Human Cytomegalovirus UL97 Kinase Activity Is Required for the Hyperphosphorylation of Retinoblastoma Protein and Inhibits the Formation of Nuclear Aggresomes[▽]

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Cells infected with human cytomegalovirus in the absence of UL97 kinase activity produce large nuclear aggregates that sequester considerable quantities of viral proteins. A transient expression assay suggested that pp71 and IE1 were also involved in this process, and this suggestion was significant, since both proteins have been reported to interact with components of promyelocytic leukemia (PML) bodies (ND10) and also interact functionally with retinoblastoma pocket proteins (RB). PML bodies have been linked to the formation of nuclear aggresomes, and colocalization studies suggested that viral proteins were recruited to these structures and that UL97 kinase activity inhibited their formation. Proteins associated with PML bodies were examined by Western blot analysis, and pUL97 appeared to specifically affect the phosphorylation of RB in a kinase-dependent manner. Three consensus RB binding motifs were identified in the UL97 kinase, and recombinant viruses were constructed in which each was mutated to assess a potential role in the phosphorylation of RB and the inhibition of nuclear aggresome formation. The mutation of either the conserved LxCxE RB binding motif or the lysine required for kinase activity impaired the ability of the virus to stabilize and phosphorylate RB. We concluded from these studies that both UL97 kinase activity and the LxCxE RB binding motif are required for the phosphorylation and stabilization of RB in infected cells and that this effect can be antagonized by the antiviral drug maribavir. These data also suggest a potential link between RB function and the formation of aggresomes.

All the human herpesviruses encode well-conserved serine/threonine protein kinases that are important in viral infection (51) and are thought to phosphorylate substrates that are also targets of cdc2 (33). Herpes simplex virus (HSV) UL13 and Epstein-Barr virus BGLF4 phosphorylate eukaryotic elongation factor 1delta (34), and HSV UL13 and human cytomegalovirus (HCMV) pUL97 both phosphorylate the carboxyl-terminal domain of RNA polymerase II (4, 12). Many other interesting activities of cellular proteins have previously been described, such as the activation of cdc2 by HSV UL13 (1) as well as the inhibition of histone acetylation (57) and activation of protein kinase A (5, 43) by HSV US3. Viral proteins can also be substrates for these kinases; the DNA polymerase processivity factors are substrates, and it appears to be a common theme in the herpesviruses (21, 27, 39, 47). Studies examining the function of these kinases suggest that they are not strictly required for viral infection; however, they perform important functions that are required for replication *in vivo* and suggest that the effects of these kinases on host and viral targets are important (53, 54, 62).

The UL97 protein kinase in HCMV is particularly important

because of its relevance to antiviral therapy. This enzyme phosphorylates and activates the antiviral drug ganciclovir, which is the treatment of choice for HCMV infections (44, 63). Although this drug is effective in treating HCMV infections, its toxicity limits its use in clinics and new drugs are required. This requirement led to the development of maribavir (MBV), which is a potent and selective inhibitor of UL97 protein kinase activity (6). This drug is active both *in vitro* and *in vivo* (35, 69) and is well tolerated in human subjects (45).

The UL97 kinase is a tegument protein expressed with early/late kinetics (52, 64); the protein autophosphorylates amino-terminal serine and threonine residues (23). A recombinant virus with a large deletion in *UL97* replicates poorly, and virus titers are reduced 100-fold in confluent cells, suggesting that *UL97* plays a critical role in the replication of the virus (59). Many defects have previously been described for the nuclei of cells infected with the null mutant, and these defects include modestly reduced DNA synthesis, inefficient DNA packaging (70), and impaired nuclear egress (38, 70). The recruitment of pUL97 by the cellular p32 protein to the lamin B receptor results in the redistribution of components of the nuclear lamina by a mechanism that requires its kinase activity (48). Virion morphogenesis in the nucleus also appears to be significantly impaired, resulting in the inappropriate aggregation and sequestration of viral proteins in the nucleus (58). This result was reproduced in a transient system in which the UL97 kinase

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inhibited the aggregation of pp65 in a kinase-dependent manner. It is possible that the phosphorylation of pp65 may mediate this effect, or it may result from the physical interaction of the two proteins during viral infection (32). Which among these defects is responsible for the critical deficiencies in viral replication is unclear, but all of them likely contribute to the poor-growth phenotype.

Investigations of the nature of nuclear aggregates presented here suggest that they are related to cellular structures called nuclear aggresomes. Aggresomes sequester inappropriately folded proteins in a dynamic manner, and the formation of these aggresomes is linked to pathogenic processes in aggregative diseases, such as Huntington's and other ataxias (reviewed in references 19 and 72). Many prion and viral proteins are also targeted to these structures, and it has previously been suggested that they may be sites for viral assembly and replication for some viruses (68). The cellular protein green fluorescent protein (GFP)-GCP170* is a marker for aggresomes and has previously been used as a tool to characterize their formation, which starts with the accumulation of small aggregates associated with promyelocytic leukemia (PML) bodies (nuclear bodies, ND10 sites, and promyelocytic oncogenic domains) (18). The aggregates then coalesce to form large aggresomes, and the coalescence results in the spatial rearrangement of PML bodies. This process is also associated with transcriptional repression, suggesting that PML bodies participate in aggresome formation (17, 18). Both PML bodies and aggresomes can be considered innate antiviral defenses, since interferons upregulate many proteins associated with these structures and the overexpression of PML inhibits the replication of many viruses, including HCMV (14, 50). Viral genomes and proteins are also recruited to PML bodies, presumably to promote viral replication (13). In HCMV, pp71 is involved in the derepression of the major immediate promoter and is dependent on its interaction with the PML-associated protein hDaxx (25, 49). In addition, IE1 directs the dispersion of PML bodies through the SUMOylation of PML (42) and the deletion of IE1 leads to a broad defect in virus delayed-early gene expression (20). The role of PML as a tumor suppressor was recently shown to involve both the p53 and retinoblastoma protein (RB) pathways and has previously been reviewed (22, 61). Of interest, RB forms stable complexes with the unphosphorylated form of PML (3) and results in increased numbers of PML bodies (15). This interaction appears to be functional, since PML increases the transcriptional repression mediated by RB (36) and the two function together to promote hematopoiesis (40). The overexpression of PML also results in senescence in primary fibroblasts in a process that appears to be mediated by RB, since papillomavirus E7, but not E6, can circumvent this event (7, 46).

Studies presented here show that pUL97 inhibits the formation of aggresomes by a kinase-dependent mechanism and that this enzyme is also required for the hyperphosphorylation of RB during viral infection. The mutation of either the lysine required for enzymatic activity or the conserved amino-terminal RB binding motif reduced the ability of the UL97 kinase to phosphorylate and stabilize RB and suggests that both of these motifs are required for the proper function of pUL97. The RB binding motifs may also play a role in the inhibition of aggresome formation, since the mutagenesis of two consensus RB

binding motifs reduced the ability of the enzyme to inhibit aggresome formation.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblast (HFF) cells and COS7 cells were routinely propagated in monolayer cultures in minimum Eagle's medium with Earle's salts supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/ml penicillin G, and 25 µg/ml gentamicin. HCMV strain AD169 was obtained from the American Type Culture Collection (Manassas, VA), virus stocks were prepared, and titers were determined as described previously (60). The construction and characterization of a UL97 kinase mutant (RCΔ97) were described previously (59). MBV was obtained through the National Institute for Allergy and Infectious Diseases.

Plasmids. Construction of the plasmids expressing the pp65-GFP fusion protein as well as those for the epitope-tagged version of the wild-type (wt) UL97 open reading frame (ORF) and the K355M mutation were described previously (58), as was the plasmid expressing the aggresome marker GFP-GCP170* (18). Mutations in UL97 were constructed by site-specific mutagenesis using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) and plasmid pMP92 as a template. The C151G mutation was constructed using the primers C151G QC F (5'-CCA CGG CTT GCG CGG CCG CGA AAC TTC-3') and C151G QC R (5'-GAA GTT TCG CGG CCG CGC AAG CCG TGG-3'), the C428G mutation was produced with primers C428G QC F (5'-AGT GGA AGC TGG CGG GCA TCG ACA GCT AC-3') and C428G QC R (5'-GTA GCT GTC GAT GCC CGC CAG CTT CCA CT-3'), the C693G mutation was constructed with primers C693G QC F (5'-GCA CCA CCA GCA TAA TCG GCG AGG AGG ACC-3') and C693G QC R (5'-GGT CCT CCT CGC CGA TTA TGC TGG TGG TGC-3'), and the K355M UL97 mutant was reproduced using the primers K355M QC F (5'-TCG CTA TCG CGT GGT CAT GGT GGC GCG-3') and K355M QC R (5'-CGC GCC ACC ATG ACC ACG CGA TAG CGA-3'). Plasmids containing two or more point mutations were mutagenized sequentially with the primers listed above. The resulting plasmids pMP305 (C428G), pMP306 (C151G), pMP310 (C693G), pMP307 (K355M), and pMP313 (C151G/C428G) were sequenced to confirm the introduction of the desired point mutations and that no other mutations were present in the UL97 ORF.

Construction of recombinant viruses. The bacterial artificial chromosome (BAC) strain HB5 was reported previously (8) and was mutated using the recombinering protocols and plasmids described previously by Warming et al. (66). Briefly, a PCR was performed using a *galK*-containing plasmid, a UL97 *galK* forward primer (5'-GGC CTT ACG TGC GAC CCG CGT ATG TTC TTG CGC CTT ACG CAT CCC GAG CTC TGC GAC CTG TTG ACA ATT AAT CAT CGG CA-3'), and a UL97 *galK* reverse primer (5'-ATC TTG TGG CAA AAA TCG TCC TCT TTG GGC ACG TAG ACC AGC AGG TAG GAG ATA GAG AGC TCA GCA CTG TCC TGC TCC TT-3'). This PCR product was electroporated into the SW102 recombinering strain containing the pHB5 BAC and plated on selective medium containing galactose. The resulting BAC contained a *galK* insertion at amino acid 304 and was designated pMP290. PCR products from plasmids containing K355M, C151G, C428G, and C693G point mutations were electroporated into the bacteria containing pMP290, and *galK*-negative BACs were grown on selective medium containing deoxygalactose and designated pMP314, pMP295, pMP312, and pMP316, respectively. Restriction digests of all BACs were conducted to confirm that no large rearrangements had occurred, the UL97 ORFs from each of the resulting BACs were sequenced, and it was confirmed that these ORFs contained only the engineered mutations. BACs with the mutations K355M, C151G, C428G, and C693G were rescued, and the viruses were designated RC314, RC295, RC312, and RC316, respectively.

Polyacrylamide gels and Western blotting. HFF cells were infected at a multiplicity of infection (MOI) of 2 PFU/cell, and at 24 and 72 h after infection they were disrupted in 2× Laemmli buffer (Sigma-Aldrich, St. Louis, MO) and separated on 10%, 7.5%, or 5% polyacrylamide gels, depending on the size of the protein to be resolved (Bio-Rad, Hercules, CA). Separated proteins were transferred to nitrocellulose membranes (Roche Applied Science, Indianapolis, IN) in a buffer containing 28 mM Tris, 39 mM glycine, 0.0375% sodium dodecyl sulfate, and 20% methanol in a semidry transfer cell (Bio-Rad). Membranes were blocked in 1% bovine serum albumin in phosphate-buffered saline (PBS), incubated with primary antibodies overnight at 4°C, and washed extensively with PBS supplemented with 0.05% Tween 20. A secondary antibody conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL) and CDP-Star (Roche Applied Science) was used to detect the bound primary antibody. Monoclonal antibodies used in these studies were directed against PML (H-238) (Santa Cruz Biotechnology, Santa Cruz, CA), DAXX (Upstate Cell

Signaling Solutions, Temecula, CA), p53, RB, β -tubulin, CREB binding protein (Chemicon International, Temecula, CA), and both V5 and Xpress (Invitrogen, Carlsbad, CA). Rabbit antisera directed against RB phosphorylated on Ser780 (Cell Signaling Technology, Danvers, MA) were used in conjunction with ImmunoPure goat anti-rabbit immunoglobulin G horseradish peroxidase and SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford IL).

Indirect immunofluorescence microscopy. Cells expressing proteins from transfected plasmids were visualized by methods published previously (58). Briefly, monolayers of COS7 or HFF cells were grown on 12-mm-diameter coverslips in 24-well plates. Transiently transfected cells were fixed for 15 min with freshly prepared 2% formaldehyde in PBS and washed two times with PBS, and membranes were permeabilized with 0.2% Triton X-100 in PBS for 15 min. Monoclonal antibodies to IE1 (63–27) and pp65 (28–19) were generously provided by Bill Britt (University of Alabama, Birmingham) and were used as culture supernatants with goat anti-mouse secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or Texas Red (Southern Biotechnology). PML domains were visualized with rabbit polyclonal antisera to SP100 (Chemicon) and a FITC-conjugated goat anti-rabbit secondary antibody. In some studies, monoclonal antibodies were labeled with the Texas Red Zenon antibody labeling kit (Invitrogen).

Aggregate formation was assayed as reported previously (58). Briefly, coexpression of pp65-GFP and viral proteins in COS7 cells were confirmed by methods described above. Cells containing fluorescent nuclear pp65-GFP aggregates were scored as positive, while those without aggregates were scored as negative and at least 60 cells were evaluated on each coverslip. The expression levels of pp65-GFP in cotransfected cells were determined in 96-well plates containing COS7 cells. Each well was transfected with 100 ng of a plasmid expressing pp65-GFP and 100 ng of a second expression construct or salmon sperm DNA complexed with 0.5 μ l of Lipofectamine 2000 (Invitrogen). Fluorescence of the pp65-GFP fusion protein was determined in a FluoStar Optima fluorometer (BMG Labtech, Durham, NC), with excitation and emission wavelengths of 485 and 520 nm, respectively.

Isolation of nuclear and cytoplasmic aggregates. Low-passage HFF cells were infected with RC497 at a low MOI in 175-cm² flasks, and infected cells were passaged at 7 days following infection (as plaques started to form) as well as on days 12 and 16 after infection (until 100% cytopathic effect was observed). Infected cells were dislodged with 0.25% trypsin-EDTA (Gibco, Grand Island, NY) and resuspended in a volume of 10 ml growth medium. Cells were collected by centrifugation and resuspended in PBS containing 0.6% NP-40, and nuclei were centrifuged through a cushion of Histopaque 1077 (Sigma-Aldrich) at 1,000 $\times$ g for 5 min. Nuclei were lysed in PBS supplemented with 2.5 M NaCl, and cellular DNA was degraded with 10,000 U of DNase I. An equal volume of 5 M urea was added to the nuclear lysate, and the nuclear aggregates were collected by centrifugation at 3,500 $\times$ g for 10 min through a Histopaque cushion. Nuclear aggregates were resuspended in PBS supplemented with 0.5% NP-40 and frozen at -80°C . Cytoplasmic aggregates were isolated from the cytoplasmic fraction by sedimentation at 3,500 $\times$ g. The sedimented material was resuspended in a PBS buffer containing 5 M urea, and aggregates were collected by sedimentation through a Histopaque cushion, resuspended in PBS with 0.5% NP-40, and frozen at -80°C .

Tryptic digestion of viral inclusions. HCMV inclusion bodies were denatured by the addition of urea to a final concentration of 8 M and heating to 37°C for 30 min. The sample was then diluted fourfold with 100 mM NH_4HCO_3 and 1 mM CaCl_2 . Methylated, sequencing-grade porcine trypsin (Promega, Madison, WI) was added at a substrate-to-enzyme ratio of 50:1 (mass to mass) and incubated at 37°C for 15 h. Sample cleanup was achieved by using a 1-ml solid-phase extraction C_{18} column (Supelco, Bellefonte, PA). The peptides were eluted from each column with 1 ml of methanol and lyophilized. The samples were reconstituted at a final concentration of 10 $\mu\text{g}/\mu\text{l}$ in a 25 mM NH_4HCO_3 buffer and frozen at -20°C until analyzed.

Tandem mass spectrometric analysis of peptides. Peptide samples were analyzed by reversed-phase capillary liquid chromatography coupled directly with electrospray tandem mass spectrometers (models LCQ Duo and DecaXP; Thermo Finnigan, San Jose, CA). Chromatography was performed on a 60-cm capillary column (inside diameter, 150- μm ; outside diameter, 360- μm ; Polymicro Technologies, Phoenix, AZ) packed with Jupiter C_{18} 5- μm -diameter particles (Phenomenex, Torrance, CA). A solvent gradient was used to elute the peptides by using 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient was linear, starting with solvent A and increasing to 5% solvent B in 20 min, 5 to 70% gradient in 80 min, and 70 to 85% gradient in 45 min. The solvent flow rate was 1.8 $\mu\text{l}/\text{min}$. The capillary liquid chromatography system was coupled to an LCQ ion trap mass spectrometer (Thermo Finnigan) by using a custom-manufactured ESI interface in which no sheath gas

TABLE 1. Effect of coexpressed viral proteins on pp65-GFP expression and aggregation in COS7 cells

Proteins expressed ^a	pp65 GFP fluorescence ^b	% Cells containing aggregates ^c
pp65-GFP, ppUL44	100 $\pm$ 3	67 $\pm$ 7
pp65-GFP, ppUL97-V5	94 $\pm$ 1	10 $\pm$ 4 ^d
pp65-GFP, UL97-V5 (K355M)	104 $\pm$ 1	57 $\pm$ 16
pp65-GFP, IE1	125 $\pm$ 1	84 $\pm$ 9 ^d
pp65-GFP, pp71-V5	116 $\pm$ 2	92 $\pm$ 5 ^d
pp65-GFP, pUL69-V5	93 $\pm$ 6	58 $\pm$ 9

^a Expression of protein from cotransfected plasmids was confirmed by fluorescence of pp65-GFP and antibody staining with antibodies to the second protein and Texas Red secondary antibody.

^b Fluorescence of cells expressing pp65-GFP was averaged from triplicate transfections and converted to percent control, with the standard deviations shown.

^c The percentages of cotransfected cells exhibiting pp65-GFP nuclear aggregates from four experiments were averaged, with the standard deviations shown.

^d Values shown were significantly different ($P < 0.05$) in a comparison with the control (pp65-GFP, ppUL44).

or makeup liquid was used. The temperatures of heated capillary and electrospray voltage were 200°C and 3.0 kV, respectively. Samples were analyzed using the data-dependent tandem mass spectrometry (MS/MS) mode over the m/z range of 300 to 2,000. The three most abundant ions detected in each MS scan were selected for collision-induced dissociation.

SEQUEST analysis. The SEQUEST algorithm was run on each of the datasets against a combined database comprised of the HCMV.fasta and the human.fasta from the National Center for Biotechnology Information. MS/MS peaks were generated by “extract_msn.exe,” part of the SEQUEST software package. A peptide was considered to be a match by using a conservative criteria set developed previously by Yates and coworkers (67). Briefly, all accepted SEQUEST results were significant and had a delta Cn of ≥ 0.1 . Peptides with +1 charge states were accepted if they were fully tryptic and had a cross correlation (X_{corr}) of at least 1.9. Peptides with +2 charge states were accepted if they were fully tryptic or partially tryptic and had an X_{corr} of at least 2.2. Peptides with +2 or +3 charge states with X_{corr} values of at least 3.0 or 3.75, respectively, were accepted regardless of their tryptic states.

RESULTS

Aggregation of pp65 is affected by pp71 and IE1. An investigation into nuclear aggregate formation was undertaken to help understand the function of the UL97 kinase in viral infection. It has previously been reported that the expression of a pp65-GFP fusion protein in COS7 cells was sufficient to induce the formation of large nuclear aggregates, which were inhibited by UL97 kinase activity (58). To identify other viral proteins that might be involved in this process, expression plasmids for 128 viral ORFs were individually cotransfected with a plasmid expressing GFP-tagged pp65 and the effect on aggregation was assessed by immunofluorescence microscopy. In these transfections, the expression of 125 viral genes did not impact the aggregation of pp65-GFP, whereas the expression of pUL97, pp71, or IE1 significantly affected the formation of pp65 aggregates (data not shown). This effect did not appear to be related to expression levels, since cotransfection with a plasmid expressing both IE1 and IE2 had no effect. These data were confirmed in a second series of cotransfections and demonstrated that the expression of either IE1 or pp71 significantly increased the proportion of pp65-expressing cells that exhibited nuclear aggregates (Table 1). While both proteins slightly increased the expression of pp65-GFP, the formation of aggregates was not strictly correlated with expression; cells coexpressing pUL97 or pUL69 expressed very similar quantities of

FIG. 1. UL97 reduces the number of PML domains in COS7 cells by a kinase-dependent mechanism. Cells were transfected with plasmids expressing ppUL44, IE1, pp71-V5, UL97-V5, UL97-V5 K355M, and UL97-V5, with the addition of MBV, as indicated in the figure. PML domains were visualized with a rabbit polyclonal antibody to SP100 and a FITC-conjugated secondary antibody (green staining), and examples of these sites are indicated by arrows. The expression of viral proteins was confirmed by staining with monoclonal antibodies labeled with Texas Red (red staining). All nuclei were stained with DAPI (4',6'-diamidino-2-phenylindole) (blue staining). Values shown in each panel are the average numbers of PML domains in cells expressing the viral proteins and reflect the average of at least 50 cells, with the standard deviations as shown. Control cells expressing ppUL44 contained an average of 4.7 PML domains (A), while positive control cells expressing IE1 contained significantly fewer PML domains (B). The expression of pp71 resulted in the recruitment of this protein to PML domains, but did not affect their number (C). Cells expressing UL97 also contained reduced numbers of PML domains (D) and was dependent on its kinase activity, since neither the K355M mutant nor UL97 in the presence of MBV significantly reduced their numbers (E and F).

pp65-GFP, yet only the UL97 kinase impacted aggregate formation (Table 1). The effect of pp71 and IE1 on aggregation was interesting, because both proteins have been reported to interact with components of PML domains (2, 25) and they also interact functionally with RB pocket proteins (31, 56).

PML domains are affected by pUL97. PML domains have been linked to nuclear aggregates and the formation of nuclear aggresomes (18). A series of transient transfection experiments in COS7 cells were conducted to investigate the possibility that the UL97 kinase might affect these domains, which were visualized using an antibody to SP100. IE1 has previously been reported to disperse the nuclear structures in cells expressing this protein (37). This result was confirmed here when PML domains were reduced in nuclei expressing the protein (Fig. 1B). The overexpression of a control viral protein (ppUL44, ICP36) did not affect the number of PML domains in cells in which it was expressed (Fig. 1A). Their numbers were also unaffected by the expression of pp71; however, it colocalized with these structures, which is consistent with previous reports (25, 26, 49). The expression of pUL97 reduced the number of PML bodies (Fig. 1D) by a mechanism that was kinase dependent, since neither the K355M mutant nor the active kinase in

the presence of MBV affected PML numbers (Fig. 1E and F). While this effect was not as robust as the dispersion by IE1, it was both significant and repeatable. These results suggested that UL97 kinase activity affected PML domains and was interesting, since pUL97, IE1, and pp71 all impact PML domains as well as the aggregation of pp65.

Aggregation of pp71 and GFP-GCP170* is inhibited by the UL97 kinase. The relationship between PML bodies and nuclear aggregation prompted an examination of the effect of the UL97 kinase on the aggregation of two other aggregating proteins, pp71 and a cellular protein, GFP-GCP170*. The latter is a marker for nuclear and cytoplasmic aggresomes and has previously been used to study their formation. These proteins were coexpressed with the active UL97 kinase and the inactive K355M mutant to examine their effects on aggregation. The formation of pp65-GFP aggregates (Fig. 2A) was inhibited by the coexpression of the UL97 kinase (Fig. 2B), whereas the kinase-negative form of UL97 (K355M) was unable to inhibit formation of the aggregates and was recruited to the large nuclear aggregates (Fig. 2C). Aggregation also occurred in the presence of MBV, a specific inhibitor of UL97 kinase activity (data not shown), and this finding is consistent with results reported previously (58). The localization of the K355M mutant protein with pp65-GFP aggregates is consistent with the results of a previous report (58) and also those of a more recent paper describing the physical interaction of pUL97 and pp65 (32). A subset of cells expressing the pp71 tegument protein have previously been reported to form large nuclear aggregates similar to those formed by pp65; this subset was affected by the presence of PML (49). The transfection of COS7 cells with an epitope-tagged version of pp71 confirmed that large nuclear aggregates were formed in some transfected cells (Fig. 2D); these aggregates resembled those formed by pp65-GFP in some respects. Aggregates induced with pp71 were visualized with antibodies that bound only the exterior of the dense aggregates and, thus, were not as bright as those formed with pp65-GFP. Nonetheless, the aggregates induced by pp71 resembled those formed by pp65 in terms of their large sizes, and this result is consistent with a previous report (49). Cytoplasmic aggregates were also observed in many cells. The UL97 kinase substantially reduced the aggregation of pp71 in cotransfected cells (Fig. 2E), and this reduction was similar to results observed with pp65-GFP. The K355M mutant was unable to inhibit the aggregation of pp71 and was also recruited to the aggregates (Fig. 2F). The UL97 kinase was unable to inhibit the aggregation of pp71 in the presence of MBV, confirming that the inhibition was dependent on its enzymatic activity and was consistent with results obtained with pp65-GFP (data not shown). These results suggested that the ability of the UL97 kinase to inhibit aggregation was not limited to pp65, but also occurred with another viral phosphoprotein. This result indicated that the UL97 kinase may impact a more general cellular function, possibly involving aggresome formation, so its effect on aggresomes induced by GFP-GCP170* was also investigated. The expression of GFP-GCP170* induced cytoplasmic and nuclear aggresomes in COS7 cells and confirmed data reported previously (Fig. 2G) (18). Coexpression of pUL97 with this aggresome marker reduced the size and number of all aggresomes and also affected their distribution in the cell (Fig. 2, compare panel H with panels G and I). This

FIG. 2. Inhibition of pp71 and GFP-GCP170* aggregate formation by UL97 kinase. Aggregating proteins pp65-GFP, pp71-V5, and GFP-GCP170* were coexpressed in COS7 cells with epitope-tagged pUL97 and the kinase-negative K355M point mutant. Aggregates formed by GFP fusion proteins pp65-GFP (A) and GFP-GCP170* (G) were visualized directly, while pp71-V5 aggregates (D) were stained with a monoclonal antibody to the epitope tag and a FITC-conjugated secondary antibody (green staining). The expressions of epitope-tagged pUL97 and the K355M point mutant were detected with the V5 monoclonal antibody labeled with the Zenon Texas Red labeling kit (red staining). Aggregate formation by pp65-GFP was inhibited in cells that also expressed pUL97 (compare panels A and B). (C) The K355M kinase-negative form pUL97 was unable to inhibit the aggregation of pp65-GFP and was recruited to the aggregates as shown by the yellow staining in the merge panel. The aggregation of pp71 was also inhibited by pUL97 (compare panels D and E). (F) This effect was kinase dependent, since the K355M mutant was unable to reduce pp71 aggregate formation and instead was recruited to these structures, as shown in the merge panel. Aggregates induced through the expression of a cellular marker for these structures, GFP-GCP170*, were also substantially reduced by the expression of pUL97 (compare panels G and H). (I) The inhibition of aggresome formation by pUL97 appeared to be kinase dependent, since their frequency and distribution were unaffected by the K355M mutant. Interestingly, the K355M mutant was specifically recruited to cytoplasmic aggresomes and was not recruited to nuclear aggresomes, as shown in the merge panel.

activity was dependent on its enzymatic activity, since the K355M mutant failed to inhibit aggregation and instead was recruited to cytoplasmic aggresomes (Fig. 2I). Similar results were also observed when the enzymatic activity was inhibited with MBV (data not shown). Taken together, these data were consistent and suggested that the UL97 kinase inhibited the formation of cytoplasmic and nuclear aggresomes. This interpretation of the data is also consistent with the observation that pp71 aggregates also occur in the cytoplasm and may reflect the recruitment of this protein to cytoplasmic aggresomes. This result was interesting because it suggested that the

inhibition of this cellular process might be one of the important functions of pUL97 during infection.

Tegument aggregates are nuclear aggresomes that contain virion proteins. One possible interpretation of the data presented above was that aggregated virion proteins were contained within nuclear aggresomes and that their disruption by the UL97 kinase prevented cells from sequestering these foreign proteins. To test this hypothesis, GFP-GCP170* was coexpressed with pp71 and pUL69 in COS7 cells to see whether they exhibited similar staining patterns. Both pp71 and pUL69 appeared to colocalize with GFP-GCP170*, suggesting that

FIG. 3. Viral proteins are recruited to aggresomes in the absence of UL97 kinase activity. Coexpression of either pp71-V5 or pUL69-V5 (red staining) with GFP-GCP170* (green staining) in COS7 cells resulted in the recruitment of viral proteins to aggresome structures. The recruitment of pp71 was apparent both in nuclear and cytoplasmic aggresomes (A, merge panel), while pUL69 was preferentially recruited to nuclear aggresomes (B, merge panel). (C) Cells infected with the UL97 K355M point mutant contained large tegument aggregates that stained with an antibody to pp65 (red staining) and recruited GFP-GCP170* (green staining) when it was expressed late in infection (white arrows). (D) Cells infected with the wt HB5 virus did not produce large aggregates, and pp65 (red staining) appeared to be uniformly distributed in the nucleus. When GFP-GCP170* (green staining) was expressed late in viral infection, it did not aggregate in the nucleus and only a few aggregates were observed in the cytoplasm.

these proteins were deposited in aggresomes (Fig. 3A and B). While pp71 was present in both nuclear and cytoplasmic aggresomes, pUL69 appeared to partition preferentially to nuclear aggresomes and may reflect the more robust nuclear localization exhibited by this protein. These data were confirmed using HFF cells infected with a recombinant virus expressing the pUL97 K355M mutant (RC314) that contained large aggregates. The transfection of infected cells with a plasmid expressing GFP-GCP170* late in infection resulted in the recruitment of the aggresome marker to some of the large tegument aggregates defined by pp65 staining (Fig. 3C). Large aggregates do not form in cells infected with the wt HB5 virus and the intracellular distribution of GFP-GCP170* expressed late in infection was much more diffuse and resulted in the formation of fewer aggregates (Fig. 3D). These data, together

with the colocalization data, suggest that the structures described previously as tegument aggregates induced in COS7 cells (58) were nuclear aggresomes containing viral proteins. The data also suggested that the inhibition of tegument protein aggregation is related to the ability of the UL97 kinase to prevent the formation of these cellular structures.

Aggresomes contain large quantities of virion proteins. To determine the protein content of the HCMV nuclear and cytoplasmic aggresomes, these structures were purified from infected cells by methods described previously and analyzed by MS (58, 65). The aggresomes were denatured and digested with trypsin, and the complex mixture of peptides was analyzed by two-dimensional liquid chromatography coupled to MS/MS. Identified peptides were compared to those in a HCMV-FASTA database. This analysis revealed that the aggresomes contained large quantities of viral structural proteins (Table 2). There were 25 HCMV proteins in the nuclear aggresomes and 19 present in the cytoplasmic aggresomes. These included the capsid proteins UL46 (minor capsid binding protein), UL48A (smallest capsid protein), UL80 (assembly protein), UL85 (minor capsid protein), and UL86 (major capsid protein) as well as a number of tegument proteins, including UL25, UL26, UL32, UL35, UL47, UL48, UL82, UL83, UL94, and US22. In addition, a number of proteins involved in transcription and DNA replication were also present, including IRS1, UL31, UL34, UL44, UL57, UL69, UL84, UL98, UL104, and UL122. Overall, the ratios of viral proteins present in the aggresomes resembled dense bodies rather than virions (65) and might suggest that immature virions were sequestered in aggresomes prior to genome packaging. This is consistent with potential packaging defects reported previously (70) as well as the apparent association of immature virions with aggregates in electron micrographs (58).

To identify cellular proteins in aggresomes, the results from the mass spectroscopy analysis were also compared to the predicted peptides of a human-FASTA database. The nuclear and cytoplasmic aggresomes contained a number of cellular heat shock proteins (HSPs), including HSP70, HSP71c, HSP70-2, and HSP60, that are known to be associated with aggresomes (Table 3). The function of these proteins in the formation of these structures remains unclear, but their presence suggests that the observed tegument aggregates are aggresomes and is consistent with previous results. The aggresome marker GFP-GCP170* was not detected in this analysis, and this was not necessarily surprising, since the abundance and function of this protein in cells has not been described previously. The cellular protein aurora-related kinase 1 is known to segregate chromosomes; the presence of this protein in the nuclear aggresomes may suggest that it plays a role in viral DNA replication. Nucleophosmin was detected in the cytoplasmic aggresomes and not in the nuclear-derived aggresomes. This result is noteworthy, since nucleophosmin is a nucleolar protein that is critical for centrosome duplication and genomic stability. The overexpression of nucleophosmin has previously been noted for a number of malignancies; this may be attributed to its ability to inactivate p53 and thus suppress apoptosis. The inactivation of p53 has previously been noted for HCMV-infected cells, although it was thought that this might only be due to the viral immediate-early proteins.

UL97 kinase activity is required for the hyperphosphorylation of RB. The effect of the UL97 kinase on PML bodies and

TABLE 2. Viral proteins identified by liquid chromatography MS/MS in cytoplasmic and nuclear aggresomes

HCMV ORF	Cytoplasmic aggresomes		Nuclear aggresomes		Description
	Max X_{corr}	No. unique peptides	Max X_{corr}	No. unique peptides	
IRS1	5.12	2	4.73	3	Transcriptional transactivator; US22 family member
UL25	4.97	14	5.54	25	Tegument protein; UL25 family member
UL26	3.46	2	4.91	4	Tegument protein; US22 family member
UL31	ND	ND	3.97	3	Hypothetical protein
UL32	4.71	4	4.38	4	pp150 tegument protein
UL34	ND	ND	4.34	2	Transcriptional repressor
UL35	ND	ND	6.58	3	UL25 family member
UL44	5.57	11	5.16	7	Processivity subunit of DNA polymerase; HSV-1 UL42 counterpart
UL46	ND	ND	4.18	3	Intercapsomeric triplex capsid protein; HSV-1 UL38 counterpart
UL47	ND	ND	3.50	1	Tegument protein; HSV-1 UL37 counterpart
UL48	4.30	1	5.68	5	Tegument protein; HSV-1 UL36 counterpart
UL48A	7.17	5	7.03	2	Capsid protein located at tips of hexons; HSV-1UL35 counterpart
UL50	6.23	1	ND	ND	Membrane protein involved in nuclear capsid egress; HSV-1 UL34 counterpart
UL57	1.91	1	ND	ND	Single-stranded DNA-binding protein; HSV-1 UL29 counterpart
UL69	ND	ND	3.28	2	Posttranscriptional regulator of gene expression; HSV-1 UL54 counterpart
UL71	5.09	2	ND	ND	Tegument protein; HSV-1 UL51 counterpart
UL77	ND	ND	4.58	1	DNA packaging protein; HSV-1 UL25 counterpart
UL80	5.60	9	4.10	2	Protease (N terminus) and minor scaffold protein (C terminus); HSV-1 UL26 counterpart
UL82	ND	ND	4.82	5	pp71 upper matrix phosphoprotein; tegumentprotein; transactivator of MIEP
UL83	6.00	60	6.56	82	pp65 lower matrix phosphoprotein; tegument protein
UL84	5.05	4	5.65	3	Transdominant inhibitor of IE2-mediated transactivation
UL85	3.00	2	2.99	3	Intercapsomeric triplex capsid protein; HSV-1 UL18 counterpart
UL86	5.59	23	6.41	21	Major hexon and penton forming capsid protein; HSV-1 UL19 counterpart
UL94	ND	ND	3.83	1	Tegument protein; HSV-1 UL16 counterpart
UL98	ND	ND	3.32	1	DNase; HSV-1 UL12 counterpart
UL104	ND	ND	3.84	2	DNA packaging protein; capsid portal protein; HSV-1UL6 counterpart
UL112	1.97	1	ND	ND	Hypothetical protein
UL115	4.16	1	ND	ND	Envelope glycoprotein; associated with gH and gO; HSV-1 UL1 counterpart; gL
UL122	4.24	3	3.23	1	Immediate-early transcriptional regulator; IE2
US22	2.48	1	3.53	1	Tegument protein; US22 family member

^a ND, not determined.

aggresome formation, taken together with the ability of pp71 and IE1 to affect this process, suggested that this enzyme might be affecting one of the many proteins associated with PML. Proteins in this complex were examined by Western blot analysis by using samples derived from HFF cells that were uninfected, infected with AD169, or infected with a kinase null mutant at an MOI of 3 PFU/cell. Infections were also conducted in the presence of 15 μ M MBV to confirm that the observed changes were due to a deficiency of UL97 kinase activity. The expression of IE1 was detected in all infected cells to confirm infection (Fig. 4). No changes were observed in the

quantity of PML, CPB, or hDAXX, but p53 appeared to be stabilized in cells infected with the wt virus (Fig. 4, lanes 9 and 10), which is consistent with the results of a previous report (28). This increase was unaffected by the deletion of the UL97 ORF or treatment with MBV, indicating that it was not impacted by the activity of the UL97 kinase (Fig. 4, lanes 11 and 12). Also consistent with the previous report was the stabilization and hyperphosphorylation of RB at 24 h following infection (Fig. 4, lane 3). However, this did not occur in cells infected with the wt virus that were treated with MBV (Fig. 4, lane 4) and suggested that UL97 kinase activity was required

TABLE 3. Notable cellular proteins identified by liquid chromatography MS/MS in cytoplasmic and nuclear aggresomes

Aggresome type	Reference	Description	Max X_{corr}	No. of unique peptides
Cytoplasmic	gi 5729877	Heat shock 70-kDa protein 10 (HSC71)	4.74	2
	gi 4758570	Heat shock 70-kDa protein 9B	5.11	2
	gi 1708307	Heat shock-related 70-KDa protein 2	3.94	1
	gi 129379	Mitochondrial matrix protein P1 precursor (HSP-60)	3.93	1
	gi 125731	ATP-dependent DNA helicase II, 80-kDa subunit	7.14	5
	gi 114762	Nucleophosmin (nucleolar phosphoprotein B23)	6.12	1
Nuclear	gi 7446411	Aurora-related kinase 1	2.29	1
	gi 5729877	Heat shock 70-kDa protein 10 (HSC71)	6.53	1
	gi 4502549	Calmodulin 2 (phosphorylase kinase, delta)	4.84	1
	gi 2119712	DNAK-type molecular chaperone HSPA1L	3.21	1

FIG. 4. UL97 kinase activity is required for the hyperphosphorylation of RB in infected cells. HFF cells were mock infected or infected at an MOI of 2 PFU/cell with AD169 or a UL97 null virus (UL97Δ), both with (+) and without (−) the addition of MBV as shown. Cell lysates were harvested at 24 and 72 h following infection, separated on polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. Shown are immunoblots, with monoclonal antibodies to the proteins indicated to the left of the figure. The accumulation of hyperphosphorylated forms of RB was reduced when the UL97 kinase was deleted or when its activity was inhibited with MBV.

for the hyperphosphorylation of RB. This suggestion was confirmed with cells infected with RCΔ97, where no hyperphosphorylated RB was detected either with or without MBV (Fig. 4, lanes 5 and 6). These data suggest that UL97 kinase activity is required for the inactivation of RB at early times in viral infection. Similar results were observed 72 h following infection, when cells infected with the wt virus had high levels of hyperphosphorylated RB that were significantly reduced by the addition of MBV (Fig. 4, lanes 9 and 10). Reduced levels of hyperphosphorylated RB were also observed in cells infected with the null mutant. While some hyperphosphorylated RB was observed in the null mutant at this point (Fig. 4, lanes 11

and 12), it was present at lower levels than in cells infected with the wt virus and was relatively unaffected by MBV. This result may be due to the poor condition of the cells following the high MOI of infection with the low-titer mutant virus, but it may also be due to a compensatory pathway in the mutant that is unaffected by MBV, possibly involving pp71. Nevertheless, UL97 kinase activity clearly induces the hyperphosphorylation of RB, which can be antagonized through pharmacologic inhibition or genetic inactivation. These data confirm that HCMV infection increases levels of hyperphosphorylated RB and suggest that UL97 kinase activity is required for this effect. This result was intriguing, since RB interacts directly with PML (3) and has previously been shown to increase the number of PML bodies (46).

UL97 kinase contains three RB binding motifs. The kinase-dependent induction of RB phosphorylation by pUL97 prompted an examination of its amino acid sequence for potential RB binding motifs. Three candidate sequences were identified (Fig. 5A). The amino terminus contained the consensus LxCxE motif and an adjacent SEED sequence conserved among proteins that bind RB, including simian virus 40 (SV40) large T antigen, adenovirus E1A, and human papillomavirus E7 (Fig. 5B) (41). This motif was also conserved in UL97 homologs from many betaherpesviruses, including human herpesvirus 6-A (HHV6-A), HHV6-B, HHV7, and chimpanzee CMV, notwithstanding the low level of amino acid identity in the amino termini of these proteins. Near the middle of the protein was a second motif that contained an LxCxD sequence, which is similar to the amino acid sequence required for RB binding in pp71 (30, 31), but was not present in the other betaherpesviruses. The third carboxyl-terminal LxCxE motif was also present in chimpanzee CMV, but not in any of the other human betaherpesviruses. The conserved RB binding domains in the UL97 kinase homologs of the betaherpesviruses were consistent with its effects on RB and suggest that this function may be important in the replication of these viruses.

Mutation of LxCxE RB binding motif and kinase motif impacts RB stabilization and phosphorylation. Four recombinant viruses were constructed in the HB5 BAC strain (8) to

FIG. 5. RB binding motifs in pUL97. (A) Amino acid sequences of viral proteins containing LxCxE and LxCxD motifs were aligned with the motifs identified in pUL97. (B) Sequences for HCMV LxCxE (NP_040032.1), chimpanzee CMV UL97 (NP_612729), SV40 large T (NP_043127), human adenovirus E1A (ABK35030.1), human papillomavirus 16 E7, (AAD33253.1), HHV-6A U69 (NP_042962.1), HHV-6B U69 (T44214), HHV-7 U69 (YP_073809.1), HCMV pp71 (NP_040017), and HCMV LxCxD (NP_040032.1) are shown, with the consensus sequence given below.

FIG. 6. Recombinant viruses with point mutations in either the LxCxE RB binding motif or the kinase motif are impaired in their abilities to stabilize and phosphorylate RB. HFF cells were infected (at an MOI of 2 PFU/cell) with the wt virus HB5 or with recombinant viruses containing the point mutations in pUL97, as shown. Cell lysates were harvested at 24 h following infection, separated on polyacrylamide gels, and transferred to nitrocellulose membranes. Shown are immunoblots, with the antibodies to the proteins indicated to the left of the figure. The accumulation of RB occurred in cells infected with HB5, but was reduced in cells infected with the K355M mutant and the C151G mutant. The phosphorylation of RB on serine 780 was determined with specific antisera. Cells infected with HB5 contained increased levels of RB phosphorylated on serine 780. Cells infected with viruses containing point mutations that abrogated UL97 kinase activity (K355M) or disrupted the LxCxE motif (C151G) exhibited reduced levels of RB phosphorylated on serine 780. The expression of IE1 confirmed that cells were infected, and tubulin (tub) was included as a loading control.

assess a potential role for the conserved RB binding domains on RB stabilization and phosphorylation. These included viruses in which the central cysteines in the LxCxE, LxCxD, and IxCxE domains were mutated to glycines as well as a K355M mutant to confirm that the kinase motif was also required. The entire *UL97* ORF was sequenced in each of the viruses to confirm that they contained only the engineered mutations, and no large-scale rearrangements were detected by restriction fragment analysis of the BAC DNA. All the viruses appeared to replicate relatively well, with the exception of the virus containing the K355M mutation. This virus replicated very poorly, and its replication kinetics were indistinguishable from those of RCΔ97 in low-MOI growth curves (data not shown), consistent with results reported by others (32). This result confirmed that the poor-growth phenotype exhibited by the RCΔ97 deletion mutant was due to a deficiency in kinase activity rather than to the disruption of other viral functions.

The effects of four recombinant viruses containing point mutations on RB stabilization and phosphorylation were assessed by Western blot analysis using cell lysates harvested at 24 h after infection (Fig. 6). Cells infected with the wt virus appeared to stabilize RB, and this was consistent with results shown in Fig. 4, although the phosphorylated forms were not resolved in this blot. This stabilization did not occur when cells were infected with the K355M mutant, confirming that kinase activity was required. The accumulation of RB was unaffected by the C693G mutant (IxCxE), but appeared to be reduced in the C151G mutant, suggesting that this motif was involved with the stabilization of this protein. To confirm these data, the phosphorylation of RB was examined by using antisera specific for RB phosphorylated on serine 780. The phosphorylation of serine 780 appeared to be reduced in the K355M and C151G mutants relative to the wt virus and resembled levels seen in

uninfected cells. These data suggest that both the conserved LxCxE RB binding motif and the kinase motif were involved in the stabilization and phosphorylation of RB. We cannot exclude the possibility that the LxCxD motif might also be involved in conjunction with the LxCxE motif, and their functions may be partially redundant. Taken together, these results are consistent with the direct interaction and phosphorylation of RB by the UL97 kinase.

RB binding motif mutations impact aggregate formation.

Initial studies suggested that pUL97 inhibited the formation of nuclear aggresomes by a kinase-dependent mechanism that likely involved PML domains. A subsequent analysis of the proteins associated with PML complexes suggested that RB was hyperphosphorylated in the presence of this kinase activity, and three putative RB binding domains were identified. We hypothesized that these motifs might also be involved in the inhibition of nuclear aggresome formation. To investigate this possibility, we examined the effect of the C151G and C428G mutations on aggregation in transient assays in COS7 cells. The expression of the wt kinase inhibited the formation of nuclear aggresomes, while cells expressing either the K355M mutant or UL27 had no effect (Fig. 7A). The disruption of either the LxCxE or the LxCxD motif in pUL97 with a C151G or C428G mutation, respectively, significantly reduced the number of cells with pp65 aggregates as did the LxCxE/LxCxD double mutant (Fig. 7A). These data suggested that the RB binding motifs can impact the inhibition of aggregate formation by UL97. They did not, however, appear to affect UL97 kinase activity, since each of the mutants appeared to be phosphorylated, as determined by the altered electrophoretic mobility (data not shown). Although the disruption of the RB binding motifs in pUL97 increased the number of cells containing aggregates, the aggregates tended to be smaller than those observed with the K355M mutant and suggested that the UL97 kinase retained some capacity to inhibit the aggregation of pp65 even without these motifs. Consistent with this result, none of the RB point mutants affected the activity of pUL97 to the same degree as that with the K355M mutant ($P < 0.05$).

The wt virus and recombinant viruses containing point mutations were used to infect confluent HFF cells at a low MOI, and infected cells were examined 8 days postinfection. Cells infected with the wt virus exhibited both nuclear and cytoplasmic staining of pp65, while those infected with the K355M mutant exhibited largely nuclear pp65 staining and contained large nuclear aggregates (Fig. 7B). These data were consistent with previous reports of the *UL97* deletion mutant and confirmed that kinase activity was required to inhibit aggresome formation in infected cells. In cells infected with either the C151G or the C428G mutants, there appeared to be an overrepresentation of aggregates in the cytoplasm, although they tended to be somewhat smaller than the nuclear aggregates observed in the K355M mutant. These results were consistent with results from the transient assays and confirmed that the RB binding motifs may be involved in the inhibition of aggresome formation in infected cells. Cells infected with either the LxCxE or the LxCxD mutants exhibited aggresomes that were predominantly cytoplasmic, while those in the absence of kinase activity were predominantly nuclear, although cytoplasmic aggregates were frequently observed. This difference was interesting and may reflect the ability of the kinases with mu-

FIG. 7. RB binding motifs in pUL97 impact the inhibition of aggresome formation. COS7 cells were transfected with plasmids expressing pp65-GFP, plasmids expressing a UL27-negative control, and plasmids expressing UL97, with the point mutations as labeled. (A) The graph depicts the percentage of cells containing visible aggregates. The values shown are the averages of six separate experiments, with the standard deviations (error bars) shown; the exception was the double mutant, for which values were determined twice. (B) Recombinant viruses with mutations in the UL97 kinase domain or the RB binding domains also form large aggregates. Viruses with point mutations in the amino acids shown were used to infect confluent HFF cells and were harvested 8 days following infection. Shown are fluorescent phase-contrast images of infected cells stained with an antibody to pp65 (green) to confirm viral infection.

tations in the RB binding domains to disrupt nuclear lamina, thus releasing the aggresomes.

DISCUSSION

The most striking feature of cells infected with HCMV in the absence of UL97 kinase activity is the formation of large refractile bodies that occur predominantly in the nucleus. The nature of these inclusions was investigated to help define the mechanism of action of MBV and to understand the function of the UL97 kinase in viral infection. The inhibition of aggregate formation was modeled in a transient expression system

by using a pp65-GFP fusion protein, and a series of cotransfection experiments implicated both pp71 and IE1 as affecting aggresome formation. This observation was fortuitous, because the functions of the viral proteins were relatively well characterized and the intersection of their reported activities suggested that PML domains and RB pocket proteins may be involved in this process. Subsequent studies revealed that UL97 kinase activity also affected PML domains, consistent with the notion that these structures were linked to the formation of aggregates. A cellular marker of aggresomes confirmed that aggregates of tegument proteins localized to these cellular structures and that pUL97 inhibited their formation in a ki-

nase-dependent manner. Aggresome formation initiates at PML domains that were altered by the UL97 kinase and suggested a potential mechanism involving cellular proteins in the PML complex. The RB tumor suppressor, which interacts with PML, was hyperphosphorylated early in infection with the wt virus. This hyperphosphorylation did not occur in cells infected with RCΔ97 or in cells infected with the wt virus when kinase activity was inhibited by MBV and suggested that the UL97 kinase was required for the hyperphosphorylation of RB. The mutation of either the conserved LxCxE RB binding motif or the essential lysine in the kinase motif impaired the ability of the virus to stabilize and phosphorylate RB. These mutations also appeared to impact the ability of the kinase to inhibit aggresome formation. These data link the stabilization and phosphorylation of RB induced by the viral kinase with the disruption of PML domains and the inhibition of aggresome formation in infected cells.

The reduction of nuclear aggresome formation in infected cells by the UL97 kinase is strongly supported by experiments demonstrating their appearance in the presence of MBV or when the UL97 kinase is genetically inactivated with a K355M mutation. The recapitulation of this effect in a transient system suggested that kinase activity was sufficient to inhibit this process and proved to be a good model to study aggregation. PML domains have previously been reported to play a role in aggresome formation, and UL97 kinase appeared to disrupt these domains. However, the precise functions that PML domains perform in the establishment of nuclear aggresomes are unclear and it is possible that the effects of the UL97 kinase on PML bodies are indirect and only tangentially related to the formation of aggresomes. The mechanism by which RB stimulates PML domain formation is also incompletely understood. Nevertheless, RB is physically associated with PML and has previously been shown to promote the formation of PML domains, suggesting a plausible mechanism that requires additional investigation. The UL97 kinase and specific inhibitors of its activity will be valuable tools in this regard and promise to provide new insights on the formation of PML domains and their relationship to nuclear aggresomes. This promise is particularly important, since elucidating mechanisms that inhibit aggregate formation will improve our understanding of this process. The identification of specific proteins and pathways affected by the UL97 kinase may reveal new pathways and targets for the treatment of aggregative diseases.

Aggresomes have been suggested to be sites of virion assembly for some viruses (68). Here, we demonstrate that considerable quantities of viral proteins are sequestered in nuclear aggresomes in the absence of kinase activity, which appears to reduce the efficiency of virion morphogenesis. We presume that it negatively impacts HCMV infection, since the virus specifically inhibits aggregation. PML domains have also been proposed to specifically recognize and isolate highly ordered proteins, such as virion proteins, and many viruses disrupt these structures (11, 18, 26). Thus, both PML domains and nuclear aggresomes can be thought of as innate antiviral defenses that inhibit viral assembly through the sequestration of viral proteins. The pp65 tegument protein appears to be particularly susceptible to this defense, since it is efficiently sequestered when expressed transiently and aggresomes are not induced efficiently in viruses that lack this protein (58). It is

possible that pp65 promotes the formation of aggresomes and that the UL97 kinase can prevent this from occurring by phosphorylation or through a direct interaction that was described recently (32). Results presented here represent a clear example of the specific sequestration of viral proteins in nuclear aggresomes by the cell and how the virus circumvents this defense by eliminating these defensive structures.

The ability of UL97 kinase to induce the hyperphosphorylation and stabilization of RB in the context of a viral infection is intriguing. The dual approach of genetic inactivation and pharmacologic inhibition of the UL97 kinase yielded independent and consistent results in support of the conclusion that the UL97 kinase is required for the hyperphosphorylation of RB in viral infection. The identification of three consensus RB binding motifs in the UL97 kinase raised the possibility that this protein may phosphorylate RB directly. Subsequent experiments with recombinant viruses containing point mutations disrupting either the conserved LxCxE motif or the lysine required for enzymatic activity showed that these viruses were impaired in their abilities to stabilize and phosphorylate RB. These results are certainly consistent with the idea that the UL97 kinase might phosphorylate RB directly; however, studies presented here provide no direct evidence that UL97 interacts with RB directly. Additional experiments will be required to test this hypothesis.

The UL97 kinase is the third CMV gene product reported to affect the RB pocket proteins. The pp71 tegument protein directs the proteasome-dependent, ubiquitin-independent degradation of active unphosphorylated RB, but its effect during infection is unclear (31). IE1 was shown to interact with the RB-related p107 pocket protein in the context of viral infection and relieved E2F transcriptional repression but did not interact with RB (31, 56). IE1 interacts with the amino-terminal portion of p107, and this interaction can alleviate inhibition of cyclin E/cdk2 (29, 71). It has also been reported to possess kinase activity and to phosphorylate the RB-related pocket proteins p107 and p130, but not RB (55). Here we report that the UL97 kinase is required for the hyperphosphorylation and stabilization of RB in viral infection, which is distinct from the activities reported for IE1 and pp71. The inactivation of RB by the UL97 kinase should result in a host of downstream effects. The dispersion of PML domains and inhibition of aggresome formation may be related to the impairment of RB function, but whether this is a direct effect is not yet clear.

It is interesting that IE1, pp71, and pUL97 appear to affect similar cellular processes, yet they impact them in different ways. Both IE1 and pUL97 reduce the number of PML domains, while pp71 is recruited to them. Similarly, IE1 and pp71 stimulate the formation of aggresomes, while pUL97 reduces their numbers. It is notable, however, that pp71, IE1, and the UL97 kinase all affect RB family members, and each of them also affects aggresome formation. These results seem to support the link between the pocket proteins and aggregation, but the exact mechanism remains to be elucidated.

Studies presented here describe an important thread in the complex phenotype observed in the absence of UL97 kinase activity. The extensive morphological changes that occur in infected cells in the absence of the UL97 kinase confirm that it exerts a powerful influence on the cell and on viral infection. The hyperphosphorylation of RB induced by the UL97 kinase

FIG. 8. Model of the effect of pUL97 on RB phosphorylation and aggresome formation. In uninfected cells, RB interacts with E2F and PML and promotes the formation of PML domains and aggresomes. In cells infected with HCMV, UL97 kinase phosphorylates RB, which leads to the dissociation of E2F and PML and impairs the ability of the cell to sequester viral proteins in aggresomes.

is entirely consistent with its profound impact on the cell. Based on the data presented here, we propose a model in which the UL97 kinase interacts directly with RB through its consensus RB binding domains (Fig. 8). This interaction could facilitate the direct phosphorylation and inactivation of RB by the UL97 kinase, resulting in a cascade of downstream effects, including changes in PML domains and the inhibition of nuclear aggresome formation. This model also predicts that the UL97 kinase promotes the disassociation of RB and E2F, resulting in the stimulation of E2F responsive promoters. This interpretation of the data is consistent with several notable aspects of the UL97 phenotype and would explain why UL97 null mutants replicate better in dividing cells (59) and why some cellular kinase inhibitors can enhance the activity of this drug (9, 24).

The hyperphosphorylation of RB induced by the UL97 kinase is similar to that induced by other viral oncoproteins, such as SV40 large T antigen, adenovirus E1A, and papillomavirus E7 (16). However, it appears to be distinct in that it has kinase activity as well as RB binding motifs and may prove to phosphorylate the RB directly. This activity might also be present in uninfected cells, since the UL97 kinase is present in dense bodies that circulate during the course of infection. This activity also raises important questions regarding the potential of HCMV to induce cell proliferation under some conditions and could possibly contribute to proliferative and inflammatory disorders. While this virus has not been associated with any tumors, there are sporadic reports of viral gene expression in gliomas and some other tumors (10). If such conditions were identified, MBV could inhibit this activity and might alter the course of the disease. But most important are the vital functions of the UL97 kinase during viral infection, underscoring the importance of this enzyme as a target for the therapy of HCMV infections.

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