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Analysis of cyclin-dependent kinase activity after herpes simplex virus type 2 infection

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Small DNA viruses (adenoviruses, simian virus 40, or human papillomaviruses) induce S-phase progression but prevent cell division to provide precursors for viral DNA replication. Herpes simplex viruses types 1 or 2 (HSV-1 or HSV-2) contain genes which encode DNA-metabolizing enzymes, for example, ribonucleotide reductase, thymidine kinase and dUTPase, suggesting that S-phase factors are not required for an efficient infection. However, several studies indicated that HSV induces some events that occur during cell-cycle progression. To determine if HSV-2 induces S-phase entry, we examined serum-arrested African green monkey kidney cells (CV-1) after infection. Two hours after infection steady-state levels of the S-phase-specific cyclin, cyclin A, increased. S-phase cyclin-dependent kinase activity (CDK2) was stimulated 10-fold 8 h after infection but decreased at 16 or 24 h after infection. Mitotic CDK activity (CDC2) was not activated after infection, in part due to decreases in CDC2 protein levels and inactivation of enzymatic activity resulting from tyrosine phosphorylation of CDC2. Furthermore, CDK4 activity was not dramatically affected by infection. These studies indicate that HSV-2 infection selectively activates CDK2 after infection but cell-cycle progression does not occur. We hypothesize that infection activates certain components of the cell cycle which enhance viral gene expression and DNA replication.

Introduction

Herpes simplex virus (HSV) gene expression can be divided into three distinct classes: immediate early (IE), early (E) and late (L) (reviewed by Hayward, 1993; Roizman & Sears, 1990). IE RNA expression is detected at 2 h post-infection (p.i.), does not require protein synthesis, and is stimulated by a virion component, VP16 or α-TIF (reviewed by O'Hare, 1993). VP16, the octamer transcription factor 1 (OTF-1), and at least one other cellular factor bind to a consensus TAATGARAT/OTF-1 motif, thus activating IE RNA expression. Each IE promoter contains one or more copies of a TAATGARAT/OTF-1 motif. E RNA expression is detected within 4 h p.i. and is dependent on at least one IE protein (reviewed by Hayward, 1993; Roizman & Sears, 1990). In general, E RNAs encode non-structural proteins which play a role in viral DNA synthesis. L RNA expression is maximal after viral DNA synthesis and the mRNA species generally encode structural proteins.

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Several independent studies have concluded that HSV-1 or HSV-2 can induce events which correlate with cell-cycle progression. For example, HSV-1 induces S-phase forms of E2F (Hilton *et al.*, 1995), a transcription factor which promotes cell-cycle progression (Wang *et al.*, 1994; Weinberg, 1995). Viral genes encoding ICP4, ICP27, the single-stranded DNA-binding protein necessary for DNA replication (UL29 or ICP8), DNA polymerase (UL30), or helicase (UL5) are necessary for induction of E2F (Hilton *et al.*, 1995). Mutations in the ICP0 (Cai & Schaffer, 1991) or VP16 gene (Daksis & Preston, 1992) of HSV-1 are complemented by cellular factors in the G_1 or S phase of the cell cycle. Promoters of IE genes are activated by cellular factors when growth arrest is released (Ralph *et al.*, 1994). HSV infection causes unscheduled DNA replication (Kulomaa *et al.*, 1992), amplification of integrated simian virus 40 (SV40) DNA (Heilbronn & zur Hausen, 1989), and can rescue non-autonomous parvoviruses which require S phase for growth (Berns, 1990). Six HSV-1-encoded genes are involved in amplification of integrated DNA: UL5, UL8, UL52 (components of the viral helicase–primase complex which are essential for viral DNA replication), UL29, UL30 and UL42 (subunits of DNA polymerase) (Heilbronn & zur Hausen, 1989). HSV-1 infection also alters the nuclear localization of Rb

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(Wilcock & Lane, 1991) and DNA replication complexes or pre-replicative site structures (de Bruyn Kops & Knipe, 1988). Rb is a tumour suppressor gene which regulates cell-cycle progression (reviewed by Wang *et al.*, 1994; Weinberg, 1995). Two recent studies have concluded that UL5, UL8, UL9, UL29, UL30, UL42 and UL52 are necessary for formation of pre-replicative site structures (Liptak *et al.*, 1996; Lukonis & Weller, 1996). Thus, it is reasonable to hypothesize that the ability of HSV to induce cell-cycle regulatory factors plays a role in regulating virus transcription and DNA replication.

Cell-cycle progression is regulated by two families of proteins, cyclins and cyclin-dependent kinases (CDK) (reviewed by Heichman & Roberts, 1994; Nurse, 1994; Sherr, 1994). The interaction of specific cyclins with CDK partners results in active protein kinases, phosphorylation of cell cycle-specific targets, and cell-cycle progression. For G_1 cell-cycle progression to occur, D-type cyclins (D1, D2, D3) assemble into holoenzymes with CDK4 or CDK6. Late in the G_1 phase, cyclin E binds to CDK2 and it appears CDK2/cyclin E activates origin of replication recognition factors, consequently DNA synthesis is initiated. Although overexpression of D- or E-type cyclins contracts G_1, decreases cell size and reduces the requirements for mitogenic stimuli, their functions are likely to be quite unique. Upon commitment to S phase, cyclin A and CDK2 complexes are detected. Cyclin A is associated with replicating DNA and is required for S-phase entry and progression (Cardoso *et al.*, 1993; Pagano *et al.*, 1992). During the G_2 and M phases of the cell cycle, mitotic CDK activity (cyclin A/CDC2 or cyclin B/CDC2 complexes) form the mitotic promoting factor (MPF). Although many of the substrates for CDK/cyclins have not been identified, Rb is known to be phosphorylated by CDK4/D-type cyclins and CDK2/cyclin A (Resnitzky & Reed, 1995; Resnitzky *et al.*, 1995). Numerous CDK/cyclin phosphorylation sites are located on Rb, and under-phosphorylated Rb represses growth.

In this study, we examined the effects that HSV-2 has on Rb phosphorylation, CDK activity, CDK levels, or cyclin levels after infection. CDK2 activity, but not CDK4 activity, increased following infection with HSV-2. In contrast to infection, serum stimulation led to prolonged activation of CDK2. Expression of cyclin A was induced 2 h after infection or serum stimulation. Cyclin B-associated kinase activity and CDC2 levels were slightly repressed by infection but were activated following serum stimulation. Taken together, the results indicate that HSV-2 induced phosphorylation of Rb and that CDK2 activation played a role in this process.

Methods

■ **Virus and cells.** Growth and maintenance of African green monkey kidney cells (CV-1 cells) were described previously (Hanson *et al.*, 1994). Cells were infected with HSV-2 (strain MS) as described previously (Hanson *et al.*, 1994). Stock virus was grown in confluent CV-1 cells in 5% FBS for 3 days. Confluent monolayers were subsequently infected with 0·001 p.f.u./cell. Seven days after infection cells were scraped, virus was released by three cycles of freezing–thawing (−80 °C to 37 °C), and cellular debris was pelleted by centrifugation (15 000 ***g***, 30 min).

Cell monolayers were trypsinized and 5×10^5 cells seeded in a 100 mM dish. The cells were incubated with EMEM containing 0·4% FBS for 72 h prior to infection with HSV-2 (2 p.f.u./cell). After 72 h, media were removed and designated 'spent media'. Virus stocks were diluted 10-fold (HSV-2) or in 0·4% spent medium. Control samples (C) were mock-infected with supernatants (1:10 dilution in spent media) which were prepared from mock-infected cells that were cultured for 7 days.

■ **Preparation of cell lysates and Western blot analysis.** Cell lysates were prepared for immunoprecipitation and CDK activity was performed as described previously (Beijersbergen *et al.*, 1995) using histone H1 as a substrate. Cell monolayers were washed once with PBS and cells were collected with a cell scraper in 500 µl of lysis buffer (20 mM HEPES pH 7·9, 0·4 M NaCl, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, 25%, w/v, glycerol). The cells were kept on ice for 20 min and lysed by two freeze–thaw cycles (−80 °C to 4 °C). After centrifugation (10 min, 15 000 r.p.m., 4 °C), the supernatant was stored at −80 °C. Cell lysates and Western blots were prepared as described before (Hossain *et al.*, 1995). Rabbit polyclonal antibodies (Santa Cruz Biotechnology) directed against CDK2 (#sc-163) or CDK4 (#sc-260) were used. Mouse monoclonal antibodies from Santa Cruz Biotechnology used for these studies were directed against: cyclin A (#sc-239), cyclin B1 (#sc-245) and CDC2 (#sc-54). The antibody which recognized the tyrosine-phosphorylated forms of CDC2 was purchased from New England Biolabs. Phosphorylated and unphosphorylated Rb were separated in a 6% polyacrylamide gel. A monoclonal antibody (#14001A; Pharmingen) was used for detection of Rb. Secondary antibody was either anti-rabbit or anti-mouse immunoglobulin whole antibody linked to horseradish peroxidase (Amersham Life Sciences). Immunodetection was carried out by the enhanced chemiluminescence system (ECL, Amersham Life Sciences). Western blots and immunoprecipitation were conducted as described previously (Schang *et al.*, 1996).

■ **Cyclin-dependent kinase activity.** Cell lysates (100–150 µg) were used to measure CDK activity following immunoprecipitation with the respective antibodies and histone H1 (2·5 µg) was the substrate (Resnitzky & Reed, 1995). For measuring CDK4 activity, Rb (2 µg; Santa Cruz Biotechnology) was used as a substrate because histone H1 is not a reliable substrate for CDK4 (Matsushime *et al.*, 1994). Cell lysates (precleared with normal rabbit serum) were incubated with the anti-CDK or cyclin antibody for 18 h at 4 °C. Immune complexes were then precipitated with Protein A–Sepharose (Sigma) and washed three times with kinase wash buffer (10 mM Tris–HCl pH 8·0, 50 mM NaCl, 1 mM EDTA and 0·5% NP40) and once with kinase buffer (50 mM Tris–HCl pH 8·0, 25 mM magnesium acetate, 2·5 mM EDTA). All washes were performed at 4 °C. The final pellet was suspended in 25 µl kinase buffer supplemented with 10 µCi [γ-^{32}P]ATP and 0·1 M ATP. After incubation for 20 min at 30 °C, the reaction was stopped by the addition of SDS–PAGE sample buffer and boiling for 5 min. Phosphorylated histone H1 was visualized by autoradiography and then quantified by a Phosphorimager (Molecular Dynamics) after separation on 10% SDS–PAGE.

■ **Flow cytometry analysis of infected CV-1 cells.** Cells were fixed with 70% ethanol on ice for 1 h. Before flow cytometry analysis, the cells were pelleted, washed once in PBS and incubated with 20 µg/ml propidium iodide containing 200 µg/ml RNAse. Flow cytometry was performed on a Becton-Dickinson FACScan machine as described previously (Schang *et al.*, 1996; Wang *et al.*, 1996). The intensity of propidium iodide staining was analysed on cell populations that were

positive for FITC staining to determine the DNA content. Cell-cycle profiles were generated using the CellFit cell-cycle analysis software.

Results

Analysis of Rb phosphorylation

To test whether infection influenced phosphorylation of Rb, serum-arrested CV-1 cells were infected with HSV-2 and Rb phosphorylation was assessed by Western blot analysis. The Rb protein contains 16 potential CDK phosphorylation sites and progressive phosphorylation of Rb correlates with cell-cycle progression (reviewed by Wang *et al.*, 1994; Weinberg, 1995). It is well established that phosphorylation of Rb leads to slower migrating forms of the protein which can be separated on SDS–polyacrylamide gels (Resnitzky & Reed, 1995; Resnitzky *et al.*, 1995; Wang *et al.*, 1994; Weinberg, 1995), making it possible to discern the various forms of Rb. CV-1 cells were used for these studies because they are contact inhibited, do not form tumours in *nu/nu* mice, have a functional *Rb* gene, and their growth is arrested if serum is withdrawn. After CV-1 cells were incubated in low serum for 72 h, more than 80% of the cells were in G_1 (Table 1) and the majority of the Rb protein migrated as the faster underphosphorylated form (Fig. 1A, lane C). After serum stimulation, most of the Rb protein migrated as the slower migrating phosphorylated form (Fig. 1A, lane S). Two hours after serum stimulation nearly all of the Rb migrated as heavily phosphorylated Rb, which is indicative of cell-cycle progression (ppRb; Fig. 1A), and by 24 h after serum stimulation more than 60% of the cells were in S or G_2/M (Table 1). At 4, 8, or 16 h p.i., most of the Rb migrated in a similar position as heavily phosphorylated Rb (Fig. 1A). In some experiments, we observed a shift in the mobility of Rb as soon as 2 h p.i. (data not shown).

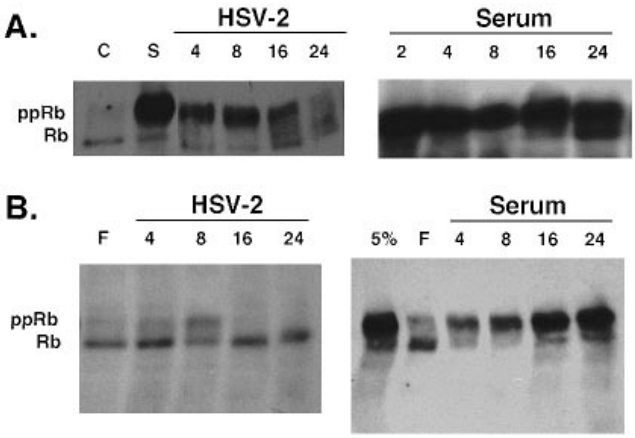

Fig. 1. Analysis of Rb phosphorylated isoforms after serum stimulation or infection. Whole cell lysates for Western blots were prepared as described in Methods. Fifty μg protein was electrophoresed in 6% SDS–PAGE and Western blots were performed. An antibody which recognizes the various phosphorylated forms of Rb was used to detect underphosphorylated Rb or phosphorylated Rb (ppRb). (A) CV-1 cells were arrested in 0·4% serum as described in Methods for 72 h and then infected with HSV-2 (2 p.f.u./cell) or fresh medium containing 5% FBS. (B) CV-1 cells were treated with FB1 (5 μM) and 0·4% serum for 72 h prior to infection (2 p.f.u./cell). The lane marked F contains cells prior to serum addition (Serum panel) or mock-infected (HSV-2 panel). The lane marked 5% contains CV-1 cells plated in 5% FBS and allowed to grow for 48 h.

Table 1. Cell-cycle distribution of serum-arrested CV-1 cells after infection with HSV-2 or serum stimulation

CV-1 cells were growth arrested in 0·4% FBS for 72 h and treated with media containing 10% FBS (serum) or infected with HSV-2 (2 p.f.u./cell). As controls, serum-arrested cells were mock-infected with media prepared from uninfected cells as described in Methods. Some cultures were treated with phosphonoacetic acid (PAA; 400 μg/ml) at the time of infection. This treatment blocked more than 90% of viral DNA replication but only inhibited cellular DNA synthesis by 15% in uninfected cells (data not shown). At 8, 16, or 24 h after infection or serum stimulation, cells were prepared for FACS analysis and at least 10000 cells were analysed for each sample. FACS was performed as described previously (Wang *et al.*, 1996; Schang *et al.*, 1996) and the values are presented as the percentage of the total.

	Experiment 1			Experiment 2			Experiment 3		
	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M
Mock-infected	81	6	13	85	8	7	82	9	9
Infected 8 h p.i.	82	7	11	83	10	7	79	12	9
Infected 16 h p.i.	73	19	8	74	16	10	77	18	5
Infected 24 h p.i.	42	46	12	38	48	14	44	43	13
PAA 8 h p.i.	79	10	11	83	9	8	80	11	9
PAA 16 h p.i.	84	9	7	78	14	8	82	12	6
PAA 24 h p.i.	82	14	4	81	11	8	78	15	7
8 h serum	78	16	6	81	16	3	83	9	8
16 h serum	63	25	12	54	27	19	49	35	16
24 h serum	30	48	22	39	40	21	23	46	31

The ability of infection to induce phosphorylation of Rb was also analysed after CV-1 cells were treated with the mycotoxin fumonisin B1 (FB1). FB1 is a sphingolipid homologue which can arrest CV-1 cells in G_1 and also leads to Rb dephosphorylation (Wang *et al.*, 1996; J. Ciacci-Zanella, A. H. Merril Jr, E. Wang & C. Jones, unpublished results; Fig. 1B). When cells were treated with FB1 (5 μM) for 48 h and then infected with HSV-2, the phosphorylated isoforms of Rb increased at 8 h p.i. At 16 or 24 h p.i., Rb migrated as unphosphorylated Rb, suggesting that phosphorylation of Rb was transient after FB1-treated cells were infected with HSV-2. Infectious virus was released from FB1-treated cells indicating that a productive infection occurred (J. Ciacci-Zanella & C. Jones, unpublished results). In summary, both studies indicated that infection with HSV-2 led to phosphorylation of Rb.

Since infection induced phosphorylation of Rb and phosphorylation of Rb is indicative of cell-cycle progression (reviewed in Wang *et al.*, 1994; Weinberg, 1995), we examined whether infection induced cell-cycle progression. This topic is somewhat controversial because one study concluded that HSV-2 induced unscheduled DNA synthesis of cervical cancer cells, in part due to S-phase entry (Kulomaa *et al.*, 1992). In contrast, infection of CV-1 cells with HSV-1 strain KOS (20 p.f.u./cell) did not lead to enhanced progression from G_1 to S (de Bruyn Kops & Knipe, 1988). Although infected CV-1 cells contained a high proportion of cells which appeared to be in S phase 24 h after infection, addition of phosphonoacetic acid (PAA) to cells at the time of infection eliminated the increase in DNA content. In CV-1 cells, we have estimated that 5×10^9 p.f.u. are produced in 1×10^6 CV-1 cells, indicating that approximately 5000 viral genomes can be present in an infected cell. Since the HSV-2 genome is 1×10^5 base pairs, approximately 5×10^8 base pairs of viral DNA would be present in an infected cell. The increase in propidium iodide staining resulting from viral DNA is close to cellular DNA (1×10^9 base pairs) given the preference for propidium iodide to intercalate into G + C-rich DNA (Reinhardt & Krugh, 1978). As judged by [^{3}H]thymidine incorporation, viral DNA synthesis was detected at 4 h p.i. and it peaked at 8 h p.i. (data not shown). Although extensive cytopathic effect was apparent at 16 and 24 h p.i., high levels of the large subunit of ribonucleotide reductase were detected at 17 h p.i. when CV-1 cells were infected with 5 p.f.u./cell (Hanson *et al.*, 1994). As judged by dye exclusion, infected cells were still alive at 16 and 24 h p.i. (data not shown). In summary, this study confirms a previous study (de Bruyn Kops & Knipe, 1988) which concluded that infection did not induce true S-phase progression and that semi-conservative replication of cellular chromosomes does not occur after infection.

Analysis of CDK4 in infected cells

CDK2/cyclin A complexes (Resnitzky *et al.*, 1995) or CDK4/D-type cyclins (Resnitzky & Reed, 1995) phosphorylate Rb during cell-cycle progression. CDK4 plays a crucial role in G_1 cell-cycle progression (Sherr, 1994) and thus its activity was examined following infection. No dramatic changes in CDK4 activity were observed following infection of serum-arrested CV-1 cells even though CDK4 levels increased at 16 and 24 h p.i. (Fig. 2A, B). In contrast, there was an obvious decline and then an increase in CDK4 activity following serum stimulation. We have not found dramatic changes in D-type cyclin-associated CDK activity or levels of D-type cyclins following infection (data not shown), which was expected because D-type cyclins bind to CDK4 (Sherr, 1994).

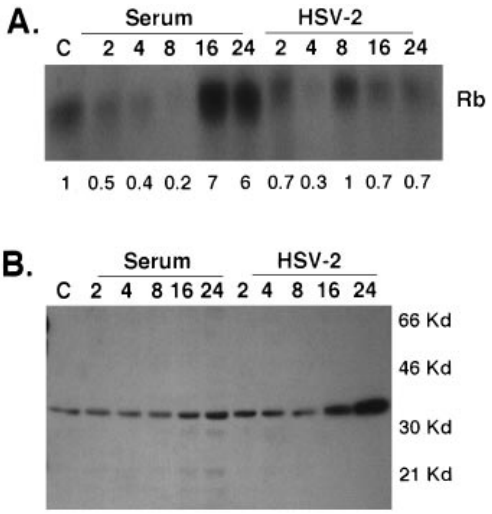

Fig. 2. Analysis of CDK4 following HSV-2 infection or serum stimulation. (A) CDK4-associated kinase activity was measured after immunoprecipitation (100 μg protein) with a CDK4 antibody as described in Methods. Rb protein (2 μg) was added to the immunoprecipitates. Samples were electrophoresed in 10% SDS–PAGE, then the gel was dried and autoradiographed. The amount of phosphorylated Rb was measured with a Phosphorimager (Molecular Dynamics). The value in the control sample (C) was designated 1 and all other values were normalized with respect to the control. The results are representative of three independent experiments. (B) Whole cell lysates for Western blots were prepared as described in Methods. Fifty μg protein was electrophoresed in 10% SDS–PAGE and Western blots were performed as described previously (Hossain *et al.*, 1995).

Analysis of CDK2 after HSV-2 infection or serum stimulation

CDK2 activity was more than 10-fold higher at 8 h p.i. but decreased at 16 and 24 h p.i. (Fig. 3A). We have also consistently detected three- or fourfold higher levels at 2–4 h p.i., suggesting activation of CDK2 is an early event after infection. CDK2 activity increased as a function of time after serum stimulation and was more than 30-fold higher after serum stimulation. In serum-arrested cells, a single band migrating with a molecular mass near 35 kDa was recognized by an antibody directed against CDK2 and the steady-state levels of CDK2 were not higher after infection (Fig. 3B). At 16 and 24 h after serum stimulation, three additional bands were

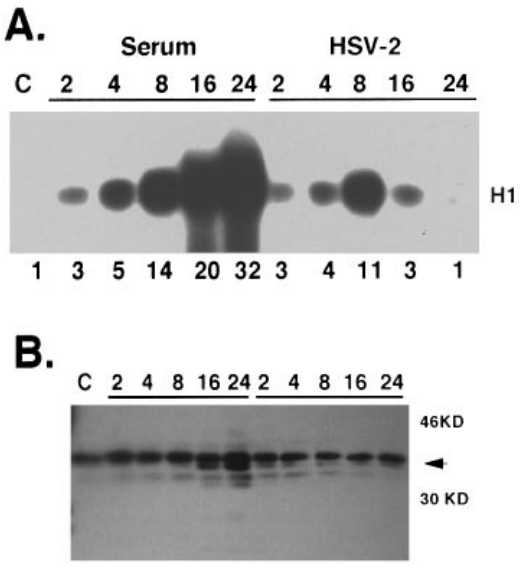

Fig. 3. Analysis of CDK2 following HSV-2 infection or serum stimulation. (A) CDK2-associated kinase activity was measured after immunoprecipitation (100 μg protein) with a CDK2 antibody as described in Methods. Histone 1 (2 μg) was added to the immunoprecipitates. Samples were electrophoresed in 10% SDS–PAGE, then the gel was dried and autoradiographed. The amount of phosphorylated histone 1 was measured with a Phosphorimager (Molecular Dynamics). The value in the control sample (C) was designated 1 and all other values were normalized with respect to the control. The results are representative of at least two determinations from three independent experiments. (B) Whole cell lysates for Western blots were prepared as described in Methods. Fifty μg protein was electrophoresed in 10% SDS–PAGE and Western blots were performed as described previously (Hossain *et al*., 1995).

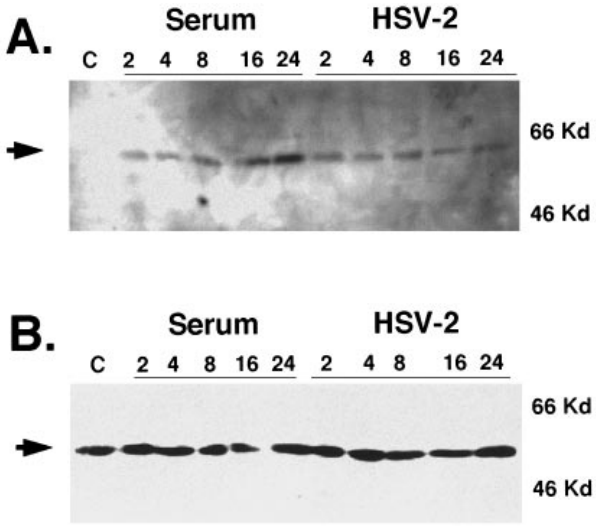

Fig. 4. Analysis of cyclin A and cyclin E levels following infection or serum stimulation. CV-1 cells were serum-arrested and infected with HSV-2 (2 p.f.u./cell) as described in Methods. Whole cell lysates for Western blots were prepared as described in Methods. Fifty μg protein was electrophoresed in 10% SDS–PAGE and Western blots were performed as described previously (Hossain *et al*., 1995). Panel A was incubated with a monoclonal antibody directed against cyclin A, panel B was incubated with a monoclonal antibody directed against cyclin E.

readily detected by the CDK2 antibody. Three phosphorylation sites exist within CDKs and phosphorylation of CDK2 leads to faster migrating species (Gu *et al*., 1992) suggesting the additional bands were phosphorylated forms of CDK2. Faster migrating forms of CDK2 are catalytically active and are only found in S or G_2 (Gu *et al*., 1992). The presence of faster migrating bands which were recognized by the CDK2 antibody suggested that CDK2 was phosphorylated after infection.

Since cyclins A or E bind CDK2 (reviewed by Heichman & Roberts, 1994), the levels of these cyclins were measured after infection and compared to serum-stimulated CV-1 cells. Cyclin A was not readily detected in serum-arrested cells but was induced by infection or serum stimulation (Fig. 4A). We have not observed a dramatic increase in cyclin E levels after serum stimulation or infection (Fig. 4B). In summary, these studies indicated that infection of serum-arrested CV-1 cells transiently stimulated CDK2 activity. In contrast, CDK2 levels steadily increased following serum stimulation and activity was more than 30-fold higher 24 h after serum stimulation.

Analysis of CDC2 and cyclin B in infected cells

CDC2 kinase activity and cyclin B comprise the MPFs which are required for G_2 and mitosis (Nurse, 1994). Since cyclin B primarily interacts with CDC2 but CDC2 can also bind cyclin A, cyclin B-associated kinase activity was used as a measure of MPF activity after infection. Cyclin B-associated kinase activity was three- to ninefold higher at 16 or 24 h after serum stimulation (Fig. 5A). However, cyclin B-associated kinase activity was not dramatically stimulated after infection. Steady-state levels of cyclin B did not decrease after infection (Fig. 5B) but CDC2 levels were lower than controls (Fig. 5C). Western blots contained faint bands which migrated slower than CDC2 late after infection (Fig. 5C). Slower migrating forms of CDC2 are the result of phosphorylation and they accumulate in S or G_2 but not M (Gu *et al*., 1992), suggesting the minor bands in infected cells were phosphorylated forms of CDC2.

It is well established that CDC2 contains a single tyrosine residue at position 15 which if phosphorylated inactivates kinase activity (Norbury *et al*., 1991). After G_2/M is completed, phosphorylation of the CDC2 tyrosine residue occurs. In G_2/M, CDC2 has low levels of phosphorylated tyrosine residues and this contributes to its increased activity. In serum-arrested cells, an antibody which recognizes CDC2 with a phosphorylated tyrosine residue detected a single band which migrated with an apparent molecular mass near 35 kDa (Fig. 5D). Following serum stimulation, the band disappeared but reappeared at 16 and 24 h after serum stimulation, confirming published results (Norbury *et al*., 1991) that tyrosine residues on CDC2 were dephosphorylated as cells progressed through G_2/M and re-entered G_1. The finding that MPF activity increased at 16 and 24 h after serum stimulation was expected because more cells were in G_2/M (Table 1). After infection, the

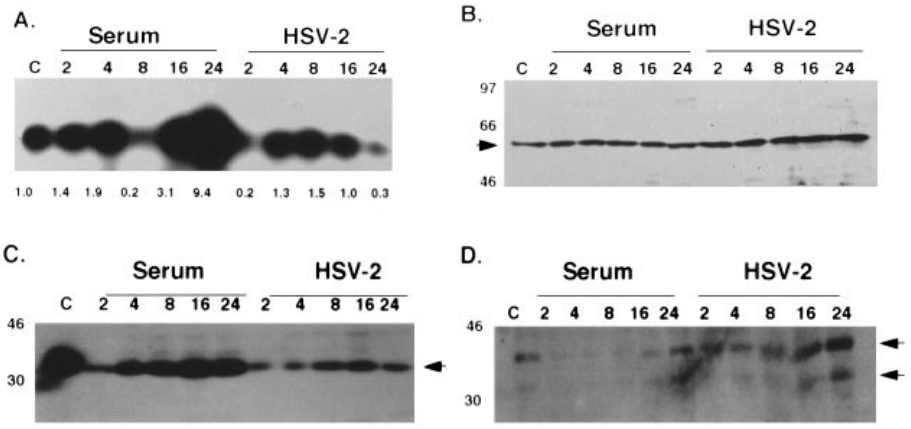

Fig. 5. Analysis of MPF after infection or serum stimulation. CV-1 cells were serum arrested and infected with HSV-2 (2 p.f.u./cell) as described in Methods. (A) Cyclin B-associated kinase activity was measured after immunoprecipitation (100 μg protein) with a cyclin B antibody as described in Methods. Histone 1 (2 μg) was added to the immunoprecipitates. Samples were electrophoresed in 10% SDS–PAGE, then the gel was dried and autoradiographed. The amount of phosphorylated histone 1 was measured with a Phosphorimager (Molecular Dynamics). The value in the control sample (C) was designated 1 and all other values were normalized with respect to the control. The results are representative of at least two determinations from three independent experiments. (B–D) Whole cell lysates for Western blots were prepared as described in Methods. Fifty μg protein was electrophoresed in 10% SDS–PAGE and Western blots were performed with an antibody which recognizes cyclin B (B), CDC2 (C), or CDC2 with a phosphorylated tyrosine (D). Arrows indicated the various isoforms of CDC2.

tyrosine-phosphorylated band was not reduced. Furthermore, the intensity of the 35 kDa band increased at 16 and 24 h p.i. and a 32 kDa band was detected suggesting that tyrosine phosphorylation of CDC2 increased during the late stages of infection. In summary, these results demonstrated that dephosphorylation of the tyrosine residue on CDC2 was inhibited after infection.

Discussion

In this study, we have provided evidence that HSV-2 infection led to phosphorylation of Rb and transient activation of CDK2 activity. In contrast, CDK4 or CDC2 was not activated and cell-cycle progression did not occur, demonstrating that certain cell-cycle regulators were stimulated. We hypothesize that selective activation of cell-cycle components facilitates viral gene expression and DNA replication in differentiated cells.

The observation that HSV-2 infection induced cyclin A protein expression at 2 h p.i. (Fig. 4) is important because cyclin A is required for S-phase entry and colocalizes with replication forks (Cardoso *et al.*, 1993; Resnitzky & Reed, 1995; Pagano *et al.*, 1992). Although infection induced CDK2 activity (Fig. 3), the kinetics of this process were different compared to serum stimulation. For example, CDK2 activity steadily increased for 24 h following serum stimulation but peaked at 8 h p.i. CDK2/cyclin A complexes are known to phosphorylate Rb directly (Resnitzky *et al.*, 1995), suggesting stimulation of CDK2 activity was important for Rb phosphorylation.

After infection, tyrosine dephosphorylation of CDC2 levels did not occur and MPF was not activated relative to serum stimulation (Fig. 5). These results are consistent with a block in cell-cycle progression following infection and support our conclusions that infection leads to selective activation of certain cell-cycle factors. CDK2 activates CDC2/cyclin B kinase activity and consequently is required for mitosis (Guadagno & Newport, 1996). Low levels of CDK2 activity at 16 and 24 h p.i. may have also prevented MPF activation and cell cycle-progression. SV40 (Gershey, 1979) and human immunodeficiency virus (HIV) (He *et al.*, 1995) repress G_2 or mitosis during a productive infection. SV40 T antigen plays a critical role in this process by inducing tyrosine phosphorylation of CDC2 (Scarano *et al.*, 1994) and binding to the yeast homologue of CDC2, $p34^{CDC28}$ (Nacht *et al.*, 1995). The HIV gene *Vpr* inactivates CDC2 by increasing phosphorylation of CDC2 but Vpr does not bind CDC2 (He *et al.*, 1995). Cycling cells and cells expressing high levels of CDC are more efficiently killed by cytotoxic T cells (Greenberg & Litchfield, 1995), suggesting this would be advantageous for the virus *in vivo*. Since DNA damage induces a G_2 arrest (Maltzman & Czyzyk, 1984; Zhan *et al.*, 1993) and HSV infection leads to DNA damage (Kulomaa *et al.*, 1992; Pilon *et al.*, 1986; Schlehofer & zur Hausen, 1982), DNA damage may also activate cellular factors which block mitosis.

The major findings in this study are that following infection, Rb was phosphorylated, CDK2 activity was increased, and steady-state levels of cyclin A were increased. When CDK2/cyclin A complexes phosphorylate Rb, transcription factors which bind to Rb, including the transcription factor E2F, are released (Zarkowska & Mittnacht, 1997). In contrast, CDK4/cyclin D1 complexes or CDK2/cyclin E complexes differen-

tially phosphorylate Rb, but Rb-binding transcription factors are not released. In general, E2F/Rb complexes repress promoters which contain consensus E2F-binding sites and thus 'free' E2F is a transcriptional activator of many genes which regulate cell-cycle progression (reviewed by Wang *et al.*, 1994; Weinberg, 1995). S-phase forms of the transcription factor E2F are present after infection with HSV-1 (Hilton *et al.*, 1995), which is consistent with release of E2F from Rb following infection. We hypothesize that CDK2/cyclin A complexes, in part, phosphorylate Rb after infection, resulting in the release of E2F. Although we have been unable to identify an HSV promoter which contains a consensus E2F-binding site, a recent study has demonstrated that E2F can activate the HSV-1 thymidine kinase promoter in the absence of IE proteins (Shin *et al.*, 1996). The *cis*-acting sequences in the thymidine kinase promoter which are activated by E2F contain an SP1-binding site. Furthermore, Rb stimulates Sp1-mediated transcription by liberating Sp1 from a negative regulator (Chen *et al.*, 1994). Previous studies have demonstrated that Rb can transactivate the HSV-2 large subunit of ribonucleotide reductase promoter (Hanson *et al.*, 1994). Since nearly every HSV promoter (IE, E, or L) contains SP1-binding sites, it is reasonable to hypothesize that phosphorylation of Rb and release of E2F would stimulate viral gene expression. Rb also binds other transcription factors, PU.1, ATF-2, UBF, Elf-1, MyoD and BRG-1 (Zarkowska & Mittnacht, 1997; reveiwed by Wang *et al.*, 1994; Weinberg, 1995), suggesting these cellular transcription factors may also stimulate viral gene expression. Experiments designed to identify viral genes which regulate CDK2 activity, Rb phosphorylation and the precise role that cell-cycle components play in the infection process are in progress.

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