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Functional regions of the human cytomegalovirus protein pUL97 involved in nuclear localization and phosphorylation of ganciclovir and pUL97 itself

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In order to identify functional regions of the human cytomegalovirus protein pUL97 (i) different 5' fragments of the UL97 open reading frame (ORF) were fused to the coding region of the green fluorescent protein and (ii) recombinant vaccinia viruses (rVV) were generated carrying two full-length and 11 mutated UL97 ORFs. The results indicated the presence of an N-terminal region within pUL97 which changed the intracellular distribution of the fusion proteins. pUL97 was localized in the nucleus, but not in the nucleoli, and was detected in the nuclear matrix fraction. Expression of all pUL97 mutants could be confirmed by Western blot analysis. pUL97-associated ganciclovir (GCV) phos-

phorylation in rVV-infected cells, determined quantitatively by HPLC analysis, was abolished completely using individual UL97 deletion mutants. Phosphorylation of full-length and some of the mutated pUL97 was detected in cells infected with the rVVs. The UL97 constructs carrying point mutations from GCV-resistant HCMV isolates at positions 460M, 520H, 594V, and the 4 aa deletion 590AACR593, also resulted in decreased but not abolished phosphorylation of GCV in the rVV system, whereas the phosphorylation of pUL97 itself was not influenced. The rVV system is a suitable method for quantitatively testing the functional relevance of pUL97 mutations.

Introduction

Human cytomegalovirus (HCMV) is a major pathogen in immunocompromised individuals. The nucleoside analogue ganciclovir (GCV) is one of the few drugs currently licensed for the treatment of HCMV diseases. The activity of GCV depends on virus-controlled initial phosphorylation in infected cells. It has been shown that open reading frame (ORF) UL97 of HCMV encodes a protein that shares homology with protein kinases but which, on the other hand, directs the phosphorylation of GCV to its monophosphate (Chee *et al.*, 1989; Littler *et al.*, 1992; Sullivan *et al.*, 1992). Several mutations in the coding region of UL97 have been described

which induce the resistance of HCMV strains to GCV in biological assays (Sullivan *et al.*, 1992; Lurain *et al.*, 1994; Wolf *et al.*, 1995; Baldanti *et al.*, 1995; Chou *et al.*, 1995; Hanson *et al.*, 1995). Until now, only point mutations between amino acids 460 and 607 have been detected in GCV-resistant clinical isolates, and neither isolation of clinical HCMV strains lacking the UL97 ORF or carrying extended deletions in the UL97 ORF has been reported, nor has anyone succeeded in generating such mutants in the laboratory (He *et al.*, 1997; Michel *et al.*, 1996). Furthermore, all GCV-resistant HCMV phenotypes examined so far exhibited a reduced phosphorylating capacity, but a complete loss of GCV phosphorylation has never been found. Thus, all data available suggest that the UL97 gene function might be essential for the HCMV life-cycle. Consequently, the question remains, whether the UL97 ORF or pUL97 itself might be a useful target for an alternative antiviral approach in the future.

It has been shown that the UL97 ORF alone is sufficient to allow GCV phosphorylation by the naturally non-GCV-phosphorylating vaccinia virus (Metzger *et al.*, 1994; Michel *et al.*, 1996). Evidence obtained from this heterologous system

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first indicated that pUL97 is not a nucleoside kinase (Michel *et al.*, 1996). It has been shown that pUL97 can partially substitute the function of the herpes simplex virus UL13 gene (Ng *et al.*, 1996) and, furthermore, that after expression in the heterologous baculovirus system, the protein autophosphorylates serines and threonines (He *et al.*, 1997). Recently, it has been published that pUL97 is also present in the virus particles and that it is posttranslationally modified by phosphorylation in HCMV-infected cells (van Zeijl *et al.*, 1997). To get further insight into the still unknown biological function of pUL97, we first wanted to determine the functional domains of this viral protein.

Here we present data showing that there exists only a partial functional overlap between regions on pUL97 which seem to be involved in the pUL97-directed GCV phosphorylation and a region which was found to be essential for phosphorylation of pUL97 itself. Using green fluorescent protein (GFP) fusion proteins we experimentally proved that the N-terminal region of pUL97 is involved in the nuclear localization of the protein.

Methods

■ **Cells and viruses.** Confluent human fibroblasts (HFF) were grown in minimal essential medium and infected with HCMV strain AD169 or clinical isolates as described previously (Michel *et al.*, 1996). 143B cells (thymidine kinase-deficient) or CV1 cells were infected with vaccinia viruses as described earlier (Metzger *et al.*, 1994; Michel *et al.*, 1996).

■ **Plasmids and recombinant vaccinia viruses.** Recombinant vaccinia viruses (rVV) were constructed using the standard vector p7.5K131 for homologous recombination as described previously (Metzger *et al.*, 1994). For cloning of point mutations 520H → Q (strain CMV-6, a kind gift from A. Erice, University of Minnesota Medical School, MS, USA) (Hanson *et al.*, 1995) and 594A → V (Chou *et al.*, 1995) (done in the laboratory of G. Gerna, Servizio di Virologia, Pavia, Italy) in the UL97 ORF of GCV-resistant clinical isolates, the previously described primers pri.5-KpnI and pri.6-EcoRI (Metzger *et al.*, 1994) were used for amplification of an appropriate fragment of DNA from the clinical isolates. The mutation at amino acid position 460M (Lurain *et al.*, 1994) was introduced by site-directed mutagenesis according to the method of Landt *et al.* (1990) by using the mutated primer 5' ATT ACA CCC GTG AAC GTG C 3' for amplification. The UL97 sequences for constructing rVV232, rVV230 and rVV231 were directly cut from the described plasmids: p214 carrying the entire UL97, p213 with a 5' deletion of 11 bp, and p221 with a 5' deletion of 314 bp (Michel *et al.*, 1996). The truncated UL97 ORFs for rVV225 (*Sall*, *EcoRI*), rVV228 (*SmaI*, *EcoRI*), rVV233 (*HindIII*, *KpnI*) and rVV235 (*HindIII*, *Sall*) were cut from plasmid p214 with the indicated endonucleases. rVV234 was obtained by exonuclease III digestion, resulting in a 183 bp (61 aa) deletion in the central region of the UL97 ORF.

For construction of fusion proteins, the plasmids pGREEN LANTERN (Gibco BRL) and pEGFP-N1 (Clontech) encoding GFP from the jellyfish *Aequorea victoria* driven by the HCMV immediate early promoter were used (Prasher *et al.*, 1992; Chalfie *et al.*, 1994). From plasmid p214 the first 720 bp (240 aa) of the UL97 ORF were isolated and translationally fused to GFP, resulting in plasmid pUL97-GFP240. From plasmid p221 a fragment from bp 315 to 720 of the UL97 ORF (aa 111 to 240) was extracted and also fused to GFP, resulting in pUL97-GFP110. Three

additional regions of the UL97 ORF were fused to GFP after amplification by PCR, using a 5' oligonucleotide (5' CTA GTG CAA GCT TAG GAA CAG G 3') and one of the three 3' oligonucleotides (5' ACT GGC CCC GGG GCG CCC T 3', resulting in fusion construct pUL97-GFP33; 5' CCT GCA CCC GGG CTT GAG CG 3', pUL97-GFP48; and 5' ACG CAT CAC GTG AAT TCG AAC G 3', pUL97-GFP110).

■ **Transient expression.** For transient expression experiments, HFF were transfected with 10 to 20 µg plasmid as described by Sambrook *et al.* (1989) by calcium phosphate precipitation followed by glycerol shock. Before transfection, cells were grown for 1 day on glass coverslips in 24-well plates. Immunofluorescence was carried out 24 or 48 h post-transfection.

■ **Indirect immunofluorescence.** For determination of intranuclear localization of pUL97, indirect immunofluorescence with double staining was performed as described previously (Michel *et al.*, 1996). As primary antibodies, we used mouse IgG₁ MAbs (Novus Molecular) against the nucleoli organizing region (NOR) of human cells, and rabbit polyclonal antiserum (Michel *et al.*, 1996) directed against pUL97. Fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG F(ab')₂ (dilution 1:30) and rhodamine-conjugated anti-rabbit IgG (Dianova, dilution 1:30) were used as secondary antibodies.

■ **Western blot analysis.** Total cell lysates were extracted as described by Sambrook *et al.* (1989) from cell monolayers infected with the indicated rVV and harvested 17 to 24 h post-infection (p.i.). The proteins were separated on a 12% SDS-polyacrylamide gel and semi-dry transferred to nitrocellulose membranes. The membranes were probed either with the pUL97 antiserum (dilution of 1:300) or with an anti-pUL97 mouse MAb (pUL97 MAb at a dilution of 1:100) and the protein-antibody complex was detected by using an enhanced chemiluminescence Western blotting detection system (Amersham). The pUL97 MAb was generated in collaboration with P. Lucin at the University of Rijeka (Croatia) using the 47 kDa pUL97 fragment expressed in *E. coli* as described previously (Michel *et al.*, 1996).

■ **HPLC analysis of GCV phosphorylation.** HPLC analysis was performed as described previously (Michel *et al.*, 1996; Zimmermann *et al.*, 1997). 143B cells were infected with the pUL97-expressing rVVs at an m.o.i. of 10.

■ **Cell fractionation.** For fractionation of nuclei and cytoplasm, CV1 cells were infected with rVV at an m.o.i. of 5, harvested 20 h p.i. and washed three times with ice-cold PBS. All steps were performed at 4 °C or on ice unless otherwise mentioned. Cells were lysed in a buffer containing 20 mM Tris-HCl pH 7.5, 0.25 M sucrose, 5 mM MgCl₂, 1% NP40, 1 mM PMSF, and nuclei were sedimented by centrifugation for 10 min at 1500 g. Nuclei were washed three times in 20 mM Tris-HCl pH 7.5, 0.25 M sucrose, 5 mM MgCl₂, 0.2 mM EGTA, 0.1 mM PMSF and digested by 250 µg/ml each of RNase A and DNase I in the same buffer for 30 min at room temperature. After centrifugation at 5000 g for 10 min, the resulting pellet was resuspended in 10 mM Tris-HCl pH 7.5, 0.2 mM MgCl₂, 0.2 mM EGTA, 0.1 mM PMSF and drop-wise adjusted to a final concentration of 1.6 M NaCl with 2 M NaCl in the same buffer. The high salt nuclear extract was clarified by centrifugation for 10 min at 10000 g and the pellet was resuspended in 10 mM Tris-HCl pH 8.5.

■ **Protein kinase assay.** Phosphorylation of pUL97 was determined according to He *et al.* (1997). In brief, the final pellet of nuclear fractionation as described above was resuspended in protein kinase buffer containing 50 mM Tris-HCl pH 9.0, 1 M NaCl, 10 mM MgCl₂ and 2 mM DTT. The protein kinase reaction was started by adding ATP to a final concentration of 5 µM and 2.5 µCi per 10 µl [*γ*-³²P]ATP (sp. act. 6000 Ci/mmol) in the same buffer. After 30 min of incubation at 37 °C

the reaction was terminated by adding an equal volume of $2 \times$ Laemmli sample buffer (Laemmli, 1970) and boiling for 3 min. The phosphorylated proteins were separated by SDS-PAGE (10% acrylamide) and detected by autoradiography.

Results

Intracellular localization of pUL97-GFP fusion proteins

We had previously shown by indirect immunofluorescence that pUL97 is located in the nuclei of infected cells. Subsequent sequence analysis had indicated that pUL97 might contain a nuclear localization signal (NLS) from aa 31 to 36 and sequence alignments even revealed identities with a second (bipartite) NLS (aa 190 to 199) (Schmolke *et al.*, 1996; Michel *et al.*, 1996). In order to experimentally prove such a functional segment in the N-terminal region, we constructed five different fusion proteins (shown in Fig. 1) using the GFP. After transient expression of the GFP alone, it is distributed throughout the cell (see Fig. 2 A). After fusion of a functionally active NLS to the GFP, the intracellular distribution should change.

As summarized in Fig. 1, no change in the intracellular distribution of GFP was observed after transient expression of a GFP fusion protein containing the first 33 aa of pUL97 (pUL97-GFP33). Similarly, the fusion protein containing the first 48 aa of pUL97 (pUL97-GFP48), thereby carrying the whole predicted NLS, was not able to clearly decrease cytoplasmic staining. However, after fusion of the first 110 aa of pUL97 (pUL97-GFP110) to the GFP, no significant signal remained visible in the cytoplasm. An identical result was obtained with the fusion protein containing the first 240 aa of pUL97 (pUL97-GFP240) (Fig. 2 B). To further elucidate a possible role of the second presumed NLS (aa 190 to 199), a truncated N-terminal region (aa 111 to 240) of the pUL97 (pUL97-GFP110) containing only the second putative NLS was used, but did not lead to decreased cytoplasmic staining.

The results suggest that a functional NLS is indeed located in the N-terminal region of pUL97, but between aa 48 and 110, and that the second region alone neither acts as an NLS, nor visibly augments the nuclear localization induced by the functionally active region in our test system.

Intranuclear localization of pUL97

The nuclear localization of pUL97 suggested a functional connection to a structure located within this compartment. With respect to the spotty fluorescence of pUL97 in the nuclei of infected cells (Michel *et al.*, 1996), we investigated whether pUL97 might be able to enter the nucleoli.

To answer this question, indirect immunofluorescence with double staining of HCMV-infected HFF was performed using the antiserum raised against pUL97 and a MAb recognizing the NOR. As shown in Fig. 2(C, D), after double staining it was clearly visible that pUL97 is not located in the nucleoli, since the pUL97 aggregates could be well distinguished from the NOR. Furthermore, during cell fractionation pUL97 was not

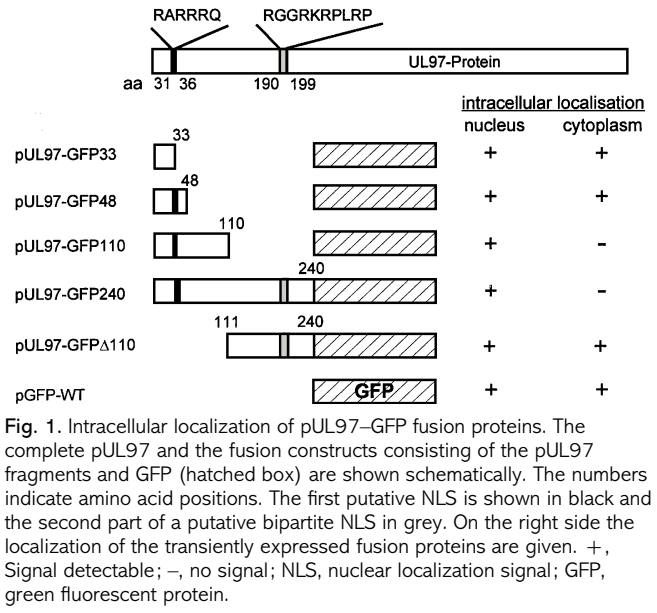


Fig. 1. Intracellular localization of pUL97-GFP fusion proteins. The complete pUL97 and the fusion constructs consisting of the pUL97 fragments and GFP (hatched box) are shown schematically. The numbers indicate amino acid positions. The first putative NLS is shown in black and the second part of a putative bipartite NLS in grey. On the right side the localization of the transiently expressed fusion proteins are given. +, Signal detectable; -, no signal; NLS, nuclear localization signal; GFP, green fluorescent protein.

extractable by DNase I or RNase A digestion from the nuclei (data not shown), but was cofractionated with the nuclear matrix both in HCMV- and rVV-infected cells.

Regions involved in the phosphorylation of GCV

Although there is growing evidence that pUL97 is an autophosphorylating protein kinase, it directs phosphorylation of the nucleoside analogue GCV (He *et al.*, 1997; van Zeijl *et al.*, 1997). We wanted to define segments of the protein involved in phosphorylation of either GCV or pUL97. A quantitative determination of the influence of mutations or deletions in the UL97 ORF on GCV phosphorylation using HCMV-infected HFF is hampered by the fact that pUL97 seems to be essential for virus replication (Michel *et al.*, 1996). Furthermore, the generation of genetically defined HCMV recombinants is laborious. However, we could show that, by using rVV, the quantification of GCV phosphorylation was possible (Michel *et al.*, 1996; Zimmermann *et al.*, 1997) in the absence of any other HCMV genes. Hence, we generated a set of rVVs carrying different wild-type or truncated UL97 ORFs, as well as UL97 constructs carrying point mutations which had been identified in GCV-resistant clinical HCMV isolates (Hanson *et al.*, 1995; Lurain *et al.*, 1994; Chou *et al.*, 1995; Sullivan *et al.*, 1992). A few of these UL97 mutants had been used in the previously described transient expression experiments (Michel *et al.*, 1996). The numbering of the constructs is identical in Figs 3 and 4 and the constructs are characterized on the left side of Fig. 4.

The expression of various sizes of pUL97 was demonstrated by Western blot analysis of extracts from rVV-infected 143B thymidine kinase-deficient cells using our polyclonal pUL97 antiserum (Fig. 3 A) as well as our pUL97 MAb (Fig. 3 C). All rVVs expressed a pUL97 in infected cells. Interestingly, neither

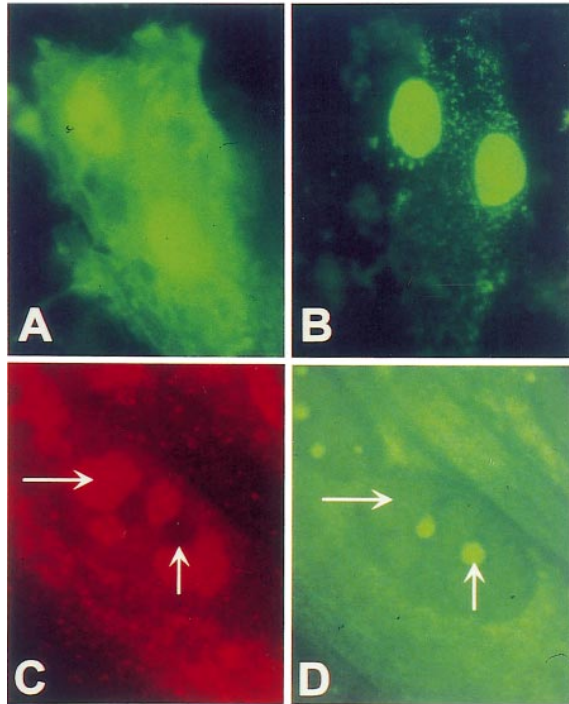


Fig. 2. Intracellular localization of transiently expressed pUL97-GFP fusion proteins (A, B) and intranuclear localization of pUL97 in HCMV-infected fibroblasts (C, D). Transiently expressed pUL97-GFP fusion proteins in fibroblasts were directly visualized by fluorescence microscopy. (A) Control with GFP wild-type. (B) GFP fusion construct containing the first 240 aa of the N-terminal region of pUL97. Indirect immunofluorescence after double staining of HCMV-infected cells. (C) With an antibody directed against the NOR, visualized by a FITC-conjugated secondary antibody, and (D) with pUL97 antiserum and a rhodamine-conjugated secondary antibody. Horizontal arrows indicate pUL97 accumulations, and vertical arrows indicate the NOR. Magnification $\times 1000$.

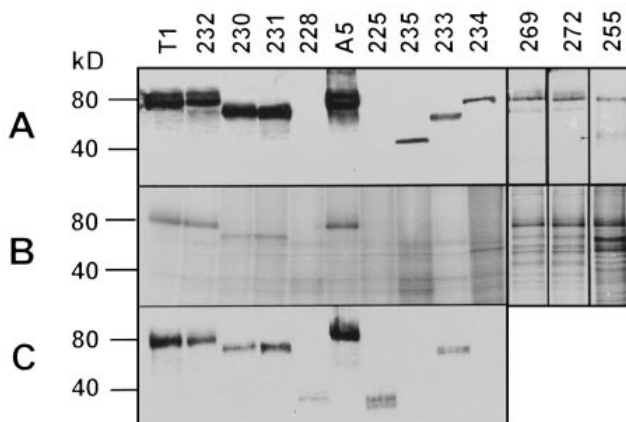


Fig. 3. Expression and phosphorylation of different UL97 proteins after infection with different rVVs. For Western blotting, 143B cells were harvested 17 to 24 h p.i. and proteins were visualized by chemiluminescence either (A) with the pUL97 antiserum or (C) with a mouse pUL97 MAb. T1 to 234 are rVVs carrying different UL97 ORFs mutated in the laboratory; 269, 272 and 255 represent point mutations found in GCV-resistant HCMV isolates. (B) For determination of pUL97 phosphorylation, CV1 cells were infected with the indicated rVV and nuclear matrix fractions were tested for protein kinase activity. Phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography.

the polyclonal antiserum nor the MAb bound to all the UL97 proteins and only by using both antibody preparations could all proteins be detected. These results suggested that the polyclonal antiserum is directed against a region between aa 110 and 292 in the N-terminal part of the protein, whereas the MAb recognizes an epitope in the central region of the pUL97 between aa 327 and 365. The fact that rVV230 and rVV231 expressed proteins of the same size confirmed our previous results obtained by transient expression in HFF, namely that the second and third AUG were not used as signals for the start of translation (Michel *et al.*, 1996). In the transient expression experiments the amounts of protein expressed from these constructs differed (data not shown). One explanation for this phenomenon could be that the different lengths of the 5' non-translated region of the mRNAs might influence the efficiency of translation or the initiation of translation.

For quantification of the pUL97-directed GCV phosphorylation, HPLC analyses of 143B cell extracts were performed after infection with the different rVVs. The results obtained from these experiments are summarized in Fig. 4 (black bars). The rVVs expressing pUL97 with N-terminal deletions (rVV230, rVV231, rVV228) were still able to phosphorylate GCV. Even the lack of 240 aa in rVV228 did not prevent phosphorylation of GCV, which was strongly reduced but still higher than in the mock-infected cell control. The point mutations and the 4 aa deletion (aa 590 to 593) in the C-terminal region, found in GCV-resistant HCMV strains, also led to decreased but not completely abrogated GCV phosphorylation in the vaccinia virus system, as described for the corresponding HCMV variants (Hanson *et al.*, 1995; Lurain *et al.*, 1994; Chou *et al.*, 1995; Sullivan *et al.*, 1992).

A larger N-terminal deletion (rVV225) and extended deletions of the C-terminal region (rVV235, rVV233) completely abolished GCV phosphorylation. Interestingly, the deletion of a central region of 61 aa (rVV234), which revealed similarities to domain I and II of protein kinases containing a nucleotide-binding site (Table 1), also resulted in the complete loss of any detectable pUL97-directed GCV phosphorylation (Fig. 4). These functional regions of protein kinases (Table 1) contain a motif that is predicted to form a secondary structure capable of binding both the phosphate and ribose of a nucleotide and contain an invariant lysine residue (Chee *et al.*, 1989; Hanks *et al.*, 1988). Compared with cellular protein kinases the consensus sequence L/IGXGS/CFGXV (aa 337 to 360) is more conserved in pUL97 than in homologous proteins of other herpesviruses (Table 1). In conclusion, these results suggest that GCV phosphorylation is quantitatively influenced by UL97 mutations in the vaccinia virus system and is not a yes or no phenomenon. Thus, the vaccinia system should be reliable for investigation of UL97 mutations in order to examine their quantitative relevance both for phosphorylation and resistance.

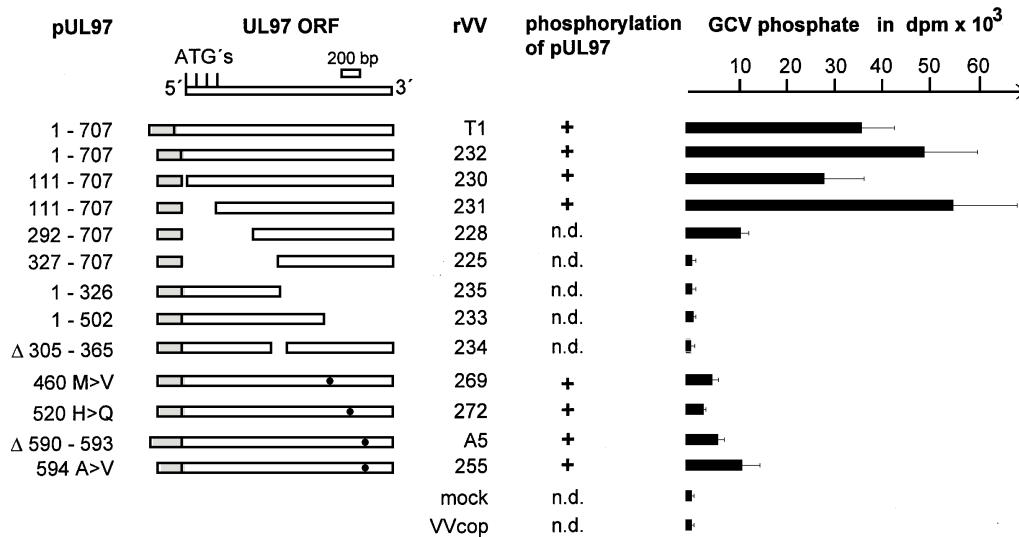


Fig. 4. GCV phosphorylation in cells infected with rVV. The first and last amino acids of the different UL97 proteins expressed after infection with the rVV are indicated on the left. The entire UL97 ORF with the first in-frame ATG codons (top), and the different truncated UL97 sequences inserted into the rVV are depicted schematically (white boxes). Transcription is driven by the 7.5 k promoter of vaccinia virus (grey boxes). The 4 aa deletion (rVVA5) and point mutations that conferred GCV resistance to the original HCMV strains are indicated by black dots. T1 and A5 contain an approximately 100 bp longer untranslated region than the other constructs (232–255). On the right, phosphorylation of GCV in 143B cells after infection with the indicated rVV is shown as black bars. Average values from four or more independent experiments performed in duplicate are presented. VVcop, cells infected with vaccinia strain Copenhagen; +, phosphorylation of the respective pUL97 was demonstrable; n.d., phosphorylation of the respective pUL97 was not detectable.

Table 1. Alignment of the pUL97 central region with nucleotide-binding sites of protein kinases (Hanks *et al.*, 1988) as well as with putative phosphotransferases of other herpesviruses (Chee *et al.*, 1989)

Amino acids are shown in the single letter code. Conserved amino acids are framed.

Protein	
pUL97	L G Q G S F G E V W P L D R Y R V V K V A R K H
SNF1	L G E G S F G K V K L A Y H T T T G Q K V A L K I
PKS	I G T G S F G T V F R G R W H G D V A V K V
Raf	I G S G S F G T V Y K G K W H G D V A V K I
cAPK- α	L G T G S F G R V M L V K H M E T G N H Y A M K I
Src	L G Q G C F G E V W M G T W N G T T R V A I K T
PKC- α	L G K G S F G K V M L A D R K G T E E L Y A I K I
Consensus	L/I G G S/C F G V V/Y A K
EBV BGLF4	L G R G S Y G A V Y A H A D N A T V K T
HSV UL13	G G S G G Y G D V Q L I R E H K L A V K T
VZV 47	A G R G T Y G R V H I Y P S S K I A V K T
HHV-6 15R	L G V G A Y G K V F D L D K V A I K T
Consensus	G G Y G V A/T V/I K T

pUL97-dependent phosphorylation of pUL97

To clarify whether phosphorylation of pUL97 itself also takes place in cells infected with rVV, protein kinase assays were done using nuclear matrix fractions. As shown in Fig. 3(B), in fractions from cells infected with rVVT1 and rVV232 a phosphorylated protein of 80 kDa could be detected. In

fractions from cells infected with rVV230 or rVV231 expressing the same N-terminal truncated pUL97 (see above) a smaller phosphorylated protein of approximately 70 kDa was observed. The point mutations (rVV269, rVV272, rVV255) and the 4 aa deletion (rVVA5) had no visible influence on the phosphorylation of pUL97. No phosphorylation of pUL97

could be detected in extracts from cells infected with the other rVVs (rVV228, rVV225, rVV235, rVV233 and rVV234).

These results showed (i) that phosphorylation of the pUL97 occurs in the heterologous rVV system and (ii) that the 61 aa central region is essential for this function, whereas (iii) the first 110 aa of the N-terminal region have no influence on this function and in addition, seem not to be a quantitatively relevant target for phosphorylation, and (iv) that the point mutations conferring GCV resistance of HCMV have no influence on the protein phosphorylation.

Discussion

Concerning UL97 of HCMV we have to face a quite unique situation. Growing evidence indicates that the gene function might be essential for virus replication and that the gene product might act as an autophosphorylating protein kinase, but the biological function and natural substrates are still unknown. On the other hand, pUL97 provides crucial enzymatic activity for monophosphorylation of the antiviral nucleoside analogue GCV and quite a number of pUL97 point mutations which lead to a GCV-resistant phenotype of the respective HCMV isolates have been described. We wanted to determine functional regions of the pUL97 in analogy to other protein kinases. This study was intended to possibly discriminate between regions involved in the phosphorylation of GCV and others responsible for phosphorylation of pUL97 itself using UL97 mutants in the rVV system. Furthermore, we wanted to experimentally confirm the presence of an N-terminal region responsible for nuclear localization and finally to determine the intranuclear localization of the pUL97. We hoped that by studying the different functional parameters of different mutants, it might also be possible to discriminate between independent and associated effects.

N-terminal region of pUL97

There are significant similarities in the N-terminal region of pUL97 with the NLS of other viral proteins, especially with the transacting proteins of the retroviruses human T-lymphotropic virus type I and human immunodeficiency virus type I, and the tegument protein pp65 of HCMV (Michel *et al.*, 1996). Using pUL97-GFP fusion proteins for transient expression we could show that pUL97 might indeed contain an NLS in the N-terminal region. We could not demonstrate an effect or additional effect of a second basic region of the UL97 protein (RGGRKRPLRP, aa 190–199). After sequence alignments, we had suspected it to be the second part of a bipartite NLS, as had already been shown for the polyoma large T antigen and for pp65 of HCMV. Both proteins contain two regions (bipartite) which can direct nuclear uptake of the respective proteins (Michel *et al.*, 1996). Surprisingly, there was no evidence of an influence on nuclear transport of pUL97 for either suspected region, but we could map an NLS function between aa 48 and 110 and show that the nuclear uptake of this karyophilic

protein is also a signal-mediated process. Although located in the nuclei, pUL97 was not found in the nucleoli, as shown by double staining experiments, but bound to the nuclear matrix fraction after biochemical fractionation of nuclei. The probable connection between localization of the protein and the biological function remains to be elucidated.

The central part of pUL97

The central region reveals similarities to domains I and II of protein kinases (Chee *et al.*, 1989), with a consensus sequence G-X-G-X-X-G located at the N-terminal side of a lysine implicated in the binding of ATP (Wierenga & Hol, 1983; Kamps *et al.*, 1984; Hanks *et al.*, 1988). Since it has been shown that proteins containing the mentioned cluster of glycines are capable of phosphorylating proteins at tyrosine residues, it had to be clarified whether or not pUL97 is also able to phosphorylate proteins. On the other hand, similar clusters of glycines have also been found in highly conserved regions of a number of regulatory proteins with DNA-binding properties, including the repressor and cro proteins of bacteriophages lambda and the Mu phage B transposition protein.

The evidence of protein phosphorylating activity came from He *et al.* (1997), who showed that pUL97 autophosphorylates threonines and serines. The assumption that pUL97 might rather be connected with protein kinase activity was supported by results showing that it partially substitutes for the function of the UL13 protein of herpes simplex virus type 1 (Ng *et al.*, 1996). This protein has been demonstrated to control the phosphorylation of the regulatory gene alpha 22 (Purves & Roizman, 1992). However, the definite biological function of UL13 in virus replication also remains to be determined.

We now provide evidence that pUL97 is also phosphorylated in cells infected by rVV. We could not formally prove that this happened due to autophosphorylation, since pUL97 was not purified from rVV-infected cells in sufficient amounts as it was strongly associated with the nuclear matrix. We clearly showed that the deletion of a central part of a wild-type pUL97 comprising the presumed ATP-binding side led to a complete loss of pUL97- and GCV-phosphorylation as well. Our results are in very good accordance with the observation of He *et al.* (1997), who expressed pUL97 in the baculovirus system. They could show by mutation analysis that a lysine located in this pUL97 region at position 355 is essential for autophosphorylation of the protein. Mutations in the central domain may interfere with a presumed essential function of pUL97 in the HCMV life-cycle for, until now, no natural mutation has been described for this region, neither in clinical HCMV isolates nor in laboratory HCMV strains.

The C-terminal part of UL97

The C-terminal portion of pUL97 is obviously involved in GCV phosphorylation and might be involved in GCV binding.

However, no direct proof for the latter assumption has been published so far. The biological relevance of this region is not clear, but an homologous region in the bovine cyclic AMP-dependent protein kinase has been implicated in binding of an inhibitory peptide (Knighton *et al.*, 1991). This could indicate that this region might be involved in substrate binding. Point mutations and small deletions in this domain have been detected in phenotypically GCV-resistant HCMV (Lurain *et al.*, 1994; Wolf *et al.*, 1995; Baldanti *et al.*, 1995; Chou *et al.*, 1995; Hanson *et al.*, 1995). The 4 aa deletion and the point mutations investigated here in the vaccinia virus system had no detectable influence on phosphorylation of pUL97. In contrast, the GCV phosphorylation was clearly impaired but not completely abolished.

Interdependence of the domains and the influence of mutations

Our results indicate that the NLS-containing region is essentially involved in neither the phosphorylation of pUL97, nor GCV phosphorylation, at least in cells infected with rVV. However, it is reasonable to assume that the biological function during HCMV infection might depend on nuclear localization and on interaction with other nuclear-localized substrates. Obviously, the presence of pUL97 in virions is in very good accordance with nuclear localization as reported by van Zeijl *et al.* (1997). But it is also possible that pUL97 may interact with other proteins in order to transport them in an ATP-dependent manner into the nucleus as shown for the adenovirus DNA polymerase (Zhao & Padmanabhan, 1988).

Deletion of the central region abolished GCV phosphorylation as well as phosphorylation of pUL97. The same is true for rVV225, representing only the C-terminal half of the protein, and rVV235, expressing the complementary part of the pUL97. Both proteins contain only half of the central region, whereby the active centre may be inactivated. All data available today indicate that this central region might be indispensable for autophosphorylation and the presumably essential biological function of pUL97. Mutations in the C-terminal region could be tolerated as long as only GCV phosphorylation is impaired. It remains unclear whether GCV has to be bound to UL97. The fact that rVV233 is not able to phosphorylate GCV could be explained by the large C-terminal deletion. It is not clear why phosphorylation of pUL97 does not take place either, but this might be due to conformational changes of the truncated protein. On the other hand, rVV228 containing the central region is able to phosphorylate GCV, but phosphorylation of the protein itself could not be detected. It could be that rVV228 lacks the target site for phosphorylation still present in rVV231, but this has to be proven. Based upon our data it is not possible to completely distinguish between the function of a central region and a C-terminal region, but we could say that (i) the N-terminal segment assures that the protein is capable of entering the nuclei of the infected cells, and one may speculate that (ii) the

C terminus is mainly involved in GCV phosphorylation or may even bind GCV, and (iii) the central region is connected with the phosphorylation of both GCV and pUL97 itself.

In the future it has to be determined whether pUL97 can bind/contact other proteins/nucleic acids, and the relevance of pUL97 phosphorylation has to be clarified. Furthermore it should be interesting to investigate whether mutations found in different pUL97 regions in clinical isolates have different quantitative influences on the phosphorylation of pUL97. Blocking UL97 expression or its function should be considered as a target for antiviral therapy.

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