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The Human Cytomegalovirus UL97 Protein Is a Protein Kinase That Autophosphorylates on Serines and Threonines

ZUWEN HE,[1] YUN-SHENG HE,[1]† YOUNHEE KIM,[1]‡ LILY CHU,[2]§ CAROL OHMSTEDE,[2]‖ KAREN K. BIRON,[3] AND DONALD M. COEN[1]*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115,[1] and Division of Cell Biology[2] and Department of Virology,[3] Glaxo Wellcome Company, Research Triangle Park, North Carolina 27709

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The product of the human cytomegalovirus (CMV) *UL97* gene, which controls ganciclovir phosphorylation in virus-infected cells, is homologous to known protein kinases but diverges from them at a number of positions that are functionally important. To investigate UL97, we raised an antibody against it and overexpressed it in baculovirus-infected insect cells. Recombinant baculovirus expressing full-length UL97 directed the phosphorylation of ganciclovir in insect cells, which was abolished by a four-codon deletion that confers ganciclovir resistance to CMV. When incubated with [γ-^{32}P]ATP, full-length UL97 was phosphorylated on serine and threonine residues. Phosphorylation was severely impaired by a point mutation that alters lysine-355 in a motif that aligns with subdomain II of protein kinases. However, phosphorylation was impaired much less severely by the four-codon deletion. A UL97 fusion protein expressed from recombinant baculovirus was purified to near homogeneity. It too was phosphorylated upon incubation with [γ-^{32}P]ATP in vitro. This phosphorylation, which was abolished by the lysine 355 mutation, was optimal at high NaCl and high pH. The activity required either Mn^{2+} or Mg^{2+}, with a preference for Mn^{2+}, and utilized either ATP or GTP as a phosphate donor, with K_ms of 2 and 4 μM, respectively. The phosphorylation rate was first order with protein concentration, consistent with autophosphorylation. These data strongly argue that UL97 is a serine/threonine protein kinase that autophosphorylates and suggest that the four-codon deletion affects its substrate specificity.

Human cytomegalovirus (CMV) is an important pathogen in immunosuppressed individuals such as those with AIDS. Ganciclovir (GCV) is an antiviral drug widely used to treat CMV infections. However, GCV and other licensed and investigational drugs against CMV are limited by toxicity, pharmacokinetic problems, and/or lack of potency or efficacy in various settings. Moreover, drug resistance is a substantial problem (3, 13, 14, 45). Thus, it would be valuable to gain further understanding of GCV resistance and to discover new anti-CMV drugs.

Information about the CMV UL97 protein may abet the achievement of both of these goals. UL97, which is homologous to protein kinases (5), controls the phosphorylation of GCV in CMV-infected cells, as *UL97* mutations confer GCV resistance and decreased phosphorylation (1, 17, 26, 43). UL97, when expressed in heterologous systems, can induce GCV phosphorylation (25, 28, 29); thus, it is the only CMV protein required for this. Littler et al. (25) reported that extracts of *Escherichia coli* expressing UL97 can phosphorylate GCV and that antisera raised against UL97 partially purified from *E. coli* can immunoprecipitate GCV kinase activity from extracts of CMV-infected human cells. Thus, it appears that *UL97* mutations exert resistance by impairing the GCV kinase activity of UL97.

Interestingly, of the 40 or so GCV-resistant (GCV^r) *UL97* mutants derived from clinical isolates or laboratory strains published to date, none is obviously a null mutant. For example, no nonsense or frameshift mutations have been published. Two classes of mutations can be discerned. Some (1–3, 6, 7, 17, 47, 48) affect a segment that corresponds to a portion of protein kinases that recognizes peptide and protein substrates (21–24, 49). Other mutations (6, 7, 26, 47) have mapped to codon 460, which corresponds to a nonconserved residue in the catalytic loops of protein kinases that is also implicated in substrate recognition (21–24, 49). This finding suggests the hypothesis that these mutations drastically affect the phosphorylation of GCV without major effects on phosphorylation of its natural substrate. The corollary of this hypothesis is that UL97 might be essential for CMV replication. In that case, UL97 could be a valid target for inhibition by antiviral drugs.

The sequence of UL97 diverges from those of protein kinases sufficiently to raise the question of whether it is in fact a protein kinase (5). Homologs of UL97 encoded by several alphaherpesviruses diverge less from known protein kinases than does UL97 (5). These proteins are phosphorylated and are required for phosphorylation of certain proteins in virus-infected cells or in extracts of infected cells (8, 9, 11, 20, 32–37, 41). When immunoprecipitated from virus-infected cells and incubated with [γ-^{32}P]ATP, these proteins become phosphorylated. However, none of these proteins has been purified and shown to contain protein kinase activity. Indeed, Stevenson et al. (41) found that heterologously expressed varicella-zoster virus (VZV) gene 47 protein (ORF 47) was unable to be phosphorylated in vitro and suggested that other proteins were involved in phosphorylation.

To begin characterization of UL97, we have raised an anti-

* Corresponding author. Mailing address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 250 Longwood Ave., Boston, MA 02115. Phone: (617) 432-1691. Fax: (617) 432-3833. E-mail: dcoen@warren.med.harvard.edu.

† Present address: Metabolism Branch, National Cancer Institute, Bethesda, MD 20892.

‡ Present address: Department of Biological Education, Seoul National University, Seoul, Korea.

§ Present address: 4735 Anglia St., Manlius, NY 13104.

‖ Present address: Worldwide Project Planning, Glaxo-Wellcome Co., Research Triangle Park, NC 27709.

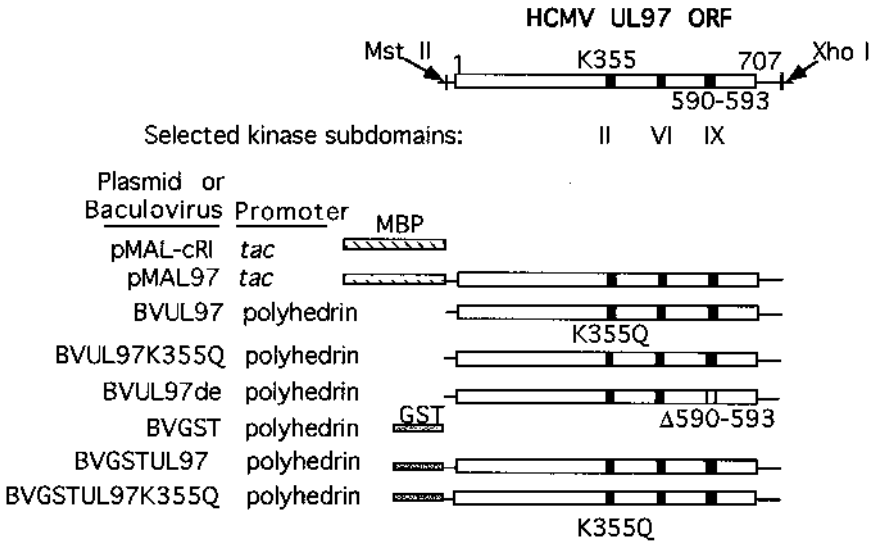

FIG. 1. Constructs for expression of wild-type and mutant forms of UL97. The top line indicates the *UL97* open reading frame (ORF) as an open box. Restriction sites for *Mst*II and *Xho*I that flank the open reading frame are shown. Segments of UL97 corresponding to protein kinase subdomains II, VI, and IX (16) are indicated as filled boxes, and the positions of lysine 355 (K355) and residues 590 to 593, which are altered in mutant forms of UL97, are shown. Below are diagrammed the constructs used for expression of UL97. The top two constructs are plasmids that were used for expression in *E. coli*, and the remaining constructs are recombinant baculoviruses. The promoter sequences used (*tac* for bacterial expression and polyhedrin for baculovirus expression) are indicated. Fusion partners are designated by hatched boxes for MBP and shaded boxes for GST. The *UL97* open reading frame is cartooned as an open box. The position of the K355Q *UL97* mutation is designated by K355Q and a shaded box in place of a filled box, and the four-codon deletion from mutant 759^{r}D100 (43) is indicated by Δ590-593 and an open box (replacing a filled box) when these mutations are present.

body against it, overexpressed it in baculovirus-infected insect cells, and purified it to near homogeneity. Our results argue that UL97 is indeed a protein kinase that can phosphorylate itself on serine and threonine residues and that at least one GCV resistance mutation affects the substrate specificity of UL97.

MATERIALS AND METHODS

Cells and viruses. *Spodoptera frugiperda* Sf9 and Sf21 cells were obtained from Invitrogen and maintained in Grace's insect medium (BioWhittaker) supplemented with 10% fetal calf serum, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. Recombinant baculoviruses derived from *Autographa californica* nuclear polyhedrosis virus were propagated by using standard methods (44).

Expression of MBP-UL97 fusion protein in *E. coli*. Plasmid pADEH contains a 3.5-kb *Eco*RI-*Hin*dIII fragment (positions 9415 to 12913 in the CMV strain AD169 genome) isolated from cosmid pCM1065 (15) (kindly provided by B. Fleckenstein) inserted into the corresponding sites of pGEM7Zf(+) (Promega Inc.). This plasmid, which contains the *UL97* open reading frame, was constructed by V. Sullivan of the Coen laboratory. A 2.25-kb fragment (positions 10376 to 12632) obtained by digesting pADEH with *Mst*II and *Xho*I was treated with Klenow fragment to generate blunt ends and cloned into the *Pvu*II site of pIng14.1 (generous gift of S. Inglis, Cantab Pharmaceuticals). The 2.25-kb *Bgl*II-*Hin*dIII (these sites flank the *Pvu*II site in pIng14.1) fragment from the resulting plasmid was then inserted between *Bam*HI and *Hin*dIII sites of pMAL-cRI (New England Biolabs) to generate pMAL97 (Fig. 1), thus placing the *UL97* gene downstream of and in frame with a gene encoding maltose-binding protein (MBP). Bacteria harboring pMAL97 were grown in LB medium to an A_{260} of 0.4 and induced with 0.1 mM isopropythiogalactopyranoside (IPTG) at 30°C for 6 h, allowing expression of a 124-kDa MBP-UL97 fusion protein at about 2 mg/liter following IPTG induction at 30°C, with about 40% of the protein remaining soluble.

Preparation of antibody. For the preparation of antibody, MBP-UL97 fusion protein was isolated. The cell paste from 500 ml of pMAL97-transformed bacteria that had been induced to express MBP-UL97 was resuspended in lysis buffer A (20 mM Tris [pH 7.4], 0.2 M NaCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 10% glycerol) and lysed by sonication. Insoluble materials were removed by centrifugation. The supernatant was loaded onto an amylose column (New England Biolabs), followed by washing with 5 column volumes of lysis buffer A. After elution with 10 mM maltose in lysis buffer A, the fusion protein was the predominant species with three or four minor species (19). Rabbit antisera against MBP-UL97 fusion protein was prepared by Promega (Madison, Wis.). The initial immunization was with 0.5 ml (~500 μg) of antigen mixed with 0.5 ml of Freund's complete adjuvant. Rabbits were boosted four times with Freund's incomplete adjuvant. Final antisera were collected on day 139.

Immunochemistry. For Western blotting, proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10% polyacrylamide gel) and then electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell). After transfer, the membrane was incubated for 30 min in 3% nonfat dry milk (Bio-Rad) in TTBS buffer (20 mM Tris [pH 7.5], 0.9% NaCl, 0.1% Tween 20) followed by washing with TTBS buffer. The membrane was then incubated for 30 min at room temperature with primary antibodies diluted 1:300 in TTBS buffer followed by incubation with alkaline phosphate-conjugated anti-rabbit antibodies in TTBS buffer for 30 min. Immunoreactive bands were detected by using 5-bromo-4-chloro-3-indolylphosphate (0.15 mg/ml) and nitro blue tetrazolium (0.3 mg/ml) in 50 mM Tris (pH 9.5)–5 mM $MgCl_2$.

To assay relative UL97 concentrations in cell extracts, Western blots of gels containing dilutions of each sample and dilutions of known amounts of UL97 were developed as described above, using incubation times so that the intensities of the UL97 signals were proportional to the amounts of UL97 loaded on the gel ($r^2 \geq 0.89$). The blots were scanned by using a Microtek scanner, a Macintosh computer, and Adobe Photoshop 3.0, and the intensities of UL97 bands were measured by using Image 1.31p.

Construction of recombinant baculoviruses. For each recombinant baculovirus, a transfer plasmid was constructed and then cotransfected with linearized A. californica nuclear polyhedrosis virus DNA (InVitrogen or PharMingen), using cationic liposomes (InVitrogen) or calcium phosphate. Relevant features of the constructs are shown in Fig. 1. To construct BVUL97, which expresses wild-type UL97, the 2.25-kb *Mst*II-*Xho*I fragment from pADEH that contains the AD169 *UL97* gene was treated with Klenow fragment to create blunt ends, ligated with a 5′-phosphorylated *Xba*I linker, and then inserted into the *Nhe*I site of pBlueBac (InVitrogen), resulting in transfer plasmid pBlueBac97. To construct BVUL97de, the 2.25-kb *Mst*II-*Xho*I fragment from pGEH7 (43), which contains the *UL97* gene from GCV^r mutant 759^{r}D100, was treated with Klenow fragment to create blunt ends, ligated with phosphorylated *Xba*I linker, and then inserted onto pBlueBac, resulting in transfer plasmid pBlueBac97de. To construct BVUL97K355Q, in which codon 355 of *UL97* was altered to encode glutamine instead of lysine (K355Q), the *Xba*I fragment containing *UL97* from pBlueBac97 was cloned into M13 mp18. Single-stranded DNA was prepared and used as a template for oligonucleotide-directed mutagenesis, using oligonucleotide 5′CGC GTGGTCCAGGTGGCGCG3′ and the method of Taylor et al. (46) with an in vitro mutagenesis kit (Amersham) according to manufacturer's recommendations. The mutation was identified and shown to be the only mutation in *UL97* sequences by DNA sequencing. The *Xba*I fragment containing the mutation was then inserted into pBlueBac to produce the transfer plasmid pBlueBac97K355Q. To construct BVGSTUL97, which expresses a glutathione *S*-transferase (GST)-UL97 fusion protein, plasmid pMAL97 was digested with *Hin*dIII and *Eco*RI,

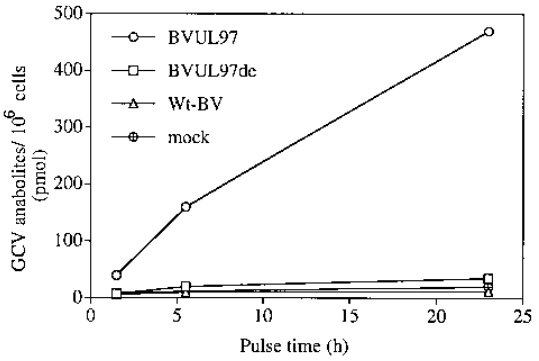

FIG. 2. Recombinant baculovirus BV97 induces GCV phosphorylation in insect cells. Insect cells that were either mock infected or infected with the indicated viruses were pulsed with radiolabeled GCV for the indicated times, and radiolabeled GCV anabolites were measured following cation-exchange chromatography as described previously (40).

treated with Klenow fragment to generate blunt ends, and then ligated with phosphorylated oligonucleotide 5′TTTGTTGAAGAATTCTCAACAAA3′ to adjust the open reading frame and create *Eco*RI sites. This DNA was then digested with *Eco*RI and inserted into the *Eco*RI site of the plasmid pAcG3X baculovirus expression vector (PharMingen) to generate transfer plasmid pGSTUL97. To construct a baculovirus expressing a GST-UL97 fusion protein with the K355Q alteration (BVGSTUL97K355Q), the *Eco*RI fragment of pGSTUL97 was subcloned into pGEX2T (Pharmacia), resulting in pGEX97. The *Sph*I-*Ppu*MI fragment from the UL97 coding region of pGEX97 was replaced with the corresponding fragment of pBlueBac97K355Q to create plasmid pGEX97K355Q. The *Eco*RI fragment of this plasmid was then inserted back into pAcG3X to generate transfer plasmid pGST97K355Q. The orientation of the insert was confirmed by restriction enzyme analysis, and the presence of the K355Q mutation was confirmed by DNA sequencing. To construct BVGST, the transfer plasmid was pAcG3x (PharMingen), which encodes GST. The recombinant viruses were plaque purified three times and expanded into virus stocks. Protein expression was confirmed by immunoblotting analysis using antibodies against either MBP-UL97 fusion protein and/or GST.

Anabolism of GCV. Sf9 monolayers in 35-mm-diameter dishes were infected at a multiplicity of infection (MOI) of 0.2. At 3 days postinfection, the cells were pulse-labeled with 50 μM ^{14}C-labeled GCV (specific activity, 52 mCi/mmol) which had been purified by high-performance liquid chromatography to remove guanine contaminants. Cells were extracted with perchloric acid, and GCV anabolites were determined with a cation-exchange column as described previously (40).

Protein kinase assays. Unless otherwise stated, individual protein samples were added to 20 μl of reaction buffer containing 50 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 5 μM ATP, 2 mM DTT, 1 M NaCl, and 5 μCi of [γ-^{32}P]ATP at 6,000 Ci/mmol. After incubation at 37°C for 30 min, the reaction was terminated by addition of 20 μl of 2× Laemmli sample buffer and boiling for 3 min. The phosphorylated protein was resolved by SDS-PAGE (10% gel) and detected by autoradiography. For quantification, the region of the gel corresponding to the labeled protein was excised and Cerenkov radioactivity was counted. For assay of extracts of insect cells infected with BVUL97 and related recombinant viruses, 2×10^7 mock-infected or infected cells were broken by sonication in 1 ml of ice-cold lysis buffer B (50 mM Tris-HCl [pH 7.6], 100 mM NaCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 10% glycerol, 10 mg of aprotinin per ml, 10 mg of leupeptin per ml, 1 mM PMSF). Insoluble material was removed by centrifugation at 12,000 × *g* for 5 min.

Identification of phosphoamino acids. To prepare phosphorylated UL97 for phosphoamino acid analysis, UL97 was partially purified from BVUL97-infected insect cells. All operations were performed at 0 to 4°C unless otherwise specified. Sf9 cells were infected with BVUL97 at an MOI of 5 and incubated at 27°C for 40 to 46 h. Infected cells were scraped into the culture medium, centrifuged for 5 min at 800 × *g*, washed with phosphate-buffered saline, and resuspended in 50 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$–0.5% Nonidet NP-40–1 mM PMSF–1 mM benzamidine–2 mM DTT (lysis buffer C). The cells were incubated for 10 min and then sheared by six strokes in a Dounce homogenizer. The nuclear fraction was collected by centrifugation for 5 min at 1,800 × *g* and then resuspended in lysis buffer C, frozen on dry ice, and stored at −80°C. The frozen pellets were subsequently thawed, and 5 M NaCl was added to a final concentration of 0.6 M. The samples were gently rocked for 40 min, and then DNase I was added to 0.1 mg/ml. Following another 40 min of incubation, the samples were centrifuged for 5 min at 10,000 × *g*. The pellet was resuspended in lysis buffer C containing 10% glycerol and stored at −80°C. Following thawing, the material was subjected to phosphorylation in a protein kinase assay as described above. Phosphoproteins were resolved by SDS-PAGE (10% gel). The portion of the gel containing UL97 was excised and ground into small pieces, and radioactive protein was eluted by boiling for 3 min in 50 mM ammonium bicarbonate–10 mM β-mercaptoethanol–0.3% SDS. The eluted protein was coprecipitated with 20 μg of bovine serum albumin in ice-cold 20% trichloroacetic acid. The pellet was washed with ethanol, dried, and then subjected to acid hydrolysis in 50 μl of 6 N HCl for 3 h at 110°C. After drying, the samples were spotted onto a cellulose thin-layer plate with a mixture of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine and subjected to electrophoresis at pH 1.9 (acetic acid-formic acid-water, 78:25:897) in one dimension and at pH 3.5 (butanol-pyridine-acetic acid-water, 15:10:3:127) in the other dimension. The plate was then stained with ninhydrin to locate the positions of the unlabeled phosphopeptides and autoradiographed.

Purification of GST-UL97 fusion protein. All purification steps were performed at 4°C. Sf21 cells were infected with BVGSTUL97 or BVGSTUL97K355Q at an MOI of 5 PFU at 27°C. At 40 to 46 h postinfection, the infected cells were harvested by scraping followed by centrifugation at 500 × *g* for 5 min and then stored as slurry at −80°C. The paste was thawed in 3 volumes of lysis buffer D (1× phosphate-buffered saline, 10 mM EDTA, 2 mM DTT, 10% glycerol, 1 mM PMSF, 1 mM benzamidine, 0.5 μg of leupeptin per ml, 20 μg of aprotinin per ml, 1 mM sodium metasulfite, 25 μg of antipain per ml, 10 μg of pepstatin A per ml, 7 μg of E-64 per ml [protease inhibitors from Sigma]) and lysed in a French press at 1,000 lb/in^2. The homogenate was centrifuged at 10,000 × *g* for 30 min, and the resulting supernatant was loaded on a 3-ml glutathione affinity column (Pharmacia). The column was washed with 20 ml of lysis buffer D, and the protein was eluted with elution buffer (50 mM Tris [pH 8.0], 10% glycerol, 2 mM EDTA, 2 mM DTT, 50 mM NaCl, 10 mM reduced glutathione). The fractions containing GST fusion proteins were pooled and applied to a Q-Sepharose Fast Flow column (Pharmacia) and eluted by using a 0.2 to 1.0 NaCl gradient in elution buffer. Fractions containing GST-UL97 were pooled and repurified by using a glutathione column to remove NaCl. The fractions containing fusion protein were then chromatographed on an SP Sepharose high-performance column (Pharmacia), using a 0.1 to 1.0 M NaCl gradient in elution buffer. The fractions containing GST-UL97 were collected and concentrated in a Centricon 30 (Amicon). Protein concentrations were determined by the Bio-Rad microassay system, using bovine serum albumin as a protein standard and a predicted molecular mass of 104 kDa for GST-UL97. Purified preparations were stored at −80°C.

RESULTS

Induction of GCV phosphorylation by UL97 in recombinant baculovirus-infected insect cells. We initially attempted to detect enzymatic activity with an MBP-UL97 fusion protein expressed in *E. coli*. Although this material was useful for preparing anti-UL97 antisera (see Materials and Methods), it contained no enzymatic activity in our hands (19). We then turned to the baculovirus system to attempt to express enzymatically active UL97. The wild-type *UL97* gene from CMV strain AD169 and the *UL97* gene containing a four-codon deletion from GCV^r mutant 759^{r}D100 (43) were each introduced into baculovirus under the control of the polyhedrin promoter. The resulting viruses, BVUL97 and BVUL97de (Fig. 1), expressed a new ~80-kDa polypeptide that reacted with anti-UL97 antisera on Western blots (19). To determine if the UL97 was biologically active, we measured the anabolism of radiolabeled GCV in mock-infected insect cells or insect

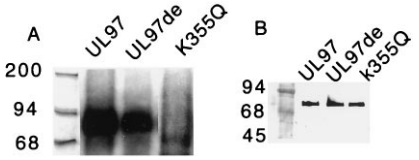

FIG. 3. Phosphorylation of baculovirus-expressed UL97. Insect cells were infected with recombinant baculovirus BVUL97 (UL97), BVUL97de (UL97de), or BVUL97K355Q (K355Q). Extracts were prepared and incubated with radiolabeled ATP. Equal aliquots of all extracts were subjected to SDS-PAGE. Gels were then subjected to either autoradiography (A) or Western blot analysis (B). The leftmost lane in each panel contains protein size markers, and the sizes of the proteins are indicated in kilodaltons to the left of the panels. The faint band below the position of UL97 in the K355Q lane was also observed in extracts of cells infected with wt-BV (19). The image in this and other continuous-tone figures was obtained by scanning with a Microtek scanner and Adobe Photoshop 3.0, using a MacIntosh computer.

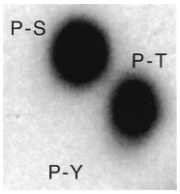

FIG. 4. Phosphoamino acid analysis of baculovirus-expressed UL97. UL97 expressed by BVUL97 was partially purified, labeled with [γ-^{32}P]ATP, gel purified, acid hydrolyzed, and mixed with unlabeled phosphoamino acids, and the mixture was subjected to two-dimensional electrophoresis. The thin-layer plate was then visualized with ninhydrin and autoradiographed. The autoradiograph is shown. The positions of unlabeled phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) are indicated.

cells infected with either BVUL97, BVUL97de, or baculovirus containing no insert (wt-BV). BVUL97-infected cells phosphorylated GCV far more effectively than did wt-BV- or mock-infected cells (Fig. 2). The rate of phosphorylation in the BVUL97-infected insect cells was similar to that observed in CMV-infected human cells (43). BVUL97de was severely impaired for induction of GCV phosphorylation (Fig. 2); thus, the four-codon deletion that impairs GCV phosphorylation in CMV-infected human cells exerts the same effect in insect cells. We conclude that baculovirus-expressed UL97 is biologically active, as measured by GCV phosphorylation.

Effects of mutations on phosphorylation of baculovirus-expressed UL97. As many protein kinases have autophosphorylating activity and there is evidence for autophosphorylation by certain alphaherpesvirus homologs of UL97 (9, 11, 32), we incubated extracts of insect cells infected with BVUL97 with [γ-^{32}P]ATP. Under the conditions used (high salt and high pH), which suppressed endogenous kinase activities, an 80-kDa polypeptide was the major labeled species (Fig. 3A). This labeled species comigrated with BVUL97-expressed UL97 detected by Coomassie blue staining or Western blot analysis (19). No labeling at this position was observed when wt-BV-infected insect cells was subjected to incubation with [γ-^{32}P]ATP (19). These data suggested that UL97 could autophosphorylate.

To examine this apparent autophosphorylation further, we tested mutant forms of UL97 for this activity. Insect cells were infected with either BVUL97, BVUL97de, or BVUL97K355Q. The latter mutant (Fig. 1) contains a glutamine in place of a lysine that corresponds to an invariant lysine in subdomain II of protein kinases that cannot be mutated without loss of activity (16). In cyclic AMP-dependent protein kinase, this lysine aligns the phosphates of ATP (23). Extracts were prepared and incubated with [γ-^{32}P]ATP. Substantial labeling of UL97 was observed in extracts of BVUL97- and BVUL97de-infected cells (Fig. 3A). Little or no labeling was observed in extracts of BVUL97K355Q-infected cells (Fig. 3A), although the extract contained a similar amount of UL97 as did the other extracts (Fig. 3B). The amounts of UL97 present in the various cell extracts were compared by semiquantitative Western blot analysis (see Materials and Methods), and the relative amounts of labeling per UL97 protein of various mutants were determined. The K355Q point mutant was impaired ≥20-fold. This finding strongly suggests that UL97 catalytic activity is required for UL97 phosphorylation, i.e., that UL97 autophosphorylates. However, the four-codon deletion in BVUL97de, which severely impaired GCV phosphorylation, had only a two- to threefold effect on UL97 phosphorylation (44% of the specific activity of BVUL97).

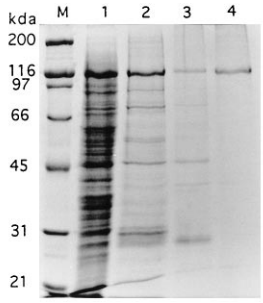

FIG. 5. Purification of GST-UL97. Shown is a Coomassie blue-stained SDS-polyacrylamide gel of aliquots of protein markers (lane M), lysate of insect cells infected with BVGSTUL97 (lane 1), eluate of a glutathione affinity column of the lysate (lane 2), pooled fractions from Q-Sepharose chromatography (lane 3), and concentrated fractions from SP Sepharose chromatography (lane 4). The sizes of protein markers are indicated to the left.

UL97 becomes phosphorylated on serine and threonine. The above data strongly suggested that UL97 autophosphorylates. To determine the amino acids phosphorylated, we partially purified UL97 from BVUL97-infected cells, incubated it in vitro with [γ-^{32}P]ATP, purified the labeled UL97 by gel electrophoresis, and subjected it to phosphoamino acid analysis (Fig. 4). About 65% of the radioactivity comigrated with phosphoserine, and about 35% comigrated with phosphothreonine. No labeled species corresponding to phosphotyrosine was observed. Thus, the protein kinase associated with UL97 is a serine/threonine kinase.

Purification of GST-UL97 fusion protein. Although we could express relatively high levels of UL97 by using BVUL97 and although our data strongly suggested that at least some of this protein was active, this expression system suffered a number of drawbacks. First, the extent of phosphorylation was less than 1% (18), suggesting that a large proportion of the protein was not active. Second, the vast majority of the protein behaved as if it were insoluble, cosedimenting with nuclei following cell lysis and sedimenting at low speed even after the nuclei were lysed with high salt and treated with DNase. Interestingly, this material did not have a specific activity meaningfully lower than that of the material that did not sediment following cell lysis (18). Third, although the rapid sedimentation of the pro-

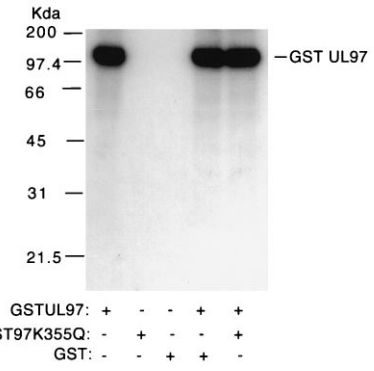

FIG. 6. Phosphorylation of purified GST-UL97. Purified GST-UL97, GST-UL97K355Q, or GST or mixtures of GST-UL97 with each of the other proteins were incubated with [γ-^{32}P]ATP and subjected to SDS-PAGE. An autoradiograph of the gel is shown, and the protein content of the mixtures is indicated at the bottom (+, protein present; −, protein absent). The positions of proteins of the indicated sizes are shown at the left, and the position of GST-UL97 is shown at the right.

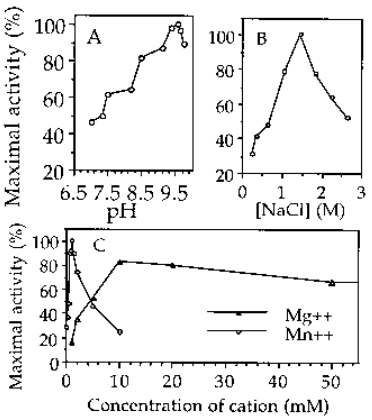

FIG. 7. Optimal conditions for GST-UL97 phosphorylation. GST-UL97 was incubated with radiolabeled ATP, using standard protein kinase assay conditions (see Materials and Methods) except that the reactions were for 1 min (to measure initial rates) and the pH (A), NaCl concentration (B), and divalent cation concentration (C) were varied as indicated. The incorporation of phosphate into GST-UL97 was determined for each condition.

tein made it easy to obtain UL97 at about 80% purity, it was difficult to purify it any further without loss of activity (18).

We therefore sought ways to increase the solubility of UL97 while retaining its activity. For this purpose, we expressed UL97 as a GST fusion protein, using recombinant baculovirus BVGSTUL97 (Fig. 1). As controls, we also expressed GST alone, using recombinant baculovirus BVGST (Fig. 1), and we expressed a mutant GST-UL97 containing the lysine-to-glutamine alteration that inactivated UL97 phosphorylation activity (Fig. 3), using recombinant baculovirus BVGSTUL97K 355Q (Fig. 1). GST and both the wild-type and mutant fusion proteins were expressed to high levels as species of the expected sizes that reacted with anti-UL97 and/or anti-GST antisera on Western blots (18). The fusion proteins could be readily purified to near homogeneity by using gluathione-Sepharose, Q-Sepharose, and SP Sepharose (Fig. 5), while the GST was purified to apparent homogeneity by simply using glutathione-Sepharose (18). When equal amounts of purified GST, GSTUL97, and GSTUL97K355Q were incubated with [γ-^{32}P]ATP, only the wild-type fusion protein became labeled (Fig. 6). The mutant protein preparation did not prevent phosphorylation of the wild-type protein, indicating that its failure to become phosphorylated was not due to a contaminating inhibitor (Fig. 6).

Properties and kinetics of the phosphorylation reaction. The phosphorylation reaction exhibited a number of interesting properties. We found that the rate of phosphorylation was linear for ~10 min and complete by ~25 min. To determine optimal conditions, we performed 1-min reactions to measure initial rates of phosphorylation. Phosphorylation was greatest at rather high pH, with maximal incorporation at about pH 9.5 (Fig. 7A). The activity was stimulated by salt, with an optimum at 1.5 M NaCl and with 50% activity remaining at 2.8 M NaCl (Fig. 7B). The activity required divalent cations, with a preference for Mn^{2+} (optimal concentration, ~1 mM; Fig. 7C). Mg^{2+} could substitute for Mn^{2+} (optimal concentration, ~10 mM), but less activity was observed (Fig. 7C). Neither Co^{2+}, Ca^{2+}, nor Zn^{2+} at concentrations of between 1 and 10 mM supported phosphorylation (18). GST-UL97 was able to utilize either GTP or ATP as a phosphate donor, with K_ms of 2 μM for ATP and 4 μM for GTP at 10 mM Mg^{2+}. Similar results were obtained with BVUL97-expressed UL97 (19).

As a final test to determine whether the phosphorylation of UL97 is due to autophosphorylation, we measured the initial rate of phosphorylation (3-min assays) of purified GST-UL97 at different concentrations of protein. If UL97 autophosphorylates, then one would expect the rate to be directly proportional to protein concentration over a wide range of concentrations. If there was a contaminating protein kinase responsible for phosphorylation, then, because both the contaminating kinase and its substrate (UL97) would vary coordinately, one would expect the rate to increase exponentially with protein concentration via second-order kinetics. We found that the rate was directly proportional to the protein concentration over a wide range (Fig. 8), with a k_{cat} of 0.18 ± 0.03 min^{-1}; i.e., the rate of incorporation per protein molecule did not vary with protein concentration. Thus, by both genetic and enzymological criteria, UL97 is a protein kinase that autophosphorylates.

DISCUSSION

Our results show that CMV UL97 becomes phosphorylated upon serine and threonine when incubated with [γ-^{32}P]ATP. This phosphorylation is abolished by a single point mutation (K355Q) that alters a lysine residue that corresponds (5) to an invariant, essential lysine in subdomain II of protein kinases (16) whose function in at least one protein kinase is to properly align the phosphates of ATP (23). The simplest interpretation of this result is that UL97 autophosphorylates. However, these data alone from less than pure protein could not completely exclude the possibility that the K355Q mutation somehow affected the ability of UL97 to serve as a substrate for a contaminating kinase. We therefore expressed UL97 as a soluble fusion protein and purified it to near homogeneity. Phosphorylation of this purified protein was also abolished by the K355Q mutation and was first order with protein concentration, which would not be expected if UL97 was phosphorylated by a contaminating kinase. We conclude that UL97 is a serine/threonine protein kinase that autophosphorylates. We discuss our results in terms of phosphorylation mechanisms, in terms of published results regarding protein kinases associated with CMV and herpesvirus-encoded homologs of UL97, and in terms of UL97 as a target for antiviral drugs.

Mechanisms of UL97 autophosphorylation. The first-order kinetics of UL97 autophosphorylation with respect to protein concentration (Fig. 8) shows that phosphorylation of UL97 occurs either by one UL97 monomer phosphorylating itself or

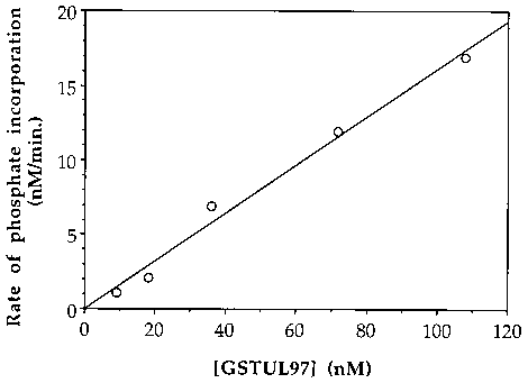

FIG. 8. Rate of GST-UL97 as a function of protein concentration. Protein kinase assays were performed for 3 min, using the indicated concentration of a purified GST-UL97 preparation. The rate of phosphate incorporation was determined for each concentration.

by UL97 molecules in some higher-order structure (e.g., a dimer) phosphorylating each other. We are investigating this question.

Our results (Fig. 4) show that UL97 expressed by BVUL97 is a serine/threonine protein kinase and suggest that there is more than one autophosphorylation site. We have confirmed this serine/threonine specificity for GST-UL97 and have identified at least three autophosphorylation sites within its UL97 moiety (18). The locations of these sites are under investigation. The serine/threonine specificity of UL97 is of interest, in part because of the lack of conservation of UL97 with other protein kinases in subdomains VI and VIII, which correspond to the catalytic and P+1 loops in protein kinase structures, respectively, and are implicated in recognition of the correct hydroxyamino acid (16, 22, 23). The sequence of subdomain VI in UL97 is Asp-456–Ile–Thr–Pro–Met–Asn, where Asp-456 corresponds to the catalytic Asp in cyclic AMP-dependent protein kinase. Pro-459 corresponds to prolines that are found in many serine/threonine kinases but are very unusual in tyrosine kinases (16). The consensus sequence of subdomain VIII for serine/threonine kinases is Gly-Thr/Ser-X-X-Tyr/Phe-X-Ala-Pro-Glu (16), with an important role for an interaction of the Thr/Ser residue with the catalytic loop in hydroxyamino acid selection (22). Subdomain VIII is difficult to locate in UL97 (5), but an alignment with its homologs with herpesviruses suggests that its sequence is Tyr-517–His–Pro–Ala–Phe–Arg–Pro–Met–Pro (12), with only Phe-521 conserved with conventional serine/threonine kinases. Perhaps it plays a key role in the hydroxyamino acid specificity of UL97. It is also interesting that both Pro-459 and Phe-521 are adjacent to residues that, when mutated, confer GCV resistance, consistent with a role in substrate selection (see below).

Relationship to CMV-associated protein kinases. There have been several reports of protein kinases induced by or associated with CMV infection (4, 27, 30, 31, 38). The properties of the UL97 protein kinase differ from those of the serine/threonine kinase associated with CMV protein p68 (4, 30, 31) in a number of features, including the high pH optima and preference for Mn^{2+} (Fig. 7). However, it does share some properties in common with the virion-associated enzyme reported by Mar et al. (27) and an enzyme associated with the particulate fraction of virions (38). In particular, like these enzymes, UL97 has a high pH optimum (Fig. 7) and phosphorylates casein poorly if at all (18). However, there are too many apparent differences between the properties of UL97 and these previously reported activities to permit the conclusion that they are the same.

Relationship to homologs of UL97 encoded by other herpesviruses. CMV UL97 is most closely related in sequence to a family of proteins encoded by all known herpesviruses (5, 11, 39). Herpes simplex virus UL13, VZV ORF 47, and pseudorabies virus UL13 are all associated with protein kinases. As a group, these associated activities exhibit, like UL97, a preference for Mn^{2+}, the ability to use either ATP or GTP, and stimulation by high NaCl concentrations, although all of these properties have not necessarily been reported for all three activities (8, 9, 12, 32, 41). However, of these, only the pseudorabies virus UL13 protein, like CMV UL97, has been shown to be active when expressed in a heterologous system (12), and none of these proteins has yet been purified to show that it does not require any cellular proteins as cofactors. Indeed, VZV ORF 47 failed to exhibit activity following heterologous expression (33, 41). Nevertheless, the results of our studies here with CMV UL97 increase the likelihood that each of the homologs of UL97 is, in fact, a protein kinase by itself.

UL97 as a drug target. UL97 controls the phosphorylation of GCV in CMV-infected cells based on studies of CMV *UL97* mutants (1, 17, 26, 43). Our results (Fig. 2) demonstrate that UL97 can control GCV phosphorylation in insect cells as well, confirming and extending work by others examining GCV phosphorylation in extracts of *E. coli* expressing UL97 and in human cells infected with vaccinia virus recombinants expressing UL97 (25, 28, 29). These results in widely divergent cell types are most simply interpreted as indicating that UL97 is a GCV kinase. Given that interpretation (although it has not yet been demonstrated that purified UL97 can phosphorylate GCV), it appears that the four-codon deletion (Δ590-593) in BVUL97de from CMV mutant 759^{r}D100 (43) drastically impairs GCV kinase activity, with only modest reductions in protein kinase activity. This finding is consistent with the location of Δ590-593 and other GCV resistance mutations in *UL97* (reviewed in the introduction) and the hypothesis that these mutations affect substrate specificity rather than inactivate UL97. We speculate that these mutations alter the substrate binding surface of UL97 so that it can no longer efficiently phosphorylate GCV yet can still phosphorylate itself and other relevant protein substrates. The two- to threefold decrease in UL97 autophosphorylation by BVUL97de is similar to the effects on thymidine phosphorylation of substrate specificity mutations that drastically impair acyclovir phosphorylation by herpes simplex virus thymidine kinase (10). Although we have recently found that UL97 can phosphorylate certain histones (18), its natural substrates in CMV-infected cells remain unknown.

The phenotype of the four-codon deletion mutation and the locations of the numerous *UL97* mutations discovered to date raise the possibility that UL97 is essential for CMV replication and, thus, that inhibitors of UL97 could serve as antiviral drugs. In that case, UL97 would differ significantly from its alphaherpesvirus homologs, which can be deleted without major effects on viral replication in many cultured cells (8, 11, 20, 35, 37) (although such mutations may have important effects on replication in certain cultured cells and on viral pathogenesis). Testing this possibility will require the isolation of CMV containing a *UL97* null mutation, which may require a complementing cell line. Indeed, we and others have obtained negative results in efforts to derive recombinant CMV mutants containing *UL97* null mutations (29, 42). Further characterization of UL97, particularly in terms of its substrate specificity, should address the interesting question of how a protein kinase can phosphorylate a nucleoside analog (GCV), should help elucidate the mechanisms of GCV resistance, and may aid in the discovery of new anti-CMV drugs.

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REFERENCES

1. **Baldanti, F., E. Silini, A. Sarasini, C. L. Talarico, S. C. Stanat, K. K. Biron, M. Furione, F. Bono, G. Palù, and G. Gerna.** 1995. A three-nucleotide deletion in the UL97 open reading frame is responsible for the ganciclovir resistance of a human cytomegalovirus clinical isolate. J. Virol. **69:**796–800.
2. **Baldanti, F., M. R. Underwood, S. C. Stanat, K. K. Biron, S. Chou, A. Sarasini, E. Silini, and G. Gerna.** 1996. Single amino acid changes in the DNA polymerase confer foscarnet resistance and slow-growth phenotype, while mutations in the UL97-encoded phosphotransferase confer ganciclovir resistance in three double resistant human cytomegalovirus strains recovered

from patients with AIDS. J. Virol. **90:**1390–1395.
3. **Biron, K.** 1994. Ganciclovir resistance of cytomegalovirus: mechanisms and prospects for rapid detection. Int. Antiviral News **2:**117–118.
4. **Britt, W. J., and D. Auger.** 1986. Human cytomegalovirus virion-associated protein with kinase activity. J. Virol. **59:**185–188.
5. **Chee, M. S., G. L. Lawrence, and B. G. Barrell.** 1989. Alpha-, beta- and gammaherpesviruses encode a putative phosphotransferase. J. Gen. Virol. **70:**1151–1160.
6. **Chou, S., A. Erice, M. C. Jordan, G. M. Vercellotti, K. R. Michels, C. L. Talarico, S. C. Stanat, and K. K. Biron.** 1995. Analysis of the UL97 phosphotransferase coding sequence in clinical cytomegalovirus isolates and identification of mutations conferring ganciclovir resistance. J. Infect. Dis. **171:**576–583.
7. **Chou, S., S. Guentzel, K. R. Michels, R. C. Miner, and W. L. Drew.** 1995. Frequency of UL97 phosphotransferase mutations related to ganciclovir resistance in clinical cytomegalovirus isolates. J. Infect. Dis. **172:**239–242.
8. **Coulter, L. J., H. W. Moss, J. Lang, and D. J. McGeoch.** 1993. A mutant of herpes simplex virus type 1 in which the UL13 protein kinase gene is disrupted. J. Gen. Virol. **74:**387–395.
9. **Cunningham, C., A. J. Davison, A. Dolan, M. C. Frame, D. J. McGeoch, D. M. Meredith, H. W. Moss, and A. C. Orr.** 1992. The UL13 virion protein of herpes simplex virus type 1 is phosphorylated by a novel virus-induced protein kinase. J. Gen. Virol. **73:**303–311.
10. **Darby, G., H. J. Field, and S. A. Salisbury.** 1981. Altered substrate-specificity of herpes simplex virus thymidine kinase confers acyclovir resistance. Nature **289:**81–83.
11. **de Wind, N., J. Domen, and A. Berns.** 1992. Herpesviruses encode an unusual protein-serine/threonine kinase which is nonessential for growth in cultured cells. J. Virol. **66:**5200–5209.
12. **de Wind, N., A. Zijderveld, K. Glazenburg, A. Gielkens, and A. Berns.** 1990. Linker insertion mutagenesis of herpesviruses: inactivation of single genes within the U_s region of pseudorabies virus. J. Virol. **64:**4691–4696.
13. **Drew, W. L., R. C. Miner, D. F. Busch, S. E. Follansbee, J. Gullett, S. G. Mehalko, S. M. Gordon, W. F. Owen, Jr., T. R. Matthews, W. C. Buhles, et al.** 1991. Prevalence of resistance in patients receiving ganciclovir for serious cytomegalovirus infection. J. Infect. Dis. **163:**716–719.
14. **Erice, A., S. Chou, K. K. Biron, S. C. Stanat, H. H. Balfour, Jr., and M. C. Jordan.** 1989. Progressive disease due to ganciclovir-resistant cytomegalovirus in immunocompromised patients. N. Engl. J. Med. **320:**289–293.
15. **Fleckenstein, B., I. Müller, and J. Collins.** 1982. Cloning of the complete human cytomegalovirus genome in cosmids. Gene **18:**39–46.
16. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1988. The protein kinase family: conserved features and deduced phylogeny of catalytic domains. Science **241:**42–52.
17. **Hanson, M. N., L. C. Preheim, S. Chou, C. L. Talarico, K. K. Biron, and A. Erice.** 1995. Novel mutation in the UL97 gene of a clinical cytomegalovirus strain conferring resistance to ganciclovir. Antimicrob. Agents Chemother. **39:**1204–1205.
18. **He, Z., and D. M. Coen.** 1996. Unpublished results.
19. **He, Z., Y. He, Y. Kim, and D. M. Coen.** 1994. Unpublished results.
20. **Heineman, T. C., and J. I. Cohen.** 1995. The varicella-zoster virus (VZV) open reading frame 47 (ORF47) protein kinase is dispensable for viral replication and is not required for phosphorylation of ORF63 protein, the VZV homolog of herpes simplex virus ICP22. J. Virol. **69:**7367–7370.
21. **Hu, S. H., M. W. Parker, J. Y. Lei, M. C. Wilce, G. M. Benian, and B. E. Kemp.** 1994. Insights into autoregulation from the crystal structure of twitchin kinase. Nature **369:**581–584.
22. **Hubbard, S. R., L. Wei, L. Ellis, and W. A. Hendrickson.** 1994. Crystal structure of the tyrosine kinase domain of the human insulin receptor. Nature **372:**746–754.
23. **Knighton, D. R., J. Zheng, L. F. Ten Eyck, V. A. Ashford, N.-H. Xuong, S. S. Taylor, and J. M. Sowadski.** 1991. Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science **253:**407–414.
24. **Knighton, D. R., J. Zheng, L. F. Ten Eyck, N.-H. Xuong, S. S. Taylor, and J. M. Sowadski.** 1991. Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science **253:**414–420.
25. **Littler, E., A. D. Stuart, and M. S. Chee.** 1992. Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. Nature **358:**160–162.
26. **Lurain, N. S., L. E. Spafford, and K. D. Thompson.** 1994. Mutation in the UL97 open reading frame of human cytomegalovirus strains resistant to ganciclovir. J. Virol. **68:**4427–4431.
27. **Mar, E. C., P. C. Patel, and E. S. Huang.** 1981. Human cytomegalovirus associated DNA polymerase and protein kinase activities. J. Gen. Virol. **57:**149–156.
28. **Metzger, C., D. Michel, K. Schneider, A. Luske, H. J. Schlicht, and T. Mertens.** 1994. Human cytomegalovirus UL97 kinase confers ganciclovir susceptibility to recombinant vaccinia virus. J. Virol. **68:**8423–8427.
29. **Michel, D., I. Pavić, A. Zimmerman, E. Haupt, K. Wunderlich, M. Heuschmid, and T. Mertens.** 1996. The UL97 gene product of human cytomegalovirus is an early-late protein with a nuclear localization but is not a nucleoside kinase. J. Virol. **70:**6340–6346.
30. **Michelson, S., M. Tardy-Panit, and O. Bârzu.** 1985. Catalytic properties of a human cytomegalovirus-induced protein kinase. Eur. J. Biochem. **149:**393–399.
31. **Michelson, S., M. Tardy-Panit, and O. Bârzu.** 1984. Properties of a human cytomegalovirus-induced protein kinase. Virology **134:**259–268.
32. **Ng, T. I., and C. Grose.** 1992. Serine protein kinase associated with varicella-zoster virus ORF 47. Virology **191:**9–18.
33. **Ng, T. I., L. Keenan, P. R. Kinchington, and C. Grose.** 1994. Phosphorylation of varicella-zoster virus open reading frame (ORF) 62 regulatory product by viral ORF 47-associated protein kinase. J. Virol. **68:**1350–1359.
34. **Overton, H. A., D. J. McMillan, L. S. Klavinskis, L. Hope, A. J. Ritchie, and P. Wong-Kai-In.** 1992. Herpes simplex virus type 1 gene UL13 encodes a phosphoprotein that is a component of the virion. Virology **190:**184–192.
35. **Overton, H., D. McMillan, L. Hope, and P. Wong-Kai-In.** 1994. Production of host shutoff-defective mutants of herpes simplex virus type 1 by inactivation of the UL13 gene. Virology **202:**97–106.
36. **Purves, F. C., W. O. Ogle, and B. Roizman.** 1993. Processing of the herpes simplex virus regulatory protein α22 mediated by the U_L13 protein kinase determines the accumulation of a subset of α and γ mRNAs and proteins in infected cells. Proc. Natl. Acad. Sci. USA **90:**6701–6705.
37. **Purves, F. C., and B. Roizman.** 1992. The U_L13 gene of herpes simplex virus 1 encodes the functions for posttranslational processing associated with phosphorylation of the regulatory protein α22. Proc. Natl. Acad. Sci. USA **89:**7310–7314.
38. **Roby, C., and W. Gibson.** 1986. Characterization of phosphoproteins and protein kinase activity of virions, noninfectious enveloped particles, and dense bodies of human cytomegalovirus. J. Virol. **59:**714–727.
39. **Smith, R. F., and T. F. Smith.** 1989. Identification of new protein-kinase related genes in three herpesviruses, herpes simplex virus, varicella zoster virus, and Epstein-Barr virus. J. Virol. **63:**450–455.
40. **Stanat, S. C., J. E. Reardon, A. Erice, M. C. Jordan, W. L. Drew, and K. K. Biron.** 1991. Ganciclovir-resistant cytomegalovirus clinical isolates: mode of resistance to ganciclovir. Antimicrob. Agents Chemother. **35:**2191–2197.
41. **Stevenson, D., K. L. Colman, and A. J. Davison.** 1994. Characterization of the putative protein kinases specified by varicella-zoster virus genes 47 and 66. J. Gen. Virol. **75:**317–326.
42. **Sullivan, V., and D. M. Coen.** 1992. Unpublished results.
43. **Sullivan, V., C. L. Talarico, S. C. Stanat, M. Davis, D. M. Coen, and K. K. Biron.** 1992. A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature **358:**162–164.
44. **Summers, M., and G. Smith.** 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station, College Station.
45. **Tatarowicz, W. A., N. S. Lurain, and K. D. Thompson.** 1992. A ganciclovir-resistant clinical isolate of human cytomegalovirus exhibiting cross-resistance to other DNA polymerase inhibitors. J. Infect. Dis. **166:**904–907.
46. **Taylor, J. W., J. Ott, and F. Eckstein.** 1985. The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. Nucleic Acids Res. **13:**8765–8785.
47. **Wolf, D. G., D. J. Lee, and S. A. Spector.** 1995. Detection of human cytomegalovirus mutations associated with ganciclovir resistance in cerebrospinal fluid of AIDS patients with central nervous system disease. Antimicrob. Agents Chemother. **39:**2552–2554.
48. **Wolf, D. G., I. L. Smith, D. J. Lee, W. R. Freeman, M. Flores-Aguilar, and S. A. Spector.** 1995. Mutations in human cytomegalovirus UL97 gene confer clinical resistance to ganciclovir and can be detected directly in patient plasma. J. Clin. Invest. **95:**257–263.
49. **Zhang, F., A. Strand, D. Robbins, M. H. Cobb, and E. J. Goldsmith.** 1994. Atomic structure of the MAP kinase ERK2 at 2.3 Å resolution. Nature **367:**704–711.