Herpes Simplex Virus Gene Products Required for Viral Inhibition of Expression of G1-Phase Functions

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INTRODUCTION

Infection with herpes simplex virus (HSV) profoundly alters the host cell and its synthetic machinery, redirecting the cell from synthesis of cellular proteins to synthesis of viral proteins. HSV encodes several genes that are involved in priming the newly infected cells to support efficient viral replication. This virus-induced switch in gene expression involves destabilization of host mRNAs, repression of host transcription, and inhibition of RNA splicing (reviewed by Roizman and Knipe, 2001). Furthermore, HSV infection alters the cell cycle regulatory molecules and mechanisms in many ways.

A variety of virion-associated regulatory proteins located in the tegument are delivered into the cytoplasm after fusion of the viral envelope with the host cell plasma membrane. VP16 is an abundant tegument protein and stimulates transcription of the viral immediate-early genes (Batterson and Roizman, 1983; Campbell et al., 1984; Pellett et al., 1985; Post et al., 1981). The virion host shutoff (vhs) protein, another tegument protein, causes degradation of mRNAs and triggers rapid shut off of host cell protein synthesis in the cytoplasm (Fenwick and McMenamin, 1984; Read and Frenkel, 1983; Schek and Bachenheimer, 1985; Strom and Frenkel, 1987). In addition, HSV infection inhibits transcription of many host genes (Long et al., 1999; Rice et al., 1994, 1995; Spencer et al., 1997). Viral immediate-early proteins (ICP0, ICP4, ICP22, and ICP27) are expressed first upon infection (Batterson and Roizman, 1983) and they act cooperatively to regulate the expression of all classes of viral genes (reviewed by Roizman and Knipe, 2001).

HSV replicates well in all phases of the cell cycle (Cai and Schaffer, 1991; Roizman and Knipe, 2001; Yeh and Knipe, unpublished results). Furthermore, HSV encodes seven proteins essential for viral DNA synthesis, including a viral DNA polymerase as well as functional homologs of several S-phase functions, including ribonucleotide reductase and thymidine kinase, further arguing that cellular S phase is not required for an efficient infection. Several different effects of HSV infection on the cell cycle have been reported. First, we had shown that HSV infection blocks cell-cycle progression into S phase (de Bruyn Kops and Knipe, 1988). Second, a study concluded that HSV-2 infection leads to increased phosphorylation of pRb and transient activation of cyclin-dependent kinase 2 (cdk2) activity, which would be consistent with cells moving into S phase (Hossain et al., 1997). Third, it has been reported that HSV-1 ICP0 protein binds to and stabilizes cyclin D3 (Kawaguchi et al., 1997) and that cyclin-dependent kinases are required for HSV replication and transcription (Jordan et al., 1999; Schang et
al., 1998, 1999, 2000), possibly indicating a need for cell-cycle progression during viral infection. Recently, several studies have shown that HSV infection blocks G1 events and arrests host cell growth in G1 phase (Ehmann et al., 2000; Olgiate et al., 1999; Song et al., 2000).

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/cdk complexes (Pines, 1993). The expression of D-type cyclins is stimulated by growth factors and mitogens (Bates et al., 1994; Matsushima et al., 1992; 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; Myerson and Harlow, 1994; Xiong et al., 1992). D-type cyclin/cdk complexes play a major role in phosphorylation of the retinoblastoma protein (pRb), which is required for the G1- to S-phase transition (Sherr, 1993, 1994). pRb is found in a hypophosphorylated form in quiescent cells and early G1 phase 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 whose products are required for S-phase progression (reviewed by Nevins, 1994; Weinberg, 1995).

We have shown that HSV infection leads to an inhibition of G1-phase-specific events, including blocking the phosphorylation of pRb and blocking the induction of cyclin D1 and cdk4, important G1-phase regulators (Song et al., 2000). To determine the viral gene products responsible for this effect, we examined the cells infected with several mutant viruses with mutations in four immediate-early proteins, vhs function, or a DNA replication function. Our results demonstrate that HSV-1 ICP27, ICP4, and ICP0 functions contribute to blocking the induction of cyclin D1 and cdk4 proteins in HSV-infected cells and that HSV-1 ICP27 protein is required for blocking the phosphorylation of pRb protein and for blocking the cell cycle in the G1 phase.

**RESULTS**

The virion host shutoff function is not required for the effects of HSV infection on pRb

HSV infection blocks G1 events in the cell cycle and arrests host cell growth in the G1 phase (de Bruyn Kops and Knipe, 1988; Ehmann et al., 2000; Song et al., 2000). To determine the viral gene product(s) responsible for this effect, we examined various HSV-1 mutant strains for their effects on the cell cycle regulatory proteins (pRb, cyclin D1, and cdk4) and on cell cycle progression into S phase.

Immediately after infection, the vhs function destabilizes mRNAs in the cytoplasm and contributes to redirecting the cell from synthesis of cellular to synthesis of viral proteins (Fenwick and McMenamin, 1984; Read and Frenkel, 1983; Schek and Bachenheimer, 1985; Strom and Frenkel, 1987).

To determine if the vhs function played a role in the effects of HSV infection on pRb, we infected CV-1 cells with vhs mutant or wild-type virus, prepared total cell extracts, and examined pRb proteins by immunoblotting (Fig. 1A). As described previously (Song et al., 2000), pRb proteins were hypophosphorylated in G0 phase and shifted toward slower-migrating, hyperphosphorylated forms as cells moved into G1 phase, while wild-type HSV infection blocked the shift and the majority of pRb remained as hypophosphorylated species (Fig. 1A). The vhs mutant virus (vhs) was as effective as wild-type virus (KOS) in shifting pRb proteins toward hypophosphorylated forms, while an ICP27 mutant virus (5d/t.2) was defective for downshifting pRb proteins (Fig. 1A). To ensure that the vhs mutant virus was defective for inhibition of host protein synthesis, we examined protein synthesis in infected and mock-infected cells (Fig. 1B). While host protein synthesis was inhibited in cells infected with wild-type virus, host protein synthesis was much less affected in cells infected with the vhs mutant virus under normal infection conditions (Fig. 1B, lanes 1 to 3) or in the presence of actinomycin D to inhibit transcription (Fig. 1B, lanes 4 to 6). Thus, the vhs mutant virus infection blocked pRb phosphorylation even though host shutoff was defective.

Viral DNA replication and late gene functions are not required for the effects of HSV infection on pRb

Expression of HSV-1 genes during a lytic infection proceeds in a temporally ordered cascade, and the late genes require viral DNA synthesis for maximal induction (Honess and Roizman, 1974, 1975; Roizman and Knipe, 2001; Wagner, 1985). To determine if viral DNA replication and late gene functions were involved in the effects of HSV infection on pRb, we infected CV-1 cells with an HSV-1 mutant virus defective for the HSV DNA replication protein ICP8 (HD2) or with KOS and examined pRb protein by immunoblotting (Fig. 2). The mutant virus HD2 was as effective as the wild-type virus in blocking the phosphorylation of pRb during infection (Fig. 2, lanes 1, 3, and 5). Similarly, wild-type virus infection in the presence of an inhibitor of viral DNA replication, PAA, effectively blocked the phosphorylation of pRb proteins (Fig. 2, lane 4). These results indicated that ICP8, viral DNA replication, and true late gene functions are not required for the effects of HSV infection on pRb.

An ICP27 mutant strain is unable to block the formation of hyperphosphorylated forms of pRb in infected cells

To examine the roles of viral immediate-early genes on the HSV-induced cell cycle block, we infected CV-1 cells with wild-type HSV-1 KOS or KOS mutant strains with
mutations in each of four viral immediate-early genes (ICP0, ICP4, ICP22, or ICP27), prepared total cell extracts, and examined pRb by immunoblotting (Fig. 3). pRb proteins were hypophosphorylated in G0 phase and shifted toward slower-migrating, hyperphosphorylated forms as cells moved to G1 phase (Fig. 3, lanes 1 and 2).

Wild-type HSV infection blocked the shift, and the majority of pRb remained as hypophosphorylated species, bands a and b' (Fig. 3, lane 3). However, an ICP27 mutant (5dl1.2) (Fig. 3, lane 4) failed to block the formation of the

FIG. 1. Effect of virion host shutoff (vhs) function on the phosphorylation of pRb protein during HSV infection. (A) Following growth arrest induced by serum starvation, CV-1 cells were infected with an HSV-1 strain as indicated or mock infected as a control and overlaid with DMEM–10% serum. Total cell extracts, harvested at 0 or 10 h p.i., were examined for pRb by immunoblot analysis. Mock, mock-infected; KOS, wild-type HSV-1; vhs, HSV-1 virion host shutoff mutant; and 5dl1.2, HSV-1 ICP27 mutant. (B) Effect of vhs function on host protein synthesis. Cycling CV-1 cells were infected with HSV-1 strains at an m.o.i. of 20 or mock infected as a control and overlaid with DMEM–10% serum for 6.5 h in the absence (−) and presence (+) of the inhibitor of RNA synthesis actinomycin D. Prior to preparing total cell extracts at 6.5 h p.i., the cells were labeled for 1 h with [35S]Met, Cys in minimal essential medium containing 1% serum, pRb bands are labeled as described in Song et al. (2000).

FIG. 2. Effect of viral DNA replication on the phosphorylation of pRb protein during HSV infection. Following growth arrest induced by serum starvation, CV-1 cells were infected with an HSV-1 strain as indicated or mock infected as a control and overlaid with DMEM–10% serum. Total cell extracts, harvested at 0 or 10 h p.i., were examined for pRb by immunoblot analysis. Mock, mock-infected; KOS, wild-type HSV-1; KOS+PAA, infected with KOS in the presence of PAA, the inhibitor of viral DNA synthesis; and HD2, HSV-1 ICP8 mutant.

Wild-type HSV infection blocked the shift, and the majority of pRb remained as hypophosphorylated species, bands a and b' (Fig. 3, lane 3). However, an ICP27 mutant (5dl1.2) (Fig. 3, lane 4) failed to block the formation of the

FIG. 3. Phosphorylation pattern of the retinoblastoma protein (pRb) in cells infected with HSV-1 mutants with mutations in immediate-early genes. CV-1 cells were growth arrested by culturing in DMEM containing 1% calf serum for 48 h to synchronize them in G0 phase and infected with an HSV-1 strain as indicated or mock infected as a control. At 1 h p.i., all cells were overlaid with DMEM containing 10% serum. Total cell extracts were prepared at 0 and 10 h postinfection and processed for immunoblotting analysis for pRb protein, as described previously. Mock, mock-infected; KOS, wild-type HSV-1; 5dl1.2, HSV-1 ICP27 mutant; n212, HSV-1 ICP0 mutant; n12, HSV-1 ICP4 mutant; and n199, HSV-1 ICP22 mutant.
hyperphosphorylated pRb forms, bands c and d, while the ICP0 (n212), ICP4 (n12), and ICP22 (n199) mutants showed wild-type phenotypes (Fig. 3, lanes 5, 6, and 7).

To confirm that ICP27 was specifically required for the effects of HSV infection on pRb, we repaired the mutation of KOS1.1 d27-1 (Rice and Knipe, 1990), which has the same deletion as KOS 5d1.2 (McCarthy et al., 1989) (Fig. 4). We cotransfected the complementing V827 cells with d27-1 DNA and plasmid pPs27pd1 DNA, which contains the wild-type ICP27 gene (Fig. 4A). Progeny viruses were plated on Vero cells to select viral recombinants in which the deletion had been repaired by recombination. After three rounds of plaque purification on Vero cells, we confirmed the wild-type ICP27 locus in the rescued virus, d27-1R, by Southern hybridization (Fig. 4B) and examined pRb from the extracts from the cells infected with the wild-type, mutant, or rescued virus by immunoblot analysis (Fig. 4C). The ICP27-rescued virus, d27-1R, blocked the phosphorylation of pRb protein like the wild-type virus, indicating that ICP27 was required for blocking the phosphorylation of pRb in HSV-infected cells. These results also showed similar phenotypes with independent ICP27 mutants, 5d1.2 and d27-1, further confirming the role of ICP27 in regulating the phosphorylation of pRb protein in HSV-infected cells.

Cyclin D1 and cdk4 expression during infection with HSV-1 strains with mutations in immediate-early genes

D-type cyclin/cdk complexes play a major role in the phosphorylation of pRb (Sherr, 1994), which is required for the G1- to S-phase transition. To determine if viral immediate-early gene products (ICP0, ICP4, ICP22, and ICP27) exerted any effect on the induction of cyclin D1 and cdk4, we infected CV-1 cells with wild-type or mutant viruses and examined cyclin D1 and cdk4 from the cell extracts by immunoblotting (Figs. 5 and 6). As cells moved from G0 to G1 phase, the levels of cyclin D1 increased (Fig. 5A, lanes 1 to 4, and Fig. 5B, lanes 1 and 2). While the wild-type (KOS), ICP0 mutant (n212), and ICP22 mutant (n199) viruses were able to block the induction of cyclin D1 (Fig. 5B, lanes 3, 4, and 6), the ICP4

FIG. 4. Rescue of an ICP27 mutant virus and analysis of pRb protein in cells infected with the rescued virus. (A) Genome structure of wild-type HSV-1 strain KOS1.1 and the DNA regions of the plasmid pPs27pd1 and the ICP27-deletion mutant virus (d27-1) are depicted. (B) Southern blot analysis of the genome structures of the wild-type (KOS1.1), ICP27 mutant (d27-1), and rescued virus (d27-1R). The viral DNAs were digested with PstI, separated in a 0.8% agarose gel, and processed for Southern blot analysis using an ICP27 gene-specific probe, a 6.1-kb PstI fragment from pPs27pd1. A 6.1-kb PstI fragment was detected in the viral DNAs from KOS1.1 and d27-1R and a smaller fragment detected in the viral DNAs from d27-1 infection. (C) Immunoblot analysis for pRb protein. Total cell extracts were prepared from the infected cells at 11 h postinfection, separated on SDS–PAGE, and processed for immunoblot analysis for pRb protein, as described in the legend to Fig. 1.

FIG. 5. Cyclin D1 protein levels in cells infected with HSV-1 mutants with mutations in the viral immediate-early genes. (A) Following growth arrest induced by serum starvation, CV-1 cells were grown in DMEM containing 10% serum and total cell extracts were prepared at 0, 3, 6, 9, 12, and 15 h after the release from serum starvation, followed by immunoblot analysis for cyclin D1. (B) Following growth arrest induced by serum starvation, CV-1 cells were infected with the indicated HSV-1 strain or mock infected as a control and overlaid with DMEM–10% serum. Total cell extracts, harvested at 0 or 10 h p.i., were examined for cyclin D1 by immunoblot analysis. Mock, mock-infected; KOS, wild-type HSV-1; n212, HSV-1 ICP0 mutant; n12, HSV-1 ICP4 mutant; n199, HSV-1 ICP22 mutant; and 5d1.2, HSV-1 ICP27 mutant.
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FIG. 6. Cyclin-dependent kinase 4 (cdk4) protein levels in cells infected with HSV-1 mutants with mutations in the viral immediate-early genes. (A) Following growth arrest induced by serum starvation, CV-1 cells were grown in DMEM containing 10% serum and total cell extracts were prepared at 0, 3, 6, 9, 12, and 15 h after the release from serum starvation, followed by immunoblot analysis for cdk4. (B) Following growth arrest induced by serum starvation, CV-1 cells were infected with the indicated HSV-1 strain or mock infected as a control and overlaid with DMEM–10% serum. Total cell extracts, harvested at 0 or 10 h p.i., were examined for cdk4 by immunoblot analysis. Mock, mock-infected; KOS, wild-type HSV-1; n212, HSV-1 ICP0 mutant; n12 HSV-1 ICP4 mutant; n199, HSV-1 ICP22 mutant; and 5d1/12, HSV-1 ICP27 mutant.

(n12) and ICP27 (5d1/12) mutant viruses were at least partially defective for this effect (Fig. 5B, lanes 5 and 7).

As cells moved from G0 to G1 phase, the levels of cdk4 increased (Fig. 6A, lanes 1 to 4, and Fig. 6B, lanes 1 and 2). While the wild-type (KOS) and ICP22 mutant (n199) viruses were able to block the induction of cdk4 (Fig. 6B, lanes 3 and 6), the ICP0 (n212), ICP4 (n12), and ICP27 (5d1/12) mutant viruses were defective for blocking the induction (Fig. 6B, lanes 4, 5, and 7).

These results indicated that ICP27, ICP4, and ICP0 were required for blocking the induction of cyclin D1 and cdk4.

The effects of the ICP27 and vhs functions on the levels of cyclin D1 and cdk4 proteins were examined in the infected cells (Fig. 7). Wild-type HSV infection blocked the induction of cyclin D1 and cdk4 but the ICP27 mutant virus was not able to block the induction (Figs. 7A and 7B, lanes 3 and 4). The effect of vhs function on cyclin D1 and cdk4 proteins was slightly less significant than the ICP27 function (Figs. 7A and 7B, lanes 4 and 5). These results demonstrated that the effects of HSV infection on the G1 regulatory proteins cyclin D1 and cdk4 were not due entirely to the general host shutoff function of the virion protein vhs and indicate that viral immediate-early protein ICP27 also plays an essential role in this process.

ICP27 plays an essential role in blocking the infected cells in the G1 phase

To confirm the role for ICP27 in cell cycle arrest, we synchronized CV-1 cells by mitotic shake-off and monitored the progression of the cell cycle by incorporation of the thymidine analogue BrdU into DNA and detection of newly synthesized DNA using an immunofluorescence assay. Preliminary results showed that the midpoint of G1 phase was 6 h after the mitotic shake-off and the midpoint of S phase was 13.5 h after the shake-off (Yeh and Knipe, unpublished results). Infection of G1-phase cells with wild-type HSV (KOS1.1) in the presence of PAA, an inhibitor of viral DNA synthesis, caused a threefold reduction in the number of cells staining with anti-BrdU antibody, compared with mock infection (Fig. 8, left). In contrast, infection with d27-1 did not reduce the number of cells entering S phase (Fig. 8, left).

Once cells had moved into S phase, infection with wild-type or d27-1 virus did not alter the number of cells synthesizing DNA (Fig. 8, right). These results confirm the previous observation that HSV infection inhibits the cell cycle progression from G1 to S phase (de Bruyn Kops and Knipe, 1988; Ehmann et al., 2000; Song et al., 2000). These results together demonstrate that ICP27 is required for the inhibitory effect of HSV infection on cell cycle progression.

Possible mechanisms for the cell cycle block induced during HSV infection

To determine the viral gene products involved in regulating the expression of cyclin D1 mRNA, total cellular RNA was prepared from CV-1 cells infected with various HSV strains or from mock-infected cells as a control and processed for Northern blot analysis of cyclin D1 mRNA (Fig. 9). As cells moved from the G0 phase to G1 phase, the levels of cyclin D1 RNA increased (Fig. 9, lanes 1 and 2). Upon wild-type HSV infection, cyclin D1 RNA was significantly reduced to levels below that in G0 phase (Fig. 9, lane 3). In cells infected with the vhs mutant (Fig. 9, lane 4) or the ICP27 mutant (Fig. 9, lane 5), only a partial reduction in cyclin D1 mRNA was observed. These results indicated that both the vhs function and...
ICP27 contributed to blocking the induction of cyclin D1 RNA.

**DISCUSSION**

Several studies have shown that HSV infection inhibits G1-specific events and blocks the infected cells in the G1 phase (de Bruyn Kops and Knipe, 1988; Ehmann et al., 2000; Olgiate et al., 1999; Song et al., 2000). To determine what viral gene product(s) was involved in this process, we examined several mutant viruses with mutations in each of the immediate-early proteins, vhs function, and viral DNA replication. We observed that ICP0, ICP4, and ICP27 mutant viruses were defective for blocking the induction of cyclin D1 and cdk4 proteins. The ICP27 mutant virus was defective for blocking the induction of cyclin D1 mRNAs, for blocking the phosphorylation of pRb proteins, and for blocking cell cycle transition from G1 to S phase. Cyclin D1 and cdk4 are major regulatory proteins during G1 progression and form a holoenzyme complex with kinase activity. Cyclin D1/cdk4 kinase phosphorylates pRb, a necessary modification for progression of the cells to S phase. The lack of the essential components of the G1 cyclin/cdk holoenzyme complexes in the HSV-infected cells is consistent with the reduced phosphorylation of pRb and the cell-cycle block in G1 phase. However, the HSV-1 vhs mutant virus also failed to decrease levels of cyclin D1 mRNA to the same extent as the ICP27 mutant virus, but the vhs mutant virus blocked pRb phosphorylation similar to wt virus. Therefore, while host shutoff may play a role in blocking G1 events, ICP27 appears to have a distinct function that is necessary for blocking or decreasing pRb phosphorylation.

**Multiple viral functions are involved in regulating the expression of G1 proteins**

Phosphorylation of pRb in the ICP27-mutant-infected cells was not equivalent to that in mock-infected cells.
This suggests that other viral proteins may be involved in blocking the phosphorylation of pRb. Indeed, the ICP4 mutant was partially defective for blocking the phosphorylation of pRb proteins, suggesting the role of the ICP4 protein. Furthermore, several viral immediate-early gene products including ICP0, ICP4, and ICP27 and virion protein vhs were involved in inhibiting the expression of cyclin D1 and cdk4 proteins, which are important G1 regulators responsible for the phosphorylation of pRb. However, vhs, viral DNA replication, and possibly true late gene functions were not required for blocking the phosphorylation of pRb in HSV-infected cells.

When we examined the expression of cyclin D1 mRNA in HSV-infected cells, the wild-type virus blocked the induction of cyclin D1 RNA even below the level in the mock-infected cells at the G0 phase. In the cells infected with the vhs mutant or the ICP27 mutant, expression of cyclin D1 RNA was partially restored. These results indicate that both vhs and ICP27 functions contributed to blocking the expression of cyclin D1 RNA in HSV-infected cells. The effects of vhs and ICP27 mutations on the levels of cyclin D1 RNA and protein are consistent with the idea that vhs degrades RNA in the cytoplasm right after infection and shuts off the synthesis of host proteins (Fenwick and McMenamin, 1984; Read and Frenkel, 1983; Schek and Bachenheimer, 1985; Strom and Frenkel, 1987) and that ICP27 inhibits host cell RNA splicing (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994) and/or host cell transcription (Long et al., 1999; Rice et al., 1994, 1995; Spencer et al., 1997). These results argue that both vhs and ICP27 are required for viral inhibition of host gene expression and suggest that both are required for viral inhibition of host cell responses to viral infection.

Potential mechanisms for the cell cycle block induced during HSV infection

It is likely that HSV affects the host cell cycle in multiple ways through its effects on several cellular events such as transcription, splicing, and regulation of protein stability. The shift in transcription from cellular to viral genomes may involve several mechanisms such as modifications to the RNA polymerase II transcription machinery, alterations in DNA conformation, or localization of viral genes in specialized nuclear compartments. It has been reported that HSV represses host RNA polymerase II transcription (Spencer et al., 1997) and that HSV immediate-early protein ICP22 is required for virus-induced modification of host RNA polymerase II (Long et al., 1999; Rice et al., 1994, 1995). Also, it has been reported that the viral immediate-early protein ICP4 interacts with the TATA box binding protein, TFIIB, and TAF250 (Carrozza and DeLuca, 1996; Smith et al., 1993). Finally, it is known that HSV immediate-early protein ICP27 inhibits host cell RNA splicing (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994) and is required for inhibition of host gene transcription (Spencer et al., 1997). These effects could contribute to the viral inhibition of G1 gene expression and block in the G1 stage of the cell cycle.

Although the role of ICP27 in blocking expression of cyclin D and cdk4 is consistent with blocking progression of G1, vhs exerts a similar effect on cyclin D mRNA levels without blocking phosphorylation of pRb. Thus, it appears that ICP27 exerts an effect on progression of the cell cycle in addition to blocking induction of expression of these two gene products. These results are consistent with the observation that the human cytomegalovirus ICP27 homolog UL69 can block the cell cycle in G1 phase (Lu and Shenk, 1999).

There are several possible mechanisms by which ICP27 could otherwise affect progression of the cell cycle and phosphorylation of pRb. First, it could inhibit the expression of cell cycle gene products other than those assayed in this work. Second, it could inhibit the activity of kinases necessary for cell cycle progression and pRb phosphorylation. In this regard, ICP27 has been shown to interact with casein kinase II (Wadd et al., 1999). Third, ICP27 could stimulate the activity of phosphatases so that pRb or other molecules are more readily dephosphorylated. ICP27 has been shown to decrease or alter the phosphorylation of ICP4 (Rice and Knipe, 1988; Su and Knipe, 1989; Xia et al., 1996) and the splicing factor SC35 (Sandri-Goldin and Hibbard, 1996). Further studies are needed to determine the portions of ICP27 needed for this effect and the biochemical activities associated with that domain.

MATERIALS AND METHODS

Viruses and cells

The parental HSV-1 wild-type strains KOS and KOS1.1 were obtained from P. Schaffer and M. Levine, respectively. The ICP0 mutant (n212), ICP4 mutant (n12), ICP22 mutant (n199), and ICP27 mutant (5d/1.2) viruses were all derived from KOS and were provided by P. Schaffer (Beth Israel–Deaconess Hospital, Boston, MA). The ICP27 mutant (d27-1), derived from KOS1.1, was described previously (Rice and Knipe, 1990). The virion host shutoff mutant (vhs-1) was derived from KOS and was provided by S. Read (Read and Frenkel, 1983). The ICP8 mutant (HD2), derived from KOS1.1, was described previously (Gao and Knipe, 1989). 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 infected at an m.o.i. of 20. Cells were growth arrested by incubation in 199 medium containing 1% calf serum for 24 to 48 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

The antibodies used for immunoblotting pRb, cyclin D1, and cdk4 were as follows. Mouse anti-pRb monoclonal antibody (PharMingen 14001A) was used at a 1:1000 dilution. Mouse anti-cyclin D1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; sc-8396) was used at a 1:500 dilution. Rabbit anti-cdk4 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; sc-8396) was used at a 1:1000 dilution.

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) bromophenol blue]. Proteins in the cell lysate were resolved by SDS–PAGE according to the procedures of the manufacturer (Bio-Rad). Expression of pRb, cyclin D1, and cdk4 was monitored by probing the blots with the primary antibodies described earlier, followed by horse-radish peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology) and enhanced chemiluminescence (Amersham).

Rescue of the ICP27 mutant virus d27-1

ICP8- and ICP27-complementing V827 cells (Da Costa et al., 2001) were cotransfected with 1 μg of d27-1 viral DNA and 1 μg of PstI-digested plasmid pPs27pd1 (Rice et al., 1989), which contains the wild-type ICP27 gene, using Lipofectamine (Life Technologies 18324-012) according to the procedures of the manufacturer. At 4 days posttransfection, the transfected cells were harvested following the formation of plaques. Progeny viruses were plated on Vero cells to select wt recombinants. After three rounds of plaque purification on Vero cells, a viral stock of the rescued virus, d27-1R, was made for further analysis. To confirm the genome structure of the rescued virus, viral DNAs were prepared from the infected cells using the DNeasy Tissue Kit (Qiagen 69504) according to the procedures of the manufacturer, digested with PstI, and processed for Southern blot analysis using an ICP27 gene-specific probe. The probe was labeled with [32P]dCTP using the RadPrime DNA Labeling System (Life Technologies).

35S labeling of proteins

CV-1 cells in a 25-mm2 flask were mock infected or infected with HSV and incubated in 2 ml of medium containing 100 μCi of Tran35S-Label (ICN 51006, 1175 Ci/mmol) containing [35S]methionine (70%) and [35S]cysteine (15%) for 1 h from 5.5 to 6.5 h p.i. Then, the cell lysates were separated on SDS–PAGE and processed for autoradiography.

BrdU labeling of the cells synchronized by mitotic shake-off

CV-1 cells were synchronized by mitotic shake-off and plated in DMEM–10% fetal calf serum. Virus infection or mock infection was initiated in the G1 phase, 6.5 h after plating, or in the S phase, 13.5 h after plating. Cells were mock infected or infected with the wild-type (KOS1.1) or the ICP27 mutant (d27) in the presence of sodium phosphonoacetate (400 μg/ml) for 5 h. Cells were then labeled with BrdU for 15 min and immunostained with anti-BrdU antibody.

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