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Human papillomavirus 16 E7 inactivator of retinoblastoma family proteins complements human cytomegalovirus lacking UL97 protein kinase

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Several different families of DNA viruses encode proteins that inactivate the cellular retinoblastoma tumor suppressor protein (pRb), which normally functions to bind E2F transcription factors and restrict expression of genes necessary for cellular processes including DNA replication. Human cytomegalovirus (HCMV) UL97, a protein kinase functionally orthologous to cellular cyclin-dependent kinases, phosphorylates pRb on inactivating residues during HCMV infection. To assess if such phosphorylation is biologically relevant, we tested whether the human papillomavirus type 16 E7 protein, which inactivates pRb family proteins by direct binding and destabilization, could substitute for UL97 during HCMV infection. In the absence of UL97, expression of wild-type E7 protein, but not a mutant E7 unable to bind pRb family proteins, restored E2F-responsive cellular gene expression, late viral gene expression, and viral DNA synthesis to levels normally observed during wild-type virus infection of quiescent cells. UL97-null mutants exhibited more pronounced defects in virus production and DNA synthesis in quiescent cells as compared to serum-fed, cycling cells. E7 expression substantially enhanced infectious virus production in quiescent cells, but did not complement the defects observed during UL97-null virus infection of cycling cells. Thus, a primary role of UL97 is to inactivate pRb family proteins during infection of quiescent cells, and this inactivation likely abets virus replication by induction of cellular E2F-responsive genes. Our findings have implications for human cytomegalovirus disease and for drugs that target UL97.

E2F | cyclin-dependent kinase | cell cycle | antiviral drugs | retinoblastoma protein

The pathway controlled by the retinoblastoma (pRb) family of tumor suppressors is dysregulated in most if not all human cancers [reviewed in (1)]. pRb proteins inhibit the ability of the E2F transcription factors to activate the expression of genes required for many important cellular processes, including DNA replication. During normal cell cycle progression, phosphorylation of pRb proteins by cyclin dependent kinases (Cdks) leads to the disruption of pRb-E2F complexes and the entry of cells into the S phase, where DNA is synthesized. Certain viruses with DNA genomes specifically target pRb proteins for inactivation, at least in part to facilitate the high level genome replication required for efficient virus production (2, 3). One such virus is human papillomavirus (HPV), of which certain subtypes cause cervical cancer (2). The HPV16 E7 protein binds and destabilizes pRb family proteins, liberating E2F to activate the expression of genes required for DNA synthesis (4–6).

Recently, we discovered a different way by which viruses inactivate pRb: direct phosphorylation by a virally encoded kinase (7). The human cytomegalovirus (HCMV) protein kinase encoded by the UL97 gene is necessary and sufficient for pRb phosphorylation on inactivating residues *in vitro* (7) and in cells (7, 8). HCMV is a ubiquitous human pathogen that establishes a lifelong infection, which is typically asymptomatic in healthy

individuals [reviewed in (9)]. Nonetheless, HCMV is a leading viral cause of birth defects and life threatening disease in immunocompromised patients, and UL97 is an important target for drugs to ameliorate HCMV disease (10–14).

HCMV mutants lacking UL97 produce approximately 10 to 1,000 fold fewer infectious particles than wild-type virus during replication in cultured cells (7, 15–18). Specific defects of these mutants during viral replication include reduced viral DNA synthesis, impaired exit of capsids from the nucleus (nuclear egress), and altered intracellular localization of virion components; pharmacological inhibition of UL97 results in similar phenotypes (8, 10, 15, 17, 19, 20). Our discovery that UL97 directly phosphorylates pRb prompted us to examine the roles that both UL97 and pRb play during HCMV infection. To determine whether UL97-mediated inactivation of pRb was important during HCMV infection, we asked if the pRb-inactivating protein from HPV16 could complement HCMV replication defects observed in the absence of UL97.

Results

The Human Papillomavirus 16 E7 Protein Expressed from a Recombinant Human Cytomegalovirus Inactivates and Degrades pRb. UL97-null HCMV fails to induce pRb phosphorylation and shows defects in the production of infectious virus progeny (7, 8, 14–17, 20). We wanted to determine whether UL97 functionally inactivates pRb and if heterologous pRb inactivation in cells infected with UL97-null virus would rescue deficiencies of UL97-null HCMV. To this end, we used *E. coli* 'recombineering' techniques on an infectious bacterial artificial chromosome (BAC) clone of HCMV strain AD169 (21) to engineer viruses in which UL97 protein-coding sequences were replaced by either a wild-type allele of the HPV16 E7 oncoprotein, or a Δ DLYC mutant allele encoding an HPV16 E7 that lacks an LxCxE motif essential for inactivation of pRb (22, 23). These manipulations resulted in two UL97-null viruses: Δ 97-E7 and Δ 97-DLYC (Fig. 1). Restriction digests (Fig. S1) and sequencing of resulting BAC clones confirmed the genomic integrity of these recombinant viruses. Electroporation of these BAC clones into permissive human fibroblast cells allowed for the recovery of infectious virus.

Upon infection of subconfluent, serum-starved (quiescent) human fibroblasts, the appropriate expression patterns of UL97 and E7 proteins were observed by Western blot (Fig. 2A). Furthermore, in mock-infected cells, or in cells infected with a

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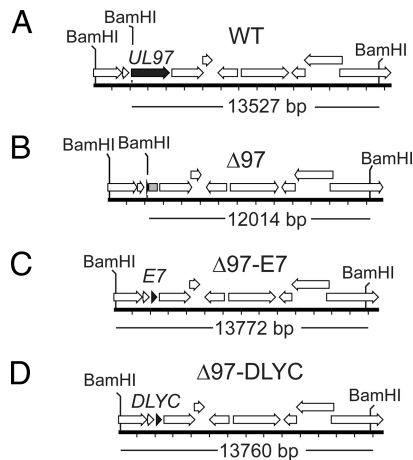


Fig. 1. *UL97*-null HCMVs that express wild-type or mutant forms of the E7 oncoprotein of HPV16. Schematic of the coding content of the *UL95–UL105* region of the viruses used in this study. (A) Wild-type HCMV strain AD169 (WT); *Bam*HI sites are labeled and an arrow representing the *UL97* ORF is shown in black (top). (B) The same region of $\Delta 97$, a *UL97* deletion virus is shown. A remnant 37-amino acid ORF, comprised of the first 23 amino acids of *UL97* and an additional 14 amino acids attributed to a frameshift after the deletion, is shown in black; a gray box represents a remnant *UL97* coding region not predicted to be translated due to a naturally occurring stop codon. (C) $\Delta 97$ -E7, a *UL97*-null HCMV that encodes the E7 protein of HPV16 (E7, black arrow) in place of *UL97*. (D) $\Delta 97$ -DLYC, a *UL97*-null HCMV that encodes a Δ DLYC mutant form of HPV16 E7 (DLYC, black arrow) in place of *UL97*.

previously characterized *UL97*-null virus, $\Delta 97$ (18), pRb remained in a hypophosphorylated state, while infection with wild-type virus (WT) resulted in pRb hyperphosphorylation (Fig. 2*A* and *B*), as previously reported (7, 8, 24). In cells infected with $\Delta 97$ -E7, pRb levels were substantially decreased, and there was some conversion of hypophosphorylated pRb into the hyperphosphorylated form at early time points (Fig. 2*A* and *B*). These changes were not observed in cells infected with $\Delta 97$ -DLYC, where pRb remained stable and hypophosphorylated throughout infection.

Induction of E2F-Responsive Cellular Genes and Viral Genes. Hypophosphorylated (active) pRb prevents the expression of E2F-responsive genes (1). Previous work has shown that HCMV infection induces E2F-responsive gene expression (25–27), but the viral protein(s) responsible have not been conclusively identified. In subconfluent, serum-starved cells, we found that the re-addition of serum or WT infection caused an increase in the steady state levels of transcripts encoding thymidylate synthase (*TS*) and uracil monophosphate synthase (*UMPS*) (Fig. 3). The *TS* promoter is known to be highly E2F-responsive (28) and the *UMPS* promoter has been shown to bind E2F-1 and E2F-4 (29). $\Delta 97$ and $\Delta 97$ -DLYC, which were unable to induce pRb phosphorylation (Fig. 2), failed to induce the expression of these two transcripts (Fig. 3). However, infection with $\Delta 97$ -E7 caused accumulation of *TS* and *UMPS* transcripts to levels similar to those during WT infection or after re-addition of serum (Fig. 3).

Like all herpesviruses, productive infection with HCMV initiates a temporal cascade of gene expression where immediate early genes are first expressed, followed next by early genes, and finally by late genes. We found that the expression of proteins encoded by immediate early gene *UL123* (IE1) and the early gene *UL57* was similar after infection of quiescent cells with WT, $\Delta 97$, $\Delta 97$ -E7, and $\Delta 97$ -DLYC viruses (Fig. 2*A*). However, expression of the pp28 protein encoded by the late gene *UL99* was markedly reduced under conditions where both *UL97* and wild-type E7 were absent, and where pRb remained present in

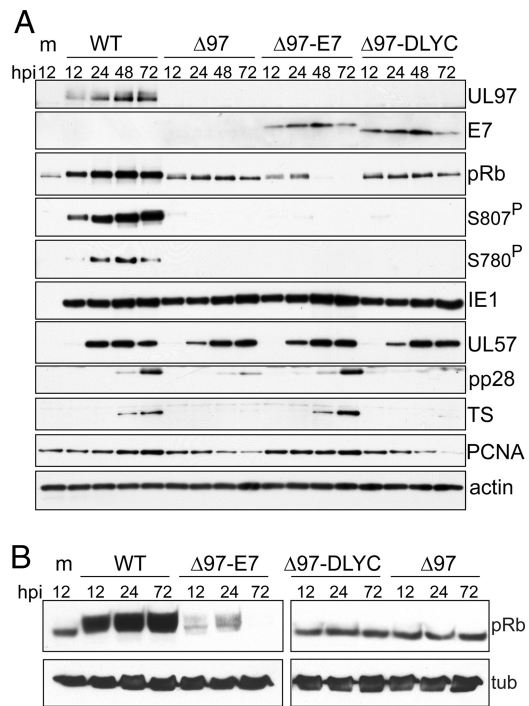


Fig. 2. Analysis of viral and cellular protein expression in quiescent cells. Western blot analyses of cellular and viral protein expression in cells infected with the viruses used in this study. (A) Serum-deprived human fibroblasts were infected at an MOI of 1 PFU/cell with WT HCMV (WT), *UL97*-null HCMV ($\Delta 97$), or *UL97*-null HCMV expressing either wild-type ($\Delta 97$ -E7) or Δ DLYC mutant ($\Delta 97$ -DLYC) forms of HPV16 E7. Lysates prepared at the indicated time points (hpi) were compared for the expression of the following viral gene products: the immediate early protein IE1–72 (IE1); *UL57*, an early protein; and pp28, a late protein. Expression levels of *TS* and *PCNA* were measured as examples of E2F-responsive cellular genes. Expression of *UL97*, E7, pRb were also monitored as controls, as was beta-actin, to indicate protein loading. Phosphospecific antibodies were used to monitor for the presence of pRb phosphorylated at Ser-807/811 (S807^P) and at Ser-780 (S780^P). (B) Serum-starved cells maintained in 0.1% FBS were either mock-infected (m) or infected with the same viruses and lysed at the indicated time points (hpi) following infection and analyzed for pRb expression and mobility by Western blot. Beta tubulin (tub) reactivity was determined as a loading control.

its hypophosphorylated (active) form (Fig. 2*A*). The defect in late gene expression correlated with a decrease in protein expression for two E2F-responsive cellular DNA synthesis genes,

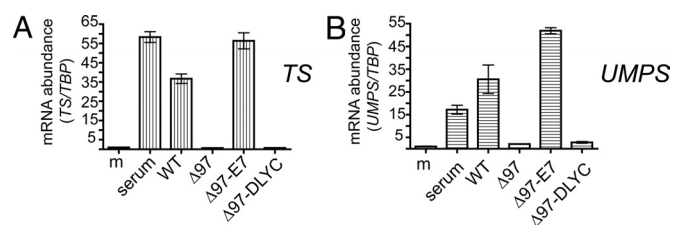


Fig. 3. Comparison of mRNA levels for *TS* and *UMPS*. Analysis of mRNA levels for two genes involved in cellular DNA synthesis whose promoters bind E2F transcription factors. (A) RT-qPCR was used to compare mRNA levels at 48 hpi for *TS* and TATA-binding protein (*TBP*) in serum-deprived cells which were either mock-infected or infected with the indicated viruses. RNA from a set of cells that were serum stimulated in parallel and harvested at 24 h post-stimulation was used as a positive control for *TS* induction. *TS* mRNA levels are displayed as normalized values after correcting for differences in *TBP* expression. (B) mRNA levels at 48 hpi for *UMPS* were compared, as above. For both panels, the data represent the average measurement from three replicates per condition, with error bars representing standard deviations.

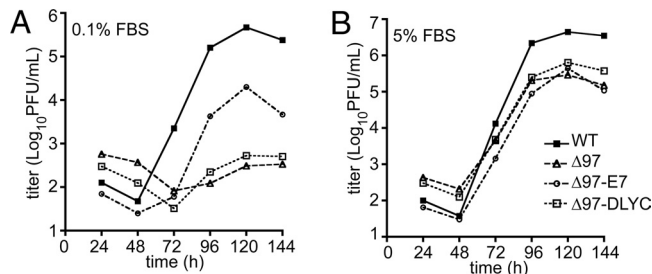


Fig. 4. Complementation of a severe replication defect in quiescent cells of *UL97*-null HCMV by wild-type HPV E7. Replication kinetics experiments comparing viral replication efficiency in quiescent and cycling cells. (A) Serum-deprived cells, maintained in medium containing 0.1% FBS were infected with WT HCMV (WT), *UL97*-null HCMV ($\Delta 97$), or *UL97*-null HCMV expressing either wild-type ($\Delta 97$ -E7) or Δ DLYC mutant ($\Delta 97$ -DLYC) forms of HPV16 E7 at an MOI of 1 PFU/cell and compared for yield of infectious virus. (B) Asynchronously dividing cells, maintained in the presence of 5% FBS, were infected as above and compared for yield of infectious virus. For both panels, because counts from duplicate titrations did not vary by more than 2-fold for any data points, error bars are not shown.

TS and proliferating cell nuclear antigen (*PCNA*) (Fig. 2A). A similar defect in pp28 expression that correlated with decreased pRb phosphorylation and TS expression was observed with an HCMV point mutant expressing a catalytically deficient *UL97* (K355Q), but no such defect was seen with a rescued virus derived from this mutant (Q335K) (Fig. S24).

HPV16 E7 Expression increases Infectious Virion Production and Viral DNA Synthesis in Quiescent Cells Infected with *UL97*-Null HCMV.

Because HPV16 E7 complemented defects in E2F-responsive gene expression and viral late gene expression observed in the absence of *UL97*, we asked if this papillomavirus protein could increase the yield of infectious *UL97*-null viruses. We found that $\Delta 97$ exhibited a replication defect of as much as 1,000-fold in quiescent cells (Fig. 4A), and no less than ≈ 100 -fold in repeated experiments. Moreover, the *UL97* point mutant virus (K355Q) replicated indistinguishably from $\Delta 97$ under these conditions (Fig. S3A). Expression of wild-type E7, but not the Δ DLYC mutant, was able to rescue infectious virus production substantially (50-fold in Fig. 4A). Thus, E7 can partially, but not fully, substitute for the loss of *UL97* during HCMV infection of quiescent cells.

As has been previously reported by others as data not shown (20), *UL97*-null viruses produced higher titers in serum-fed cycling cells than in quiescent cells, consistently showing only a ≈ 10 -fold replication defect relative to WT in cycling cells (Fig. 4B). Interestingly, E7 expression was unable to complement this smaller defect of $\Delta 97$ in cycling cells (Fig. 4B).

To control for the possibility that HPV16 E7 expression nonspecifically enhances replication of wild-type HCMV, we prepared two viruses, WT-E7 and WT-DLYC, which in addition to expressing *UL97* from its native locus, express either the wild-type or the Δ DLYC mutant form of HPV16 E7, respectively, under the control of a duplicated *UL97* promoter in the unique short (*U_S*) region of the HCMV genome (Fig. S4) from an intergenic locus between *US9* and *US10*, a site that has been used by others to express heterologous genes (30).

We confirmed that these viruses express E7 (Fig. S2B). To examine the biological activity of the E7 expressed by these viruses, we used the *UL97* inhibitor maribavir (10). In quiescent cells, treatment with 1 μ M maribavir reduced the yield of parental WT and WT-DLYC by approximately 100-fold, but reduced the yield of WT-E7 by less than 10-fold (Fig. S3B). Maribavir treatment inhibited pRb phosphorylation in WT- and WT-DLYC-infected cells; however, in WT-E7-infected cells, as

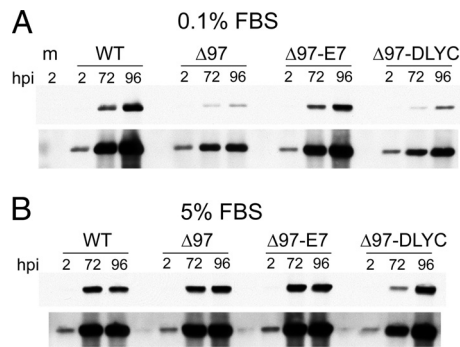


Fig. 5. Comparison of viral DNA synthesis in dividing versus non-dividing cells by Southern blot. Southern blot analysis of viral DNA synthesis in non-dividing, serum-deprived cells and asynchronously dividing cells maintained in 5% FBS. (A) Equal quantities of total DNA, prepared at the indicated time points from quiescent cells maintained in 0.1% FBS which were infected with WT HCMV (WT), *UL97*-null HCMV ($\Delta 97$), or *UL97*-null HCMV expressing either wild-type ($\Delta 97$ -E7) or Δ DLYC mutant ($\Delta 97$ -DLYC) forms of HPV16 E7, were analyzed by Southern blot using a probe against a viral gene (*UL83*). (B) Samples from a parallel experiment in asynchronously dividing cells maintained in 5% FBS were subjected to Southern blot analysis, as above. In both panels, a longer exposure is shown immediately beneath the normal exposure so that levels of DNA from input virus can be observed.

expected, pRb levels were drastically reduced, which correlated with increased expression of TS and pp28 (Fig. S24), akin to what was seen in $\Delta 97$ -E7-infected cells (Fig. 2A). Furthermore, under serum-deprivation conditions, WT-E7 replicated to yield three-fold lower levels of infectious virus than its parental WT (Fig. S3B) and similar results were seen during replication in 5% FBS (Fig. S3C). Thus, the complementation of the $\Delta 97$ defect by HPV16 E7 is not due to nonspecific enhancement of HCMV replication.

UL97 has been previously reported to be important for viral DNA synthesis (10, 17). As noted above, in the absence of *UL97* activity or E7, we detected reduced levels of pp28, a viral protein whose expression requires viral DNA synthesis (31) (Fig. 2A and Fig. S24). While it is possible that *UL97* may have a direct effect on HCMV late gene expression, we thought it unlikely that a HPV protein would, and therefore sought an alternative explanation for this observation. Since many E2F regulated cellular genes have clear roles in cellular DNA synthesis (1), we decided to investigate whether differences in viral DNA synthesis might correlate with the complementation of the *UL97*-null phenotype by E7 that we observed in quiescent cells.

Southern blotting experiments revealed that in quiescent cells, viral DNA synthesis during lytic infection with either $\Delta 97$ or $\Delta 97$ -DLYC was substantially reduced compared to that seen with WT or $\Delta 97$ -E7 (Fig. 5A). Thus, the Δ DLYC mutant form of E7 was apparently unable to complement the viral DNA synthesis defect observed in the absence of *UL97*, while wild-type E7 was able to restore viral DNA synthesis to wild-type levels (Fig. 5A). In contrast, the DNA synthesis defect of *UL97*-null viruses was not as dramatic in cycling cells, and expression of wild-type E7 did not appear to provide any notable advantage in viral DNA synthesis under these conditions (Fig. 5B).

We thus conclude that the HPV16 E7 protein, through its ability to inactivate pRb family members, can replace some, but not all of the functions of the HCMV *UL97* protein. Thus, pRb family protein inactivation by *UL97* protein kinase is required for efficient HCMV replication in quiescent cells. Furthermore, our results suggest that a function of pRb-inactivation by *UL97* is to facilitate viral DNA synthesis, most likely by activating the expression of E2F-responsive cellular genes.

Discussion

Papillomaviruses and cytomegaloviruses each face the challenge of amplifying their genomes in non-dividing cells that are incompetent for rapid and efficient DNA synthesis. These two viruses have evolved independent mechanisms to inactivate the pRb tumor suppressor protein and activate E2F-mediated gene expression. HPV16 E7 binds and destabilizes pRb, and HCMV UL97 phosphorylates pRb. In quiescent cells, expression of wild-type HPV16 E7 by *UL97*-null HCMV restored viral DNA synthesis and gene expression to wild-type or near wild-type levels, and enhanced production of infectious virus substantially.

However, HPV16 E7 failed to fully complement *UL97*-null HCMV in production of infectious particles, and HPV16 E7 did not appear to enhance replication of *UL97*-null virus in cycling cells, which may underscore the relevance of functions of *UL97* other than inactivation of pRb family members. In particular, *UL97*-mediated phosphorylation of lamin A/C has been posited to play a role in altering the nuclear lamina to promote nuclear egress (15, 32–34). A defect in nuclear egress largely explains the replication defect of *UL97*-null mutants in cycling cells (15), which would in turn explain why HPV16 E7 expression does not complement the loss of *UL97* in these cells. It is also possible that toxicity from HPV16 E7 expression [reviewed in (35)] may account for some portion of the replication defect observed for $\Delta 97$ -E7 in quiescent cells. Further studies will be needed to demonstrate whether the failure to phosphorylate lamin A/C accounts for the *UL97*-null replication defect in quiescent cells that cannot be complemented by HPV16 E7 expression.

There is considerable variability reported in the literature regarding the degree and nature of replication defects caused by genetic ablation or pharmacological inhibition of *UL97*, and some of this variability appears to depend on cell type and culture conditions (10, 14–18, 36). Nonetheless, we are unaware of any previously presented data directly comparing the replication of viruses lacking *UL97* activity in dividing versus resting cells. Our results suggest that at least some of the variability in the literature may be explained by the relative proportion, at the time of infection, of resting cells, which contain hypophosphorylated, active pRb, and cycling cells, which contain inactivated pRb. Indeed, we have observed variability in yield of *UL97*-null viruses that correlated with cultivation of cells in different lots of serum. Other researchers have used serum-fed confluent cells in their assays (10, 36, 37). Since confluent cells can express increased levels of Cdk inhibitor proteins that prevent cellular kinases from efficiently inactivating pRb (38, 39), such conditions might also be expected to exacerbate pRb-dependent replication defects of *UL97*-null mutants.

While this manuscript was being completed, Gill et al. reported that HCMV mutants bearing mutations in *UL97* that alter putative pRb binding motifs replicated well in human fibroblasts (40). However, the viruses were assayed in serum-fed cells and these mutations were not shown to abolish pRb inactivation.

As recently reviewed (3), HCMV encodes at least three other genes that have been reported to antagonize the function of pRb family proteins or otherwise up-regulate E2F-responsive cellular genes; these include IE1, IE2, and pp71. Since the viruses used in this study were wild-type with regard to IE1, IE2, and pp71, our results suggest that pRb family member inactivation by these proteins is functionally or temporally distinct from that mediated by *UL97*, or by HPV16 E7, when expressed in place of *UL97*. Moreover, our results show that *UL97* is responsible for activating at least a subset of E2F-responsive genes when HCMV infects quiescent cells. Thus, it seems likely that *UL97*-null viruses grow better in serum-fed cycling cells than in quiescent cells because serum induces E2F-responsive gene expression.

Unlike cells infected with $\Delta 97$ or $\Delta 97$ -DLYC, where pRb remains hypophosphorylated, there was evidence of pRb phosphorylation in cells infected with $\Delta 97$ -E7 (Fig. 2 *A* and *B*). Because HCMV encodes no other conventional protein kinases, and because E7 is not known to have kinase activity, such phosphorylation might be attributed to increased activity of Cdk2-cyclin E complexes following pRb inactivation by E7, as cyclin E expression is up-regulated following pRb inactivation (41–43) and Cdk2-cyclin E activity has been shown to be induced by HCMV infection (24, 44). Moreover, HPV16 E7 preferentially associates with and destabilizes hypophosphorylated pRb (45). Nonetheless, precisely why Cdks might phosphorylate pRb in cells infected with $\Delta 97$ -E7 but fail to do so in $\Delta 97$ and $\Delta 97$ -DLYC infected cells remains unclear.

To successfully infect and spread within its natural host, HCMV is likely to depend on its ability to replicate in non-dividing cells. Our results argue that a major role of *UL97* is to inactivate pRb family proteins in non-dividing cells, where these proteins are not readily inactivated by host cell kinases. Since the vast preponderance of cells in an adult vertebrate are quiescent and contain unduplicated DNA (46), the role of *UL97* as an inactivator of pRb family proteins is likely to be of great relevance for HCMV disease.

UL97 is an important drug target in therapeutic strategies against HCMV infection (10–13). Maribavir, a specific inhibitor of *UL97* kinase activity, is currently being evaluated for approval as an antiviral agent against HCMV disease (10, 11, 47, 48). Thus, the results presented here help establish a likely mechanism by which inhibition of *UL97* may provide its therapeutic efficacy in patients (11, 48). Furthermore, our data suggest that *UL97* inhibition may be more efficacious at treating HCMV disease caused by viral infection of resting cells, and less efficacious when disease results from infection of dividing cells. It is also of interest that investigators have reported an association between HCMV infection and certain cancers (49), particularly glioblastoma (50–52), a brain tumor. While a role for HCMV in the etiology of any human malignancy remains to be demonstrated, the ability of *UL97* to inactivate an important tumor suppressor provides an impetus to consider that *UL97* may potentially act as an oncogene, and that *UL97* inhibitors such as maribavir might conceivably inhibit tumor progression in certain cancers.

Materials and Methods

Plasmids, Cells, and Viruses. Sequences encoding wild-type and Δ DLYC mutant E7 were each PCR amplified from pOZ-CE7 and pOZ E7 DLYC (53) and adapted for “*en passant*” mutagenesis, as previously described (18, 54). Details are provided in *SI Materials and Methods*. $\Delta 97$, a recombinant HCMV bearing a large deletion in *UL97*, has been previously described (18). All HCMV infection experiments were performed in human foreskin fibroblasts (ATCC) at an MOI of 1 PFU/cell, as previously described (18), with the exception that all virus preparations used in this study were concentrated by ultracentrifugation and resuspended in Dulbecco’s modified Eagle’s Medium (DMEM) containing 0.1% FBS before use. Ultracentrifugation of virus was performed as described previously (55), except 25 mM Na-HEPES pH 7.4 was used instead of Tris-HCl to buffer the 20% D-(+) sorbitol cushion. In experiments where serum-deprivation was used, cells were maintained in DMEM containing 0.1% FBS and 20 μ g/mL gentamicin from 48 h before infection until the end of the experiment.

Construction of Recombinant Viruses. Recombinant viruses were constructed using two-step Red, “*en passant*” recombination (54), as described previously (18). $\Delta 97$ -E7 and $\Delta 97$ -DLYC were constructed from AD169rv (21), a BAC clone of HCMV strain AD169. WT-E7 and WT-DLYC were derived from pBAC/AD169, a close predecessor of pAD/Cre (56). See *Table S1* for a list of primers used, and *SI Materials and Methods* for further details.

Western Blotting. Western blotting was carried out as described previously (7, 18). To detect E7, monoclonal antibodies ED19 (57) or ED17 (Santa Cruz Biotechnology), in combination with 8C9 (Invitrogen), were used. pRb, tubulin, *UL97*, IE1, *UL44*, and actin were detected as previously described (7, 18, 33).

UL57 and pp28 were detected using mouse monoclonal antibodies (Virusys Inc). TS and PCNA were detected using monoclonal antibodies TS 106 (Abcam Inc.), and PC10 (Santa Cruz Biotechnology), respectively.

RNA Quantification. To quantify mRNA levels of *TS*, *UMPS*, and *TBP*, reverse-transcriptase (RT) qPCR was performed on DNase treated total RNA isolated from infected cells using TaqMan Gene Expression Assay (Applied Biosystems) primer and probe sets for *TS*, *UMPS*, and *TBP*, as per the manufacturer's recommendations. Additional details are provided in *SI Materials and Methods*.

Viral Replication Assays. Replication kinetics assays were performed as described previously (18), except supernatants of infected cells were collected without first scraping cells. Where indicated, maribavir was used at 1 μ M.

Southern Blotting. Southern blots were performed with chemiluminescent detection, as described previously (58). A probe that encompassed the entire

UL83 ORF was used to detect viral DNA. Additional details are provided in *SI Materials and Methods*.

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