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26. Formation of a delocalized chlorophyll dimer would depend on occupancy of both A5 and B5 sites by chlorophyll a. Although the A5 site exclusively binds chlorophyll a, B5 is a mixed site of chlorophylls a and b in minor complexes (CP29, CP26, and CP24) (*21*). Therefore, a delocalized chlorophyll a dimer can form within the A5 and B5 sites in CP29. Moreover, this dimer structure could also explain why Z^{+} formation is not favored in LHCII (*14*), in which the A5 and B5 sites specifically bind chlorophylls a and b, respectively (*12*). Because the large spectral absorbance gap (~700 cm^{-1}) between chlorophylls a and b prohibits delocalization over a chlorophyll heterodimer, no CT state can be produced in the L2 site of LHCII. One additional factor of specificity is that the capacity of LHCII to undergo exchange of V to Z in site L2 in vivo is very low relative to minor complexes (*22*).
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30. We thank D. Zigmantas for helpful discussions. Supported by Korea Research Foundation grant KRF-2006-214-C00037 funded by the Korean Government (T.K.A.); USDA National Research Initiative competitive grant 2006-03279 (T.J.A.); Office of Basic Energy Sciences, Chemical Sciences Division, U.S. Department of Energy contract DE-AC03-76SF000098 (G.R.F. and K.K.N.); and Italian Basic Research Foundation contract RBLA0345SF and Trento Research Council contract SAMBA (R.B.).

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Phosphorylation of Retinoblastoma Protein by Viral Protein with Cyclin-Dependent Kinase Function

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As obligate intracellular parasites, viruses expertly modify cellular processes to facilitate their replication and spread, often by encoding genes that mimic the functions of cellular proteins while lacking regulatory features that modify their activity. We show that the human cytomegalovirus UL97 protein has activities similar to cellular cyclin–cyclin-dependent kinase (CDK) complexes. UL97 phosphorylated and inactivated the retinoblastoma tumor suppressor, stimulated cell cycle progression in mammalian cells, and rescued proliferation of *Saccharomyces cerevisiae* lacking CDK activity. UL97 is not inhibited by the CDK inhibitor p21 and lacks amino acid residues conserved in the CDKs that permit the attenuation of kinase activity. Thus, UL97 represents a functional ortholog of cellular CDKs that is immune from normal CDK control mechanisms.

Cyclin–cyclin-dependent kinase (CDK) complexes are found in all eukaryotes and control cell cycle progression and other processes (*1*). In higher eukaryotes, a major target of the CDKs is the retinoblastoma (Rb) tumor-suppressor protein that controls progression through G_1 phase of the cell cycle. The pathway controlled by Rb may be aberrant in most human cancers (*2*). Unphosphorylated Rb binds to E2F transcription factors, thus inhibiting the expression of genes required for DNA replication and arresting cell cycle progression in G_0 or G_1 phase. During normal cell cycle progression, Rb is functionally inactivated by multiple phosphorylations mediated sequentially by a series of CDK complexes (*3*). Phosphorylation of Rb disrupts complexes with E2Fs, allowing for cell cycle progression into S phase. To create an advantageous cellular environment for viral replication, viruses can inactivate Rb through direct binding of viral proteins to Rb and the consequent disruption of Rb-E2F complexes, by causing Rb degradation, or through constitutive activation of cellular CDKs by virally encoded cyclin proteins (*4*, *5*). Here, we describe a virally encoded protein kinase that directly phosphorylates Rb, and we show that this kinase can substitute for CDKs during cell cycle regulation.

Upon infection of quiescent cells with human cytomegalovirus (HCMV), unphosphorylated Rb is first degraded by pp71 (*6*) and then phosphorylated (*7*, *8*) (Fig. 1A and fig. S1). Phosphorylated Rb migrates more slowly during SDS–polyacrylamide gel electrophoresis than does the unphosphorylated form of the protein, and it can be detected in lysates from HCMV-infected cells within 4 hours after infection (Fig. 1A). Three small-molecule inhibitors of CDK activity (roscovitine, olomoucine, and flavopiridol) inhibited Rb phosphorylation induced by serum stimulation, but not phosphorylation induced by HCMV infection (Fig. 1B). Additional experiments with a panel of 20 kinase inhibitors (table S1) revealed that only 2, Gö6976 and NGIC-I, inhibited Rb phosphorylation during HCMV infection (Fig. 1C). These drugs inhibit both cellular protein kinase C (PKC) and the HCMV protein kinase UL97 (*9*). However, Gö7478, an inhibitor of PKC that does not inhibit UL97, did not reduce Rb phosphorylation in HCMV-infected cells (Fig. 1C). Because an HCMV mutant lacking the UL97 gene (*10*) did not induce Rb phosphorylation (Fig. 1, D and E) and because of the presence of three potential Rb-binding motifs in UL97 (fig. S2), we suspected that UL97 was required for Rb phosphorylation during HCMV infection.

Phosphorylation of Rb on Ser^{780}, Ser^{807}, Ser^{811}, and Thr^{821} inactivates the cell cycle–inhibitory and tumor-suppressor functions of Rb by disrupting Rb-E2F complexes (*3*). All of these residues are phosphorylated in HCMV-infected cells (Fig. 2A). Residues not known to modify Rb function upon phosphorylation, such as Ser^{249} and Thr^{252}, were not phosphorylated in HCMV-infected cells but were phosphorylated in serum-stimulated cells (Fig. 2A). A recombinant HCMV in which the wild-type (WT) UL97 gene was replaced with an allele encoding a UL97 protein substituted at the active site Lys (Lys^{355} → Gln^{355}; K355Q) failed to induce phosphorylation of Rb, but a WT revertant virus derived from the K355Q mutant did induce the phosphorylation of Rb (Fig. 2B). The UL97-K355Q mutant virus exhibited a growth defect similar to that of the UL97-null virus, and the growth defect was rescued in the revertant virus (fig. S3). The CDK inhibitor flavopiridol again failed to prevent HCMV-induced phosphorylation of Rb in HCMV-infected cells, but two drugs that inhibit UL97 kinase activity (Gö6976 and maribavir) did inhibit such phosphorylation (fig. S4). Thus, in HCMV-infected cells, kinase activity of UL97 is necessary for Rb phosphorylation on residues that inactivate its function. Rb degradation and phosphorylation in HCMV-infected cells are independent events (fig. S1).

We also tested whether UL97 alone is sufficient to induce Rb phosphorylation. Transfection of expression plasmids for epitope-tagged wild type [but not a catalytically inactive (Lys^{355} → Met^{355}; K355M) mutant (*11*)] induced the phosphorylation of cotransfected Rb on inactivating residues (Fig. 3A) in Saos-2 cells that are intrinsically unable to phosphorylate Rb. Drugs that inhibit UL97 partially suppressed Rb phosphorylation when added to UL97-expressing

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Saos-2 cells, but those that inhibit the CDKs or only PKC did not (Fig. 3B). The K355M mutant protein was readily detected by protein immunoblotting, but WT UL97 was consistently expressed in lower amounts. Inhibition of kinase activity of WT UL97 with drugs also seemed to allow accumulation of more protein (Fig. 3B), perhaps indicating that active UL97 is toxic to mammalian cells. Nevertheless, UL97 expression was sufficient to induce Rb phosphorylation. Purified UL97 phosphorylated an Rb fragment in vitro, as assayed by the incorporation of ^{32}P (Fig. 3C), or with an antibody that specifically recognized Rb phosphorylated on Ser^{807} and Ser^{811} (Fig. 3D). Autophosphorylation was also detected (fig. S5). UL97 showed the same spectrum of sensitivity to kinase inhibitors in vitro as was observed for Rb phosphorylation in HCMV-infected cells (Fig. 3, C and D), and purified catalytically inactive (K355Q) UL97 failed to phosphorylate Rb in vitro (Fig. 3, C and D). These results make it unlikely that a copurifying insect kinase phosphorylated Rb in this assay.

UL97 directly phosphorylates Rb on inactivating residues, an activity shared by cellular CDKs. Therefore, we tested whether UL97 represents a functional CDK ortholog by testing if

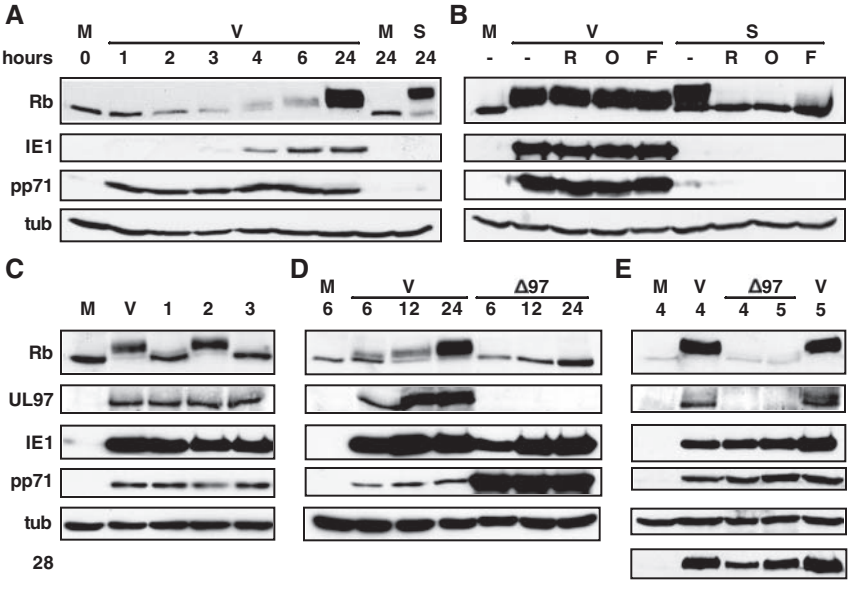

Fig. 1. Requirement of viral UL97 protein kinase, but not cellular CDKs, for Rb phosphorylation in HCMV-infected cells. (**A**) Subconfluent human fibroblasts deprived of serum (ssHFs) were mock-infected (M), infected with HCMV (V) at a multiplicity of infection (MOI) of 3, or stimulated with serum (S). Lysates prepared at the indicated times (in hours) were analyzed by Western blots. The Rb antibody (4H1) detects all forms of the protein. Virion-delivered pp71 (71) and newly synthesized IE1 are viral proteins, and tubulin (tub) is a loading control. (**B**) Cells treated as in (A) were also incubated with the CDK inhibitors roscovitine (R), olomoucine (O), or flavopiridol (F). Proteins in lysates harvested at 6 hours from virus-infected cells or 24 hours from uninfected cells were analyzed by Western blots. Dashes indicate no drug treatment. (**C**) ssHFs treated as in (A) were also incubated with the PKC inhibitors Gö6976 (1), Gö7874 (2), or NGIC-I (3), and proteins from lysates collected after 6 hours were analyzed by Western blots. Expression of the viral UL97 protein is confirmed with a UL97 specific antibody. (**D**) ssHFs were mock infected or infected with WT HCMV or a UL97-null virus (Δ97) at an MOI of 1. Proteins from lysates collected at the indicated hour were analyzed by Western blots. (**E**) ssHFs were treated as in (D), and proteins from lysates harvested on the indicated day after infection were analyzed by Western blots. Viral late gene expression was confirmed by detection of the viral pp28 protein (28).

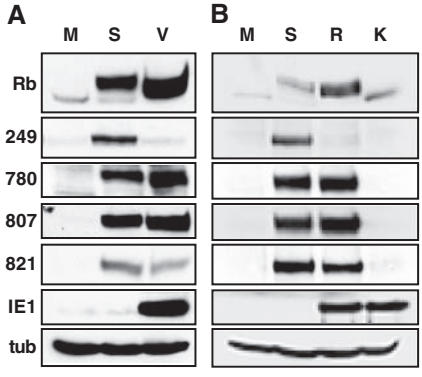

Fig. 2. Phosphorylation of Rb on residues that inactivate its tumor-suppressor function in HCMV-infected cells. (**A**) ssHFs were mock infected (M), infected with HCMV (V) at an MOI of 3, or stimulated with serum (S). Proteins from lysates prepared at 24 hours were analyzed by Western blots with control antibodies (IE1 and tub) and antibodies specific for all forms of Rb (Rb), Rb phosphorylated on Ser^{249} and Ser^{252} (249), on Ser^{780} (780), on Ser^{807} and Ser^{811} (807), or on Thr^{821} (821). (**B**) ssHFs were mock-infected, serum-stimulated, infected with a recombinant HCMV expressing a substituted form of UL97 (K355Q) that lacks kinase activity (K) or a WT revertant of the K355Q virus (R). Proteins from lysates harvested at 24 hours were analyzed by Western blots.

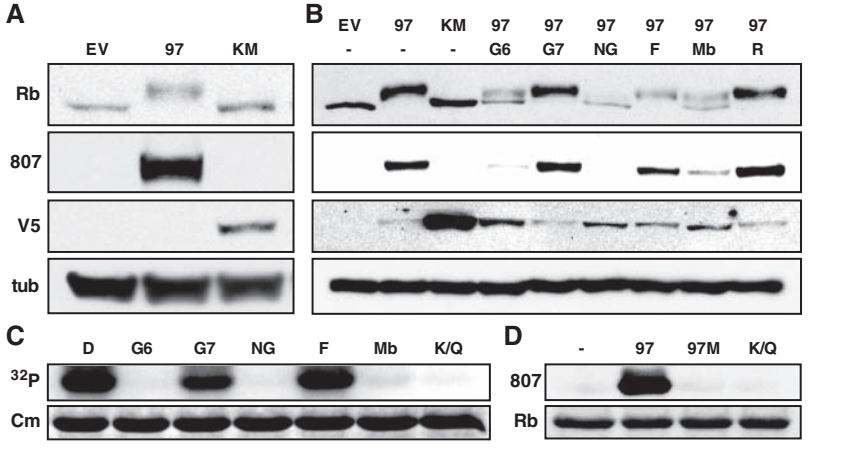

Fig. 3. Phosphorylation of Rb in vivo and in vitro by UL97. (**A**) Proteins from lysates of Saos-2 cells transfected with an Rb expression plasmid and either an empty vector (EV) or expression plasmids for V5-epitope–tagged WT (97) or catalytically inactive (KM) UL97 were analyzed by Western blots with the indicated antibodies. (**B**) Thirty hours after transfection as above, cells were left untreated (–) or treated with Gö6976 (G6), Gö7874 (G7), NGIC-I (NG), flavopiridol (F), maribavir (Mb), or roscovitine (R) for 18 hours before harvesting lysates and analysis of proteins by Western blots. (**C**) Purified GST-UL97 kinase was incubated in vitro with a His-tagged Rb fragment in a kinase reaction supplemented either with dimethyl sulfoxide [DMSO (D)] or the indicated drugs. Purified catalytically inactive GST-UL97-K355Q was also analyzed (K/Q). Transfer of radiolabeled phosphate (^{32}P) to Rb was detected by phosphorimaging, and total Rb was detected by Coomassie staining (Cm). (**D**) Samples from an in vitro kinase assay containing no added kinase (–), WT GST-UL97 (97), WT GST-UL97 plus maribavir (97M), or the catalytically inactive mutant UL97 (K/Q) were analyzed by Western blots for total Rb or Rb phosphorylated on Ser^{807} and Ser^{811} (807).

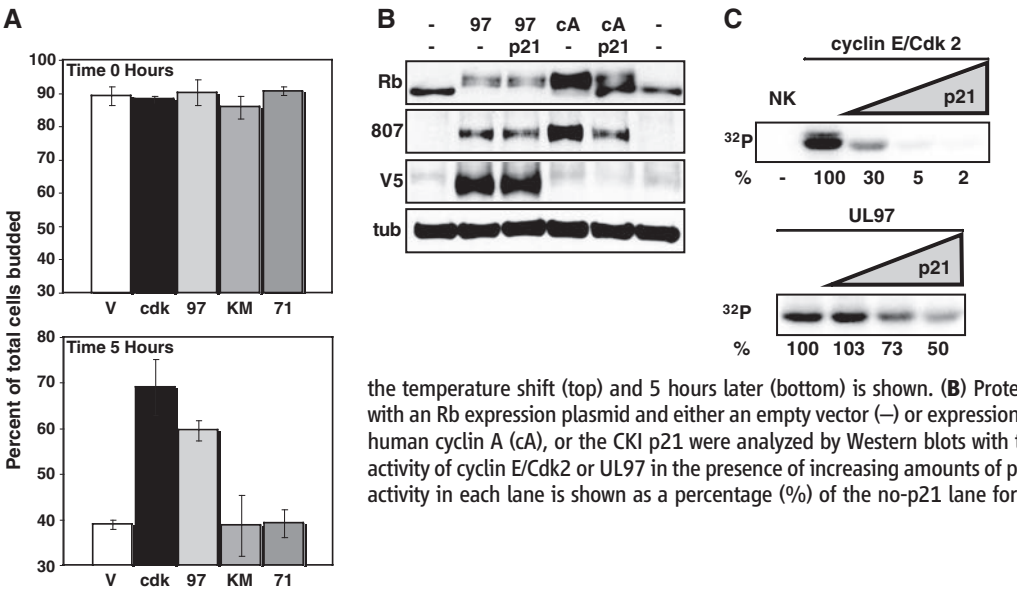

Fig. 4. CDK-like activity of HCMV UL97. (**A**) Saturated cultures of the yeast temperature-sensitive strain carrying *cdc28-13* and harboring a plasmid without a transgene (V) or one expressing WT UL97 (97), catalytically inactive UL97 (KM), HCMV pp71 (71), or cellular Cdk1 (CDK), each under the control of a galactose-inducible promoter, were diluted into galactose-induction medium for one doubling at the permissive temperature and then switched to the restrictive temperature. The number of budded cells [presented as a percentage of total cells with SDs (error bars)] present at the time of the temperature shift (top) and 5 hours later (bottom) is shown. (**B**) Proteins from lysates of Saos-2 cells transfected with an Rb expression plasmid and either an empty vector (–) or expression plasmids for V5-epitope–tagged WT UL97, human cyclin A (cA), or the CKI p21 were analyzed by Western blots with the indicated antibodies. (**C**) In vitro kinase activity of cyclin E/Cdk2 or UL97 in the presence of increasing amounts of p21 was determined as in Fig. 3C. The kinase activity in each lane is shown as a percentage (%) of the no-p21 lane for each set of reactions. NK, no kinase.

UL97 could rescue the cell cycle defect of yeast lacking CDK activity. A yeast mutant (*12*) with a temperature-sensitive allele of the single CDK gene (*cdc28-13*) arrests in G_1 as unbudded cells at the restrictive temperature (Fig. 4A and fig. S6). Expression of human Cdk1 or WT UL97 allowed the cells to remain cycling at the restrictive temperature (Fig. 4A). Catalytically inactive UL97, or the viral pp71 protein (*6*), failed to rescue the cell cycle defect. UL97 also stimulated the cell cycle of mammalian cells (fig. S7). The functional properties presented here lead us to conclude that UL97 is a viral CDK functional ortholog.

We expanded an alignment (*13*) of the kinase subdomains of UL97 with four cellular Ser/Thr kinases (including Cdk2) to include the intervening sequences (fig. S8). Our analysis suggested that UL97 is not subject to the regulatory mechanisms that control CDK activity (*14*), and we confirmed that prediction. UL97 lacks most of the residues of Cdk2 that make contacts with cyclin A, including the conserved PSTAIRE (*15*) helix required for cyclin binding (*16*), indicating that UL97 is unlikely to bind to cyclins. Because cyclins did not copurify with UL97 during tandem affinity purification (*17*) and because of the high activity of UL97 on Rb in vitro (Fig. 3, C and D) in the absence of a cyclin, we conclude that UL97 does not require cyclin binding for activity. The affinity of CDKs for cyclins is enhanced by CDK activating kinase (CAK)–mediated phosphorylation (*18*) of CDKs on Thr^{160}. Neither the Thr nor any of the three Arg residues of CDKs that coordinate the phosphate (*16*) are conserved in UL97 (fig. S8). Because flavopiridol and roscovitine, both CAK inhibitors (*18*), do not inhibit the ability of UL97 to phosphorylate Rb in vivo (Figs. 1B and 3B and fig. S4), we conclude that CAK-mediated phosphorylation does not activate UL97. CDK activity is attenuated by phosphorylation on Tyr^{15} during G_2 phase of the cell cycle and in response to radiation (*19*). UL97 has a Phe substitution (fig. S8) and thus cannot be phosphorylated at this site. UL97 (fig. S8) lacks most of the conserved CDK residues (*1*, *14*) that interact with members of both classes of CDK inhibitors (CKIs). p21, a potent CKI (*20*), did not efficiently inhibit UL97-mediated phosphorylation of Rb in vivo (Fig. 4B) or in vitro (Fig. 4C).

UL97 is a viral CDK ortholog that is immune from normal cellular control mechanisms that attenuate CDK activity and represents a previously unknown mechanism through which viruses regulate the cell cycle. The remote sequence similarity between CDKs and conserved herpesvirus protein kinases (CHPKs) such as UL97 led others to speculate that CHPKs may mimic CDK function (*13*). We provide direct experimental evidence that UL97 is functionally orthologous to cellular CDKs. UL97 is a target for anti-HCMV therapies because it phosphorylates (and thus activates) the antiviral drug ganciclovir (*21*) and because it is inhibited by maribavir (*22*). UL97 may also be useful as a tool to study the inactivation of the Rb pathway by phosphorylation, to identify other critical substrates of CDKs, and to probe the evolutionary relationships between viral and host cell kinases.

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15. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Supporting Online Material

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