Human cytomegalovirus mediates cell cycle progression through G₁ into early S phase in terminally differentiated cells

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Introduction

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that, like other members of the herpesvirus family, persists after infection throughout the lifetime of the host. Although primary infection of healthy individuals is usually asymptomatic, infection or reactivation in immunocompromised individuals can cause severe or fatal disease and CMV disease is in fact a major cause of morbidity and mortality in such individuals (Griffiths & Grundy, 1988). Following infection of permissive cells, viral gene expression follows a regulated cascade through three distinct phases, immediate-early (IE), early and late, resulting in the release of infectious virions. The most abundant IE transcripts arise from the major IE region in the unique-long (U₃) region of the genome: transcripts undergo differential splicing to give two major IE proteins, the 72 kDa IE1 and the 86 kDa IE86 (Stenberg et al., 1989; Stinski et al., 1983; Wathen et al., 1981). Studies from many laboratories, including our own, have shown that these proteins are transcriptional regulators that transactivate a number of HCMV and cellular promoters, in addition to their autoregulation of the major IE promoter–enhancer (Cherrington et al., 1991; Cherrington & Mocarski, 1989; Colberg-Poley et al., 1992; Hagemeier et al., 1992a, b; Pizzorno et al., 1988, 1991; Stenberg et al., 1990). Many of the cellular gene products up-regulated by HCMV infection are implicated in control of cell proliferation, e.g. c-fos, c-myc, dihydrofolate reductase and DNA polymerase α (Boldogh et al., 1990; Geist & Dai, 1996; Hayhurst et al., 1995; Monick et al., 1992; Wade et al., 1992), and it is now clear that HCMV infection can result in perturbation of the normal cell cycle (Albrecht et al., 1976; Bresnahan et al., 1996; Dittmer & Mocarski, 1997; Jault et al., 1995; Lu & Shenk, 1996; Morin et al., 1996; Poma et al., 1996), presumably in order to optimize the cellular environment for virus replication.

It was reported several years ago that HCMV infection results in stimulation of cellular DNA synthesis (Albrecht et al., 1976), implying overriding of normal cell cycle control. This effect has been studied recently in greater detail in quiescent fibroblast cells that have been withdrawn reversibly from the cell cycle by serum deprivation or contact inhibition (Bresnahan et al., 1996; Dittmer & Mocarski, 1997; Jault et al., 1995; Morin et al., 1996; Poma et al., 1996). In these analyses, infection resulted in cell cycle progression through G₁ leading eventually to an arrest of the cell cycle in either late G₁ or G₂/M (Bresnahan et al., 1996; Dittmer & Mocarski, 1997; Jault et al., 1995; Morin et al., 1996; Poma et al., 1996; Wiebusch & Hagemeier, 1999). Also, in cycling cells, it has been reported that HCMV may block the cell cycle at multiple points,
depending on the stage of the cell cycle at which infection occurred (Lu & Shenk, 1996; Salvant et al., 1998). To date, all studies on the effects of HCMV infection on the cell cycle of arrested cells have been carried out in quiescent primary fibroblasts. However, in vitro and in vivo, there is good evidence that permissiveness for HCMV infection in a number of cell types is dependent on terminal differentiation (Sinclair & Sissons, 1996; Soderberg-Naucler et al., 1997) and terminal differentiation essentially blocks cells in the G<sub>0</sub>/early G<sub>1</sub> phase of the cell cycle (el-Deiry et al., 1995). So far, there have been no reports analysing the effect of HCMV infection on cells withdrawn irreversibly from the cell cycle by differentiation, a cell type that clearly plays an important role in vivo in HCMV infection (Sinclair & Sissons, 1996; Soderberg-Naucler et al., 1997).

Progression through the cell cycle is regulated by cyclins and their associated cyclin-dependent kinases (Cdks). Cdks are only active when complexed with their particular cyclin partner (Cordon-Cardo, 1995; Pines, 1993; Sherr, 1993) and progression through the cell cycle occurs as a result of phosphorylation by Cdks of specific substrate molecules such as the retinoblastoma-susceptibility protein Rb. This in turn results in a specific sequence of cellular events related to the particular stage of the cell cycle. However, although formation of a cyclin–Cdk complex is a requirement, kinase activity does not simply follow cyclin levels. Other regulatory steps are also involved; these include activation of the cyclin–Cdk complex by phosphorylation and de-phosphorylation of specific sites on Cdks or inhibition by interaction with so-called Cdk inhibitors such as p21<sup>Cip1</sup>, p16<sup> Ink4a</sup> and p27<sup>Kip1</sup> (Cordon-Cardo, 1995; Pines, 1993; Sherr, 1993). Generally, high levels of Cdk activity are indicative of cell cycle progression, while terminally differentiated or quiescent cells have low levels of Cdk activity and elevated levels of mitotic inhibitors.

In recent years, it has become clear that many viral oncoproteins act by targeting cell cycle regulatory factors (DeCaprio et al., 1988; Whyte et al., 1988). For instance, adenovirus E1A can induce cell cycle progression in G<sub>0</sub>-arrested cells that have withdrawn from the cell cycle as a result of serum-deprivation or differentiation (Crescenzi et al., 1995; Rao et al., 1992; Stein et al., 1990). In these arrested cells, Rb is hypophosphorylated and, in this underphosphorylated form, Rb binds and inactivates the cellular transcription factor E2F. As E2F activates expression of a number of S phase genes, Rb plays a major role in repressing gene expression associated with the S phase of the cell cycle. Induction of cell cycle progression by E1A is believed, in part, to be due to the fact that E1A interacts physically with members of the Rb family of proteins (Hu et al., 1990; Whyte et al., 1989). This results in a release of functional E2F transcription factor, which would normally be kept in an inactive form by interaction with Rb family proteins during G<sub>0</sub>. This release of E2F then results in the expression of E2F-dependent S phase genes. More recently, it has also been reported that, as well as targeting Rb directly to release active E2F, adenovirus E1A can interact directly with Cdk inhibitors, such as p27<sup>Kip1</sup>, resulting in p27<sup>Kip1</sup> inactivation and Rb phosphorylation, which can overcome TGF-β-mediated cell cycle arrest (Mal et al., 1996b). Similarly, human papillomavirus (HPV) E7 has been shown to prevent inhibition of Cdk activity by both p21<sup>Cip1</sup> and p27<sup>Kip1</sup> (Funk et al., 1997; Keblusek et al., 1999; Zerfass-Thome et al., 1996).

We and others have reported previously that E1A and IE86 have a number of properties in common. For instance, IE86 is also known to interact physically and functionally with Rb and p53 (Hagemeier et al., 1994; Muganda et al., 1994; Poma et al., 1996; Sommer et al., 1994; Speir et al., 1994) in an manner analogous to the known interaction of adenovirus E1A with Rb. As discussed above, such interactions would be consistent with inducing progression through G<sub>1</sub> into early S phase.

Consequently, we have asked what effect HCMV infection has on cells that have withdrawn irreversibly from the cell cycle due to differentiation and, if HCMV does advance the cell cycle in these cells, whether IE86 plays a similar role to E1A in targetting inhibitors of Cdks. Here, we show that, in cells arrested irreversibly in G<sub>0</sub> as a result of terminal differentiation, HCMV is able to induce cell functions associated with progression of the cell cycle through G<sub>1</sub> into early S phase and that this is correlated with a direct physical and functional interaction between the HCMV 86 kDa IE86 protein and the Cdk inhibitor p21<sup>Cip1</sup>.

Methods

**Cell culture and virus infection.** Cells of the human embryonal carcinoma cell line NT2D1 (T2) were cultured in minimal essential medium containing 10% foetal calf serum (MEM-10) as described previously (Kothari et al., 1991) and differentiated by addition of 10<sup>–6</sup> M all-trans retinoic acid (RA) for 5 days. Primary human fibroblast cells (MRC5) were cultured in MEM-10. Cells were infected with human cytomegalovirus (AD169 strain) at an m.o.i. of 5 for 3 h. Virus was then washed from infected cells and replaced with fresh medium. As controls, cells were mock-infected with virus-conditioned control medium or infected with UV-inactivated virus as described previously (Poma et al., 1996).

**Immunofluorescence.** MRC5 or T2 RA cells were infected with HCMV for 24, 48, 72 and 96 h. Cells were fixed for 15 min, permeabilized in 70% ethanol at –20 °C and then stained with mouse monoclonal antibodies to UL69 (Winkler et al., 1994), a 55 kDa viral late protein (Chemicon) or IE72 (Biosys). Antibodies were detected by using anti-mouse FITC conjugate.

**Analysis of DNA content.** Infected or uninfected cells were fixed in 70% ice-cold ethanol, treated briefly with RNase and stained with propidium iodide (PI) as described previously (Poma et al., 1996). Infected or uninfected cells were also treated for 24 h with 10 µM 5-bromo-2′-deoxyuridine (BrdU) at 24 h post-infection (p.i.). Cells were then harvested and total DNA was isolated. DNA was restricted with Smal and separated on 0.6% agarose gels and DNA fragments were transferred to nitrocellulose by blotting. Blots were then probed with a monoclonal antibody to BrdU (Harlan Sera Laboratories) and antibody was detected by chemiluminescence with an ECL detection kit, as detailed by the manufacturer (Amersham).
Western blotting. Infected or uninfected cells were harvested by scraping, washed once in ice-cold PBS and resuspended in SDS–PAGE sample buffer. Protein samples were separated on 10 or 15% polyacrylamide gels, blotted onto nitrocellulose and probed with monoclonal antibodies to Rb protein (Santa Cruz) or a monoclonal antibody that recognizes exon 2 of the HCMV IE72 and IE86 proteins (Hayhurst et al., 1995).

GST-fusion proteins. pGEX-3X.IE2, used to generate recombinant IE86 protein, has been described previously (Caswell et al., 1993). pGST-p21, p-GST-p21N and pGST-p21C were gifts of A. Dutta and have also been described previously (Chen et al., 1996). GST-p21BN was a gift of Tony Hunter (Mal et al., 1996b). Recombinant GST or GST-fusion proteins were purified by using glutathione–Sepharose beads and eluted with glutathione (Smith & Johnson, 1988).

GST-fusion protein interaction assays. Protein–protein interaction assays were carried out exactly as described previously (Caswell et al., 1993). Vectors to generate [35S]methionine-labelled IE72, IE86, gelolin and E1A (13S) by coupled in vitro transcription/translation (Promega) have also been described previously (Caswell et al., 1993).

Immunoprecipitations and histone H1 kinase assays. Total cellular extracts were prepared from uninfected or differentiated cells in EBC buffer as described previously (Hagemeier et al., 1994). Extracts were immunoprecipitated for 2–3 h at 4 °C with anti-Cdk2, anti-Cdk4, anti-cyclin E or anti-cyclin A antibodies (Santa Cruz) followed by incubation at 4 °C for 1 h with protein A-Sepharose (Pharmacia). Immunoprecipitates were then assayed for their kinase activity by using histone H1 as substrate, essentially as described previously (Mal et al., 1996b).

Yeast two-hybrid interaction assay. Experiments were carried out using the Matchmaker yeast two-hybrid system (Clontech) with expression vectors that had been modified in the multiple cloning site. Plasmid pGAD424 was digested with EcoRI and BamHI and then ligated with a double-stranded adaptor of sequence 5′ AATTTCGATTCGGGATCCCGGGGAATTC3′ (sense strand) and 5′ GATCGAATTCCGGGGAATTC3′ (antisense strand). The recombinant plasmid, pGAD425, was then digested with BamHI and EcoRI and ligated with a BamHI–EcoRI IE2 cDNA fragment from pGEX3X-IE2 (Caswell et al., 1993) to generate pGAD-IE2. Similarly, pGAD-IE1 was made by cloning the BamHI–EcoRI IE1 cDNA fragment from pGEX3X-IE1 (Caswell et al., 1993) into pGAD425. The GAL4 DNA-binding domain–p21 fusion was made by digestion of pGST-p21 (Chen et al., 1996) with BamHI and SalI and cloning of the p21 cDNA fragment into the BamHI and SalI sites of pGBT10, which has been described previously (Caswell et al., 1996). pGBT10-IE86 has also been described previously (Caswell et al., 1996). Other plasmids used were supplied with the Matchmaker kit. Yeast transformations and quantitative liquid β-galactosidase assays were carried out in Saccharomyces cerevisiae strain SPY526 exactly as described by the manufacturers of the kit.

Results

HCMV induces cellular DNA synthesis in differentiated embryonal carcinoma cells

The human embryonal carcinoma cell line NT2D1 (T2) represents a good model system for differentiation-dependent permissiveness for HCMV infection. Undifferentiated T2 cells are non-permissive for HCMV infection due to a blockage in major IE expression. However, differentiation of T2 cells for 5 days with RA (T2+RA), to a neuronal phenotype, lifts this repression and cells become fully permissive for IE expression and productive infection, although viral DNA replication is delayed (Gonczol et al., 1984; LaFemina & Hayward, 1986; Nelson & Groudine, 1986). Consistent with previous observations (Maerz et al., 1998; Spinella et al., 1999), differentiation of T2 cells with RA for 5 days resulted in their arrest in the G0/G1 phase of the cell cycle (Fig. 1b). However, infection with HCMV resulted consistently in a substantial increase in the population of cells with a greater than G0/G1 content of DNA as early as 24 h p.i. (Fig. 1c). No such increase was observed in cells infected with UV-inactivated virus (Fig. 1b, d, f, h).

Whilst as many as 80% of the T2+RA cell population expressed viral IE antigens (Fig. 1i), we wanted to determine, specifically, whether the cells that were advancing through the G0/G1 restriction point were cells expressing IE antigens or were just bystander, uninfected cells. Consequently, we carried out the analysis with T2+RA cells infected at a lower m.o.i., such that only 10–20% of the cell population were infected with HCMV. Fig. 1(k–m) clearly shows that, when these cells were analysed for DNA content as well as their expression of IE1 protein, it was specifically the cells expressing IE1 that had the greater than G0/G1 content of DNA (compare Fig. 1f and m).

In order to rule out that this apparent increase in cells in S phase was due to viral DNA replication, we examined the extent of viral DNA replication directly in infected T2+RA cells at 48 h p.i. Fig. 2 shows that primary fibroblast cells (MRC5) infected for 48 h with HCMV and labelled with BrdU gave rise to specific viral DNA restriction fragments when analysed by Southern blot detection for BrdU-labelled DNA with an anti-BrdU antibody. This confirms high levels of viral DNA replication in these fibroblast cells, as expected. In contrast, however, T2+RA cells showed no such viral DNA replication at 24–48 h p.i., consistent with the known extended virus life cycle in these cells (Gonczol et al., 1984). Consequently, the increase in DNA content of T2+RA cells upon infection with HCMV at 48 h p.i. cannot be attributed to viral DNA synthesis.

It has been suggested that HCMV may induce cellular DNA synthesis only in cells that are abortively infected (Bresnahan et al., 1996). Whilst it has already been shown that differentiated T2+RA cells are fully permissive for HCMV infection (Gonczol et al., 1984; LaFemina & Hayward, 1986), we wanted to confirm that the infected T2+RA population we analysed was indeed productively infected. Fig. 3(a) shows that, consistent with this cell population undergoing productive infection, viral DNA replication centres were present extensively in these infected T2+RA cells at 72 h p.i., as determined by the presence of UL69, a known DNA replication accessory protein (Sarisky & Hayward, 1996). Note that, compared with fibroblast cells, the presence of viral DNA replication centres occurred in T2+RA cells at later times of infection, consistent with the delay in viral DNA replication in
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Fig. 1. For legend see facing page.

these cells. Fig. 3(b) also confirms that these cells were productively infected, as shown by the presence of high levels of true late viral gene products in the infected population.

HCMV induces Rb phosphorylation in arrested differentiated T2+RA cells

The Rb protein plays a major role in controlling progression through the cell cycle. Rb is known to be hypophosphorylated in the G₀ and early G₁ phases of the cell cycle and progression through G₁ into S phase is associated with increased Rb phosphorylation (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1992). Similarly, hypophosphorylated Rb protein induces cell cycle arrest (Goodrich et al., 1991; Hinds et al., 1992). Consequently, levels of Rb phosphorylation have been suggested to play a pivotal role in cell cycle progression through the G₁/S checkpoint. Consistent with the known G₀/G₁ arrest of T2 cells by RA (Maerz et al., 1998; Spinella et
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Fig. 1. Cytomegalovirus infection induces cell cycle progression.

(a)–(i) Undifferentiated (T2) or differentiated (T2 + RA) cells were infected with virus or UV-inactivated virus (AD169) at an m.o.i. of 10, as shown. Cells were stained with PI and their DNA content was analysed by FACS. The proportions of cells in S phase were 31% (a; T2), 2% (b; T2 + RA, 24 h UV-HCMV infection), 16% (c; T2 + RA, 24 h HCMV infection), 19% (e; T2 + RA, 48 h HCMV infection), 21% (g; T2 + RA, 72 h HCMV infection) and 23% (i; T2 + RA 96 h HCMV infection).

(j) T2 + RA cells were infected at an m.o.i. of 10 with virus or UV-inactivated virus for 24 or 96 h. Cells were fixed and then stained with an anti-IE1/2 antibody and analysed by FACS. The numbers of cells expressing IE antigens are shown as percentages of the total cell population. (k)–(m) T2 + RA cells infected for 48 h (as shown in e) at an m.o.i. of 2 were doubly stained with PI and an antibody specific for HCMV IE1/2 (IE). IE-expressing cells were sorted (k) and cells expressing IE (gate 2) or not expressing IE (gate 1) were analysed for their DNA content. The DNA profile of cells in gate 1 is shown in (l) and the DNA profile of cells in gate 2 is shown in (m).

Fig. 2. Cellular DNA synthesis is induced by HCMV. T2 + RA cells or MRC5 cells were mock infected (lanes 1) or infected with HCMV for 48 h (lanes 2). Cells were labelled with BrdU for 24 h prior to harvest and then DNA was isolated, cut with SmaI and analysed by Southern blot with an anti-BrdU antibody as probe.

al., 1999), Fig. 4(a; lanes 1 and 3) shows that differentiated T2 + RA cells contained predominantly hypophosphorylated Rb, consistent with their withdrawal from the cell cycle. Infection of these cells with HCMV resulted in an increase in the hyperphosphorylated form of Rb as early as 24 h p.i. (Fig. 4a; lane 2). Levels of HCMV infection of the T2 + RA cells were confirmed by Western blotting with an anti-IE72/IE86 antibody (Fig. 4b).

HCMV infection induces cyclin-associated kinase activity in differentiated T2+RA cells

Rb phosphorylation and subsequent progression through the cell cycle are dependent on functional Cdks. In cells arrested in G0/G1, Cdk activity is low. Cdk4 associated with the D-type cyclins is believed to be essential for G1 progression, whereas Cdk2 associated with cyclin E is believed to be important for progression through the G1/S phase
Fig. 3. For legend see facing page.
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Fig. 4. HCMV infection induces Rb phosphorylation. Differentiated T2 + RA cells were mock-infected (lanes 1 and 3) or infected with HCMV at an m.o.i. of 5 (lanes 2 and 4) for 24 h (lanes 1 and 2) or 48 h (lanes 3 and 4). Total cellular protein was analysed by Western blot with antibodies specific for Rb (a) or the HCMV major IE1/2 protein (b).

check point (Sherr, 1993). We therefore asked whether the increase in phosphorylated Rb in T2 + RA cells resulting from HCMV infection was a result of increased Cdk activity in these arrested cells.

Fig. 5 confirms the known reduction in cyclin-associated kinases that occurs upon differentiation of T2 cells with RA (Spinella et al., 1999; Maerz et al., 1998). As expected, differentiation led to a decrease in Cdk2 activity, which is consistent with the G<sub>0</sub>/G<sub>1</sub> arrest of these cells (Fig. 5a, lane 2). Similarly, cyclin E-associated kinase activity (Fig. 5b, lane 2) as well as Cdk4 and cyclin A-associated kinase activity (Fig. 5c and d, respectively; lanes 2) were also reduced in differentiated T2 + RA cells, as expected. However, infection with HCMV resulted in a clear increase in functional Cdk2 and cyclin E-associated kinase activity (Fig. 5a and b, respectively; lanes 3) as early as 17 h p.i., consistent with cell cycle progression through the G<sub>1</sub>/S checkpoint. This high level of Cdk2 and cyclin E-associated kinase activity was maintained through the initial 48 h of virus infection.

Similarly, cyclin A-associated kinase activity (Fig. 5d) was also induced by HCMV infection, but this induction was delayed and first occurred at 48 h p.i.

Fig. 3. MRC5 or T2 + RA cells were infected with HCMV at an m.o.i. of 10 as shown, fixed and stained with monoclonal antibodies to UL69 (a) or a 55 kDa true late protein (b). Antibodies were detected by using an anti-mouse FITC second layer. Arrows in (b) show staining consistent with virus production centres.
As expected, we have observed that infection of T2 + RA cells with HCMV resulted in Cdk4-associated kinase activity that phosphorylated a GST–Rb target as early as 17 h p.i. (data not shown). Interestingly, an increase in Cdk4 activity targeting histone H1 also appeared to be induced by HCMV infection (Fig. 5 c), although this induction was only observed at 48 h p.i. We are aware that Cdk4 is not normally associated with H1 kinase activity. We do not know why the immunoprecipitated Cdk4 complex is able to phosphorylate histone H1 at this time of infection. It is possible that a viral product or virus-induced product is able to target Cdk4 during infection, allowing this complex to re-target kinase activity.

HCMV IE86 binds p21<sup>Cip1</sup> <i>in vitro</i>

Two classes of inhibitors of cyclin–Cdkks are present in multicellular organisms, the INK family, which includes p15 and p16, and a second family of proteins that includes p21<sup>Cip1</sup> and p27<sup>Kip1</sup>. p27<sup>Kip1</sup> and p21<sup>Cip1</sup> inhibitors are known to interact with and inhibit Cdk2, Cdk4 and Cdk6, whereas the INK family of inhibitors has no effect on Cdk2 (Pines, 1993; Sherr, 1993).

Recently, it has been shown that TGF-β-mediated arrest of mink lung cells in late G<sub>1</sub>, which is due to inactivation of cyclin–Cdk complexes by the p27<sup>Kip1</sup> inhibitor, can be
reverses by adenovirus 12S E1A protein, resulting in cell cycle progression (Mal et al., 1996b). This reversal of Cdk inhibition has been shown to be mediated by a direct interaction between E1A and p27\(^{Rb}\) proteins, whereby E1A prevents p27\(^{Rb}\) from inhibiting the cyclin–Cdk2 complex. As we and others have shown a number of functional similarities between adenovirus E1A and HCMV IE86, we asked whether IE86 could also interact physically with cyclin–Cdk inhibitors. We carried out protein–protein interaction assays with GST–p21 fusion proteins as bait for \(^{35}\)S)methionine-labelled proteins.

Fig. 6(a) shows that, in contrast to adenovirus 12S E1A, which has been shown to interact specifically with p27\(^{Rb}\) (Mal et al., 1996b), IE86 interacted specifically with GST–p21 (lane 3) but not with GST–p27 or an additional negative control, GST–IE1. In contrast, experiments using in vitro transcribed and translated cyclin A or Cdk2 showed clear specific binding to both GST–p27 and GST–p21, as expected (data not shown). We observed no such interaction between GST–p21 and a number of negative-control proteins such as gelsolin or HCMV IE1 (lanes 1 and 4, respectively). Interestingly, in our experiments, in which adenovirus 13S E1A protein was used as a positive control, we observed a specific interaction between 13S E1A and GST–p21, but not GST–p27 (lane 2). We observed no interaction between IE86 and GST–p16 (data not shown).

We next determined which region of IE86 was responsible for this interaction with GST–p21. Fig. 6(b) shows that the C-terminal domain of IE86 (amino acids 290–579) was able to bind efficiently to GST–p21 (lane 6). In contrast, the N-terminal domain of IE86 (amino acids 1–290) showed no specific binding to GST–p21 (lane 5). Interestingly, this same C-terminal domain of IE86 is also responsible for contacting Rb, TATA box-binding protein and TFIIIB (Caswell et al., 1993; Hagemeier et al., 1994). Similar experiments were also carried out to determine which region of the p21 protein was contacted by IE86 (Fig. 6c). GST–p21 N- and C-terminal fusions were used as targets for binding in vitro transcribed and translated IE86. Fig. 5(c; lane 3) shows that IE86 bound the N-terminal domain of p21 (amino acids 1–90), which is known to contain both cyclin- and Cdk-binding domains of p21 (Chen et al., 1996). Little or no binding of IE86 was observed to the C-terminal domain of p21 (amino acids 87–164).

**IE86 interacts with p21\(^{Cip1}\) in vivo**

In order to confirm that the physical interaction between IE86 and p21\(^{Cip1}\) that we observed in vitro also occurred in vivo and that this did not depend upon any other mammalian protein, we analysed the interaction between IE86 and p21\(^{Cip1}\) in a yeast two-hybrid assay. Fig. 7 shows that a two-hybrid system in which full-length IE86 was fused to the GAL4 activation domain and full-length p21 was fused to the GAL4 DNA-binding domain (p21/IE2) resulted consistently in

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**Fig. 7.** HCMV IE86 interacts directly with p21\(^{Cip1}\) in vivo. Fusions between the GAL4 DNA-binding domain (GBT) and the GAL4 activation domain (GAD) were tested for \(^\beta\)-galactosidase expression in yeast. In all cases, full-length p21 protein was fused to GBT and full-length IE72 (IE1) or IE86 (IE2) was fused to GAD. Additional negative and positive controls included human lamin C (LamC)/GBT or IE86/IE86 double transforms, respectively. Assays were developed for 4 h.

**Fig. 8.** IE86 recovers Cdk activity in arrested T2 + RA cells and can prevent p21-mediated inhibition of Cdk2 in cycling T2 cells. (a) Cellular extracts from differentiated T2 + RA cells were incubated with 0 (lane 1), 10 (2), 50 (3), 100 (4) or 200 (5) ng purified recombinant GST–IE86 fusion protein. In all cases, additions were made up to 200 ng total protein with purified GST. Extracts were immunoprecipitated with anti-Cdk2 antibody and analysed for kinase activity by standard H1 kinase assays.

(b) Cellular extracts from undifferentiated T2 cells (lane 1) were incubated with approximately 200 ng purified recombinant GST–p21 protein together with 0 (lane 2), 10 (3) or 200 (4) ng purified recombinant GST–IE86 fusion protein. In all cases, additions were made up to 200 ng total protein with purified GST. Extracts were immunoprecipitated with anti-Cdk2 antibody and analysed for kinase activity by standard H1 kinase assays.
activated β-galactosidase expression. No such activation was observed with a comprehensive panel of controls.

Purified IE86 protein recovers cyclin–Cdk2 activity in differentiated T2+RA cells

Mal et al. (1996b) have shown that purified E1A (12S) protein is able to displace p27^Kip1 inhibitor from cyclin–Cdk2 complexes and recover kinase activity from arrested cell extracts. Fig. 8(a) shows that, as expected, differentiated T2+RA cells contained low cyclin–Cdk2 activity (lane 1). However, IE86 protein was able to recover cyclin–Cdk2 activity from these arrested T2+RA cells in a dose-dependent manner (lanes 2–5). We also routinely observed a phosphorylated protein in these H1 kinase assays that migrated at the same relative mobility as IE86 (data not shown). Interestingly, Mal et al. (1996a) have shown that Cdk2 is able to phosphorylate adenovirus E1A protein in vitro and Harel & Alwane (1998) have shown that IE86 is a target for extensive phosphorylation during infection. Experiments are in progress to confirm that the high molecular mass phosphorylated protein that we observed is IE86 and to analyse any effect of Cdk2-mediated IE86 phosphorylation on IE86 function.

Similarly, we next determined whether IE86 was able to prevent inhibition by recombinant p21^Kip1 of cyclin–Cdk2 activity in cycling T2 cell extracts (Fig. 8b). As expected, cycling T2 cells contained high Cdk activity (lane 1) and purified p21^Kip1 was able to inhibit cyclin–Cdk2 activity specifically in dividing T2 cells (lane 2). However, recombinant IE86 protein was able to prevent recombinant p21^Kip1-mediated inhibition of cyclin–Cdk2 kinase activity in undifferentiated T2 cells (lanes 3 and 4).

Discussion

HCMV encodes a number of genes (such as viral DNA polymerase) that encode functions that permit viral DNA replication to take place independently of the replication of cellular DNA. However, it is clear that viral DNA replication is linked extensively to cellular DNA replication and is also likely to depend on the cellular functions that optimize the cell for DNA synthesis in general. For this reason, HCMV has evolved a number of functions that perturb normal cellular controls in order to enhance its own replication. It has become clear that terminally differentiated cells represent an important cell type for HCMV infection in vitro (Sinclair & Sissons, 1996). However, such cells have withdrawn irreversibly from the cell cycle (el-Deiry et al., 1995) and are unlikely to represent an optimal environment for viral DNA synthesis. Here, we have asked what effect HCMV infection has on such arrested, terminally differentiated cells in order to alter cellular conditions to allow productive virus infection.

HCMV infection of these irreversibly arrested cells induces cell cycle progression through the G1/S phase checkpoint, into early S phase. This occurs as early as 48 h p.i. and is not attributable to viral DNA replication, as the virus life cycle is extended in T2+RA cells and no viral DNA replication could be detected at the time of this advance in cell cycle progression. These T2+RA cells also underwent full productive infection, as determined by the detection of viral DNA replication centres and late viral gene expression.

We also confirmed that, at low m.o.i. at which only 10–20% of the cell population was infected with HCMV, induction through the G0/G1 checkpoint correlated with IE expression. This also rules out that the induction occurs in bystander cells as a result of, for instance, mitogen or cytokine induction by virus.

We have analysed the increase in cellular DNA content induced by HCMV infection solely by PI staining and FACS analysis for specific reasons. Recently, Morin et al. (1996) have shown that HCMV encodes functions that prevent cellular thymidine salvage and that channel exogenous thymidine exclusively into viral DNA. Consequently, the use of thymidine incorporation to analyse cellular DNA synthesis during virus infection is clearly problematic. Similarly, it is established that virus-specific DNA synthesis inhibitors such as gancyclovir and phosphonoformate do have discernible effects on cellular DNA synthesis (Albert & Gudas, 1986; Isley et al., 1995; Sabourin et al., 1978), and we have observed inhibition of serum-induced S phase entry of serum-deprived fibroblasts by gancyclovir at concentrations as low as 100 μg/ml (data not shown). Consequently, it may be difficult to interpret results when these drugs are used as a means of analysing cellular DNA synthesis in the absence of viral DNA synthesis. Instead, in our experiments, we have used a cell line in which the HCMV life cycle is extended such that the induction of cell cycle progression through the G1/S phase checkpoint that we observe is quite clearly separated in time from viral DNA synthesis and cannot be attributed to an increase in viral DNA in the cell.

Consistent with observations from experiments using infection of reversibly arrested fibroblast cells (Bresnahan et al., 1996; Jault et al., 1995), HCMV infection of irreversibly arrested T2+RA cells resulted in increased Rb phosphorylation and the induction of Cdk2 and cyclin E-associated kinase activity. However, in contrast to observations in infected quiescent fibroblasts (Bresnahan et al., 1996), we did observe clear induction of cyclin A-associated kinase activity by HCMV, consistent with the induction of the start of cellular DNA synthesis. All these observations are consistent with HCMV inducing cell cycle progression of arrested T2+RA cells through the G1 checkpoint into early S phase. Interestingly, a small but reproducible transient induction of Cdk4-associated kinase activity by HCMV was also observed.

The ability of adenovirus E1A to induce cell cycle progression through the G1/S phase checkpoint of the cell cycle has been shown to be mediated by a direct physical interaction between E1A and the Cdk inhibitor p27^Kip1. This interaction prevents p27^Kip1-mediated inhibition of cyclin-
associated kinases (Mal et al., 1996b). Because of the many functional similarities between HCMV IE86 and adenovirus E1A, we were prompted to look at whether IE86 could also interact physically with p27Kip1. In contrast to E1A, we observed no interaction between p27Kip1 and IE86. However, we did observe a strong physical interaction between IE86 and the p21Cip1 Cdk inhibitor. This interaction mapped to the N-terminal domain of p21Cip1. Interestingly, the N-terminal domain of p21Cip1 is known to contain domains for binding cyclins and Cdkks (Chen et al., 1996). Consequently, the ability of IE86 to contact p21Cip1 via these cyclin-/Cdk-binding domains would be consistent with the ability of IE86 to prevent p21Cip1 from interacting with cyclin–Cdk complexes.

Although it has been shown that the E7 protein of HPV-16 can associate with p27Kip1 and prevent p27Kip1-mediated inhibition of cyclin E-associated kinase activity (Zerfass-Thome et al., 1996), a direct functional interaction between E7 and p21Cip1 has also been described more recently (Martin et al., 1998). In this study, Martin et al. (1998) showed that the E7 protein of HPV-16 interacted directly with p21Cip1 and prevented p21Cip1-mediated inhibition of cyclin E-associated kinase in cells arrested by DNA damage, so inducing the cell cycle. In contrast to our observations for IE86, the interaction between E7 and p21Cip1 was mapped to the C-terminal PCNA-binding domain of p21Cip1, which resulted in E7 preventing p21Cip1-mediated inhibition of PCNA-dependent DNA replication (Martin et al., 1998). Consequently, whilst E7 and IE86 both appear to target p21Cip1 in order to prevent Cdk inhibition, the exact mechanisms by which they achieve this appear not to be identical.

The domain of IE86 that interacts with p21Cip1 was also determined. Using GST–IE86 domain fusions, we mapped the p21Cip1 interaction domain of IE86 to the C terminus (amino acids 290–579). This region of IE86 has already been shown to be important for the interaction between IE86 and a number of cellular proteins (Caswell et al., 1993; Hagemeier et al., 1994).

We confirmed a direct physical interaction between IE86 and p21Cip1 by using a yeast two-hybrid system. This analysis showed clearly that IE86 and p21Cip1 interacted directly in vivo and did not require interactions with any other mammalian protein.

Finally, this physical interaction between IE86 and p21Cip1 was reflected in the ability of purified IE86 protein to recover cyclin-associated kinase activity in arrested cells and to prevent inhibition of cyclin-associated kinases by recombinant p21Cip1. In these experiments, differentiated T2 + RA cells regained cyclin–Cdk2 activity upon the addition of bacterially expressed IE86 in a dose-dependent manner. Similarly, inhibition of cyclin–Cdk2 activity resulting from addition of bacterially expressed p21Cip1 to extracts from cycling T2 cells was also overcome by the addition of recombinant IE86.

Recently, Wiebusch & Hagemeier (1999) have shown that IE86 is able to block the cell cycle in permissive osteosarcoma cells in late G1, which is at odds with the apparent effect of virus-expressed IE86 in T2 + RA cells. We have not tested the effect of expression of IE86 in isolation in T2 + RA cells directly, as transient transfection of T2 + RA cells by IE86 expression vectors in our hands results in substantial death of both bystander cells and cells expressing IE86 (data not shown).

Results from a number of laboratories have shown that infection with HCMV of quiescent fibroblast cells that have withdrawn reversibly from the cell cycle as a result of serum starvation or contact inhibition can lead to the induction of cell cycle progression, but that this progression appears to be arrested at G1. In contrast, we show here that, in cells that have withdrawn irreversibly from the cell cycle as a result of terminal differentiation, HCMV can overcome the known G1/S block in these cells. As HCMV infection of arrested cells does not lead to an increase in cell proliferation (data not shown), we presume that infected cells are blocked at a later stage in their cell cycle, as has been suggested for fibroblasts (Jault et al., 1995; Lu & Shenk, 1996). This is under investigation.

Our experiments point to a possible role for the IE86 protein in this induction of S phase, which may involve a direct physical and functional interaction between IE86 and the Cdk inhibitor p21Cip1. We presume that, in contrast to reversibly arrested quiescent fibroblasts, in cells arrested irreversibly by terminal differentiation, HCMV must target cellular functions involved in restriction of G1/S phase in order to optimize the cell for high levels of viral DNA replication. Whether this reflects a fundamental difference in the requirements of HCMV infection of irreversibly arrested versus quiescent cells awaits further analysis.

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