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Cytomegalovirus Infection Induces High Levels of Cyclins, Phosphorylated Rb, and p53, Leading to Cell Cycle Arrest

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Human cytomegalovirus (HCMV) infection stimulates cellular DNA synthesis and causes chromosomal damage. Because such events likely affect cellular proliferation, we investigated the impact of HCMV infection on key components of the cell cycle. Early after infection, HCMV induced elevated levels of cyclin E, cyclin E-associated kinase activity, and two tumor suppressor proteins, p53 and the retinoblastoma gene product (Rb). The steady-state concentration of Rb continued to rise throughout the infection, with most of the protein remaining in the highly phosphorylated form. At early times, HCMV infection also induced cyclin B accumulation, which was associated with a significant increase in mitosis-promoting factor activity as the infection progressed. In contrast, the levels of cyclin A and cyclin A-associated kinase activity increased only at late times in the infection, and the kinetics were delayed relative to those for cyclins E and B. Analysis of the cellular DNA content in the infected cells by flow cytometry showed a progressive shift of the cells from the G_1 to the S and G_2/M phases of the cell cycle, leading to an accumulation of aneuploid cells at late times. We propose that these HCMV-mediated perturbations result in cell cycle arrest in G_2/M.

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is the leading viral cause of birth defects and poses a serious health threat to immunocompromised persons (for reviews, see references 4, 33, 51, 60, and 67). In AIDS, HCMV is a common opportunistic infection (12, 44, 45, 68, 69) and is implicated as a possible cofactor in the progression of the disease. Although HCMV infection is usually asymptomatic in the healthy individual, it is suspected that activation of latent virus may trigger cellular damage in infected organs of certain susceptible individuals. Relevant to this, it has recently been proposed that the induction and inactivation of the tumor suppressor protein p53 by HCMV in smooth muscle cells may contribute to coronary restenosis in patients following angioplasty (70).

One of the distinctive features of HCMV is that it affects many aspects of host cell metabolism, including the stimulation of cellular DNA, RNA, and protein synthesis (2, 3, 11, 17, 18, 72, 73, 75, 83). Moreover, HCMV-induced stimulation of cellular DNA synthesis is associated with chromosomal damage, premature condensation of chromosomes, metaphase abnormalities, and induction of cellular mitotic arrest (1, 3, 36, 43, 52). Because these cellular perturbations by HCMV are likely to affect the proliferative capacity of the cell and may be crucial for HCMV replication and pathogenesis in vivo, it is important to define the effects of HCMV infection on the progression of the cell cycle and the molecular basis of these effects.

Progression through the successive phases of the cell cycle is triggered by the activation of protein kinases called cyclin-dependent kinases (cdks). The activities and specificities of these kinases are determined by phosphorylation of their catalytic subunits and by their association with various inhibitors and cyclins which are differentially expressed during the cell cycle. There appear to be two major checkpoints to ensure the proper temporal order of the cycle (reviewed in references 15, 29, 55, and 63). One occurs at the G_1/S transition and controls the initiation of DNA replication, and the other occurs just prior to mitosis and cell division at the G_2/M transition.

Cyclins expressed during the G_1 phase promote the progression from G_1 to S phase and include mainly the D-type cyclins and cyclin E. The former interact with various G_1 cdks, whereas the latter associates with cdk2 and attains peak levels at the G_1/S transition (reviewed in references 55 and 63). In addition to the G_1 cyclins, two tumor suppressor proteins, the retinoblastoma gene product (Rb) and p53, play a pivotal role in the control of cell proliferation through their ability to prevent the G_1/S transition. p53 is induced as a result of cellular DNA damage and promotes G_1 arrest through activation of the expression of $p21^{CIP1/WAF1}$, an inhibitor of cyclin E-dependent kinase activity (13, 14, 25, 28, 82). Rb is underphosphorylated in G_0 and G_1 phases of the cycle, and the level of Rb phosphorylation increases as the cells move into S phase. Rb accumulates in a highly phosphorylated form until late in mitosis at anaphase, where dephosphorylation occurs (5, 6, 9, 10, 40–42). Overexpression of underphosphorylated Rb induces cell cycle arrest in G_1, and thus it has been suggested that Rb phosphorylation plays a role in the control of the G_1/S transition (24, 32). DNA viruses such as human papillomavirus, adenovirus, and simian virus 40, which depend on the host cell entering S phase for their replication, have therefore developed mechanisms to counter the inhibitory effects of these proteins on the cell cycle (reviewed in references 30, 58, and 80). Recently, it has been shown that elevated levels of p53 are induced by HCMV infection in both permissive and nonper-

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missive asynchronous cell cultures, but its effects may be counteracted by interaction with the HCMV immediate-early protein IE2 86 (48, 70). In addition, we and others have demonstrated that IE2 86 can bind to Rb (27, 66).

A second class of cyclins, cyclins A and B, regulates the S and G_2/M phases of the cycle. Cyclin A begins to be expressed near the G_1/S transition and peaks in S phase (47, 57). Cyclin A interacts with both cdk2 and cdc2 and appears to be required to ensure completion of DNA replication before the onset of mitosis (22, 54, 77, 84). Cyclin B is synthesized throughout the S and G_2 phases and associates with cdc2 to form an inactive complex, called the pre-MPF (mitosis-promoting factor). During the G_2/M transition, dephosphorylation of cdc2 in the inactive complex by specific phosphatases induces the activation of pre-MPF to MPF, which in turn promotes the onset of mitosis (46, 56, 65, 78). MPF inactivation occurs during anaphase by degradation of cyclin B through the ubiquitin pathway, allowing the exit from mitosis and cell division (23, 31, 49).

The aim of this work was to explore further the effects of HCMV on the cell cycle, with emphasis on the patterns of expression of the tumor suppressor proteins Rb and p53, cyclins, and cyclin-associated kinase activities as the infection progressed. In this paper, we demonstrate that early after infection, HCMV induced elevated levels of p53, cyclins E and B, cyclin E- and B-associated kinase activity, and highly phosphorylated Rb. In contrast, the expression of cyclin A and cyclin A-associated kinase activity was delayed, with increased levels appearing only at late times in the infection. Analysis of the DNA content by flow cytometry also showed that the infected cells progressively shifted from the G_1 to the S and G_2/M phases of the cell cycle, with an accumulation of aneuploid cells at late times. Our results suggest that HCMV infection results in cell cycle arrest in G_2/M. Possible molecular mechanisms underlying these effects and their implications for HCMV pathogenesis are discussed.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblasts were obtained and propagated in Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum (FBS) and Mito+ serum extender (Collaborative Research Incorporated). HCMV Towne was obtained from the American Type Culture Collection. Methods for cell culture and viral infection have been described elsewhere (74).

Analysis of DNA content. Human foreskin fibroblasts were growth arrested by incubation in medium containing 0.5% FBS for approximately 45 h. The cells were subsequently infected with cell supernatants containing HCMV Towne at a multiplicity of infection of 10 PFU per cell or mock infected with conditioned medium containing 10% FBS as previously described (34) except that at least an equivalent volume of fresh medium containing 10% FBS was added to the virus stock or the conditioned medium at the time of infection. At different times postinfection (p.i.), cells were harvested with Passage Ease (VEC TEC, Inc.) and washed twice in phosphate-buffered saline (PBS). The cells were counted and subsequently stored at 4°C in PBS containing 40% ethanol. The cells were then stained with propidium iodide, and the relative DNA content was determined by flow cytometric analysis as previously described (8, 76), with slight modifications (7).

Western blot (immunoblot) analysis. Following serum starvation, human foreskin fibroblasts were infected or mock infected as described above. At different times p.i. or poststimulation, the cells were harvested, pelleted, and subsequently lysed on ice for 30 min in lysis buffer containing 10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, 10 mM NaH_2PO_4, 10 mM sodium pyrophosphate, 20 mM sodium fluoride, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μM benzamidine, and 10 μg each of aprotinin, leupeptin, pepstatin A, and phenanthroline per ml. Lysates were cleared by centrifugation for 10 min at 14,000 rpm at 4°C, and equal amounts of crude cell lysates (100 μg) were subsequently separated by electrophoresis on sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels, transferred to Immobilon membranes, and probed with specific antibodies as described previously (34). Antibodies used included anti-Rb monoclonal antibody G3-245 (PharMingen), anti-p53 monoclonal antibody DO-1 (Santa Cruz Biotechnologies), anti-cyclin E polyclonal antibody sc 198 (Santa Cruz Biotechnologies), anti-cyclin B1 monoclonal antibody GNS-1 (PharMingen), and anti-cyclin A, anti-cdk2, and anti-cdc2 antisera, kindly provided by T. Hunter, M. Pagano, and J. Y. J. Wang, respectively. Corresponding proteins were detected with an enhanced chemiluminescence Western blotting detection kit (Amersham) according to the manufacturer's instructions.

Assays of cyclin-associated histone H1- and Rb-kinase activities. Cell lysates were prepared as described above, and identical amounts of protein were immunoprecipitated with agarose-coupled anti-cyclin E, A, or B antibody (Santa Cruz Biotechnologies) for 1.5 to 3 h at 4°C and then washed three times in lysis buffer. Protein samples for Western blot analysis were resolved on SDS–12.5% polyacrylamide gels, transferred to Immobilon membranes, and assayed with the appropriate antibodies as described above. Aliquots for the assays of cyclin-associated histone H1- and Rb-kinase activities were washed twice in 50 mM Tris (pH 7.4)–10 mM $MgCl_2$–1 mM dithiothreitol. The immunoprecipitated complexes were then incubated at 37°C in 15 μl of a mixture containing 20 mM Tris (pH 7.4), 7.5 mM $MgCl_2$, 20 μM ATP, 1 mM dithiothreitol, 10 μCi of [γ-^{32}P]ATP (7,000 Ci/mmol; ICN), and either histone H1 (167 μg/ml; Boehringer Mannheim) or glutathione *S*-transferase (GST)-Rb fusion protein which was obtained by expression of pGEX-Rb/Ssp1-end in bacteria and purified as described previously (26, 79) (50 μg/ml, as estimated by SDS-polyacrylamide gel electrophoresis [PAGE]). Incubations were for 15 min for determination of histone H1-kinase activity and 45 min for determination of Rb-kinase activity. The products of the reaction were separated on SDS–12.5% polyacrylamide gels and detected by autoradiography. The histone H1 or the GST-Rb bands were excised, and the amount of radioactivity incorporated was determined with a scintillation counter.

RESULTS

HCMV infection leads to cell cycle arrest. To investigate the effects of HCMV on the cell cycle, human foreskin fibroblasts were growth arrested by incubation in medium containing 0.5% FBS for approximately 45 h. They were then infected with cell supernatants containing the Towne strain of HCMV or mock infected with conditioned medium containing 10% FBS, and samples were collected at different times after infection for determination of cell number and DNA content. By 48 h poststimulation, the number of cells in the mock-infected culture had increased twofold, and by 96 h, the cells had undergone approximately two rounds of division. In contrast, there appeared to be no significant increase in cell number in the HCMV-infected cultures (data not shown).

Analysis of the relative DNA content by flow cytometry indicated that a fraction of the mock-infected cells entered S phase at approximately 24 h poststimulation (Fig. 1). In contrast, the amount of DNA synthesized in the infected cells did not begin to increase until 48 h p.i. By 72 h, most infected cells had a DNA content greater than 2n and some had a DNA content of greater than 4n. This increase in DNA content is not due to accumulation of viral DNA, as similar results were observed when viral DNA synthesis was inhibited with ganciclovir (data not shown). Thus, in the infected cell population, there was a progressive shift from the G_1 phase toward the S and G_2/M phases of the cell cycle. At 72 h poststimulation, approximately 72% of the control cells but only 9% of the infected cells were in G_1. This minor subpopulation in the infected cultures formed a distinct peak in G_1 and may represent either uninfected cells or a fraction of infected cells which were blocked at the G_1/S boundary. The observations that cell number did not significantly increase in the infected cultures, that the majority of the infected cells were located in S, G_2, and M phases of the cycle at 72 h, and that some cells had greater than 4n content of DNA at later times strongly suggested that mitosis was blocked in the infected cells. In addition, preliminary results obtained after infection of actively growing cells by the AD169 strain of HCMV showed that the majority of the HCMV-infected cells were arrested in S and G_2/M phases of the cell cycle even at 144 h p.i. (data not shown).

The patterns of expression of cyclins E, A, and B and of the tumor suppressor proteins p53 and Rb are altered by the HCMV infection. To define the molecular events underlying the above-described effects of HCMV infection on the cell

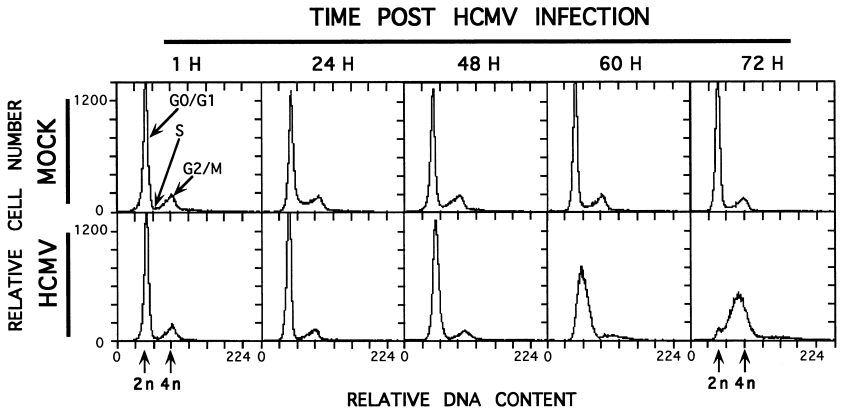

FIG. 1. HCMV infection induces cellular DNA synthesis and cell accumulation in S, G_2, and M phases. Human foreskin fibroblasts were growth arrested in 0.5% FBS and subsequently infected by HCMV or mock infected with conditioned medium containing 10% FBS as described in Materials and Methods. At different times after infection, cells were harvested, counted, and stained with propidium iodide, and the relative DNA content was determined by flow cytometric analysis.

cycle, we assayed by Western blot analysis the steady-state levels of the tumor suppressor proteins p53 and Rb (Fig. 2A), of the cyclins E, A, and B, and of cdc2 and cdk2 kinases (Fig. 2B) at different points after infection or mock infection. In the infected cells, there was induction of highly phosphorylated Rb by 4 h p.i. and a notable increase in Rb steady-state levels by 24 h p.i. (Fig. 2A). Rb protein remained in the hyperphosphorylated state throughout the infection, further indicating that the infected cells did not return to the G_1 phase. In the mock-infected cells, highly phosphorylated forms of Rb began to accumulate at 24 h poststimulation, reached a maximum at 36 h, and then declined at 48 h; a second peak of highly phosphorylated Rb was also seen at 60 h as uninfected cells progressed through the cell cycle again. Consistent with the progression of the cells through the cell cycle, the levels of cyclin A and cyclin B in the uninfected cells also increased at 36 and 60 h poststimulation (Fig. 2B). In accord with previous reports (48, 70), we also observed an increase in p53 steady-state levels in HCMV-infected cells. This increase was maximal between 4 and 12 h p.i., and although the levels decreased somewhat as the infection progressed, they remained higher in the infected cells than in the uninfected cells at all time points.

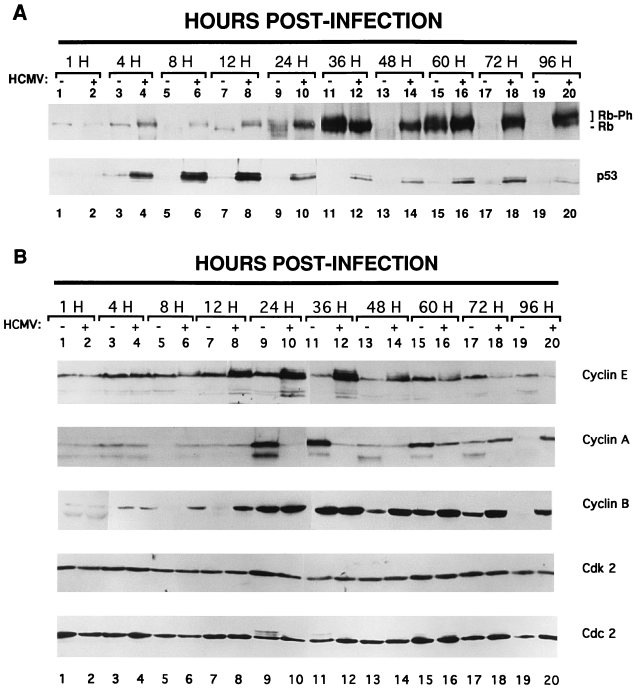

FIG. 2. Steady-state accumulation of Rb, p53, cyclins E, A, and B, and cyclin-dependent kinases cdk2 and cdc2 in HCMV-infected cells. Human foreskin fibroblasts were infected or mock infected as described in the legend to Fig. 1. At different times after infection, cells were harvested, cell lysates were prepared, and equal amounts of crude cell lysates (100 μg) were subsequently separated by gel electrophoresis and transferred to Immobilon membranes. (A) Western blot analysis with anti-Rb and anti-p53 monoclonal antibodies. (B) Western blot analyses were performed by sequential probing of different parts of the membrane with a anti-cyclin E polyclonal antibody, with an anti-cyclin B1 monoclonal antibody, and with anti-cyclin A, anti-cdk2, and anti-cdc2 antisera, kindly provided by T. Hunter, M. Pagano, and J. Y. J. Wang, respectively. Corresponding proteins were detected with enhanced chemiluminescence reagents.

As anticipated, the levels of the two protein kinases analyzed, cdk2 and cdc2, remained relatively constant during cell cycle progression in the control cells, and no apparent modification of their steady-state levels was induced by HCMV infection (Fig. 2B). In contrast, HCMV infection markedly affected the expression of the cyclins. A significant increase in the amount of cyclin E protein was observed in HCMV-infected cells between 12 and 36 h p.i., while cyclin E levels remained relatively low in uninfected cells. Preliminary experiments with the AD169 strain of HCMV indicated that steady-state levels of cyclin E mRNA also were augmented following HCMV infection, suggesting that transcriptional activation might be responsible for the increase in cyclin E levels in the infected cells (data not shown). The pattern of expression for cyclin A was opposite that observed for cyclin E. A major peak of cyclin A protein accumulation was detected in the uninfected cells between 24 and 36 h poststimulation, whereas only very low amounts of cyclin A were observed in the infected cells. The amount of cyclin A protein remained low in the infected samples until 48 h p.i. and then increased approximately threefold as the infection progressed. Accumulation of cyclin A was thus delayed in the infected cells and approached the peak level seen in the control cells only at late times. The peak of cyclin B protein accumulation in control cells was detected between 24 and 36 h poststimulation, coincident with cyclin A expression. In the infected cells, however, cyclin B did not show the same delay in kinetics of appearance as cyclin A. An increase in the steady-state amount of cyclin B was detected at early times, between 8 and 36 h p.i., and the cyclin B

level then remained roughly constant until 96 h p.i., suggesting that a balance between cyclin B protein synthesis and degradation was maintained in the infected cells.

Effect of HCMV infection on association of the cyclins with cdk2 or cdc2 kinase and their specific kinase activities. As a complement to the experiments described above, we assayed each of the cyclins for complex formation with cdk2 or cdc2 kinase and for their associated histone H1- and Rb-kinase activities. For these experiments, cell lysates were prepared at different times p.i. or poststimulation and immunoprecipitated with agarose-coupled anti-cyclin E, A, or B antibody (Fig. 3 to 5). Samples of the immunoprecipitates were then analyzed for the ability to phosphorylate exogenously added histone H1 or a GST-Rb fusion protein (Fig. 3A and B, 4A and B, and 5A and B). Following separation of the products of the reactions by SDS-PAGE and detection by autoradiography, the amount of radioactivity incorporated into the bands corresponding to histone H1 or GST-Rb was quantified. Figures 3A, 4A, and 5A show the means of incorporated radioactivity detected in two separate sets of experiments, while Fig. 3B, 4B, and 5B each show the autoradiogram of one representative experiment. Aliquots of the immunoprecipitated proteins were also assayed by Western blotting with appropriate antibodies to determine the relative amount of cyclin and cdk2 or cdc2 kinase present (Fig. 3C, 4C, and 5C).

Figure 3 shows that cyclin E-associated histone H1- and Rb-kinase activities were maximal at 24 h poststimulation in both infected and uninfected cells, but this activity was at least sevenfold greater in the HCMV-infected cells at 24 h and remained significantly higher through 96 h. Although there was good correlation between the amount of cdk2 and cyclin E protein in the complexes, this relationship was not always maintained in the case of the measured kinase activities, indicating that other levels of control might be affected by the HCMV infection. In particular, in the infected cells there was a steady increase in the specific activity of the complexes between 12 and 48 h p.i., and this activity remained elevated until 96 h p.i. despite a decrease in cyclin E protein levels. These results suggested that either the stability of the active cyclin E-cdk2 complexes was preferentially enhanced in the infected cells or there was increased stimulation of kinase activity in the complexes as the infection progressed.

We show in Fig. 4 that both cyclin A and its associated kinase activity peaked at 24 h poststimulation and then decreased to the basal level in the uninfected samples. In HCMV-infected cells, cyclin A-dependent kinase activity was initially low and then followed the same pattern of induction as cyclin A protein expression. In general, for both the infected and uninfected samples, the relative amount of cdk2 protein in the complexes correlated with the amount of cyclin A. Although we also assayed the blots with an antibody to cdc2 protein, if the protein was present in the complexes, the levels were below our limits of detection in these experiments (data not shown).

The induction of cyclin B-dependent kinase activity followed the pattern of accumulation of cyclin B protein with much earlier expression in HCMV-infected cells than in control cells (Fig. 5). In the infected cells, this activity was sustained at high levels, which were approximately 10-fold greater than in the control cells. Although the amount of cdc2 protein in the complexes remained proportional to the amount of cyclin B, it also appeared that, as noted for the cyclin E-associated kinase, the specific activity of the cyclin B-dependent kinase increased in the infected cells at late times. The activated form of MPF may thus predominate in HCMV samples while representing only a small fraction of the immunoprecipitated complexes in

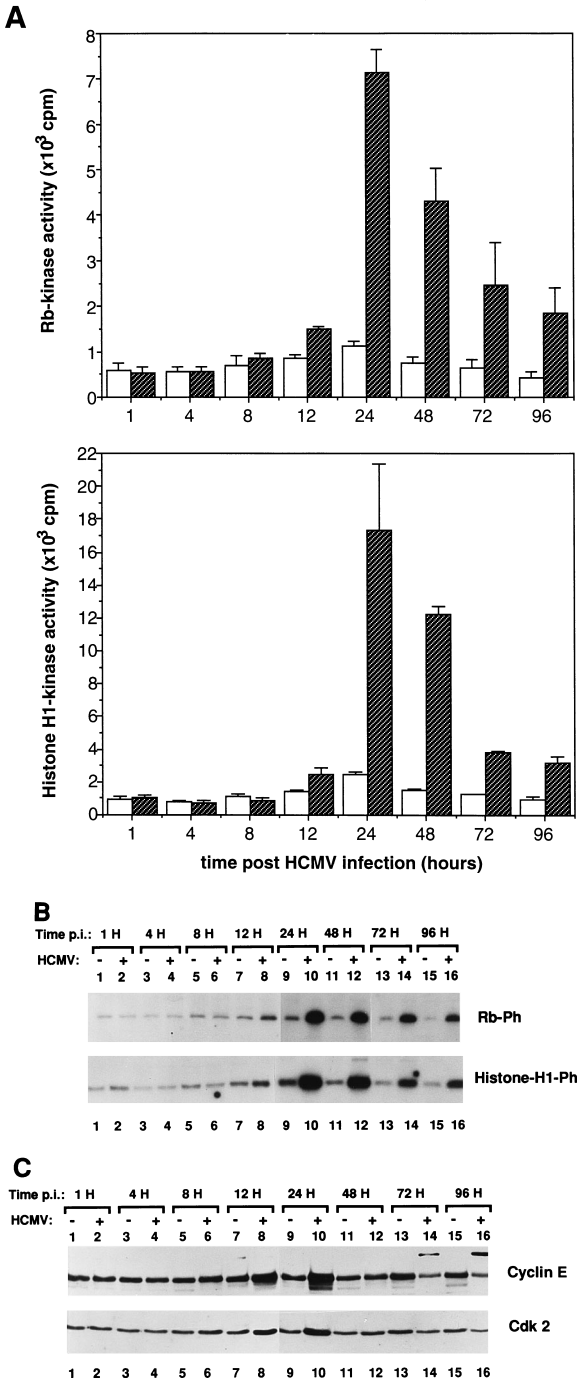

FIG. 3. Cyclin E-associated kinase activity following HCMV infection. Determination of cyclin E-associated kinase activity and analysis of the amount of cyclin E and cdk2 in the immunoprecipitated complexes were performed as described in Materials and Methods. Aliquots of immunoprecipitated complexes, corresponding to 20 and 10 μg of protein in the original lysate, were used for Rb- and histone H1-kinase activities, respectively, and aliquots corresponding to 160 μg of protein in the original lysate were used for Western blot analysis. (A) Cyclin E-associated Rb- and histone H1-kinase activities are expressed as the means of the incorporated radioactivity detected in two separate sets of experiments in mock-infected (□) or HCMV-infected (▨) samples. (B) Phosphorylation of histone H1 and of GST-Rb by cyclin E-associated kinase in one representative experiment observed after 1.5 h of autoradiography. Rb-Ph and Histone-H1-Ph, phosphorylated Rb and histone H1, respectively. (C) Western blot analysis of the amounts of cyclin E and cdk2 proteins present in the immunoprecipitated complexes.

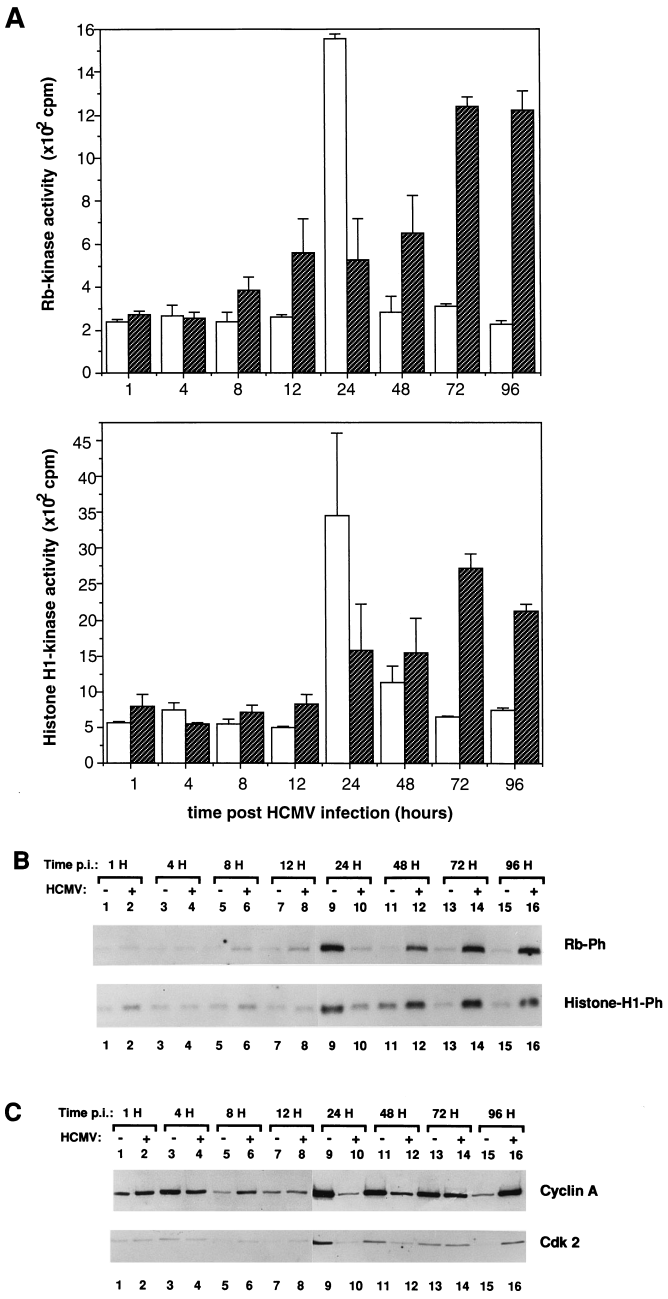

FIG. 4. Cyclin A-associated kinase activity following HCMV infection. The procedure was as described for Fig. 3 except that aliquots of the immunoprecipitated complexes corresponding to 10 μg of protein in the original lysate were used to determine Rb-kinase activity. (A) Cyclin A-associated Rb- and histone H1-kinase activities are expressed as the means of the incorporated radioactivity detected in two separate sets of experiments in mock-infected (□) or HCMV-infected (▨) samples. (B) Phosphorylation of histone H1 and of GST-Rb by cyclin A-dependent kinase in one representative experiment. Autoradiography was for 1.5 h for histone H1 and for 3 h for GST-Rb. Rb-Ph and Histone-H1-Ph, phosphorylated Rb and histone H1, respectively. (C) Western blot analysis of the amounts of cyclin A and cdk2 proteins present in the immunoprecipitated complex. No cdc2 proteins were detected in the cyclin A complexes (data not shown).

the controls. To further address this hypothesis, we analyzed the amount of cyclin B-cdc2 immunoprecipitated complexes by gel electrophoresis under conditions which favored separation of the differentially phosphorylated and activated forms of cdc2 (46, 56, 65, 78). Early after infection, a triplet band corresponding to cdc2 was detected in both infected and control cells (data not shown). From 24 to 96 h, a shift to the two faster-migrating forms was detected in infected cells, whereas the two upper forms of the cdc2 triplet bands predominated in the control cells (Fig. 5C, lower panel). This finding suggested that the increase in MPF activity detected in HCMV-infected cells resulted primarily from an early activation of cdc2 by specific dephosphorylation (46, 56, 65, 78).

DISCUSSION

The results presented here suggest that overexpression of both cyclin E and the hyperphosphorylated forms of Rb induced by HCMV infection might be the key events initiating the cascade of subsequent modifications of cyclin expression and regulation of the cell cycle. Cyclins D1 and E have been shown to be rate limiting for S-phase entry and, when overexpressed, to accelerate the G_1/S transition (35, 50, 53, 59, 61, 81). In our system, changes in cyclin D1 expression were not detected early after infection, suggesting that cyclin D1 may not play a major role, if any, in the G_1/S transition induced by HCMV. However, a decrease in cyclin D1 steady-state amount was noticeable in the infected cells at late times p.i. (data not shown). This finding further emphasizes that the majority of the infected cells do not return to G_1 phase late after infection, as would be expected with normal cycling and division. Interestingly, adenovirus, which also stimulates host cell DNA synthesis, seems to mediate its mitogenic effect through activation of cyclin E and downregulation of cyclin D1 expression (71). However, in contrast to HCMV, adenovirus does not appear to inhibit cyclin A expression and can even induce it in quiescent cells.

Although we and others (48, 70) have detected increased steady-state levels of p53 protein after HCMV infection, p53 induction did not result in cell cycle arrest at the G_1/S transition and did not interfere with HCMV induction of Rb phosphorylation or elevated cyclin E levels. Furthermore, no induction of the $p21^{CIP1/WAF1}$ inhibitor was detected in our system (data not shown). Since it has been recently reported that the HCMV immediate-early protein IE2 86 binds to p53 and inactivates its transcriptional ability (70), it is possible that this interaction is responsible for the apparent stabilization of p53 levels and its inability to induce the $p21^{CIP1/WAF1}$ inhibitor.

Several lines of evidence indicate that although cyclin E overexpression is associated with a more rapid progression through G_1 into the S phase, the lengths of the S and G_2 phases are then prolonged in a compensatory fashion, thus maintaining the overall length of the cycle. This observation has led to the idea that accumulation of factors required for DNA replication may be impaired by premature entry into S phase (53, 61, 81). In addition, depletion of cyclin A before S phase results in inhibited or delayed DNA synthesis, indicating that cyclin A is required for DNA replication (22, 54, 77, 84). Accordingly, the delay in cyclin A accumulation observed in HCMV-infected cells might be a consequence of premature entry into S phase in response to cyclin E overexpression. Cyclin A is also required for the G_2/M transition. Depending on the system involved, its depletion or absence results either in a cellular block in G_2 phase with no mitosis (39, 54) or in an activation of the cyclin B-dependent kinase MPF and premature entry into mitosis before completion of DNA replication

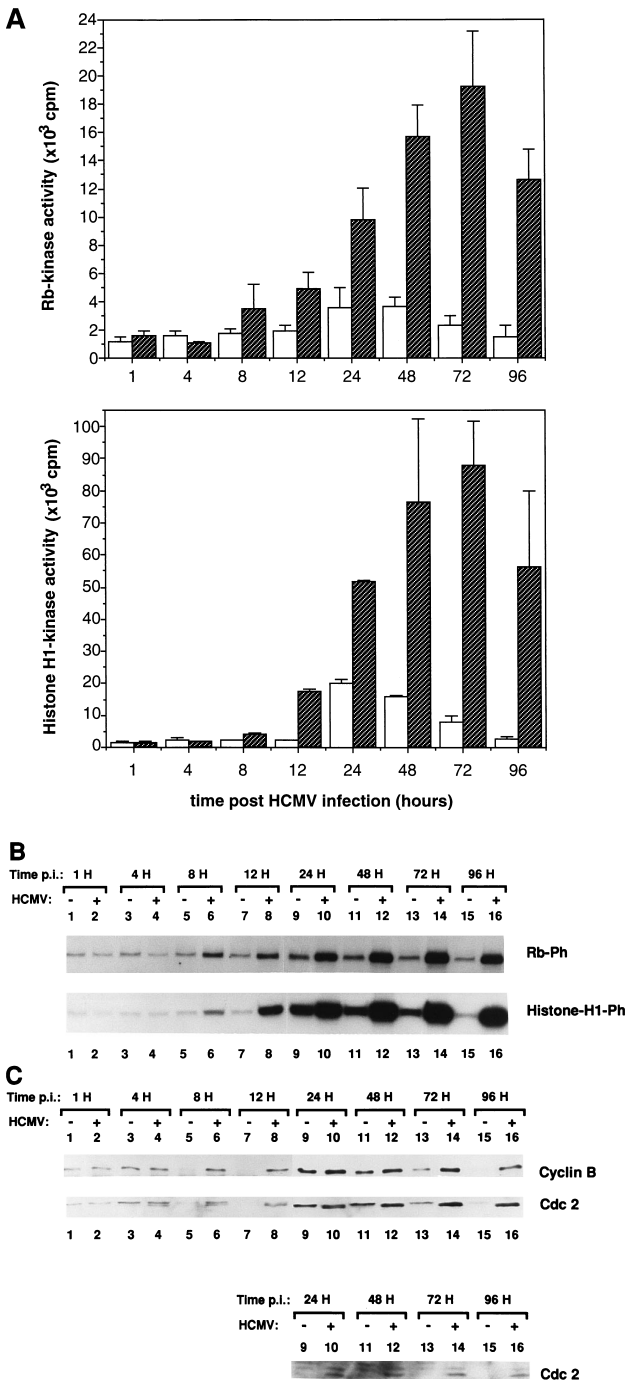

FIG. 5. Cyclin B-associated kinase activity following HCMV infection. The procedure was as described for Fig. 3 except that aliquots of the immunoprecipitated complexes corresponding to 10 μg of protein in the original lysate were used to determine Rb-kinase activity. (A) Cyclin B-dependent Rb- and histone H1-kinase activities are expressed as the means of the incorporated radioactivity detected in two separate sets of experiments in mock-infected (□) or HCMV-infected (▨) samples. (B) Phosphorylation of histone H1 and of GST-Rb by cyclin B-associated kinase in one representative experiment. Autoradiography was for 0.5 h for histone H1 and for 1.5 h for GST-Rb. Rb-Ph and Histone-H1-Ph, phosphorylated Rb and histone H1, respectively. (C) Detection of the amounts of cyclin B and cdc2 proteins present in the immunoprecipitated complexes as determined by Western blotting (top) and detection of the different forms of cdc2 present in cyclin B complexes (bottom). The complexes were subjected to electrophoresis on an SDS–7.5% polyacrylamide gel to separate the various phosphorylated forms of cdc2. Following transfer of the proteins to Immobilon membranes, cdc2 protein was detected by Western blotting as described above.

(77). It is therefore tempting to speculate that the delay in cyclin A synthesis observed in our system is responsible for the induction of MPF at early times and the premature entry into mitosis. Alternatively, HCMV infection might affect additional aspects of the numerous control systems which regulate the G_2/M transition and ensure that accurate DNA replication takes place prior to the initiation of mitosis (15, 29).

It is noteworthy that the levels of cyclin B and its associated kinase remain high in the HCMV-infected cells, suggesting that an absence of degradation through the ubiquitin pathway might contribute to the mitotic arrest in metaphase previously observed in infected cells (1, 3, 36). In accord with this notion, others have noted that the lack of degradation of cyclin B in mammalian cells or of the cyclin B homolog in yeast cells, in association with a persistent activation of cdc2, blocks mitosis at the metaphase-anaphase transition (19, 21, 23, 49, 64). Interestingly, simian virus 40 infection of monkey kidney cells results in continued stimulation of cellular DNA synthesis without mitosis, leading to an accumulation of a greater than G_2 amount of DNA (20, 37, 38). However, in this case, there is an accumulation of hypophosphorylated Rb, and the $p34^{cdc2}$ associated with cyclin B remains in its tyrosine-phosphorylated inactive form (16, 62).

Regardless of specific mechanisms, it seems likely that the induction of cyclin E overexpression and Rb phosphorylation by HCMV infection are among the key events which lead to disregulation of the cell cycle. Moreover, the disruption of the temporal order between DNA replication and MPF induction coupled with the lack of cyclin B degradation may precipitate the observed chromosome damage and mitotic arrest. The clinical consequences of such effects may include the induction of developmental abnormalities in the fetus or of coronary artery occlusion in the susceptible adult.

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J.-M.J. and F.R. contributed equally to this work.

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