Herpes Simplex Virus Infection Blocks Events in the G1 Phase of the Cell Cycle

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Infection of cells in G1 phase with herpes simplex virus (HSV) prevents their progression into S phase (de Bruyn Kops, A., and Knipe, D. M., 1988, Cell 55, 857–868). We have examined G1-phase events in infected cells to determine whether this effect was the result of inhibition of G1 phase progression or of entry into S phase. We observed that HSV infection decreased pRb phosphorylation and induced a new phosphorylated form of pRb. Furthermore, HSV infection prevented the normal G1 increases in cyclin D1 and D3 protein levels, and blocked the normal G1 appearance of new electrophoretic forms of cdk2 and cdk4. Thus, HSV infection inhibits several events that normally occur in the cell cycle during G1 phase, arguing that the HSV-induced block in the cell cycle occurs in early to mid-G1 phase.

INTRODUCTION

Viruses modify their host cells in many ways to optimize their own replication. In return, the host cell modifies its metabolism in response to stresses such as viral infection in an attempt to protect itself. As part of their manipulation of host cells, viruses often modify the host cell-cycle regulatory machinery to facilitate their replication. For example, DNA tumor viruses such as simian virus 40 (DeCaprio et al., 1988), adenovirus (Whyte et al., 1989), and papilloma virus (Dyson et al., 1989) encode proteins that bind to and eliminate the growth-inhibitory function of the cellular pRb protein. This stimulates cells to move into S phase so that S-phase functions needed for genomic viral DNA replication of these viruses are available (Knipe, 1996).

In contrast, herpes simplex virus replicates equally well in all phases of the cell cycle (Cohen et al., 1971; Cai and Schaffer, 1991; Yeh and Knipe, unpublished results). Furthermore, herpes simplex viruses encode seven proteins essential for viral DNA synthesis, including a DNA polymerase as well as functional homologs of several S-phase functions, including ribonucleotide reductase and thymidine kinase, suggesting that cellular S-phase factors are not required for an efficient infection. Indeed, we have shown that HSV infection of cells in the G1 phase prevents those cells from entering S phase (de Bruyn Kops and Knipe, 1988). Recently, several groups have reported various interactions of HSV with the cell cycle. First, one study concluded that HSV-2 infection leads to increased phosphorylation of pRb, which would be consistent with cells moving into S phase, transient activation of cdk2 activity, but lack of cell-cycle progression (Hossain et al., 1997). Second, it has been reported that HSV-1 ICP0 protein binds to and stabilizes cyclin D3 (Kawaguchi et al., 1997). Third, it has been reported that cyclin-dependent kinases are required for HSV replication and transcription (Schang et al., 1998), possibly indicating a need for cell-cycle progression during viral infection. In contrast, a fourth paper reported that HSV induces the accumulation of a hypophosphorylated form of pRb and the accumulation of E2F-pRb DNA binding complexes (Olgiate et al., 1999), consistent with our observation that HSV infection inhibits cell-cycle progression. Thus, the effects of HSV on the cell cycle are not well defined.

Progression of the cell cycle from G0 to S phase is regulated by a series of events, including the sequential assembly, activation, and subsequent inactivation of a series of cyclin-dependent kinase (cdk) complexes (Pines, 1993). The expression of D-type cyclins is stimulated by growth factors and mitogens (Matsushima et al., 1992; Bates et al., 1994; Myerson and Harlow, 1994). The D-type cyclins associate with and activate the cyclin-dependent kinases cdk2, cdk4, cdk5, and cdk6 (Matsushima et al., 1992; Xiong et al., 1992; Myerson and Harlow, 1994). Of these enzymes, cdk4 and cdk6 appear to be the most abundant D-type cyclin partners. Cyclin E appears to be the major cdk2 regulatory subunit in vivo (Dulic et al., 1992; Koff et al., 1992), and this complex is also necessary for completion of the G1/S-phase transition (Hatakeyama et al., 1994).

Some of the major targets of the cdk–cyclin complexes are the retinoblastoma family of proteins, pRb, p107, and...
p130 (reviewed by Beijersbergen and Bernards, 1996). Phosphorylation of pRb in mid-G1 is required for cells to exit G1 into S phase (Sherr, 1993). pRb is found in a hypophosphorylated form in quiescent cells and early G1 and then becomes phosphorylated on several cdk consensus sites during mid- to late-G1. The hypophosphorylated form of pRb binds to and inhibits several cellular proteins that are involved in activating the transcription of genes whose products are required for S-phase progression (reviewed by Nevins, 1994; Weinberg, 1995). G1 arrest is known to be associated with accumulation of unphosphorylated pRb (Buchkovich et al., 1989; DeCaprio et al., 1989).

To clarify the effects of HSV on the host-cell cycle and to determine whether HSV infection blocks the cell cycle immediately before S phase or within the G1 phase, we examined the effects of HSV infection on the host-cell proteins involved in the G1 progression and G1/S transition. Our results demonstrate that HSV infection leads to an inhibition of G1 phase-specific events, consistent with a block in early- to mid-G1 phase of the cell cycle.

RESULTS

HSV infection causes a change in the electrophoretic mobility of the cellular protein pRb

Previously, we had shown that HSV infection blocks cell-cycle progression into S phase (de Bruyn Kops and Knipe, 1988). To determine whether this was the result of cell-cycle arrest in the G1 phase and to explore the molecular mechanisms for this effect, we examined the effects of HSV infection on the host-cell proteins involved in G1 progression and the G1/S transition, including pRb, cyclins, and cdk.

To examine the effects of HSV infection on pRb, we infected CV-1 cells with HSV-1 or mock-infected the cultures, prepared total cell extracts, and examined pRb by immunoprecipitation and immunoblotting (Fig. 1). Four distinct species of pRb, ranging in size from 105 to 120 kDa (bands a, b, c, and d), were apparent in the mock-infected cell extracts (Fig. 1, lane 1). On HSV infection, there was a noticeable shift in the mobility of pRb toward the higher mobility species and the appearance of a new band, labeled b’, the letter migrating slightly faster than the b band (Fig. 1, lane 2). Although the relative intensity of each pRb species varied slightly from one experiment to the other, the overall downward shift of the pRb species and the appearance of the new b’ species were observed consistently on HSV infection. Although the total amount of pRb recovered from infected and mock-infected cultures varied somewhat (Fig. 1), there was no consistent effect of HSV infection on pRb levels (Liu and Knipe, unpublished results).

To confirm the alteration in the phosphorylation status of pRb, we performed phosphopeptide analysis of 32P-labeled pRb immunoprecipitated from infected and mock-infected CV-1 cells. Following immunoprecipitation with mouse antihuman Rb monoclonal antibody, each sample was resolved in an SDS–polyacrylamide gel, transferred onto a nitrocellulose membrane, and analyzed by immunoblotting using the same monoclonal antibody. The immunoblot image is shown. The species of pRb in the mock-infected cell extracts are labeled as a–d. The new species of pRb appearing in HSV-infected cell extracts is labeled as b’.
mock-infected cells. Representative autoradiographic maps are shown (Fig. 3), and the detected phosphopeptide spots have been designated numerically (Fig. 3D). Interestingly, the intensity of some spots changed on HSV infection (Figs. 3A and 3C). Spots 1 and 2 were absent in pRb from mock-infected cells (Fig. 3A) but present in large quantity in pRb from HSV-infected cells (Fig. 3C). Spots 4 and 12, clearly observed in mock-infected cells, disappeared on HSV infection. The intensity of spot 6 increased slightly on viral infection, and that of spots 7, 8, 9, 10, 11, and 13 decreased slightly on viral infection. In total, these results show that phosphorylation of pRb changes on HSV infection and indicate that this alteration in the phosphorylation is responsible for...
the change in the electrophoretic mobility of pRb following viral infection.

HSV effects on pRb in growing and resting cells

To further examine the effects of HSV infection on pRb, we performed experiments under two different conditions: (1) infection of actively growing cells and (2) infection of growth-arrested cells. In actively growing cells, HSV infection shifted the majority of pRb species to the higher mobility species by 4–6 h postinfection (hpi), while the electrophoretic profile of pRb species remained the same in mock-infected cells during the time course of this experiment (Fig. 4). In contrast, in serum-starved cells, the major band was the a band (Fig. 5, lane 1). Addition of medium containing 10% serum allowed the cells to reenter the cell cycle and to accumulate the slower-moving, more highly phosphorylated pRb species (Fig. 5, even-numbered lanes). In contrast, HSV infection prevented the serum-induced shift of pRb to the c and d bands but induced the formation of the b' species (Fig. 5, odd-numbered lanes). Furthermore, HSV infection induced the b' form even in cells maintained in the growth-arrested state (Liu and Knipe, unpublished results).

The shift of pRb to the faster-moving species was observed by 4 hpi. (Fig. 4, lane 4), suggesting the possible involvement of a virion component, a viral intermediate-early, or an early protein in this process. Consistent with these kinetics, the pRb mobility shift was also observed in infections maintained in medium containing phosphonoacetate to inhibit viral DNA synthesis (Yeh and Knipe, unpublished results). This mobility shift continued during the course of viral infection and became more prominent at late times postinfection (Figs. 4 and 5). At very late times postinfection (24 hpi), the fastest-moving species were the major species and the slow-moving species were not detectable (Liu and Knipe, unpublished results).

To determine if the effect was specific for HSV-1, we examined cells infected with HSV-2 (Fig. 6). In actively growing cells, HSV-2 infection shifted pRb to the faster-moving species by 6 hpi, while the slower-moving species were the major species in mock-infected cells (Fig. 6, lanes 1 to 3). In actively growing cells, HSV-2 infection shifted pRb to the faster-moving species by 6 hpi, while the slower-moving species were the major species in mock-infected cells (Fig. 6, lanes 1 to 3). In growth-arrested cells, the effect of viral infection on the electrophoretic mobility of pRb was similar to that observed with HSV-1.
mobility of pRb was more dramatic than in actively growing cells (Fig. 6, lanes 4 to 6). pRb shifted to the slower-migrating forms when the growth-arrested cells were grown for 6 h in medium supplemented with 10% fetal bovine serum, but HSV-2 infection actively repressed the conversion and the majority of pRb remained as fast-migrating species (Fig. 6, lanes 4 to 6). Thus, both HSV-1 and HSV-2 caused a change in the electrophoretic mobility of pRb toward the fast-migrating forms.

G1 cyclins and cyclin-dependent kinases (cdks) in HSV-infected cells

Phosphorylation of pRb in the G1 phase of the cell cycle is performed by cyclin-dependent kinases activated by binding of their cyclin partners. Thus, to explore the molecular mechanisms responsible for the altered phosphorylation of pRb in HSV-infected cells, we examined the levels of the G1 cyclins, cyclin D1, cyclin D2, cyclin D3, and cyclin E, and their catalytic partners, cdk2, cdk4, cdk5, and cdk6, in HSV- and mock-infected cells.

As expected, the levels of cyclin D1 (Fig. 7A, lanes 1 and 2) and cyclin D3 (Fig. 7C, lanes 1 and 2) increased as cells moved from G0 through G1 phase. In contrast, the level of cyclin D2 was constant as the cells moved through G1 (Fig. 7B). However, the induction of cyclin D1 and cyclin D3 was blocked in HSV-2-infected cells (Figs. 7A and 7C), while the level of cyclin D2 was not significantly affected by viral infection (Fig. 7B). The effect of viral infection was most dramatic with cyclin D1, and cyclin D1 was almost undetectable in HSV-infected cells by 6 hpi, even at a multiplicity of infection (m.o.i.) of 3.3 (Fig. 7A, lane 3).

Cdk4 protein, a major catalytic partner of D-type cyclins, ran as a doublet (Fig. 8). The level of the faster-migrating form of cdk4 increased as cells moved from G0 through G1 phase (Fig. 8, lanes 4 and 5). The increase of the faster-migrating species of cdk4 was blocked in HSV-infected cells both in actively growing cells (Fig. 8, lanes 1 to 3) and in growth-arrested cells (Fig. 8, lanes 4 to 6).

Two other cyclin D-dependent kinases, cdk5 (Fig. 9A) and cdk6 (Fig. 9B)
and cdk6 (Fig. 9B), increased slightly during G1 phase. Following viral infection, the levels of cdk5 (Fig. 9A) and cdk6 (Fig. 9B) were not changed reproducibly. The level of another G1 cyclin, cyclin E, remained unchanged for at least 6 h after the release from serum starvation, and there was no effect of HSV infection on the level of cyclin E (Fig. 10A).

Finally, we examined cdk2, a catalytic partner of cyclin E. As with cdk4, cdk2 ran as a doublet and the level of the faster-migrating species of the protein increased as cells moved from G0 through G1 phase (Fig. 10B, lanes 1 and 2). The induction of the faster-migrating species of cdk2 was blocked in HSV-infected cells (Fig. 10B, lanes 3 to 5 versus lane 2). There is experimental evidence that the protein with the faster migration is the active form of cdk2 (Gu et al., 1992). Thus, HSV infection appears to block the normal appearance of the active form of cdk2 in G1.

In conclusion, our results suggest that the normal G1 increases in protein levels or electrophoretic forms of G1 cyclins and cdks are inhibited by HSV infection, and this may inhibit the phosphorylation of the retinoblastoma protein (pRb) required for the G1/S transition, therefore blocking HSV-infected cells in the G1 phase.

DISCUSSION

This work was intended to extend our previous observation that HSV-1 infection in the G1 phase of the cell cycle prevented cells from entering S phase (de Bruyn Kops and Knipe, 1988). To determine whether G1 events were inhibited or infection was blocking cellular entry into S phase, we first examined the effect of HSV infection on the pRb protein. We observed that HSV infection blocked the phosphorylation of pRb in G1, a necessary modification for progression of the cells to S phase. Thus, we observed that HSV infection reduced the amount of hyperphosphorylated pRb observed late in G1 phase, but we also observed a new phosphorylated form of pRb, called b′, that accumulated after viral infection. The loss of hyperphosphorylated forms of pRb is consistent with blockage of the cell cycle in G1 phase rather than at the start of or within S phase. Further studies of the G1 regulatory proteins showed that viral infection prevented the normal increases in levels of cyclins D1 and D3 and the appearance of new electrophoretic forms of cdk4 and cdk2 that occur in the G1 phase of the cell cycle. The lack of these essential components of the cdk holoenzyme complexes is likely to explain the reduced phosphorylation of pRb in G1 and the cell-cycle block.

Similar effects of HSV infection on G1 cyclins and cdks have been observed by Ehmann et al. (2000). We conclude that HSV infection blocks the cell cycle in early- to mid-G1 phase. Nevertheless, HSV may induce G1- or S-phase functions by mechanisms other than progression of the cell cycle.

Although we have not yet directly measured the kinase activity levels to show that these correlate with protein levels, we have observed that the amount of the higher electrophoretic mobility form of cdk2 observed in G1-stage cells and associated with enzymatic activity (Gu et al., 1992) is decreased in HSV-infected cells. Furthermore, Ehmann et al. (2000) have observed that cdk2 activity levels are reduced in HSV-infected human U20S, C33, and human embryonic lung cells. This supports the idea that HSV reduces the level of functional cyclin–cdk complexes in infected cells, contributing to the block of the cell cycle in the G1 stage.

Multiple interactions of HSV with the cell-cycle regulatory machinery

Previous studies had suggested that HSV-2 increased the phosphorylation of pRb (Hossain et al., 1997), while HSV-1 decreased the phosphorylation of pRb (Olgiate et al., 1999). In our studies, we observed equivalent effects of HSV-1 and HSV-2 on the G1/S transition and pRb phosphorylation, so the explanation for the observed differences is not likely to be type-specific differences. Our resolution of multiple pRb species and a new electrophoretic form in HSV-infected cells may explain the paradox. Hossain et al. (1997) infected serum-starved CV-1 cells and observed a decrease in electrophoretic mobility of pRb in infected cells. Their work did not resolve the multiple pRb species; thus, it seems likely that the decreased mobility of pRb observed in that study was due to the new b′ species that we observed in HSV-infected cells and not the hyperphosphorylated form of pRb formed in late-G1 phase. Our results are consistent with those of Olgiate et al. (1999), so the apparent differences may be reconciled.
Similarly, it has been concluded that HSV ICP0 stabilizes the cell-cycle regulator cyclin D3 (Kawaguchi et al., 1997; Van Sant et al., 1999), which appears to differ from our results, showing that HSV infection decreases cyclin D3 levels. In fact, the previous work also observed that wild-type HSV-1 decreased cyclin D3 levels, but ICP0 mutant viruses showed more drastically reduced levels of cyclin D3. Hence, they concluded that ICP0 stabilizes cyclin D3. While ICP0 may indeed exert an effect on the stability of cyclin D3, the total effect of HSV infection is to decrease its level in the host cell.

Schang et al. (1998) have reported that cdk inhibitors block HSV productive infection, and they have concluded that one or more cdks active from late G1 onward are required for HSV transcription and replication. There are many possible explanations that might reconcile these observations with a G1-phase block. First, the essential cdk might not be a G1-phase function or might not be altered in activity levels through the cell cycle. Second, HSV might induce a cdk by a mechanism independent of the cell cycle. Third, the inhibitors might not be specific for cdk molecules. Identification of the cellular target(s) of the cdk inhibitors that are essential for viral replication will be necessary to distinguish between these possibilities.

Potential roles of cell-cycle blocks in herpesvirus replication

HSV can productively infect cells independently of the stage of the cell cycle (Cohen et al., 1971; Cai and Schaffer, 1991; Yeh and Knipe, unpublished results). Therefore, it is not clear that it would be beneficial for the virus to block the cell cycle, and indeed, the effect may be secondary to some other effect(s) on the host cell. However, several herpesviruses and herpesvirus gene products have been shown to block the cell cycle in the G1 phase, including Epstein–Barr virus (Cayrol and Flemington, 1996a,b; Takese et al., 1996), human cytomegalovirus (Lu and Shenk, 1996; Dittmer and Mocarski, 1997), and bovine herpesvirus 1 (Schang et al., 1996). Thus, there may be some potential benefit of the cell-cycle block for viral replication that is realized in vivo or in other cell types. Obviously, inhibition of cellular DNA synthesis could provide metabolites or cellular components that enhance viral DNA synthesis. Alternatively, blocking the host cell from entering S phase would prevent the synthesis of histones, which could bind to viral DNA and decrease its transcription or replication.

On the other hand, the cell-cycle block may represent a host response to viral infection to protect the cell by removing it from the cell cycle until homeostasis is restored. For example, G1 arrest has been associated with the unfolded protein response resulting from a block in cyclin D1 translation (Brewer et al., 1999). By this model, the G1 block might not be advantageous for the virus but rather for the host cell.

An understanding of the exact nature of this virus–host cell interaction may come from further studies to define the mechanism of viral-mediated inhibition of cyclin and cdk expression and the viral gene products involved. This work shows that accumulation of cyclins D1 and D3 is blocked by viral infection and that G1-specific forms of cdk4 and cdk2 do not accumulate in infected cells. One recently published study has shown that ICP0, over-expressed as the sole IE protein by a mutant HSV strain, can induce cell-cycle blocks at both the G1/S and G2/M boundaries (Hobbs and DeLuca, 1999). A recent second study has shown that ICP0 expressed by transfection inhibits cells in mitosis and at the G1/S-phase boundary (Lomonte and Everett, 1999). It remains to be shown that ICP0 is sufficient for cell-cycle arrest when expressed at normal levels in the context of other IE proteins. Thus, further studies are needed to identify the mechanism(s) by which HSV infection leads to the block in expression or modification of these molecules to gain further insight into this virus–host cell interaction.

MATERIALS AND METHODS

Virus and cells. The HSV-1 strain KOS1.1 (Hughes and Munyon, 1975) was originally obtained from M. Levine (Univ. of Michigan). The HSV-2 strain 186 syn-1 was originally isolated as a nonsyncytial plaque (Spang et al., 1983) from an HSV-2 strain 186 (Rawls et al., 1968) stock, kindly provided by P. Schaffer (University of Pennsylvania). CV-1 monkey kidney cells (American Type Culture Collection, Rockville, MD) were grown as described in Dulbecco’s modified minimal essential medium (Cellgro, Atlanta, GA), supplemented with 5% fetal bovine serum (Gibco-BRL, Grand Island, NY) and 5% newborn calf serum (Hyclone, Provo, UT) as described (Knipe and Spang, 1982). Cells were growth-arrested by incubation in 199 medium containing 1% calf serum for 24 h. Prior to incubation in this medium, 48% of the cells were labeled with BrdU and in S phase. After 24 h incubation in the 199 medium, less than 4% of the cells were labeled with BrdU, indicating a cell-cycle arrest.

Antibodies. Immunoprecipitation of pRb was performed with mouse anti-pRb monoclonal antibody (PharMingen 14001A) at a dilution of 1:100. The antibodies used for immunoblotting pRb, cyclins, and cdks were as follows. Mouse anti-pRb monoclonal antibody (PharMingen 14001A) was used at a 1:1000 or 1:2000 dilution. Mouse anti-cyclin D1 monoclonal antibody (Santa Cruz sc-8396) was used at a 1:500 dilution. Rat anti-cyclin D2 monoclonal antibody (Santa Cruz sc-452) was used at a 1:500 dilution. Mouse anti-cyclin D3 monoclonal antibody (Santa Cruz sc-6283) was used at a 1:250 or 1:500 dilution. Rabbit anti-cyclin E polyclonal antibody (Santa Cruz sc-198) was used at a 1:500 or 1:1000 dilu-
tion. Mouse anti-cdk2 monoclonal antibody (Santa Cruz sc-6248) was used at a 1:500 or 1:1000 dilution. Rabbit anti-cdk4 polyclonal antibody (Santa Cruz sc-260) was used at a 1:1000 dilution. Mouse anti-cdk5 monoclonal antibody (Santa Cruz sc-6247) was used at a 1:500 dilution. Rabbit anti-cdk6 polyclonal antibody (Santa Cruz sc-177) was used at a 1:2000 dilution.

Phosphatase treatment. pRB immunocomplexes were prepared as described earlier and resuspended in 60 μl of a modified New England Biolabs (Beverly, MA) bacterial phage λ protein phosphatase buffer (50 mM Tris–HCl, 5 mM DTT, 2 mM MnCl₂, 100 μg/ml BSA, 1 mM Leupeptin, 1 mM Pepstatin [Boehringer Mannheim, Indianapolis, IN], 100 μM EDTA, and 200 μM PMSF). Five hundred units of λ protein phosphatase (New England Biolabs) were used to treat pRB present in the immunocomplex for 1 h at 30°C as reported previously (Vairo et al., 1995). Phosphatase inhibitors, 10 mM sodium vanadate and 5 mM NaF, were also included in control reactions to demonstrate the specificity of the phosphatase reaction.

Phosphopeptide analysis. Mock-infected or HSV-1 KOS11-infected CV-1 cells were labeled with 32P-phosphate. pRB was immunoprecipitated with mouse anti-pRB monoclonal antibody, resolved by SDS–PAGE, and analyzed by autoradiography. Each sample was exposed to X-ray films with intensifying screens for autoradiography. Each sample contained 500 Cerenkov cpm.

Immunoblot analysis. The harvested cell pellets, from mock-infected and HSV-infected cells, were lysed in SDS-containing sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% [w/v] SDS; 20% glycerol; 0.5% [v/v] 2-mercaptoethanol; 0.1% [w/v] bromphenol blue). Proteins in the cell lysate were resolved by SDS–PAGE (Knipe and Spang, 1982) and electrophoretically transferred from the gels onto nitrocellulose filters in a transfer apparatus according to the procedures of the manufacturer (Bio-Rad). Expression of cyclins and cdkks was monitored by probing the blots with the primary antibodies described earlier, followed by horseradish peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and enhanced chemiluminescence (Amersham).

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