4 ± 1.6	7.5 ± 1.1	n.d.	n.d.	n.d.[b]
143B control[c]	4.2 ± 0.4	4.1 ± 0.2	5.2 ± 0.2	4.5 ± 0.2	5.1 ± 0.6	9.0 ± 0.7
HSV1 K1	700 ± 84	9.2 ± 0.5	1050 ± 115	450 ± 44	55 ± 4.1	n.d.

[a] Experiments were done with 143B cells, each value represents the total amount of phosphorylated compound (mono-,di- and triphosphates) as the mean $\pm$ S.D. from triplicate determinations of at least three separate experiments (and from five to nine separate experiments for the phosphorylation of the different antiviral compounds by rVVs).
[b] n.d., not determined.
[c] Mock-infected 143B cells.

UL97-expressing rVV T1. Examination of phosphorylation of GCV and PCV in HSV1-infected cells revealed a 10- and 30-fold higher value, indicating a higher phosphorylation capacity of the HSV-TK. Interestingly, ACV was phosphorylated to the same low amount in both, HSV1- and rVV T1-infected cells. Although the amount of phosphorylated ACV found in rVV-infected cells was low, the quantitative relation comparing rVV T1, A5 and T1opal was entirely reproducible throughout all experiments performed. In HCMV-infected HFF a significant phosphorylation (15 pmol/10^5 cells) was only detected for GCV (data not shown). The total amount of detectable radioactivity was much lower than in the rVV system and did not allow a reproducible quantification of ACV and PCV phosphorylation. A further problem was the fact that the uptake of GCV seemed to be increased by HCMV infection (Lurain et al., 1992). In our hands we found a 2- to 4-fold higher amount of unphosphorylated compound in HCMV-infected HFF (data not shown) which may severely interfere with an exact quantification of UL97-dependent phosphorylation in HCMV-infected HFF.

Analysis of rVV A5 revealed an impaired phosphorylation of GCV, ACV and PCV but it was repeatedly somewhat higher than the cellular background (Table 1). In contrast to this, the phosphorylation induced by rVV T1opal strain was almost identical to the values obtained with mock or vaccinia wild type-infected cells. Meanwhile, we confirmed the baseline level of rVV T1opal GCV phosphorylation by construction of other rVVs containing deletions which result in the expression of truncated UL97 proteins with molecular weights similar to rVV T1opal (unpublished data). Since the mutation in rVV A5 had a comparable quantitative effect on phosphorylation of GCV, PCV and ACV, we concluded that there is probably a common mechanism responsible for the phosphorylation of these compounds by the UL97 protein.

Since S2242 was found to be specifically phosphorylated in HCMV-infected cells (Neyts et al., 1995a, 1997), we studied the phosphorylation of this compound in 143B cells infected with the rVVs. However, no significant phosphorylation of S2242 was observed using this system.

Table 2
Antiviral activities of compounds against HCMV and HSV1

Virus	IC_{50} (μM)			
	GCV	ACV	PCV	S2242
HCMV AD169	3 ± 1	40 ± 10	150 ± 44	0.5 ± 0.4
HSV1 K1	0.17 ± 0.1	0.41 ± 0.05	16.6 ± 2.7	0.6 ± 0.2
HSV1 K1r	80.3 ± 6.0	330 ± 10	>800	2.2 ± 0.9

IC_{50}, the concentration of drug required to reduce focus formation (HCMV) or cytopathic effect (HSV) by 50%; each value represents the mean $\pm$ S.D. from at least three separate experiments

As expected, the natural substrates of TK, deoxythymidine (dT) and deoxycytidine (dC) were phosphorylated in HSV1-infected cells. In contrast, phosphorylation of dT and dC by the UL97 protein could not be detected. The TK-deficient HSV1 strain K1r did also not show any phosphorylation of all substances tested (data not shown). Summarizing the phosphorylation experiments we provided direct evidence that UL97 protein also induces phosphorylation of PCV and ACV, but at levels 2- and 10-fold lower than GCV. This is in line with drug susceptibility data of other authors who observed a decreased ACV sensitivity in HCMV strains resistant to GCV (Biron et al., 1986; Lurain et al., 1994). We also provided additional evidence that the experimental compound S2242 is not phosphorylated by the UL97 protein.

3.2. In vitro susceptibility

The results of the susceptibility of the HSV and HCMV strains to the antiviral compounds in vitro are summarized in Table 2. The sensitive HSV1 K1 strain was inhibited by all substances tested whereas resistant HSV1 K1r was cross-resistant to all substances except for S2242. The experimental compound S2242 was also active against the TK-deficient resistant HSV1 strain. The activity of S2242 was obviously neither HSV TK- nor UL97-dependent, indicating that S2242 utilizes different mechanism(s) for its antiviral activity. HCMV strain AD169 was sensitive to

GCV and S2242. ACV showed only weak activity against HCMV whereas PCV was virtually inactive.

4. Discussion

4.1. Vaccinia virus UL97 recombinants as a tool for analysis of drug phosphorylation

After detection of the GCV-phosphorylating capacity of the HCMV UL97 protein (Littler et al., 1992; Sullivan et al., 1992), some GCV-resistant HCMV strains have been characterized and mutations in the UL97 ORF responsible for resistance have been described. However, results obtained with these strains only allowed to conclude that the phosphorylation activity of GCV by these viruses is impaired (Baldanti et al., 1995; Chou et al., 1995; Stanat et al., 1991). Other researchers also found that the UL97-dependent phosphorylation in HCMV-infected cells is low (Biron et al., 1985; Lurain et al., 1994, 1992). In addition, exact quantification of GCV phosphorylation in HCMV-infected cells is hampered due to some HCMV-specific limitations (slow replication, difficulty to obtain HCMV stocks with high titer, increased GCV uptake in infected cells) as well as by the fact that only diploid human fibroblasts can be used for such experiments. In order to overcome these problems, we introduced the UL97 ORF into recombinant vaccinia viruses. Vaccinia recombinants can be produced rather easily and allow a very efficient infection of fast growing immortalized cell lines. Additionally, by using rVV, the expression and function of UL97 is independent of other HCMV proteins. We could show that expression of HCMV UL97 by recombinant vaccinia viruses allows a fast, sensitive, reproducible and quantitative determination of intracellular drug phosphorylation. The phosphorylation levels in UL97-expressing vaccinia recombinants are high enough to quantitatively analyze the phosphorylation of alternative antiherpetic compounds, even when they are phosphorylated less efficiently than GCV.

4.2. Quantitative phosphorylation of GCV, ACV, PCV and S2242

The antiviral activity of the nucleoside analogs is based on inhibition of the viral DNA polymerization process by the respective nucleoside triphosphates. The paradigm is the antiviral action of ACV against HSV in which triphosphate is a very potent competitive inhibitor of HSV DNA polymerase (Reardon and Spector, 1989). This is underlined by the fact that, although the antiviral activity against HSV1 is very good, the level of ACV phosphates in infected cells is low as compared to GCV- and PCV phosphates, confirming that the avidity to the respective polymerase is also crucial concerning antiviral activity.

By studying the phosphorylation of nucleoside analogs in rVV T1-infected as well as in HCMV-infected cells, GCV appears not only to be the most suitable substrate for phosphorylation directed by the UL97 protein but has also the best inhibitory effect on HCMV. The anti-HCMV activity of ACV is superior to that of PCV in spite of the fact that PCV is 3-fold more efficiently phosphorylated in cells infected with the UL97 carrying vaccinia virus. Thus, an inefficient phosphorylation of ACV in HCMV-infected cells may possibly be responsible for its weak anti-HCMV activity, especially since ACV triphosphate was shown to be a better inhibitor of HCMV DNA polymerase than GCV triphosphate (Mar et al., 1985).

S2242 has been shown not to be specifically phosphorylated in HSV1 (and VZV and HHV6)-infected cells, and the compound is not a substrate for the HSV1- or HSV2-encoded TK (Neyts et al., 1995b, 1997). Activation of the compound by cytoplasmic deoxycytidine (dCyd) kinase appears responsible for the activation of the compound, yielding sufficient amounts of metabolites to specifically inhibit the DNA polymerization process. In cells infected with HCMV, however, the compound is phosphorylated to high levels but not by dCyd kinase. The anti-HCMV activity of the compound is also not reversed by exogenously added dCyd or by any other nucleoside (Neyts et al., 1997). Here we investigated whether the increased phosphorylation in HCMV-infected

cells may be caused by the HCMV UL97 protein. However, the enzyme did not prove to be responsible for this activation. This is also in agreement with the recent observation that S2242 is highly active against GCV-resistant strains of HCMV with impaired UL97-dependent phosphorylation (Andrei, Snoeck, De Clercq, unpublished data).

4.3. Mutations of UL97 and phosphorylating activity

The intracellular level of GCV phosphates in cells infected with GCV-resistant HCMV strains was determined by other authors and was shown to be significantly lower than in the case of phosphorylation competent strains (Baldanti et al., 1995; Lurain et al., 1994). However, in these studies phosphorylation of GCV was reduced but still appeared higher than the cell control. Since rVV allowed discrimination between modifications of the UL97 protein with impaired phosphorylation capacity (rVV A5) and UL97-mutants which were totally incapable to phosphorylate GCV (rVV T1opal), we were able, for the first time, to prove this impaired but not totally abolished GCV phosphorylation. Additionally, vaccinia wild type phosphorylates GCV to a comparably low level. This rules out the possibility that the altered GCV anabolism of rVV T1opal is due to mutations in the vaccinia virus independent of UL97. However, such mutations appear to be possible since it has been shown recently (Ng et al., 1996) that UL97 can confer GCV susceptibility to recombinant TK-deficient HSV but not if TK-deficient cell lines are used.

Interestingly, until now exclusively HCMV-mutants with impaired phosphorylation capacity due to point mutations and small deletions have been described (Baldanti et al., 1995; Lurain et al., 1994). So far, UL97-negative mutants have not been described by other authors and isolation of UL97-negative HCMV by drug pressure or by homologous recombination also failed in our laboratory. Possibly one may speculate that UL97 may be essential for the HCMV life cycle. Also in view of this limitation, rVVs seemed to be a suitable system for analysis of UL97-dependent drug phosphorylation. The construction of a variety of rVVs by mutagenesis in vitro allows investigation of the regions of the UL97 protein responsible for drug phosphorylation.

It has been shown recently that UL97 is a protein kinase which autophosphorylates on serine and threonine residues (He et al., 1997) and is able to substitute protein kinase functions exhibited by the HSV UL13 gene product (Ng et al., 1996). Therefore, UL97 may be supposed to be a protein kinase homolog accidentally phosphorylating ganciclovir (Koyano et al., 1996; Michel et al., 1996; Sullivan et al., 1992). Thus, the possibility of a direct effect of GCV or GCV phosphates on UL97 cannot be excluded completely. More information about the role of UL97 in the viral life cycle should be obtained. A study of the functional domains of HCMV UL97 by using site directed mutagenesis of vaccinia recombinants is currently under investigation in our laboratory.

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