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Inhibition of Cellular Cdk2 Activity Blocks Human Cytomegalovirus Replication

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Human cytomegalovirus is a herpesvirus that induces numerous cellular processes upon infection. Among these are activation of cyclin-dependent kinase 2, which regulates cell cycle progression in G1 and S phase. We report here that inhibition of cellular Cdk2 activity blocks HCMV replication. Inhibition of Cdk2 activity by roscovitine inhibits HCMV DNA synthesis, production of infectious progeny, and late antigen expression in infected cells in a dose-dependent manner. HCMV replication is also inhibited by the expression of a Cdk2 dominant negative mutant, whereas expression of wild-type Cdk2 has no effect on viral replication. These data indicate that activation of cellular Cdk2 is necessary for HCMV replication.

INTRODUCTION

Human cytomegalovirus (HCMV)[2] is a ubiquitous pathogenic herpesvirus that infects over 80% of the population and causes a variety of disease conditions after prenatal infection or in patients with suppressed immune functions (organ transplantation, HIV infection) (Rubin, 1990; Schooley, 1990; Alford *et al.,* 1990). Increases in the frequency of organ transplantation and the incidence of AIDS have focused attention upon the HCMV life cycle, with a view toward discovering potential means of attenuating primary and/or reactivated infection. It has been determined that productive HCMV infection of postmitotic cells stimulates cell cycle progression (Bresnahan *et al.,* 1996; Jault *et al.,* 1995; Lu and Shenk, 1996; Poma *et al.,* 1996; Dittmer and Mocarski, 1997). Although there appears to be a general agreement that productive HCMV infection induces cellular processes that resemble progression through G1 phase upon infection of arrested cells, it is still controversial whether or not these cells are capable of entering S phase. Several reports have suggested that productively infected cells proceed through S phase and subsequently arrest (Jault *et al.,* 1996; Poma *et al.,* 1996), while a number of more recent (Bresnahan *et al.,* 1996; Lu and Shenk, 1996; Dittmer and Mocarski, 1997) and earlier reports (Albrecht *et al.,* 1989; DeMarchi, 1983) have demonstrated that productive HCMV infection inhibits cellular DNA synthesis, so that infected cells arrest in late G1 (Bresnahan *et al.,* 1996; Lu and Shenk, 1996; Dittmer and Mocarski, 1997). These latter findings are consistent with the observation that HCMV infection does not alter the abundance or activity of cyclin A/Cdk2 (Bresnahan *et al.,* 1996), whose activity is necessary for entry into and progression through S phase (Resnitzky *et al.,* 1995; Girard *et al.,* 1991).

We have proposed that the late G1-arrested state induced by productive HCMV infection provides a cellular environment that is advantageous for HCMV replication in that metabolic precursors (i.e., nucleotides) are abundantly available for viral replication, while cellular DNA synthesis is inhibited, thus allowing uncompeted viral access to these precursors. A prominent aspect of this G1-arrested state is a substantial increase in the abundance and activity of cyclin E/Cdk2 (Bresnahan *et al.,* 1996; Jault *et al.,* 1995). The increase in cyclin E kinase activity results from an increase in the abundance of cyclin E (Bresnahan *et al.,* 1996; Jault *et al.,* 1995), the translocation of Cdk2 from the cytoplasm to the nucleus,[3] and inhibition of the Cdk2 inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ (Bresnahan *et al.,* 1996). These findings suggest that HCMV replication may be related to the virus' ability to activate cyclin E/Cdk2 kinase activity. If this hypothesis is correct, inhibition of Cdk2 activation should inhibit HCMV replication.

MATERIALS AND METHODS

Cell culture and transfections

Human diploid embryonic lung fibroblasts (LU), passage 12–20, or U-373 astrocytoma cells from the Ameri-

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[2] Abbreviations used: HCMV, human cytomegalovirus; Cdk2, cyclin-dependent kinase 2; PAA, phosphonoacetic acid; EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PFU, plaque forming units; wt, wild-type; HA, hemagglutinin.

[3] Bresnahan *et al.,* submitted for publication.

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can Type Culture Collection (ATCC HTB-17) were cultured in Eagle's minimum essential medium with Earle's salts (EMEM) with 10% fetal bovine serum (FBS) and penicillin (100 units/ml)/streptomycin(100 μg/ml) at 37° in a 5% CO_2 atmosphere. LU cells were density arrested and infected as previously described (Bresnahan *et al.,* 1996). For treatment of infected cells with roscovitine or olomoucine LU cells were density-arrested and infected as described previously (Bresnahan *et al.,* 1996). Following incubation with HCMV stock the virus inoculum was removed and replaced with medium containing various concentrations of either roscovitine or olomoucine. Roscovitine or olomoucine stocks were prepared in dimethylsulfoxide (DMSO) at a concentration of 10 m*M.* Infected cultures not treated with drug always contained a volume of DMSO (vehicle) equal to that contained in the highest concentration of roscovitine used. For transfection experiments, U-373 cultures were subcultured 24 hr prior to transfection into 100-mm dishes. Cells were transfected with 10 μg of pCMVCdk2-wt-HA or pCMVCdk2-dn-HA with Tfx-50 lipofection reagent (Promega) for 2 hr with a 3:1 lipofectin:DNA ratio. Cells were harvested 48 hr after transfection and assayed for HA expression and kinase activity. For immunofluorescent staining, transfected cells were dissociated with trypsin, seeded into 35-mm dishes containing sterile glass coverslips, and cultured for 24 hr before being infected as described previously (Bresnahan *et al.,* 1996).

Immunofluorescent staining, kinase assays, and Western blotting

Coverslips were processed for immunofluorescent staining as previously described (Bresnahan *et al.,* 1996). For Western blotting and kinase assays, cells were harvested by trypsinization, collected by sedimentation, and lysed in NP-40 lysis buffer (50 m*M* Tris, pH 7.4, 150 m*M* NaCl, and 0.5% NP-40, with 1 m*M* $NaVO_3$, 50 m*M* NaF, 1 m*M* PMSF, 1 m*M* DTT, 25 μg/ml leupeptin, 25 μg/ml trypsin inhibitor, 25 μg/ml aprotinin, 1 m*M* benzamide, and 25 μg/ml pepstatin A added just before use). Cellular debris was removed by sedimentation and the reserved supernatant fluids were assayed for protein concentration (Bradford, 1976), and then used for kinase assays or immunoblotting as described previously (Bresnahan *et al.,* 1996; Dulic *et al.,* 1992). Cyclin-dependent kinase activity *in vitro,* in the presence of roscovitine, was determined as described previously (Bresnahan *et al.,* 1996; Dulic *et al.,* 1992) with slight modifications. Briefly, following immunoprecipitation, kinase reactions were carried out in a total reaction volume of 10 μl. Each reaction contained 5 μl of 2× kinase buffer (40 m*M* Tris–HCl, pH 7.5, 8 m*M* $MgCl_2$), containing 2.4 μg of histone H1 as substrate. Roscovitine was added to the reaction along with 1 μl [γ-^{32}P]ATP (10 Ci/mmol) to obtain a final concentration of 20 μM ATP. Double-distilled H_2O was added to a final reaction volume of 10 μl. Kinase reactions were carried out at 37° for 30 min and subsequently stopped by addition of 2× sample buffer.

Slot blot hybridization and virus yield reduction assays

Total DNA from infected cells was isolated by phenol extraction. Equal aliquots of DNA (2 μg) were heated to 95° and transferred to Hybond+ (Amersham) membranes in 10× SSC (1× SSC: 0.15 *M* NaCl, 0.015 *M* sodium citrate) buffer, using a slot blot apparatus. Membranes were denatured for 5 min in 0.5 *M* NaOH–1.5 *M* NaCl, neutralized in 1.5 *M* NaCl, 0.5 *M* Tris–HCl, pH. 7.2, 0.001 *M* EDTA for 5 min, and then dried for 10 min in a vacuum oven at 80°. Filters were prehybridized in Rapid-Hyb buffer (Amersham) for 3 hr at 60°. Hybridization was carried out by overnight incubation in the same buffer at 60°. A 253-bp PCR amplified immediate early fragment from HCMV strain AD169 was ^{32}P-labeled by random priming and used as probe. Filters were washed twice in 0.1% SDS in 2× SSC at room temperature for 15 min and then 0.1% SDS in 0.2× SSC at 60° for 20 min and exposed to film (Kodak XAR-5) at −80°. Virus yields were assayed as previously described (Albrecht and Weller, 1980).

Flow cytometry

LU cells were assayed for DNA content as described previously (Bresnahan *et al.,* 1996). Briefly, cells were harvested by trypsinization, washed in PBS, collected by sedimentation, suspended in low salt buffer [3% polyethylene glycol, propidium iodine (5 μg/ml), 0.1% Triton X-100, 4 m*M* sodium citrate, RNase A (100 μg/ml, added just before use)], and incubated for 20 min at 37°. High salt buffer [3% polyethylene glycol, propidium iodine (5 μg/ml), 0.1% Triton X-100, 400 m*M* NaCl] was then added, and the cells were maintained at 4° overnight. The cellular DNA content was analyzed using a Becton–Dickinson FACScan flow cytometer. For bromodeoxyuridine incorporation LU cells treated with 15 μM roscovitine for 96 hr were washed twice with PBS and then cultured in fresh EMEM containing 10% FBS and 0.1 m*M* bromodeoxyuridine for 24 hr. The cells were fixed in acetone:methanol (1:1) at −20° for 10 min and stained for bromodeoxyuridine incorporation as described by Cardoso *et al.* (1993).

RESULTS

Activation of cyclin E/Cdk2 by HCMV

The data shown in Fig. 1 illustrate HCMV's ability to activate Cdk2. LU cells were arrested by contact inhibition and then infected with HCMV. Cell lysates were prepared before infection (0 hr) and 48 hr postinfection and immunoprecipitated with anti-Cdk2 antibody, and the

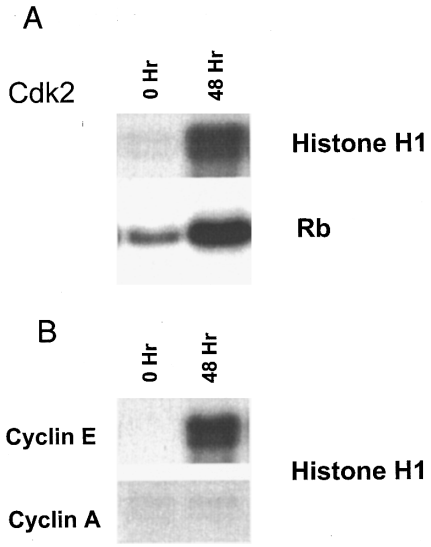

FIG. 1. Cdk2 activation in HCMV-infected cells. LU cells were growth arrested by contact inhibition and infected with HCMV as described previously (Bresnahan *et al.,* 1996). Prior to infection (0 hr) or 48 hr postinfection cells were harvested and equal amounts (100 μg) of cell lysates immunoprecipitated with Cdk2 antibody (A). The resulting immunoprecipitates were assayed for kinase activity using either Rb or histone H1 as a substrate as previously described (Bresnahan *et al.,* 1996; Dulic *et al.,* 1992). Cell lysates were also immunoprecipitated with either cyclin E or cyclin A antibodies and assayed for kinase activity using histone H1 as a substrate (B).

precipitates were assayed for Cdk2 kinase activity. Figure 1A shows that HCMV infection results in a dramatic increase in Cdk2 kinase activity using both histone H1 and Rb as substrates. Kinase assays were also done on cyclin E and cyclin A immunoprecipitates from HCMV-infected cells. HCMV infection results in an increase in cyclin E kinase activity with no induction of cyclin A kinase activity (Fig. 1B). These results demonstrate that the Cdk2 activity that is induced in HCMV-infected cells is due to cyclin E/Cdk2 complexes and not cyclin A/Cdk2 complexes (Bresnahan *et al.,* 1996).

Roscovitine inhibits HCMV replication

We have used an inhibitor of Cdk2 activity, roscovitine (Meijer, 1996; Rudolph *et al.,* 1996), to determine if Cdk2 activity is necessary for HCMV replication. The IC_{50} for cyclin E/Cdk2 inhibition *in vitro* by roscovitine is 0.7 μM (Meijer, 1996; Rudolph *et al.,* 1996). We determined if roscovitine could inhibit cyclin E/Cdk2 activity from HCMV-infected cells at similar concentrations. Cell lysates were prepared from LU cells that had been infected with HCMV for 48 hr and assayed for cyclin E/Cdk2 kinase activity in the presence of various concentrations of roscovitine. Figure 2A shows that roscovitine inhibited cyclin E/Cdk2 kinase activity in a dose-dependent manner with cyclin E/Cdk2 activity being inhibited by greater than 95% at 2.5 μM. We also determined if roscovitine could inhibit accumulation of HCMV DNA in a dose-dependent manner after addition to infected LU cells.

Density-arrested LU cells were infected for 1 hr after which the virus inoculum was removed and replaced with medium containing various concentrations of roscovitine. Total DNA was isolated from LU cells that had been infected for 72 hr, and the abundance of HCMV DNA was determined by slot blot hybridization. As Fig. 2B shows, HCMV DNA abundance was reduced by ~50% in the presence of 1 μM roscovitine; and viral DNA abundance was reduced by >90% by 10 μM inhibitor. Consequently, roscovitine significantly reduced the production of infectious HCMV progeny, as shown in Fig. 2C. Infectious HCMV yields were reduced by >90% after addition of

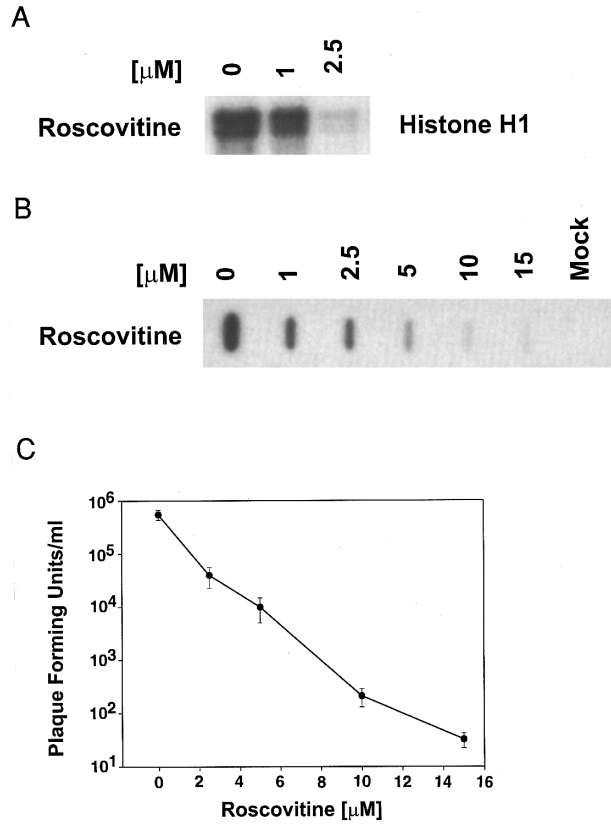

FIG. 2. Inhibition of cyclin E/Cdk2 activity, HCMV DNA synthesis and virus yields by roscovitine. LU cells were growth arrested by contact inhibition and infected with HCMV. 48 hr postinfection cell lysates were prepared, 100 μg of total protein was immunoprecipitated with cyclin E antibody, and *in vitro* kinase activity was determined in the presence of the indicated concentration of roscovitine (A). Cells were also infected and treated with various concentrations of roscovitine following infection. (B) 72 hr postinfection, the cells were harvested, total DNA was isolated, and the abundance of HCMV DNA was determined by slot blot hybridization using a specific HCMV DNA probe. (C) 96 hr postinfection, infected cells were lysed by freeze–thaw, followed by sonication. Cellular debris was removed by sedimentation and the HCMV containing supernatant fluids were assayed for infectivity by plaque assay as described previously (Albrecht and Weller, 1980). Values represent the average of three independent experiments with standard errors shown.

2.5 μM roscovitine, and >99.9% inhibition occurred at 10 μM. Since the IC_{50} for roscovitine inhibition of Cdk2 *in vitro* is 0.7 μM (Meijer, 1996; Rudolph *et al.,* 1996), the observation that both viral DNA synthesis and production of infectious virus particles is inhibited 50% at about 1 μM roscovitine suggests that inhibition of Cdk2 accounts for inhibition of viral DNA replication. Similar results were obtained when olomoucine, a Cdk2 inhibitor that is structurally related to roscovitine (Meijer, 1996), was used (data not shown); however, the concentration of olomoucine that was required to inhibit viral DNA synthesis and virus yield was about 10-fold higher than the corresponding concentration of roscovitine. The IC_{50} for olomoucine-mediated inhibition of Cdk2 *in vitro* is 7 μM (Vesely *et al.,* 1994), 10-fold higher than that of roscovitine.

To test whether drug-associated cellular toxicity was responsible for the reduced HCMV replication we investigated the effects of both roscovitine and olomoucine on noninfected cells. In these experiments, neither roscovitine nor olomoucine demonstrated any detectable toxic effects on noninfected cells. No changes in cell morphology were observed over 96 hr in the presence of 15 μM roscovitine (data not shown). In addition, more than 70% of the cells that had been exposed to roscovitine or olomoucine in this fashion were able to incorporate bromodeoxyuridine (BrdU) within 24 hr after removal of the inhibitor (Fig. 3B). Similar results were obtained when roscovitine- or olomoucine-treated cells were analyzed for cell cycle progression (using flow cytometry) after removal of the drug (Fig. 3A).

HCMV, like other herpesviruses, undergoes a sequential order of gene expression whose phases have been designated immediate early, early, and late following infection of permissive cells (Wathen and Stinski, 1982). HCMV immediate early genes are expressed within 1 hr after infection (Boldogh *et al.,* 1991), well before viral activation of cyclin E/Cdk2 (Bresnahan *et al.,* 1996), suggesting that immediate early expression is not dependent on Cdk2 activation. Indeed expression of the HCMV major immediate early proteins (IE72 and IE86) was not inhibited by roscovitine, in two cell lines (LU and U-373) which support productive HCMV infection (Bresnahan *et al.,* 1996; Ripalti *et al.,* 1995; Dal Monte *et al.,* 1996) as shown in Fig. 4A. On the other hand, expression of UL80.5 late HCMV gene products (Welch *et al.,* 1991) was inhibited by roscovitine (Fig. 4B). The results obtained with phosphonoacetic acid (PAA) are shown for comparison. PAA, a well-characterized inhibitor of HCMV DNA polymerase, inhibits viral DNA synthesis (Huang, 1975). PAA inhibits expression of HCMV late genes (e.g., UL80.5; Fig. 4B) since expression of late genes is, by definition, dependent upon viral DNA synthesis (Wathen and Stinski, 1982; Honess and Roizman, 1975). However, PAA has no effect on those HCMV genes that are expressed prior to initiation of viral DNA synthesis (Wathen and Stinski, 1982). The results shown in Figs. 2A and 4

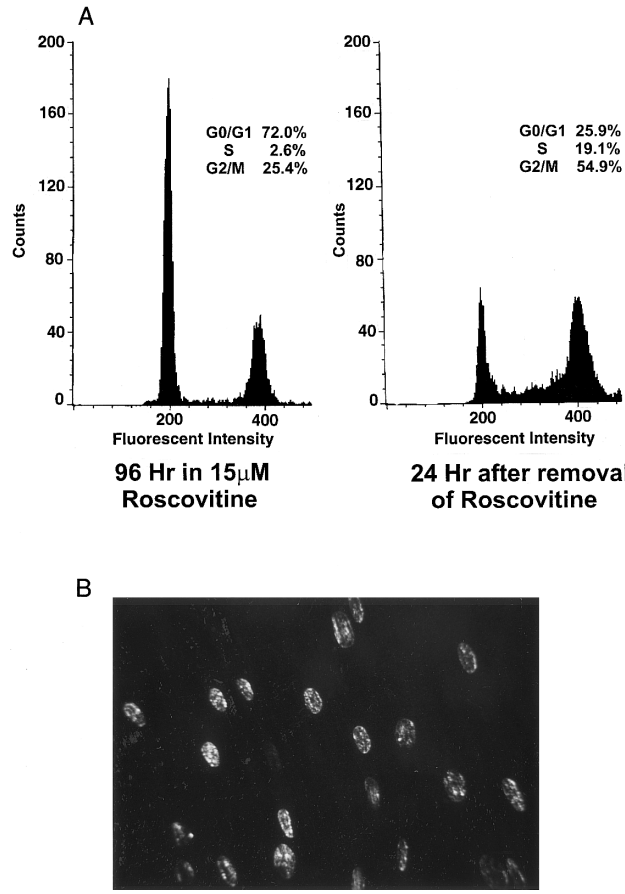

FIG. 3. Effects of roscovitine on noninfected LU cells. LU cells were treated with 15 μM roscovitine for 96 hr. After 96 hr, the cells were stained with propidium iodide and analyzed by flow cytometry (A). In parallel, the roscovitine containing medium was removed and replaced with fresh EMEM containing 10% FBS or EMEM containing 10% FBS and bromodeoxyuridine. Cells were harvested 24 hr after removal of roscovitine and analyzed for cell cycle progression by flow cytometry (A) and bromodeoxyuridine incorporation (B).

are consistent with the hypothesis that roscovitine, like PAA, blocks viral DNA replication at some point after expression of immediate early genes, but prior to the initiation of the late phase of HCMV replication. This effect can be demonstrated in at least two cell lines that are known to be productive for HCMV infection.

Expression of dominant negative mutant Cdk2 inhibits HCMV replication

Both roscovitine and olomoucine inhibit Cdk1, as well as Cdk2 (Meijer, 1996; Vesely *et al.,* 1994). Both Cdk1 and Cdk2 are inhibited at similar concentrations of roscovitine, and inhibition of HCMV replication could be due to inhibition of either enzyme. A previously characterized dominant negative Cdk2 mutant (van den Heuvel and Harlow, 1993) was used to test directly the hypothesis that the effects of roscovitine on HCMV replication are due to inhibition of Cdk2. This hypothesis predicts that expression of the dominant negative Cdk2, which results

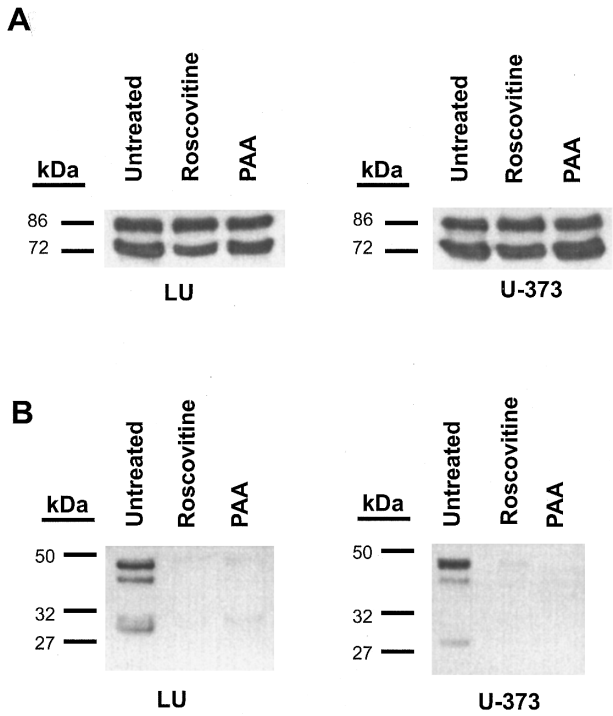

FIG. 4. Expression of HCMV immediate early and late antigens following treatment with roscovitine. LU or U-373 cells were infected with HCMV. Infected cells were either untreated or treated with roscovitine (15 μM) or phosphonoacetic acid (750 μM). (A) Infected cell lysates were prepared 8 hr postinfection. Equal amounts of protein (40 μg) were separated by SDS–PAGE, transferred to nitrocellulose, and probed with antibody against HCMV immediate early proteins. (B) Lysates were prepared 72 hr postinfection and assayed by immunoblotting for HCMV UL80.5 abundance as described above.

in inhibition of Cdk2 activity (van den Heuvel and Harlow, 1993), should be sufficient for inhibition of HCMV replication. U-373 cells were used in these experiments because of the low efficiency of transfection of LU cells. U-373 cells were transiently transfected with expression vectors encoding hemagglutinin (HA)-tagged wild-type Cdk2 (pCMVCdk2-wt-HA) or HA-tagged dominant negative Cdk2 (pCMVCdk2-dn-HA). The hemagglutinin tag allows us to distinguish between endogenous and exogenous Cdk2 by the use of a specific hemagglutinin antibody. Cells were harvested 48 hr after transfection and assayed for Cdk2-wt-HA and Cdk2-dn-HA expression and kinase activity. Western blotting with HA antibody showed that cells transfected with either wild-type or dominant negative Cdk2 expressed the exogenous protein (Fig. 5). Histone H1 kinase activity was associated with Cdk2 wild-type HA immunoprecipitates, but not with the Cdk2 dominant negative HA precipitates (Fig. 5).

To determine if Cdk2 activity is required for HCMV replication, dual immunofluorescent staining was used to assay for expression of the HA-tagged Cdks and for the HCMV late antigens encoded by UL80.5, in transfected U-373 cells that were also infected with HCMV. As shown in Fig. 6, cells that expressed the dominant negative Cdk2 mutant (shown in Fig. 6B) did not express UL80.5 late antigens (Fig. 6D). Cells that expressed wild-type Cdk2 (Fig. 6A) supported viral replication, as evidenced by expression of the UL80.5 late gene products (Fig. 6C). Three independent experiments of this kind were done; and the percentage of infected cells, cells expressing Cdk2-wt-HA plus UL80.5, and cells expressing Cdk2-dn-HA plus UL80.5 were determined. Transfection efficiencies were similar for both wild-type and dominant negative Cdk2 (data not shown). As shown in Table 1, about 33% of the cells in the infected cultures expressed UL80.5 late antigens. This efficiency of infection was observed irrespective of whether the cells were transfected with wild-type or dominant negative derivatives of Cdk2. The susceptibility of U-373 cells to HCMV in our experiments is consistent with published data (Ripalti *et al.*, 1995). The efficiency of transient expression of Cdk2 derivatives is much lower than the frequency of virus infection. We estimate that $<5\%$ of the U-373 cells, and therefore $<2\%$ of the infected cells, express HA-tagged Cdk2 derivatives. Similar results were obtained with β-galactosidase expression vectors (data not shown). Nevertheless, about 37% of the cells that expressed the wild-type HA-tagged Cdk2 derivative also expressed UL80.5 late antigens. The percentage of wild-type Cdk2-transfected cells that expressed viral late antigens was not significantly different from the percentage of HCMV-infected cells in the culture ($P > 0.05$). This observation demonstrates that transient expression of HA-tagged wild-type Cdk2 derivative has no significant effect on viral replication, as assessed by expression of viral late antigen. On the other hand, only 2% of cells that expressed the dominant negative Cdk2 mutant also expressed UL80.5 late antigens ($P < 0.0001$). This observation indicates that Cdk2 activity is vital for HCMV replication and inhibition of Cdk2 is sufficient to inhibit viral replication.

DISCUSSION

A number of studies have revealed that productive HCMV infection provokes arrested cells to enter the cell

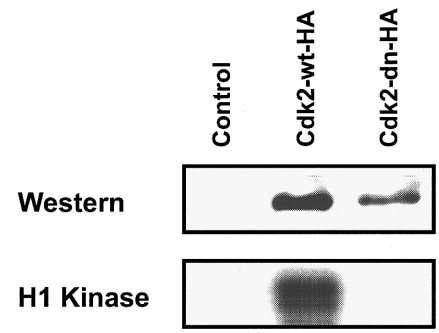

FIG. 5. Expression and activity of wild-type and dominant negative cdk2. U-373 cells were transiently transfected with either HA-tagged wild type Cdk2 (Cdk2-wt-HA) or dominant negative Cdk2 (Cdk2-dn-HA). Cells were then harvested 48 hr later and assayed for HA expression (Western) and HA-associated kinase activity (Histone H1).

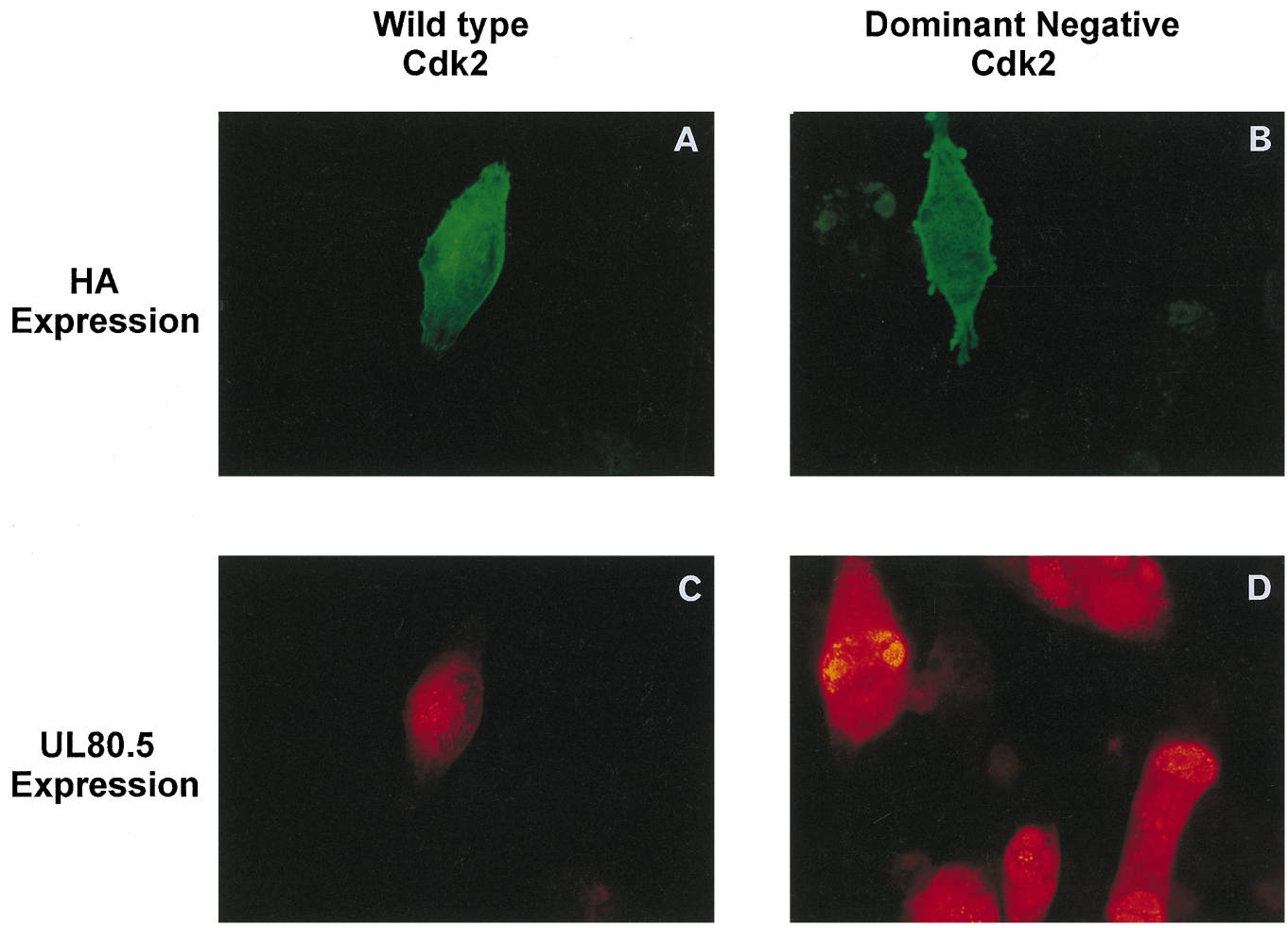

FIG. 6. Inhibition of HCMV late antigens in cells expressing dominant negative Cdk2. U-373 cells were transiently transfected with either HA-tagged wild-type Cdk2 (Cdk2 wild type) or dominant negative Cdk2 (Cdk2 dominant negative). Cells were then seeded onto glass coverslips and infected with HCMV 24 hr after transfection. The cells were fixed 72 hr postinfection with acetone:methanol (1:1) and dual immunofluorescent staining was done for HCMV UL80.5 (rhodamine) and HA (FITC) expression. (A and B) HA antigen (Cdk2) was detected by fluorescein fluorescence to demonstrate cells expressing either wild-type or dominant negative Cdk2. (C and D) HCMV UL80.5 late antigens were detected by rhodamine fluorescence. The identical field of cells is shown in A and C. An identical field of cells is also illustrated in B and D.

cycle (reviewed in Albrecht *et al.,* 1989). Recent studies have confirmed these early observations and demonstrated that productively infected cells traverse the cell cycle through at least late G1 (Bresnahan *et al.,* 1996; Lu and Shenk, 1996; Jault *et al.,* 1995; Poma *et al.,* 1996; Dittmer and Mocarski, 1997) and induce cyclin E/Cdk2 activity (Bresnahan *et al.,* 1996; Jault *et al.,* 1995). Previously, we have shown that HCMV infection causes the translocation of Cdk2 into the nucleus,[3] reduces the abundance of two Cdk2 inhibitors (Cip1 and Kip1), and induces a robust increase in cyclin E abundance and its associated kinase activity (Bresnahan *et al.,* 1996). In cells stimulated to traverse the cell cycle by serum growth factors, cyclin E/Cdk2 acts in part by phosphorylating pRB, which results in the release of the transcription factor E2F (Nevins, 1992; Weinberg, 1995). E2F can then transcriptionally activate a number of E2F responsive genes that are required for efficient cellular DNA synthesis. Among those genes whose expression is regulated by E2F are c-*myc,* c-*myb,* dihydrofolate reductase, thymidine kinase, and DNA polymerase α (see Weinberg, 1995; Farnham *et al.,* 1993 and references therein). Increased expression of these genes is required for postmitotic cells to enter S phase and support DNA synthesis. Productive HCMV infection also results in increased expression of c-*myc* (Boldogh *et al.,* 1990; Monick *et al.,* 1992), dihydrofolate reductase (Wade *et al.,* 1992), and thymidine kinase (Estes and Huang, 1977). In the case of dihydrofolate reductase, activation by HCMV requires E2F in combination with an immediate early viral gene product IE-72 (Margolis *et al.,* 1995). Phosphorylation of pRB has also recently been observed in HCMV-infected cells (Jault *et al.,* 1995; our unpublished studies). Collectively, these findings suggest an important role for Cdk2 in HCMV replication.

The present findings clearly demonstrate the require-

TABLE 1

Percentage of Cells Expressing Cdk2-HA and HCMV UL80.5 Antigens

Cells expressing	Experiment 1	Experiment 2	Experiment 3	Mean % (+SD)
UL80.5 antigens	52/156 33%	46/156 30%	53/150 35%	33 (± 2)
pCMVCdk2-wt-HA + UL80.5 antigens	58/148 39%	56/154 37%	53/147 36%	37 (±1) $P > 0.05$
pCMVCdk2-dn-HA + UL80.5 antigens	3/151 2%	5/161 3%	4/159 3%	2.6 (±0.6) $P < 0.0001$

Note. U-373 cells were transiently transfected with HA-tagged wild-type or dominant negative Cdk2 and subsequently infected with HCMV. The percentage of infected cells was determined by measuring expression of HCMV UL80.5 antigens. Multiple random fields were counted to accumulate about 150 total cells, about one-third of which expressed UL80.5. The percentage of cells expressing UL80.5 antigens was also determined from cells expressing HA-tagged wild-type or dominant negative Cdk2. In this case, multiple fields were counted to accumulate about 150 HA-positive cells, which were scored for expression of UL80.5. Statistical significance was estimated by Student's *t* test, comparing infected cells expressing HA to the percentage of infected cells in the cultures.

ment for Cdk2 activity in the replication of HCMV. Inhibition of Cdk2 activity by drugs or a dominant negative inhibitor blocks HCMV replication. However, these studies do not elucidate the precise mechanism by which Cdk2 participates in HCMV replication. We have hypothesized that Cdk2 activity may be required to phosphorylate the retinoblastoma tumor suppressor protein allowing for release of E2F and transcription of cellular and/or viral genes required for HCMV replication (Bresnahan *et al.,* 1996). To investigate this hypothesis further it will be necessary to determine if Cdk2 is responsible for the phosphorylation of pRb that one observes during HCMV infection and that upon phosphorylation of Rb there is a subsequent release of E2F. However, Cdk2 activity may also play another role in HCMV replication. Cdk2 activity has been reported to be involved in the initiation of cellular DNA synthesis at origins of replication (Jackson *et al.,* 1995). It is entirely possible that Cdk2 activity may also play a similar role in initiating HCMV DNA synthesis. The fact that inhibition of Cdk2 activity by chemical inhibitors blocks HCMV replication sometime after IE gene expression, but prior to initiation of viral DNA synthesis, is consistent with either hypothesis.

If cyclin E/Cdk2 activity is involved in phosphorylating Rb and releasing E2F, it may not be the only mechanism whereby the virus accomplishes release of E2F in HCMV-infected cells. Poma *et al.* (1996) have reported that one of the HCMV IE proteins (IE72) interacts with the RB-related protein p107 and relieves the transcriptional repression of an E2F-responsive promoter mediated by p107. Both p107 and pRB are proposed to undertake important roles in the regulation of cell cycle progression (Cao *et al.,* 1992; Weinberg, 1995). The data provided by Poma *et al.* (1996) and those reported here suggest that HCMV infection may utilize more than one mechanism for relieving the impediments to cell cycle progression in postmitotic cells. Furthermore, since IE72 expression is not blocked by the inhibitors of Cdk2 activity used in this study, our data suggest that the presence of IE72 in the absence of Cdk2 activity may not be sufficient for efficient HCMV replication in postmitotic cells.

Although several studies have suggested that substantial cellular DNA synthesis occurs in cells productively infected by HCMV (Jault *et al.,* 1995; Poma *et al.,* 1996; Morin *et al.,* 1996), most recent studies in which the confounding effects of serum have been avoided and the contributions of cellular and viral DNA have been evaluated carefully have observed that cellular DNA synthesis is inhibited in cells productively infected by HCMV (Bresnahan *et al.,* 1996; Lu and Shenk, 1996; Dittmer and Mocarski, 1997). The absence of an increase in the abundance or activity of cyclin A in cells productively infected by HCMV (Bresnahan *et al.,* 1996) would seem to preclude the possibility of substantial levels of cellular DNA synthesis in these infected cells. These findings suggest that HCMV has a distinctly different mechanism for circumventing the constraints placed upon viral replication in postmitotic cells from those recognized for small DNA viruses such as papovaviruses. Even though HCMV replication does not require cellular DNA synthesis, the present findings, as well as earlier studies, indicate that HCMV replication does depend on those cellular events that prepare postmitotic cells for DNA synthesis (reviewed in Albrecht *et al.,* 1989).

The sensitivity of HCMV replication to the Cdk2 inhibitors roscovitine and olomoucine is noteworthy. It has not escaped our attention that the use of the Cdk2 inhibitors roscovitine or olomoucine may provide novel drugs to help fight HCMV infections. These and other findings (Margolis *et al.,* 1995; Boldogh *et al.,* 1990; Jault *et al.,*

1995) suggest that a better understanding of the interactions of HCMV and cellular processes required for viral replication may lead to the development of more effective means to control HCMV infection. Further, elucidation of the mechanisms that provide for activation of nucleotide biosynthetic processes in postmitotic cells by HCMV may contribute to a better understanding of how cell cycle progression is constrained in G0 and how these normal constraints are subverted in pathologic circumstances.

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