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Herpes Simplex Virus Type 1 Infection Imposes a G_1/S Block in Asynchronously Growing Cells and Prevents G_1 Entry in Quiescent Cells

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Herpes simplex virus type 1 (HSV-1) infection disrupted cell cycle regulation in at least two ways. First, infection of quiescent human embryonic lung cells simultaneously with readdition of serum caused inhibition of cyclin D/cyclin-dependent kinase (CDK) 4,6-specific and cyclin E/CDK2-specific phosphorylation of the retinoblastoma protein pRb. The inhibition of cyclin D/CDK4,6 kinase activity corresponded to a loss of cyclin D1 protein and a failure of CDK4 and CDK6 to translocate to the nucleus. Failure to detect cyclin E/CDK2 kinase activity was accompanied by a loss of cyclin E protein and a failure of CDK2 to translocate to the nucleus. Levels of pocket protein p130 persisted, whereas p107 did not accumulate. As a result of these effects on cyclin kinase, G_0-infected cells failed to reenter the cell cycle. The second type of HSV-induced cell cycle dysregulation was observed in asynchronously dividing cell cultures. A rapid inhibition of preexisting cyclin E/CDK2 and cyclin A/CDK2 activities was observed in human embryonic lung cells, as well as two other human cell lines: C33 and U2OS. HSV-1 immediate-early gene expression was necessary for the inhibition of CDK2 kinase activity. Cyclin and CDK subunit protein levels, intracellular localization, and complex stability were unaffected by infection. In addition, levels of cyclin-dependent kinase inhibitors, p27 and p21, were not affected by HSV-1. Previous experiments demonstrated that in asynchronous infected cells, hypophosphorylated pRb and pocket protein–E2F complexes accumulated, and cellular DNA synthesis was rapidly inhibited. Coupled with the present results, this indicates that HSV-1 has evolved mechanisms for preventing cells in G_1 from proceeding through the restriction point and for cells in S from completing a round of DNA replication.

Key Words: herpes simplex virus type 1; retinoblastoma protein; CDk4; CDk6; CDK2; cyclin E; cyclin D; cell cycle.

INTRODUCTION

A family of cyclin-dependent kinases (CDKs) is responsible for the orderly progression of cells through the cell cycle. Cyclin D/CDK4,6 and cyclin E/CDK2 are necessary for progression through G_1 phase. Cyclin E/CDK2 activity increases during mid-G_1 phase and is thought to target functions necessary for the initiation of DNA replication (Sauer and Lehner, 1995), driving cells to late G_1 phase and across the G_1/S boundary. Both cyclin D/CDK4,6 and cyclin E/CDK2 activities are known to be rate limiting for G_1 phase progression, as overexpression of these kinases resulted in a shortened G_1 phase (Resnitsky and Reed, 1995). Cyclin A expression increases in an E2F-dependent manner in late G_1 phase (Liu *et al.,* 1998). Cyclin A/CDK2 kinase activity is rate limiting for the initiation of S phase (Resnitzky *et al.,* 1995) and is necessary for activities throughout S phase (Desdouets *et al.,* 1995). In late S phase, cyclin A/CDK2 complexes are replaced by cyclin A/CDC2 and cyclin B/CDC2 complexes. These kinases regulate the S/G_2 transition and finally carry cells into M phase.

Binding of the catalytic cyclin-dependent kinase subunit to its corresponding regulatory cyclin subunit and a subsequent activating phosphorylation event allow for the phosphorylation of target proteins. One such target protein is the E2F-inhibitory protein, pRb (Pines, 1995; Sherr, 1994, 1996). Phosphorylation of pRb is critical to the progression of cells from G_1 to S (Mittnacht, 1998). During G_0 and early G_1, pRb exists in an unphosphorylated state complexed with the E2F transcription factor. As cells progress through G_1, pRb is sequentially phosphorylated by cyclin D/CDK4,6 and cyclin E/CDK2 (Ezhevsky *et al.,* 1997; Knudsen and Wang, 1997; Lundberg and Weinberg, 1998). Once hyperphosphorylated, pRb releases E2F, resulting in the transcription of E2F-responsive genes necessary for the initiation of DNA replication in S phase. Included among these are genes encoding cyclin A, cdc2, DNA polymerase α, and thymidine kinase (Dyson, 1998).

The effects of an alphaherpesvirus, herpes simplex virus (HSV), infection on cell cycle regulation are not precisely understood. Although it is known that HSV encodes proteins essential for replication of viral DNA, at least one cell cycle-regulated function can enhance replication of a mutant virus. Specifically, a cellular function present during the transition from G_0 to late G_1 can

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complement an ICP0-deficient virus, allowing for activation of viral gene expression in the absence of the viral encoded transactivator (Cai and Schaffer, 1991). Interestingly, interactions between the viral protein ICP0 and cell cycle protein cyclin D3 have also been observed (Kawaguchi *et al.,* 1997; Van Sant *et al.,* 1999). More recent studies have suggested that wild-type (WT) HSV requires the activities of G_1-specific cellular proteins to create an environment favorable for WT viral replication (Hossain *et al.,* 1997; Schang *et al.,* 1998). In one, infection of serum-arrested CV-1 cells with HSV-2 revealed an induction of CDK2-dependent kinase activity up to 8 h postinfection (p.i.), coincident with an increase in cyclin A expression and pRb phosphorylation. However, HSV-2 did not result in elevated levels of cyclin E, and infection did not induce cells to enter S (Hossain *et al.,* 1997). In a second set of studies, the treatment of cells with drugs reported to inhibit late G_1- and S-phase cyclin kinases resulted in an inhibition of HSV-1 viral replication, viral DNA synthesis, and viral immediate-early (IE) and early (E) gene transcription (Schang *et al.,* 1998, 1999).

Although these results suggest that some G_1/S-cyclin kinase activity is necessary for efficient viral infection and replication, it has previously been shown that HSV-1 imposes a G_1/S block on infected cells. HSV-1 infection of cells synchronized in G_1 prevented progression into S and altered the formation of cellular DNA replication compartments (de Bruyn Kops and Knipe, 1988). In addition, the infection of asynchronous cell populations with HSV-1 revealed an accumulation of hypophosphorylated pRb; an increase in repressive E2F/pRb and E2F/p107 complexes, which are characteristic of G_0- and early G_1-phase cells; and a decrease in E2F-dependent reporter gene activity (Hilton *et al.,* 1995; Olgiate *et al.,* 1999). Accompanying the increase in repressive E2F–pocket protein complexes, E2F4 protein translocated to the nucleus of infected cells and was hyperphosphorylated compared with E2F4 of uninfected cells (Olgiate *et al.,* 1999). Recent investigations with viral mutants suggested a role for ICP0 in HSV-1-induced cell cycle blocks at both the G_1/S and G_2/M boundaries (Hobbs and DeLuca, 1999; Lomonte and Everett, 1999). The viral-induced inhibition of cell cycle progression across the G_1/S boundary and the induction of repressive E2F–pocket protein complexes prompted us to examine further the effects of HSV-1 infection on proteins implicated in regulation of the progression of the mammalian cell cycle. Specifically, we wanted to directly assay the effects of viral infection on the activities of cyclin kinases regulating the G_1/S transition.

We examined the effects of HSV-1 infection on the progression of cell cycle under two cell culture conditions: reentry of quiescent cells into the cell cycle by mitogenic stimulation and asynchronous growth. G_0 cells that were simultaneously infected and serum stimulated failed to reenter the cell cycle, whereas uninfected cells demonstrated markers of entry and progression through G_1 and S by 20 h after serum stimulation. In contrast, and consistent with our previous finding of an accumulation of hypophosphorylated pRb and a decrease in E2F-dependent promoter activity, total CDK2-dependent, cyclin E-associated, and cyclin A-associated kinase activities were reduced up to 80% after the infection of asynchronous cell cultures. These results indicate that HSV-1 infection of asynchronously growing cells inhibits the activities of preexisting G_1-cyclin kinases, with consequent effects on pRb phosphorylation and E2F activity, blocking cell cycle at the G_1/S boundary. In addition, HSV-1 infection can prevent the accumulation of cyclin kinase activity at the outset of G_1, preventing cell cycle reentry.

RESULTS

HSV-1 infection of quiescent cells prevents cell cycle reentry

Our primary aim was to understand the effects of HSV-1 on mammalian cell cycle, so we first characterized the effects of HSV-1 infection on progression of cells from quiescence into G_1 phase. Human embryonic lung (HEL) cells are useful for the analysis of virus effects on cell cycle because they are primary human diploid fibroblast cells that encode WT pRb and p53 proteins, are permissive for HSV-1 infection, and are easily synchronized by serum starvation. We synchronized HEL cells in G_0 by serum starvation for 3 days and then induced cell cycle progression by the readdition of serum. Cells were mock or HSV-1 infected at the 0-h time point (simultaneous with serum addition) and then harvested at various times after restimulation and assayed for DNA content by flow cytometry. Figure 1 shows the histograms generated by a representative experiment, displaying DNA content at various time points after serum stimulation (Fig. 1A) or serum stimulation and infection (Fig. 1B). Serum stimulated cells displayed a 2 N DNA content at 0 h, whereas an increase in cells with greater than 2 N DNA content first appeared at 16 h, indicating progression into S phase (Fig. 1A). By 20 and 24 h, a more pronounced increase in S phase cells was observed, and by 24 h, the appearance of cells in the G_2/M phase had occurred, as indicated by a 4 N DNA content (Fig. 1A). Cells that received HSV-1 simultaneous with serum addition maintained a 2 N DNA content at all time points after infection/stimulation (Fig. 1B). The quantitative results of this experiment are displayed in Table 1 (Experiment 1). Data from two similar experiments are included (Table 1). These data indicate that in the face of a mitogenic stimulus, virus-infected cells are unable to progress from G_0/G_1 into S phase, consistent with the observations of de Bruyn Kops and Knipe (1988) that cells synchronized in M phase by mitotic shake-off are

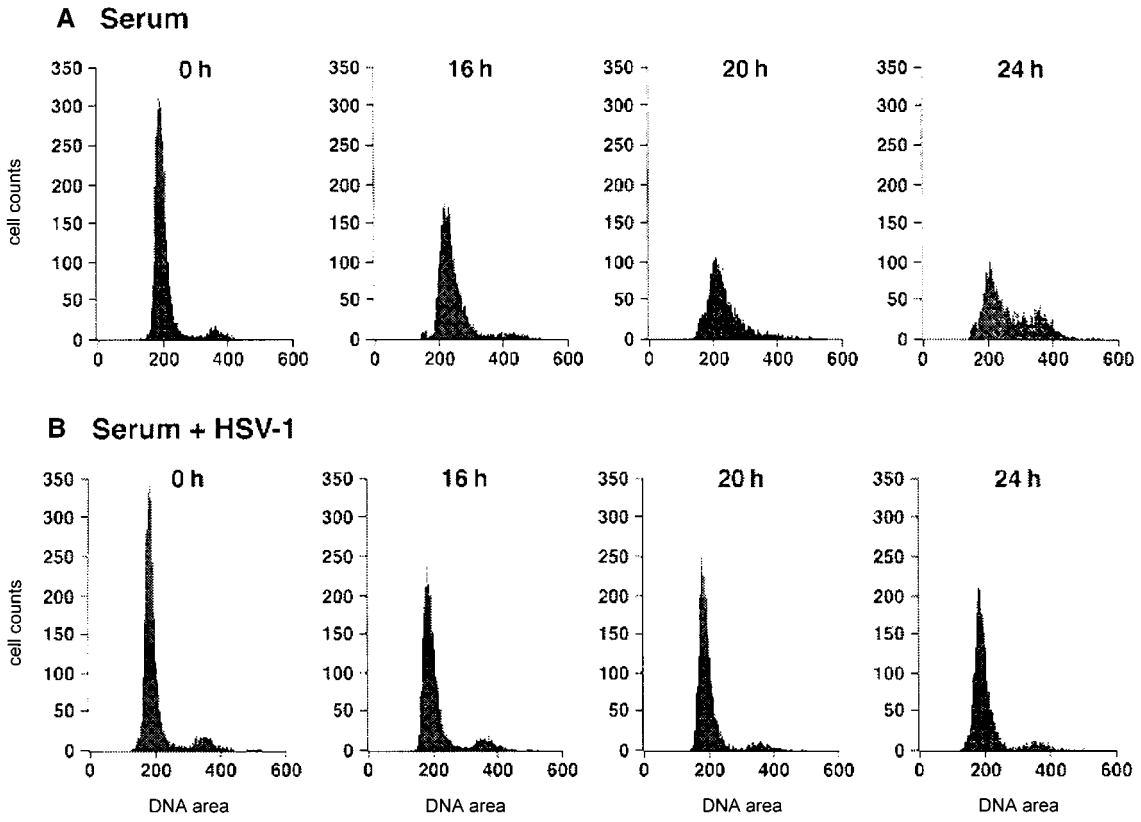

FIG. 1. HSV-1 infection of quiescent cells prevents cell cycle reentry. Cell cycle analysis of synchronized/infected cells. Subconfluent cultures of HEL cells were serum starved (0.5% FCS) for 3 days and then (A) released by replacement with medium containing 10% FCS. (B) Serum-starved cells were released by replacement with medium containing 10% FCS in the presence of HSV-1. Cellular DNA content was determined at the indicated times by FACS analysis as described in Materials and Methods. The *y* axis represents the number of cells counted, and the *x* axis represents relative DNA content.

unable to progress from G_1 to S when infected during G_1. Serum-starved cells that were HSV-1 infected without the readdition of serum displayed the same inability to progress out of G_0/G_1 phase (data not shown). Taken together, these results suggest that not only does HSV-1 not induce G_1/S progression but in fact it inhibits the

TABLE 1

Cell Cycle Profile of Serum Starved HEL Cells After Serums Stimulation or Serum Stimulation Simultaneous with HSV-1 Infection

	Experiment 1			Experiment 2			Experiment 3		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
0-h serum	88	8	4	86	3	11	92	8	0
16-h serum	86	13	1	86	4	10	83	17	0
20-h serum	72	28	0				72	28	0
24-h serum	45	36	19	40	51	9	51	37	12
30-h serum	62	24	14	34	32	34	70	25	5
0-h serum + HSV-1	87	8	5	84	3	13	92	8	0
16-h serum + HSV-1	85	9	6	81	6	13	94	4	2
20-h serum + HSV-1	87	9	4				94	4	2
24-h serum + HSV-1	87	9	4	76	11	11	95	3	2
30-h serum + HSV-1	87	10	3	81	6	13	95	3	2

Note. Subconfluent cultures of HEL cells were serum starved (0.5% FCS) for 3 days, then released by replacement with medium containing 10% FCS in the absence or presence of HSV-1. Cell DNA content and cell cycle profiles were determined as described under Materials and Methods. Experiment 1 provides cell cycle distributions as percentages for the histograms presented in Fig. 1.

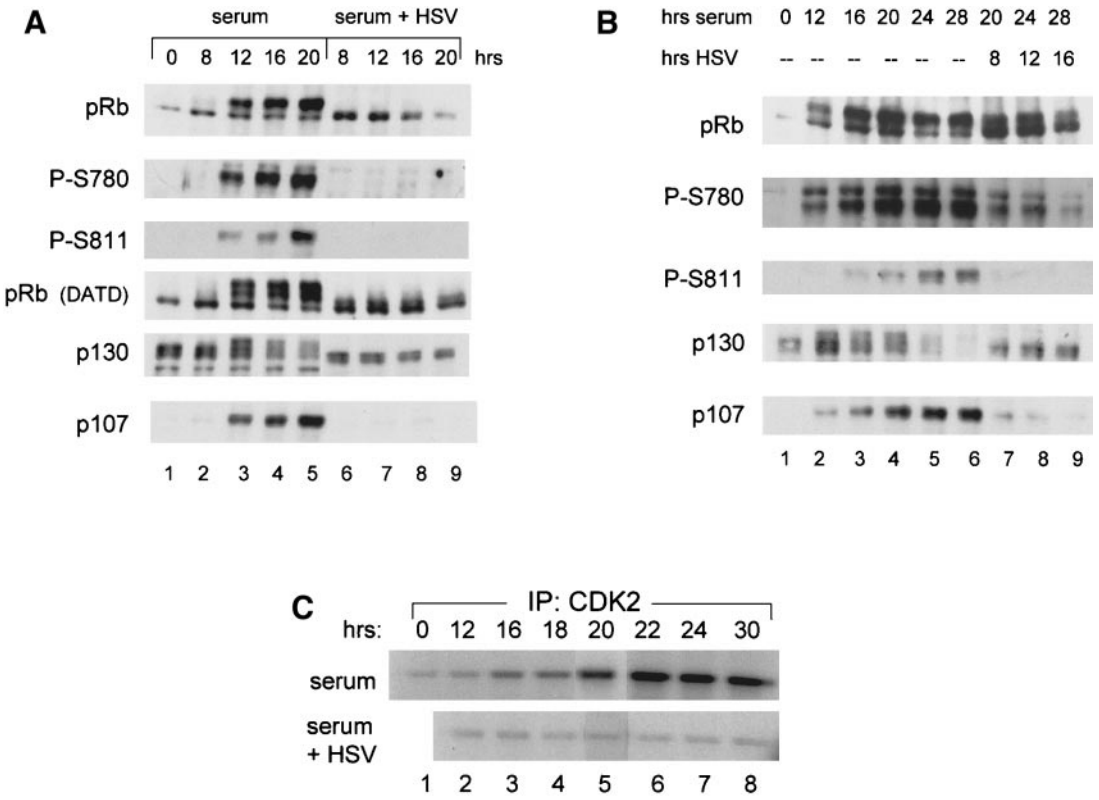

FIG. 2. HSV-1 infection of quiescent cells prevents pRb hyperphosphorylation and CDK2-dependent kinase activation. (A) Subconfluent cultures of HEL cells were synchronized by serum starvation, restimulated with serum, and mock or HSV-1 infected as described in the legend to Fig. 1. Cells were harvested at 0, 4, 8, 16, and 20 h after serum stimulation in the presence (lanes 6–9) or absence (lanes 1–5) of HSV-1. For Western blot analyses of pocket proteins, protein equivalents of nuclear lysates were separated by 6% SDS–PAGE, transferred to PVDF membrane, and probed with the antibodies indicated. pRb (DATD) indicates protein separated by DATD cross-linked 9.25% SDS–PAGE. (B) Cells were synchronized as described for A. Cells were restimulated with serum for 12 h and then mock or HSV-1 infected and harvested at the times indicated. Western blot analyses of pocket proteins were performed as described for A. (C) CDK2 was immunoprecipitated with polyclonal antibody (M2: sc-163) from whole-cell lysates obtained from synchronized HEL cells as described in the legend to Fig. 1. Kinase activity was determined using histone H1 as substrate as described in Materials and Methods.

ability of mitogenic stimulation to induce such progression.

Hypophosphorylated pRb accumulates after HSV-1 infection

The assessment of the phosphorylation state of pRb allows for an additional and specific characterization of cell cycle status. As reviewed earlier, pRb exists as an unphosphorylated form in G_0 cells, becomes hypophosphorylated during mid-G_1, and is subsequently hyperphosphorylated by late G_1. These three forms of pRb are distinguishable by their different mobilities on a polyacrylamide gel, presumably due to the total amount of phosphorylated serine (and threonine) residues. The more highly phosphorylated forms of pRb have progressively slower mobilities in SDS–PAGE.

We first assayed the effects of infection on the ability of a synchronized population of cells to generate hyperphosphorylated pRb after mitogenic stimulation. Soluble cell lysates collected from cells analyzed in the experiment described in Fig. 1 were examined by Western blot analyses for the levels and relative mobility of pRb. Uninfected cells, at 12, 16, and 20 h after serum stimulation, displayed both unphosphorylated and hyperphosphorylated forms of pRb (Fig. 2A, pRb panel, lanes 3–5). Cells stimulated with serum in the presence of HSV-1 infection failed to induce hyperphosphorylated pRb even as late as 20 h after stimulation and infection (Fig. 2A, pRb panel, lanes 6–9). Interestingly, analyses of pRb on a DATD cross-linked gel resulted in the resolution of four distinct forms of pRb (Fig. 2A, pRb DATD panel; also see Song *et al.,* 2000) as opposed to the two forms apparent on the bisacrylamide cross-linked gels (Fig. 2A, pRb panel). The DATD gel revealed unphosphorylated pRb in all lanes and hypophosphorylated and hyperphosphorylated pRb in serum-stimulated cells (Fig. 2A, pRb DATD panel, lanes 1–5). In addition, we saw a virus-specific hypophosphorylated pRb with a faster mobility than the hypophosphorylated pRb found in serum-stimulated

cells (Fig. 2A, pRb DATD panel, lanes 6–9; also see Song *et al.,* 2000).

As discussed earlier, pRb phosphorylation and the release and activation of the E2F transcription factor are dependent on the activity of at least two cyclin kinases: cyclin D/CDK4 and cyclin E/CDK2. The phosphorylation of pRb by these two kinases can be distinguished, therefore, by antibodies that recognize specific phosphorylated residues targeted by cyclin D/CDK4 or cyclin E/CDK2. pRb residue Ser780 is preferentially phosphorylated by cyclin D/CDK4 (Kitagawa *et al.,* 1996), and pRb residue Ser811 is preferentially phosphorylated by cyclin E/CDK2 (Connell-Crowley *et al.,* 1997). We examined the cyclin D/CDK4-specific and cyclin E/CDK2-specific phosphorylation of pRb in mock- or HSV-1-infected cells using two such phospho-specific antibodies. Both CDK4-specific (S780) and CDK2-specific phosphorylation (S811) of pRb appeared by 12 h after serum stimulation (Fig. 2A, lane 3). CDK4-specific phosphorylation was abundant at 12 h and increased out to 20 h (Fig. 2A, lanes 3–5), whereas CDK2-specfic phosphorylation appeared at low levels at 12 and 16 h and was more abundant at 20 h (Fig. 2A, lanes 3–5). Again, phospho-specific pRb antibodies failed to detect any CDK4-specific or CDK2-specific pRb phosphorylation in HSV-1-infected cells (Fig. 2A, lanes 6–9). The appearance of high levels of CDK2-dependent pRb phosphorylation in mock-infected cells corresponded to a time when the proportion of cells in S phase was increasing (Fig. 1A, Table 1). These phospho-pRb antibody analyses suggest that both G_1-cyclin kinase activities, cyclin D/CDK4 and cyclin E/CDK2, are inhibited in HSV-1-infected cells. An alternative conclusion is that activities of phosphatases that normally dephosphorylate these residues are increased after HSV-1 infection.

To investigate the possibility of an HSV-induced increase in phosphatase activities directed toward pRb, we examined the effects of HSV-1 infection on synchronized cells that had been allowed to progress sufficiently into late G_1 phase to allow for the presence of some hyperphosphorylated pRb at the time of infection. If an increase in phosphatase activity was responsible for the failure to detect hyperphosphorylated pRb after HSV-1 infection of cells synchronized in G_0, then we would expect to see a loss of hyperphosphorylated pRb after HSV-1 infection of cells already possessing a population of phosphorylated pRb. HEL cells were synchronized by serum starvation, released into medium containing 10% FCS, and infected after 12 h of mitogenic stimulation. Western blot analyses of pRb protein revealed that at the 12-h time point, approximately 50% of pRb had the hyperphosphorylated mobility (Fig. 2B, pRb panel, lane 2). With additional incubation in serum alone, the proportion of hyperphosphorylated pRb increased (Fig. 2B, lanes 2–6). After the addition of HSV-1 at 12 h after serum addition, we saw that the amount of hyperphosphorylated pRb present at 8 and 12 h p.i. (20 and 24 h of serum) reflected that generated by the 12-h time point (Fig. 2B, compare lanes 7 and 8 with lane 2), ruling out the possibility of virus-induced phosphatase activity targeted toward pRb. At 16 h p.i. (28 h of serum), we observed a general decrease in the amount of pRb, without effects on the proportion of phosphorylated and unphosphorylated forms (Fig. 2B, lane 9). Our data indicate that in synchronized cell populations, HSV-1 infection prevented the accumulation of hyperphosphorylated forms of pRb reflecting a disruption in G_1/S progression and inhibition of the activities of the G_1-cyclin kinases, cyclin D/CDK4 and cyclin E/CDK2.

HSV-1 prevents p130 hyperphosphorylation and p107 accumulation in synchronized cells

Pocket proteins p130 and p107 are members of the pRb protein family, and like pRb, they are important for cell cycle progression as they target and repress the E2F transcription factors. During quiescence, p130/E2F4 complexes prevail, but as cells cycle into late G_1 and approach the G_1/S boundary, p107 and pRb replace p130 in E2F4 complexes (Moberg *et al.,* 1996). Also like the pRb protein, p130 and p107 are regulated by the activities of G_1-cyclin kinases (Beijersbergen *et al.,* 1995). Having shown that infection caused a block in cell cycle progression of cells synchronized in G_0 and inhibition of pRb phosphorylation, we next investigated the effects of HSV infection on the abundance and phosphorylation status of p130 and p107.

HEL cells were synchronized as described earlier and stimulated to reenter the cell cycle by the addition of 10% FCS in the presence or absence of HSV-1. Western blot analyses of cells harvested at various times after stimulation/infection revealed the following. First, p130 was readily detectable in the nucleus of G_0 cells (Fig. 2A, lane 1). As cells progressed through cell cycle, p130 became hyperphosphorylated and protein levels decreased (Fig. 2A, lanes 1–5), which is consistent with earlier analyses in which p130 became hyperphosphorylated and degraded as cells approached the G_1/S boundary (Smith *et al.,* 1996). Infection of cells in G_0, simultaneous with serum addition, prevented p130 hyperphosphorylation, and the level of nuclear p130 protein remained unchanged (Fig. 2A, lanes 6–9). When cells were allowed to progress into late G_1 phase and then infected at 12 h after serum stimulation, hyperphosphorylation and degradation of p130 were still prevented (Fig. 2B, compare lane 2 with lanes 7–9).

Second, in serum-stimulated cells, p107 was not detectable in nuclei until late G_1 (12 h after serum addition), and levels increased steadily thereafter (Fig. 2A, lanes 1–5). Infection with HSV-1 at G_0 prevented the accumulation of p107, even after 20 h of serum stimulation (Fig.

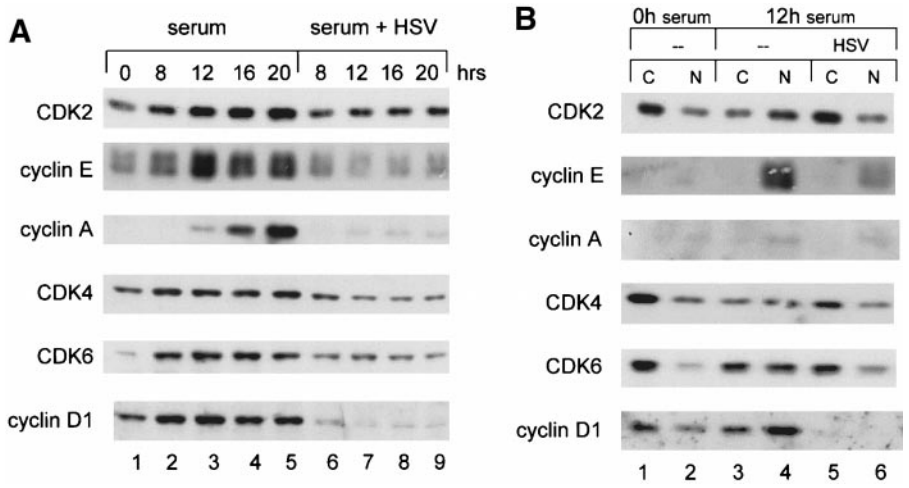

FIG. 3. Western blot analyses of CDK and cyclin proteins. (A) Protein equivalents of nuclear lysates or (B) cell equivalents of cytoplasmic and nuclear lysates (prepared as described for Fig. 2A) were separated by 15% DATD cross-linked SDS–PAGE, transferred to PVDF membrane, and probed for the indicated proteins.

2A, lanes 6–9). Cells infected after 12 h of serum stimulation contained a low level of p107; however, as infection progressed, the level of p107 declined (Fig. 2B, lanes 7–9). Thus HSV-1 infection prevents the hyperphosphorylation and degradation of p130, as well as the late G_1 phase accumulation of p107. Both of these results support the observation that HSV-1 infection prevented cell cycle reentry of cell populations that were infected during G_0.

HSV-1 infection of quiescent cells prevents activation of G_1-cyclin kinases

Results of FACS analysis and pRb Western blots indicated that HSV-1-infected quiescent cells did not progress through G_1 and across the G_1/S boundary. These data, especially the reduction in cyclin D-specific and cyclin E-specific pRb phosphorylation, suggested that the activation of the G_1-cyclin kinases was inhibited by HSV-1. We therefore wanted to corroborate the results of our phospho-pRb Western blots, suggesting inhibition of both G_1-cyclin kinases, with direct analysis of CDK2 kinase activity by immunocomplex kinase assays.

HEL cells were synchronized by serum starvation and then released by the readdition of serum in the presence or absence of virus. Cells were harvested at various times after stimulation/infection. CDK2 was immunoprecipitated out of equal amounts of protein derived from whole-cell lysates, and its relative kinase activity was determined by *in vitro* kinase assay using histone H1 as substrate. In the absence of virus, CDK2 kinase activity was apparent at low levels at 16 h poststimulation (3-fold induction), and this activity peaked at 22 and 24 h poststimulation (13- and 12-fold induction) (Fig. 2C, lanes 3, 6, and 7). These kinetics of CDK2 kinase activity are concurrent with the observed progression of cells through S phase (24 h) and into G_2 phase (30 h) (Fig. 1A) and the hyperphosphorylation of pRb (Fig. 2A, lanes 3–5). In G_0 cells, CDK2 kinase activity was low and did not increase after mitogenic stimulation in the presence of HSV-1 infection even as late as 30 h after serum stimulation (Fig. 2C, lanes 1–8).

The phospho-pRb Western blots and cyclin-dependent kinase assays indicated that HSV-1 infection of G_0 cells prevented the activation of both G_1-cyclin kinases, cyclin D/CDK4 and cyclin E/CDK2. An examination of the levels of relevant proteins revealed that the kinase activity inhibition was in large part due to a failure of G_0-infected cells to generate G_1-cyclin kinase complexes. We first examined the nuclear levels of CDK4, CDK6 and cyclin D1 subunits by Western blot analyses of synchronized HEL cells that were mock or HSV-1 infected at G_0 phase. All three subunits, but especially cyclin D1, were found to decrease, relative to mock-infected cells, in the presence of HSV-1 infection (Fig. 3A, compare lanes 1–5 with lanes 6–9). An analysis of cell equivalent amounts of nuclear and cytoplasmic extracts revealed that in uninfected quiescent cells, all three subunits were largely cytoplasmic, and on serum stimulation translocated to the nucleus (Fig. 3B, lanes 1–4). In contrast, CDK4 and CDK6 failed to localize to the nucleus (Fig. 3B, compare lanes 3 and 4 with lanes 5 and 6), and cyclin D1 decreased to undetectable levels in both the cytoplasm and nucleus of HSV-1-infected cells (Fig. 3B, lanes 5 and 6). The inhibition of cyclin D/CDK4,6 kinase activity observed in HSV-1-infected cells (Fig. 2A), therefore, was likely caused by a decrease in the amount of cyclin D/CDK4,6 kinase complex present in those cells (Figs. 3A and 3B) due to a failure of CDK4 and CDK6 to localize to the nucleus and a general loss of cyclin D1 protein.

We next asked whether the cyclin E/CDK2 activity was

down-regulated in the same manner by examining the nuclear levels of CDK2 and cyclin E in mock- or HSV-1-infected, synchronized HEL cells. CDK2 was present in G_0 cells and increased in abundance with mitogenic stimulation (Fig. 3A, lanes 1–5). When virus was added concurrently with serum, less nuclear CDK2 accumulated (Fig. 3A, lanes 6–9). An analysis of both cytoplasmic and nuclear fractions of mock- or G_0-infected HEL cells revealed that although serum stimulation resulted in the nuclear translocation of CDK2 (Fig. 3B, lanes 1–4), CDK2 failed to translocate to the nucleus in HSV-1-infected cells (Fig. 3B, lanes 5 and 6). Low levels of nuclear cyclin E protein were present in G_0 cells, peaked at 12 h after mitogenic stimulation, and then declined as cells entered S phase (Fig. 3, lanes 1–5, and Fig. 1A). In addition, accumulation of nuclear cyclin A began at 12 h and continued through 20 h of serum, consistent with its role in regulation of S phase CDK2 activity (Fig. 3A, lanes 1–5). After virus infection, nuclear levels of both cyclin E and cyclin A did not rise with serum stimulation (Fig. 3A, lanes 6–9). An analysis of both cytoplasmic and nuclear fractions revealed that the decrease in cyclin E and cyclin A in infected cells was due to a failure to up-regulate these proteins, rather than a disruption in cellular localization (Fig. 3B, compare lanes 3 and 4 with lanes 5 and 6). It appears that two effects of HSV-1 infection contributed to the resulting inhibition of cyclin E/CDK2 kinase activity in G_0 infected cells. First, the catalytic subunit of the kinase, CDK2, failed to translocate to the nucleus where the regulatory (cyclin) subunit resides. Second, HSV-1 infection prevented the up-regulation of the cyclin E component of the kinase, probably due to the failure of G_0 infected cells to phosphorylate pRb and release the E2F transcription factor, which is involved in the cyclin E positive feedback loop. Hence, the inhibition of these two events resulted in less kinase complex forming in the nucleus of virus-infected cells.

Infection of asynchronous cells results in a loss of hyperphosphorylated pRb

Our experiments to this point indicated that HSV-1 infection of quiescent cell populations inhibited cell cycle reentry as marked by the failure to observe events indicative of G_1 phase and G_1/S progression. What effect, then, would HSV-1 infection have on cycling cells expressing cyclin kinase activities? We endeavored to characterize the effects of infection on asynchronously cycling populations of cells to answer this question.

We demonstrated previously that although pRb found in the nucleus of asynchronous cultures of U2OS cells existed in a predominantly hyperphosphorylated state, infection of cells with HSV-1 resulted in a shift to predominantly hypophosphorylated and unphosphorylated pRb (Olgiate *et al.,* 1999). U2OS cells express WT pRb and p53 proteins. Although it has been shown that these cells encode an ICP0-complementing function (Yao and Schaffer, 1995), this should not obscure observations of the effects of WT HSV-1 infection. The state of pRb phosphorylation in nuclear extracts derived from asynchronous cultures of U2OS cells was compared with that of whole-cell extracts from synchronized HEL cells that were harvested at 0 h post-serum stimulation (G_0 phase, unphosphorylated pRb) and 18 h post-serum stimulation (S phase, hyperphosphorylated pRb) (Fig. 4A, lanes 1 and 2). Analyses of Western blots with phospho-specific pRb antibodies (as described earlier) confirmed that both the cyclin D/CDK4 (S780)- and the cyclin E/CDK2 (S811)-dependent phosphorylation of pRb was inhibited or reduced after HSV-1 infection of asynchronous U2OS cells (Fig. 4A). Quantification of band intensity by laser densitometry revealed that the pRb Ser780 phosphorylation of the infected sample (Fig. 4A, lane 4) was reduced to 33% of the phosphorylation of the mock-infected sample (lane 3), whereas the pRb Ser811 phosphorylation of the infected sample (Fig. 4A, lane 4) was reduced to 23% of the phosphorylation of the mock-infected sample (lane 3). These results are significant compared with the fact that the total amount of nuclear pRb protein remained unchanged between the mock-infected and the infected cell extracts (data not shown, see Olgiate *et al.,* 1999). These results further indicate that in asynchronously growing cell populations, HSV-1 infection resulted in a net loss of pRb phosphorylation, representing a loss in both cyclin D/CDK4 and cyclin E/CDK2 kinase activities. The cell cycle perturbation suggested by the decrease in the proportion of cells containing hyperphosphorylated pRb after HSV-1 infection was reflected in a disruption in the cell cycle distribution of the culture. FACS analysis of asynchronously growing U2OS and HEL cells treated with phosphonoacetic acid, an inhibitor of the viral DNA polymerase, revealed that HSV-1 infection resulted in an increase in the proportion of cells in G_1, a decrease in the proportion of cells in G_2, and no change in the proportion of cells in S compared with uninfected, phosphonoacetic acid-treated cells (data not shown).

HSV-1 infection results in the rapid inhibition of preexisting cyclin E/CDK2 and cyclin A/CDK2 kinase activities

We next characterized the effects of virus on G_1-cyclin kinase activity preexisting in the asynchronous population of cells. Immunoprecipitations for CDK2 were performed on protein equivalent aliquots of whole-cell extracts derived from mock-infected or HSV-1-infected C33, U2OS, and HEL cells. The level of CDK2 kinase activity was assayed using histone H1 as substrate. In all three cell types, we observed a reduction in CDK2 kinase activity after 8 h of HSV-1 infection (Fig. 4B). The analysis of the human cervical carcinoma cell line, C33, was added in these assays to extend our previous analyses

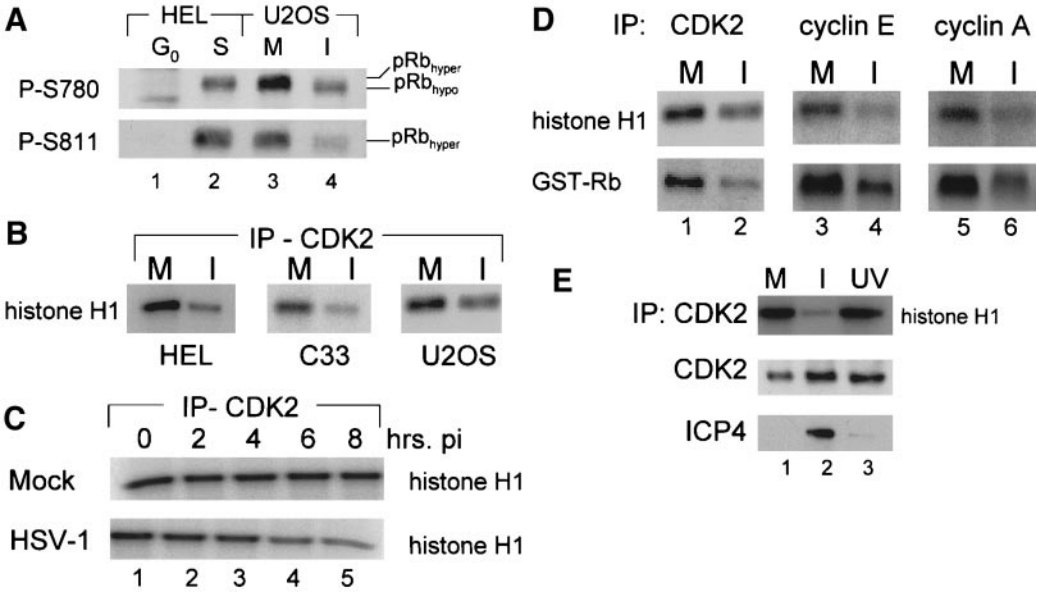

FIG. 4. Decrease in hyperphosphorylated pRb- and CDK2-dependent kinase activities in asynchronously growing cell cultures infected with HSV-1. (A) Western blot analyses of nuclear pRb. Asynchronously growing U2OS cells were mock (M) or HSV-1 (I) infected for 8 h. Protein equivalents of nuclear lysates were separated by 6% SDS–PAGE, transferred to PVDF membrane, and probed for phospho-specific pRb as indicated. Whole-cell extracts from synchronized HEL cells harvested in G_0 phase (serum starved, lane 1) or S phase (18h after serum stimulation, lane 2) served as references for unphosphorylated and hyperphosphorylated pRb mobility. (B) Immunocomplex kinase assay. CDK2 was immunoprecipitated from whole-cell lysates derived from asynchronous HEL, C33, and U2OS cells that were mock (M) or HSV-1 (I) infected for 8 h. Kinase activity was assayed as described in Materials and Methods using histone H1 as substrate. (C) U2OS cells were mock (M) or HSV-1 (I) infected and harvested at 2, 4, 6, and 8 h p.i. CDK2 was immunoprecipitated from whole-cell extracts, and kinase activity was determined using histone H1 as substrate. (D) CDK2 (lanes 1 and 2), cyclin E (lanes 3 and 4), or cyclin A (lanes 5 and 6) was immunoprecipitated from whole-cell lysates derived from asynchronous U2OS cells harvested 8 h after mock (M) or HSV-1 (I) infection. Kinase activity was determined using both histone H1 and GST-Rb as substrate. (E) Asynchronously growing U2OS cells were mock (M), HSV-1 (I), or UV-irradiated HSV-1 (UV) infected for 8 h. CDK2 was immunoprecipitated from protein equivalent amounts of whole-cell lysates, and kinase activity was determined using histone H1 as substrate (top panel). As controls for levels of CDK2 and for virus inactivation by UV-irradiation, Western blot analyses were performed. Equal protein amounts of whole-cell lysate were separated by SDS–PAGE, transferred to PVDF membrane, and probed for CDK2 (middle panel) and ICP4 (bottom panel).

of the effects of HSV-1 on cell cycle activities in this cell line (Hilton *et al.,* 1995). The use of C33 cells additionally provides an opportunity to examine the effects of HSV-1 infection on a cell type that encodes mutant forms of pRb and p53. Kinase assays of immunoprecipitates in the presence of competing CDK2 peptide, and on samples with no antibody added, resulted in no detectable kinase activity, confirming the specificity of our antibody for CDK2 kinase activity alone and the absence of nonspecific activity precipitating with the agarose beads (data not shown).

To determine the kinetics of this decline in CDK2 kinase activity after infection, asynchronously growing U2OS cells were infected, and cultures were harvested at 2, 4, 6, and 8 h after infection. Again, total CDK2 kinase activity from mock or infected cell lysates was assayed using histone H1 as substrate. As early as 2 h p.i., we observed a reduction in kinase activity, and by 8 h p.i., the kinase activity was reduced 80% compared with that of mock-infected cells (Fig. 4C, lane 5). Over the same time period, levels of cyclin E and CDK2 protein remained unchanged (see later).

To more fully characterize the effects of HSV-1 infection on CDK2 kinase activity, we examined total CDK2, cyclin E-associated, and cyclin-A associated kinase activity immunoprecipitated from U2OS whole-cell lysates on both histone H1 and the more relevant pRb substrate. Both cyclin E/CDK2 and cyclin A/CDK2 were dramatically reduced after infection as assayed (Fig. 4D), suggesting that HSV-1 may be targeting the CDK subunit specifically, thus affecting both the G_1-phase and S-phase forms of the kinase. Alternatively, virus-induced inhibition of the G_1 kinase may be responsible for the inhibition of the S-phase form, as cyclin A is dependent on cyclin E/CDK2 kinase activities for its E2F-dependent up-regulation (Liu *et al.,* 1998).

G_1-cyclin kinase inhibition in asynchronous cells is dependent on HSV-1 gene expression

To determine whether viral gene expression was necessary for the HSV-1-induced inhibition of CDK2 kinase activities, we used UV-irradiated virus to infect cells and assay for CDK2 kinase activity. UV-irradiated HSV-1 can bind to cell surface receptors and enter the cell but is unable to express viral genes. Asynchronously growing

cultures of U2OS cells were either mock-infected, infected with WT HSV-1, or infected with UV-irradiated HSV-1. Cells were harvested after 8 h of infection, and equal amounts of protein derived from whole-cell lysates were evaluated for CDK2 kinase activity. Infection with UV-irradiated virus failed to reduce CDK2 kinase activity as seen in cells infected with WT HSV-1 and maintained levels of CDK2 kinase activity comparable to that of the mock-infected cells (Fig. 4E, compare lanes 1 and 3).

Western blot analyses were performed on aliquots of whole-cell extracts. The blots were then probed for IE viral protein ICP4 to confirm that UV-irradiated virus was impaired in viral gene expression (Fig. 4E). Indeed, only minimal amounts of ICP4 were detected in cells infected with the UV-irradiated virus. This residual amount of viral protein synthesis may have been due to a low level of replication-competent virus that was not inactivated. Alternatively, the low level of ICP4 detected may represent virion-associated protein (Yao and Courtney, 1989). The levels of CDK2 protein were assayed to confirm that the effects on CDK2 kinase activity were not due to a loss of CDK2 protein or unequal protein loading (Fig. 4E). These results suggest that the virus-induced inhibition of CDK2 kinase activity requires the expression of one or more viral genes and that a virion protein or proteins alone are not sufficient to impose the block to G_1-cyclin kinase activity.

HSV-1 infection of asynchronous cells does not disrupt cyclin kinase complexes

Having established that preexisting G_1-cyclin kinase activity was inhibited in asynchronous cells infected with HSV-1, we wanted to understand by what mechanism kinase inhibition was occurring. Because one major determinant of CDK activity is the availability of cyclin and CDK subunits in the nucleus where the kinase complex assembles, we considered whether HSV-1 may be affecting the levels of cyclin or CDK subunits, the intracellular localization of the subunits, or the stability of the cyclin–CDK complexes.

We examined the levels of CDK2, cyclin E, and cyclin A proteins in mock- or HSV-1-infected, asynchronously growing U2OS and C33 cells. Although CDK2 kinase activity decreased 80% by 8 h p.i. (Fig. 4B), CDK2 and cyclin protein levels remained intact (Fig. 5A). These results suggested that unlike the case for G_0-infected cells, the inhibition of CDK2 kinase activity was not due to the limited availability of cyclin or CDK subunits. In addition, we determined whether the cellular localization of the cyclin and CDK2 subunits was altered after infection. Asynchronously growing C33 cells were harvested and then fractionated. The cellular localization of the cyclin and CDK2 subunits was examined by Western blot analyses of cytoplasmic and nuclear fractions and found to be unchanged after infection (Fig. 5B).

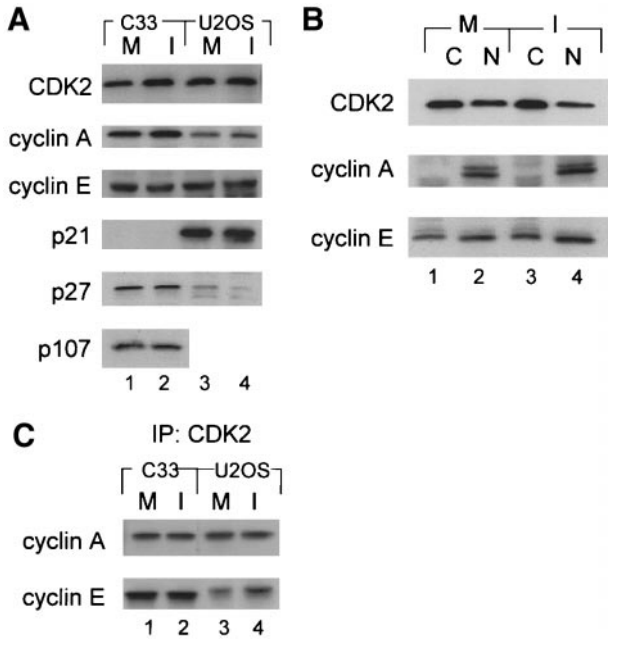

FIG. 5. HSV-1 infection of asynchronous cells does not disrupt cyclin kinase complexes or induce CKIs. Asynchronous subconfluent cultures of C33 and U2OS cells were mock (M) or HSV-1 (I) infected and harvested at 8 h p.i. (A) Western blot analyses of CDK, cyclin, and CKI proteins. Protein equivalent amounts of whole-cell extracts were separated by SDS–PAGE, transferred to PVDF membrane, and probed for the indicated proteins. (B) Cyclin–CDK complex stability. Cyclin–CDK complexes were immunoprecipitated from protein equivalent amounts of whole-cell extracts. Immunocomplexes were separated by SDS–PAGE and transferred to PVDF membrane. Cyclin A–CDK2 and cyclin E–CDK2 complexes were detected by probing for the indicated cyclin proteins. (C) Intracellular localization of cyclin–CDK subunits in C33 cells. Cytoplasmic (C) and nuclear (N) fractions were made from mock and infected cell lysates of asynchronous C33 cells. Fractionated extracts were separated by SDS–PAGE, transferred to PVDF membrane, and probed for the indicated proteins.

In addition, the decrease in CDK2 kinase activity may have resulted from a decrease in cyclin–CDK complexes. To assess cyclin–CDK complex stability, we immunoprecipitated CDK2 from whole-cell lysates of mock- or HSV-1-infected U2OS cells, performed Western blot analyses, and then probed the blots for cyclin E or cyclin A, as indicated. The coimmunoprecipitation analyses showed that the levels of cyclin E and cyclin A associated with CDK2 did not change after HSV-1 infection (Fig. 5C). This result indicates that the virus had not altered the stability of the cyclin–CDK2 complexes or prevented the assembly of those complexes.

Conversely, Western blot analyses of whole-cell lysates derived from asynchronously growing C33 and U2OS cells showed that the levels of cyclin D1 decline in HSV-1-infected cells compared with that of mock-infected cells (data not shown.) This suggests that the loss of cyclin D1/CDK4-specific phosphorylation of pRb (Fig. 4A) after HSV-1 infection of asynchronous U2OS cells was due to a loss of cyclin D1/CDK4,6 kinase complexes.

CDK inhibitor levels in HSV-1-infected cells

Because the activity of cyclin-dependent kinases can be negatively regulated by direct association with CDK-inhibitors (CKIs), we examined the level of relevant CKIs in mock and infected C33 and U2OS cells. We hypothesized that HSV-1 might be down-regulating CDK2 kinase activity by inducing the CIP/KIP family of CKIs. Western blot analyses of protein lysates from asynchronous, subconfluent cell cultures (Fig. 5A) demonstrated that CDK2 inhibitors $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ were not induced by HSV infection. In C33 cells, p21 levels were undetectable, whereas p27 levels remained unchanged. In U2OS cells, p21 levels remained unchanged and p27 levels declined after infection. In U2OS cells, the level of WT p53 was unaffected by infection (data not shown). Thus the levels of relevant CKIs in mock and infected cells do not suggest their involvement in the observed inhibition of CDK2 activity. The pocket proteins p107 and p130 are also known to bind and inhibit cyclin A or E/CDK2 activities (Castaño *et al.,* 1998; De Luca *et al.,* 1997; Woo *et al.,* 1997; Zhu *et al.,* 1995). Cycling cells possess low levels of p130, whereas p107 is abundant. By Western blot analyses of asynchronously growing C33 cells, we found that the levels of p107 remained unchanged after infection (Fig. 5A). The implications of this finding for CDK2 regulation are discussed later.

DISCUSSION

In this study, we investigated the effects of HSV-1 infection on cell cycle progression; the status of cell cycle regulatory proteins, including the retinoblastoma family; and the activities of G_1-specific cyclin-dependent kinases cyclin D/CDK4,6 and cyclin E/CDK2. After the infection of growth-arrested cells simultaneous with the readdition of serum, we observed that HSV-1 infection resulted in the persistence of a hypophosphorylated form of pRb, inhibition of cyclin D/CDK4-specific and cyclin E/CDK2-specific phosphorylation of pRb serine residues, and failure to detect CDK2-dependent kinase activities. In addition, there was a failure to hyperphosphorylate and degrade p130, accompanied by a failure to accumulate p107. These results, along with our flow cytometry data, suggest that when infected with HSV-1, G_0 cells fail to reenter the cell cycle even in the presence of mitogenic stimulation. By infecting asynchronous cell populations, we detected rapid inhibition of G_1- and S-phase CDK2-dependent kinase activity and found this inhibition to be independent of the pRb and p53 status of the cell. Coupled with our previous observations that hypophosphorylated pRb accumulated, E2F activity decreased, and cellular DNA synthesis was rapidly inhibited after infection (Olgiate *et al.,* 1999), we conclude that HSV-1 can block cell cycle progression at the restriction point near the G_1/S boundary and also in S phase.

Previously, de Bruyn Kops and Knipe (1988) showed that the infection of cells in G_1 phase prevented the progression of cells into S phase. Infection of cells during S, although resulting in the same number of cells labeled with bromodeoxyuridine (BrdU) as uninfected controls, resulted in a decrease in the level of DNA synthesis. Similarly, we have seen that cells infected in late G_1 appeared to enter S but were unable to complete DNA replication as measured with flow cytometry (G. L. Ehmann and S. L. Bachenheimer, unpublished observations; Olgiate *et al.,* 1999). It appears, therefore, that HSV-1 infection results in a halt to cell cycle progression regardless of whether the cells have transitioned through G_1. Indeed, infection of asynchronous populations of cells resulted in a general decrease in G_1- and S-specific cyclin kinase activities, revealing that regardless of the status of the cell at the time of infection, cyclin kinase inhibition ensues.

Given these observations, it is interesting to speculate as to how virus infection leads to a cell cycle block in any given phase. For example, the infection of quiescent cells resulted in the failure to target and degrade p130 and to accumulate p107. It is unclear whether the retention of p130 and prevention of p107 induction is a result of the cell cycle block or a mechanism by which the virus accomplishes the block. Indeed, when cells are in a quiescent state, p130 abounds. This population of p130 must be targeted for degradation for E2F to be released and hence begin transcription of genes necessary for cell cycle progression. If HSV-1 infection inhibits the degradation of this pocket protein, cells may be blocked in a G_0-like state. On the other hand, degradation of p130 at the initiation of cell cycle entry requires phosphorylation by G_1-cyclin kinases (Smith *et al.,* 1996). If HSV-1 is preventing cell cycle progression by inhibiting G_1-cyclin kinase activities, then p130 would persist. This second possibility is supported by our experiments in which infection of quiescent cells resulted in the failure of mitogenic signals to up-regulate cyclin D1 and cyclin E proteins, resulting in the absence of kinase activity necessary for pRb phosphorylation. The virus could be intervening at any number of points in the regulatory pathway. Activation of the RAS–RAF–MEK–ERK pathway is responsible for the up-regulation of cyclin D1 and, consequently, D-type kinases (Aktas *et al.,* 1997). Conversely, activation of glycogen synthase kinase-3β via a RAS-dependent pathway involving phosphatidylinositol 3-kinase and Akt (protein kinase B) results in cyclin D1 phosphorylation at Thr286, nuclear export, and subsequent ubiquitin-dependent proteasomal degradation (Sherr and Roberts, 1999). D-type kinases are responsible for phosphorylation of pRb, the ensuing release of E2F, and up-regulation of E2F-dependent genes necessary for cell cycle progression, including *cyclin E* and *cyclin A*. We recently reported on the induction of stress-activated protein kinases JNK and p38 and the absence of mitogen-activated protein kinase ERK induction

(McLean and Bachenheimer, 1999) after the infection of asynchronous cells. Our present work reveals the failure of quiescent cells to display markers of cell cycle progression when exposed to growth factors in the presence of virus, and our future efforts will be directed at determining where HSV-1 is blocking mitogenic stimulation of cell cycle reentry.

Our finding that HSV-1 infection inhibited both cyclin E/CDK2 and cyclin A/CDK2 kinase activities differs from that of an earlier study. It was reported that infection of serum-starved CV-1 cells by HSV-2 resulted in the transient induction of CDK2-dependent kinase activity (Hossain *et al.,* 1997). Although these authors reported an induction of CDK2-dependent kinase activity beginning at 2 h p.i. that peaked at 8 h p.i., the levels of CDK2 kinase activity in infected cells were still reduced compared with those of serum-stimulated cells, and the kinase activation in infected cells was significantly inhibited compared with that of serum-stimulated cells at 16 and 24 h post-stimulation/infection. Using synchronized CV-1 cells and HSV-2 strain G, we have been unable to reproduce the results reported by Hossain *et al.* (1997) (data not shown). In addition, Song *et al.* (2000) report that infection of CV-1 cells with HSV-2 resulted in the failure of cells to accumulate hyperphosphorylated pRb in response to mitogen, suggesting an inhibition of G_1-cyclin kinase activities. In our studies of synchronized HEL cells, we have never observed an induction of CDK2-dependent kinase activity after infection with HSV-1 (G. L. Ehmann and S. L. Bachenheimer, unpublished observation).

One previous report suggested that HSV-1 required the induction of one or more late-G_1/S cyclin-dependent kinase activities to promote viral replication (Schang *et al.,* 1998). In their study, Schang *et al.* (1998) presented evidence that viral DNA replication and viral RNA transcription were reduced in the presence of the cell cycle-inhibiting drugs roscovitine and olomoucine. These authors concluded that HSV-1 requires one or more CDK activities present during G_1 and beyond that are inhibited by these drugs (including CDK1, CDK2, or CDK5). However, no assay for the effects of roscovitine or olomoucine on levels of CDK kinase activity in normally cycling, mock- or HSV-1-infected cells was presented.

The results presented here plus those of additional studies conducted in our laboratory indicate that, in fact, HSV-1 may inhibit G_1-cyclin kinase activity to create an environment favorable to virus replication. The infection of cells ectopically overexpressing cyclin E and CDK2 from adenovirus vectors resulted in a 90% reduction in virus yield. The coexpression of the CDK2 inhibitor p21 with cyclin E and CDK2 resulted in the recovery of virus production to WT levels (S. L. Bachenheimer and G. L. Ehmann, unpublished observation).

Our assays indicated that both cyclin D/CDK4,6 and cyclin E/CDK2 kinase activities were inhibited as a result of infection of both synchronized G_0 cells and asynchronously growing cells. An examination of the protein levels of these kinase complexes revealed two potentially different mechanisms for inhibition of the kinases. Western blot analyses of proteins from synchronized cells demonstrated that cyclin E and especially cyclin D1 declined after HSV-1 infection. In addition, CDK4, CDK6, and CDK2 did not translocate to the nucleus of infected cells. In agreement with our data, Song *et al.* (2000) report that G_0 infection of synchronized CV-1 cells with HSV-2 resulted in the failure to accumulate cyclins D1 and D3, no effect on the levels of cyclin D2, and failure to accumulate G_1-specific forms of CDK4 and CDK2. We conclude that the failure to accumulate cyclin D1 and cyclin E, coupled with the failure of all three CDKs to translocate to the nucleus, resulted in a decrease in kinase complex formation and therefore a failure to detect kinase activity by phospho-pRb Western blots or immunocomplex kinase assays. However, in the second system we investigated, infection of asynchronously growing cells, we observed rapid inhibition of preexisting cyclin E/CDK2 kinase activity but without a loss of cyclin/CDK subunits or complexes.

CDK2 protein level remains constant while its activity fluctuates throughout the cell cycle. At the present, it is clear that CDK2 kinase activity is controlled by (1) the availability of corresponding cyclin subunits (Morgan, 1995), (2) its intracellular localization (Bresnahan *et al.,* 1996), (3) the phosphorylation state of Thr160, Thr14, and Tyr15 (DeBondt *et al.,* 1993; Gu *et al.,* 1992; Poon and Hunter, 1995; Sebastian *et al.,* 1993), (4) the binding stoichiometry of associated CKIs of the CIP/KIP family (Chen *et al.,* 1995; LaBaer *et al.,* 1997; Zhang *et al.,* 1994), and (5) its association with the pocket proteins p107 and p130 (Castaño *et al.,* 1998; De Luca *et al.,* 1997; Hauser *et al.,* 1997; Woo *et al.,* 1997). As reported earlier, Western blot analyses revealed that neither the protein levels nor the intracellular localization of the CDK2, cyclin E, and cyclin A subunits was affected in asynchronous cells after infection. However, cyclin D1 levels were lower in HSV-1-infected cells compared with mock-infected cells. Immunoprecipitation/Western blot analyses confirmed that cyclin/CDK complexes remained intact after infection. Thus HSV-1 does not appear to be targeting the formation or stability of the preexisting cyclin–CDK complexes. One explanation for the stability of cyclin E relative to cyclin D1 may be that targeting of cyclin E for ubiquitin-dependent degradation is regulated by autophosphorylation (Clurman *et al.,* 1996; Won and Reed, 1996). Thus loss of cyclin E/CDK2 kinase activity in HSV-1-infected cells may stabilize existing cyclin E protein.

In addition, we have been unable to detect any change in the amount of CDK2-associated tyrosine phosphorylation as judged by Western blot analyses with anti-phosphotyrosine antibody on immunoprecipitated CDK2 protein (G. L. Ehmann and S. L. Bachenheimer, unpub-

lished observation). Western blot analyses of related CKIs, p21 and p27, showed that levels of these CDK inhibitory proteins did not increase after HSV-1 infection. However, the binding stoichiometry of CKI to CDK determines whether these interactions are inhibitory. For example, it is thought that two molecules of p21 are required to inactivate a cyclin/CDK2 kinase (Zhang *et al.,* 1994), whereas a 1:1 ratio may be required for cyclin—CDK complex formation. We have not yet assessed the binding stoichiometry of the CKI–CDK2 interactions in HSV-1-infected cells, and the possibility of an increase in the ratio of p21 to CDK2 in complexes remains a potential mechanism of CDK2 inhibition to explore.

We reported previously that infection of asynchronously growing cells with HSV-1 results in the accumulation of E2F complexes containing the pocket protein p107 and cyclin A/CDK2 (Hilton *et al.,* 1995; Olgiate *et al.,* 1999). An increase in this complex may explain the decrease in CDK2 kinase activity that we observed, because previous reports had indicated that CDK2 activity bound to p107 and p130 was inactive (Castaño *et al.,* 1998; Woo *et al.,* 1997). However, on further analyses of p107 and its association with CDK2 after HSV-1 infection, we found that the total level of p107 within asynchronously growing cells was not changed after infection. Furthermore, the amount of CDK2 that coimmunoprecipitated with p107 remained unchanged in infected cells (G. L. Ehmann and S. L. Bachenheimer, data not shown). One possible explanation for these results is that the accumulation of E2F complexes containing p107 and cyclin A/CDK2 seen in infected asynchronous cell populations is actually due to the inhibition of CDK2 kinase activity. In uninfected cells, active CDK2 is bound to and phosphorylates p107, preventing recruitment of E2F. As HSV-1 infection progresses, phosphorylation of p107 by bound CDK2 decreases as CDK2 kinase activity is downregulated. Hypophosphorylated p107 accumulates and is subsequently recruited to E2F, which can be monitored by an E2F-dependent gel shift assay. This model is also supported by the observation that hypophosphorylated pRb (Olgiate *et al.,* 1999) and hypophosphorylated p130 persist after infection, coincident with an increase in E2F–pRb complexes (Olgiate *et al.,* 1999) and the persistence of E2F–p130 complexes (G. L. Ehmann and S. L. Bachenheimer, unpublished observation).

To investigate the role of virus-encoded proteins in the observed cell cycle disruptions, we determined whether viral gene expression was necessary for the inhibition of CDK2 kinase activity. Cells infected with UV-irradiated HSV-1 displayed no loss of CDK2 kinase activity. We suspect that one or more IE gene is responsible for the kinase inhibition, because CDK2 kinase activity declines as early as 2 h after infection, a time when only IE genes are being expressed. Indeed, recent studies by Hobbs and DeLuca (1999) and Lomonte and Everett (1999) indicated a role for IE gene product ICP0 in a viral-induced G_0/G_1, as well as a G_2/M, cell cycle block. Using a panel of mutant viruses, we will perform further analyses of the roles of IE gene products in the disruption of G_1-cyclin kinases and the prevention of G_1/S progression.

MATERIALS AND METHODS

Cells and virus

Human cervical carcinoma C33 cells (ATCC HTB31; mut p53 and mut pRb; American Type Culture Collection, Rockville, MD), human osteosarcoma (U2OS) cells (ATCC HTB96; WT p53 and WT pRb), and HEL fibroblast 299 cells (ATCC CCL137; WT p53 and WT pRb) were maintained in DMEM-H, supplemented with 10% FCS. HSV-1 strain KOS 1.1 was used for all experiments. Unless otherwise specified, all infections were performed at a multiplicity of infection (m.o.i.) of 5 PFU/cell.

Cell synchronization and FACS analysis

Subconfluent cultures of HEL cells were synchronized in G_0 phase by serum starvation. Cell monolayers were washed once with serum-free medium and then overlaid with medium containing 0.5% FCS and incubated at 37°C for 3–4 days. Starved cells were restimulated with medium containing 10% FCS in the presence or absence of HSV-1 and then harvested at various time points postrestimulation.

Cells were harvested by trypsinization and pelleted through 1 ml of calf serum. Cell pellets were then washed once with 1× PBS. Some cell pellets were processed to yield soluble protein lysates for Western blot analysis, as described later. Cells for FACS analysis were washed with 5 ml of 1× PBS, repelleted, and then resuspended in 1 ml of 1× PBS. Cells were then fixed with the slow addition of 4 ml of ice-cold 95% ethanol and stored at −20°C. At least 2×10^6 fixed cells were pelleted and washed with 5 ml of 1× PBS–1% BSA. Cells were pelleted again and then resuspended in 500 μl of RNase A solution (1.12% Na citrate, 20 μg/ml RNase A) and 500 μl PI solution (1.12% Na citrate, 50 μg/ml propidium iodide, 0.1% Triton X-100) and transferred to Falcon 2054 tubes. DNA content was determined using an FACScan instrument (Becton Dickinson (San Jose, CA). Cell cycle profile was determined by analysis of listmode data with ModFit software.

Preparation of cell extracts

To prepare whole-cell extracts, cells were harvested by being scraped into medium and rinsing the plate with 5 ml of 1× PBS. Cells were pelleted and then washed twice with 1× PBS. Cell pellets were resuspended in 0.2% Tween 20 lysis buffer (50 mM HEPES, pH 7.3, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.2% Tween 20 with 1 mM DTT, 1 mM PMSF, 1μg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, and

1 mM NaF) and lysed for 4–5 h at 4°C. Cellular debris was removed by centrifugation for 10 min at 12,000 × *g* at 4°C. The supernatant was recovered and stored at −70°C.

Fractionated cytoplasmic and nuclear extracts were prepared by a rapid lysis protocol. Cells were harvested by trypsinization and pelleted through 1 ml of bovine calf serum. Cell pellets were washed twice in 1× PBS and resuspended in 3 packed cell volumes (PCV) of CE buffer [10 mM HEPES, pH 7.8, 1 mM EDTA, 60 mM KCl, 1 mM PMSF, 0.1% Nonidet P-40, 25% glycerol, 0.4 mM NaF, 0.4 mM sodium orthovanadate, 10 μM pepstatin, and 4% Complete Protease Inhibitor Cocktail (Boehringer-Mannheim Biochemicals, Indianapolis, IN)]. Cells were incubated on ice for 4 min, and then nuclei were spun out with a 10-s spin in a bench top microfuge. The supernatant was recovered and clarified by centrifugation at 12,000 × *g* for 10 min at 4°C. The recovered supernatant was the cytoplasmic extract. Pelleted nuclei were resuspended in CW buffer (same as CE buffer, except lacking Nonidet P-40 and glycerol) and then subjected to 20 strokes in a 1-ml Dounce homogenizer and then repelleted at 12,000 × *g* for 10 min at 4°C. The nuclear pellet was then resuspended in 2 PCV of Moberg (Moberg *et al.,* 1996) nuclear lysis buffer (0.5 M KCl, 35% glycerol, 100 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM NaF, 1 mM PMSF, 1 mM DTT, and phosphatase and protease inhibitors as described for CE buffer). The suspended nuclei were incubated on ice for 10 min and then pelleted at 60,000 rpm for 20 min at 4°C with a Beckman TL100.3 rotor. The recovered supernatant represented the nuclear extract. Both cytoplasmic and nuclear extracts were stored at −70°C. Protein concentration of all cell extracts was determined according to the Bradford assay (Bio-Rad, Hercules, CA).

Detection of proteins

Infected cell proteins were detected by Western blot analysis. Briefly, aliquots of cell extracts representing equal total cell protein were mixed with 2× sample buffer (Harlow and Lane, 1988) and denatured by boiling for 5 min. Proteins were separated by bisacrylamide cross-linked (1:37.5) or DATD-acrylamide cross-linked (1:38) SDS–PAGE and transferred to PVDF membrane (New England Nuclear, Boston, MA). Membranes were blocked for 30 min in TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dried milk. Proteins were detected by incubation with specified rabbit polyclonal or mouse monoclonal antibody, followed by incubation with the appropriate secondary antibody conjugated to horseradish peroxidase. Antibodies were detected by incubation in Renaissance chemiluminescent reagent (New England Nuclear) and visualized on Biomax-MR film (Kodak).

Antibodies used for all experiments included CDK2 (M2, sc-163), CDK4 (sc-260), cyclin A (BF683, sc-239), cyclin E [HE111, sc-248 (for immunoprecipitation)], cyclin E [HE12, sc-247 (for Western blots)], and anti-mouse IgG-HRP (sc-2005; all from Santa Cruz Biotechnology, Santa Cruz, CA). The pRb antibody (14001A) was from PharMingen (San Diego, CA). Phospho-Rb Ser780 (no. 9307S) and phospho-Rb Ser811 (no. 9308S) were from New England Biolabs (Beverly, MA). Anti-rabbit Ig-HRP (NA 934) was from Amersham (Arlington Heights, IL). Cyclin D1 (342) was obtained from Yue Xiong (University of North Carolina at Chapel Hill). ICP4 (H943) was obtained from Lenore Pereira (University of California at San Francisco).

Immunoprecipitations

Cell extracts of 100–500 μg of protein were first precleared with protein A agarose beads (Boehringer-Mannheim) for 30 min at 4°C. Beads were spun out, and then precleared cell extract was incubated with rabbit polyclonal or mouse monoclonal antibody for 2–15 h at 4°C on an orbital mixer. Protein complexes were then precipitated by incubation with protein A agarose beads (Boehringer-Mannheim) for 1–4 h at 4°C. Precipitated complexes were washed three times with 0.2% Tween 20 lysis buffer and then resuspended in 40 μl of sample buffer and boiled for 5 min. Proteins were separated by SDS–PAGE and visualized by Western blotting as described earlier.

Immunocomplex kinase assays

CdK2-specific cyclin kinase complexes were immunoprecipitated from 100–500 μg of total cell protein with 10 μl of anti-cdk2 (M2) rabbit antibody, anti-cyclin A (BF683) mouse monoclonal antibody, or anti-cyclin E (HE111) mouse monoclonal antibody (Santa Cruz) for 12 h at 4°C and collected on 20-μl 50% protein A agarose beads (Boehringer-Mannheim) for 1 h at 4°C. Immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer (50 mM HEPES, pH 7.3, 10 mM $MgCl_2$) and resuspended in 25 μl of kinase buffer (containing 1 mM DTT, 1 mM *o*-Na vanadate, 2.5 mM EGTA, 20 μM ATP, 10 mM β-glycerophosphate, 1 mM NaF) with substrate (10–25 μg GST-Rb or 1.5 μg of histone H1) and 5 μCi of [γ-^{32}P]ATP. The kinase reaction was allowed to proceed for 30 min at 30°C and was stopped by the addition of 25 μl of 2× SDS sample buffer. Samples were boiled for 5 min and then separated by SDS–PAGE. Gels were vacuum dried, and phosphorylated substrate was visualized by autoradiography using BioMax-MR film (Kodak) and quantified by laser densitometry. *Escherichia coli* transformed with pGEX-Rb (379-928) was obtained from J. DeGregori (University of Colorado Health Science Center), and GST-Rb substrate was prepared as described previously (Matsushime *et al.,* 1994).

Generation of figures

Figures 2–5 were created using Microsoft PowerPoint. Images of original autoradiograms were generated using a desktop scanner, saved as Corel PhotoPaint files and then imported into Microsoft PowerPoint.

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