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Interaction of the human cytomegalovirus particle with the host cell induces hypoxia-inducible factor 1 alpha

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ABSTRACT

The cellular protein hypoxia-inducible factor 1 alpha (HIF-1α) was induced after infection of human fibroblasts with human cytomegalovirus (HCMV). HCMV irradiated with ultraviolet light (uv-HCMV) also elicited the effect, demonstrating that the response was provoked by interaction of the infecting virion with the cell and that viral gene expression was not required. Although induction of HIF-1α was initiated by an early event, accumulation of the protein was not detected until 9 hours post infection, with levels increasing thereafter. Infection with uv-HCMV resulted in increased abundance of HIF-1α-specific RNA, indicating stimulation of transcription. In addition, greater phosphorylation of the protein kinase Akt was observed, and the activity of this enzyme was required for induction of HIF-1α to occur. HIF-1α controls the expression of many cellular gene products; therefore the findings reveal new ways in which interaction of the HCMV particle with the host cell may cause significant alterations to cellular physiology.

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Introduction

The herpesvirus human cytomegalovirus (HCMV), a member of the subfamily *Betaherpesvirinae*, is an important human pathogen with potentially severe effects in newborn infants and immunocompromised individuals. HCMV gene expression is characterized by the sequential transcription of immediate early (IE), early and late genes over a period of many days after the initial infection (Wathen and Stinski, 1982). The HCMV genome encodes approximately 170 proteins, and 71 of these have been identified as components of the virus particle (Dolan et al., 2004; Varnum et al., 2004). The virion also contains cellular proteins, and RNAs of viral and cellular origin (Bresnahan and Shenk, 2000; Greijer et al., 2000; Varnum et al., 2004). Infection with this complex virus results in major alterations to the host cell, including increases or decreases in the abundance of individual cell transcripts and proteins (Browne et al., 2001; Chan et al., 2008; Hertel and Mocarski, 2004; Kenzelmann and Muhlemann, 2000; Stanton et al., 2007; Zhu et al., 1998).

Many of the effects of HCMV on the host cell result from the actions of virus-encoded gene products synthesized *de novo*, but the interactions of the infecting virion with the host cell can also initiate significant changes to cellular gene expression. The best studied of these is the induction of innate immune responses, which occurs even when viral gene expression is prevented by irradiation of virus with ultraviolet (uv) light. These responses include activation of the transcription factors NF-κB and interferon regulatory factor 3, which individually and in cooperation stimulate the production of a range of inflammatory cytokines, including type 1 interferons (Abate et al., 2004; Boehme et al., 2006; Boehme et al., 2004; Browne et al., 2001; DeFilippis et al., 2010; Juckem et al., 2008; Navarro et al., 1998; Netterwald et al., 2004; Noyce et al., 2009; Paladino et al., 2006; Preston et al., 2001; Rodriguez et al., 1987; Yurochko et al., 1995; Zhu et al., 1997), albeit at relatively low levels (Rodriguez et al., 1987). Other cell responses attributed to the initial interaction of the virus particle with host cell include the induction of specific chromosome breaks (Fortunato et al., 2000), activation of an angiogenic response in endothelial cells (Bentz and Yurochko, 2008), and resistance to apoptotic death of monocytes (Chan et al., 2010). Most of the effects of incoming virions described to date occur very shortly after infection, resulting either from engagement with cell receptors or a response to virus components by various pattern-recognition sensors.

The findings described here characterize the induction of hypoxia-induced factor 1 alpha (HIF-1α) after HCMV infection. Mammalian cells are constantly exposed to varying oxygen levels, and they have evolved mechanisms to ensure rapid adaptation to conditions of hypoxia. A primary level of control is through the "master" transcription factor hypoxia-inducible factor (HIF), which consists of a DNA-binding element, HIF-1β (also named ARNT), and a regulatory component, HIF-1α (Bruick, 2008; Semenza, 1998; Semenza, 2001; Walmsley et al., 2007). The levels of HIF-1α are tightly controlled by rapid turnover of the protein, mediated by the E3 ubiquitin ligase von Hippel–Lindau factor

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(VHL). At normal oxygen levels, this process operates continuously due to the action of proline hydroxylases that use oxygen, iron, ascorbate and 2-oxoglutarate as cofactors. This results in hydroxylation of two proline residues of HIF-1α that target the protein for ubiquitylation by VHL and subsequent proteolytic degradation. Proline hydroxylation slows when oxygen levels fall, and as a consequence HIF-1α accumulates and associates with HIF-1β to form an active HIF-1 complex that directs the transcription of hypoxia-responsive genes. The products of these genes coordinately mediate many changes including enhanced glucose uptake and glycolysis, angiogenesis, and release of inflammatory cytokines, each of which has major effects on cell physiology that contribute to cell survival in the hypoxic environment.

Induction of HIF-1α can occur through non-hypoxic mechanisms. Degradation of HIF-1α by the proline hydroxylase/VHL pathway is maximal at normal oxygen concentration; therefore an increase in mRNA amount or translational efficiency results in a rise in HIF-1α protein levels. This can be provoked by various stimuli including oxidative stress, exposure to hormones, growth factors or cytokines, and infection by pathogens (Dery et al., 2005; Hellwig-Burgel et al., 2005; Qutub and Popel, 2008; Werth et al., 2010; Zinkernagel et al., 2007). A variety of signaling cascades activate transcription of the *HIF1-α* gene, but stimulation of translation, when it occurs, depends on the pathway controlled by the protein kinases phosphoinositide-3-kinase (PI3K), Akt (also named protein kinase B) and mammalian target of rapamycin (mTOR) (Fukuda et al., 2002; Hellwig-Burgel et al., 2005; Laughner et al., 2001; Page et al., 2002). This is a well characterized cascade that, in general terms, promotes cell survival in response to external stimuli [reviewed by (Alessi and Cohen, 1998; Bahaskar and Hay, 2007; Bai and Jiang, 2010; Engelman et al., 2006; Shaw and Cantley, 2006)]. PI3K phosphorylates membrane phospholipids, and the products of the reaction, phosphatidylinositide di- and triphosphates, act as second messengers that permit phosphorylation of Akt at threonine 308 (T308) by 3-phosphoinositide kinase 1 (PDK1), inducing a conformational change that allows subsequent full activation through phosphorylation of Akt serine residue 473 (S473). Activated Akt exerts numerous effects on cells of which one, phosphorylation of mTOR, results in increased translation of many mRNAs including that encoding HIF-1α. Recently, it has become apparent that Akt can be activated by enzymes classed as PI3K-related kinases (PIKKs), suggesting alternative mechanisms for integrating stimuli with cellular changes (Boehme et al., 2008; Bozulic and Hemmings, 2009; Bozulic et al., 2008). The PIKKs include ataxia telangiectasia-mutated kinase (ATM), ATM and Rad3-like kinase, and DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and these enzymes coordinate the initial cellular response to DNA damage. There is a direct evidence that DNA-PKcs can activate Akt by catalyzing phosphorylation at S473 (Feng et al., 2004).

There are few studies that address the effects of viruses on HIF-1α. Infection with respiratory syncytial virus or hepatitis C virus results in an increase in HIF-1α levels that is attributed to stabilization of the protein (Kilani et al., 2004; Nasimuzzaman et al., 2007; Ripoli et al., 2010). Similarly, greater amounts of HIF-1α were detected after expression of hepatitis B virus protein X or hepatitis E virus ORF3 protein, due predominantly to reduced turnover (Moin et al., 2009; Yoo et al., 2003). The human immunodeficiency virus 1 protein Vpr activates transcription of HIF-1α (Deshmane et al., 2009). Increased HIF-1α expression was observed in biopsies of skin tissues infected with the herpesviruses varicella-zoster virus or Kaposi's sarcoma-associated herpesvirus (KSHV) (Werth et al., 2010), and KSHV latency-associated nuclear antigen (LANA) mediated transcriptional activation of HIF-1α (Cai et al., 2006). In the case of KSHV, induction is linked to a positive feedback loop, since HIF-1α cooperates with LANA to stimulate expression of the viral RTA transcriptional activator during hypoxia-induced reactivation (Cai et al., 2006). The Epstein–Barr virus latent membrane protein 1 (LMP1) enhances HIF-1α expression at the translational level, as a consequence of LMP1-induced oxidative stress (Wakisaka et al., 2004). There are no reports describing HIF-1α levels in HCMV-infected cells.

Results

In initial experiments, extracts of HFFF2 cells were analyzed for the presence of HIF-1α. At 24 h post infection (pi) with HCMV strain AD169, using virus either partially purified by centrifugation of medium harvested from infected cells or virions banded by centrifugation. A significant increase in HIF-1α levels was found in cells infected with either virus preparation (Fig. 1A). The requirement for viral gene expression was investigated by irradiation of the virus stock with ultraviolet (uv) light, using a dose that abolished detectable IE gene expression at 24 h pi (Fig. 1B). HCMV that was irradiated (uv-HCMV) was able to provoke the increase in HIF-1α levels, typically more effectively than unirradiated virus (Fig. 1B, lanes 2 and 3). This observation suggests that the increased amount of HIF-1α is a response to the input virus rather than to viral products synthesized after infection. In view of the fact that HIF-1α levels during hypoxia are

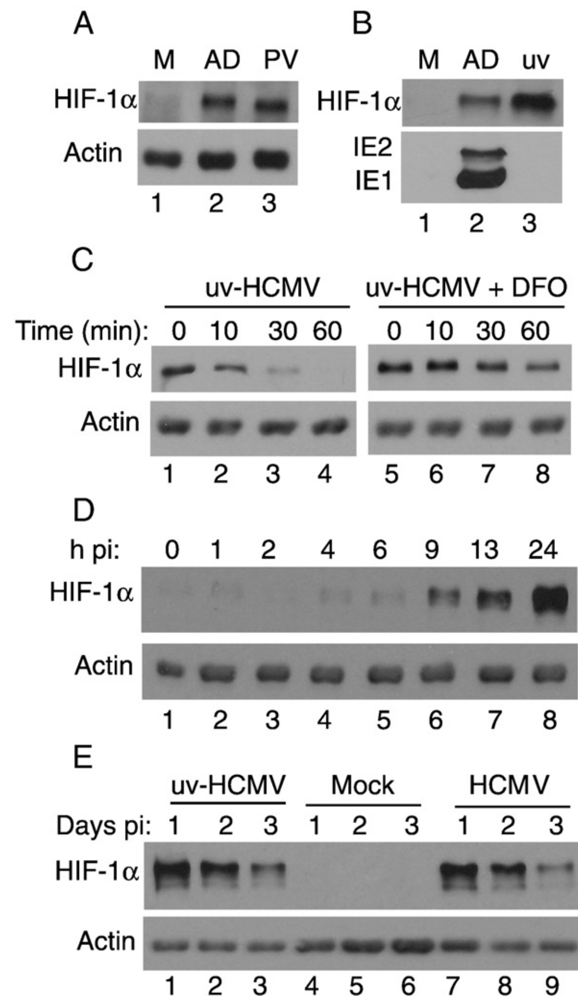

Fig. 1. Induction of HIF-1α by HCMV. A. HFFF2 monolayers were mock-infected (lane 1) or infected with partially purified HCMV AD169 (AD, lane 2) or gradient purified virions (PV, lane 3). Extracts were analyzed at 24 h pi for the presence of HIF-1α. B. HFFF2 monolayers were mock-infected (lane 1) or infected with HCMV (AD, lane 2) or uv-HCMV (uv, lane 3). Extracts were analyzed at 24 h pi for the presence of HIF-1α or immediate early (IE) proteins 1 and 2. C. HFFF2 monolayers were infected with uv-HCMV. At 22 h pi, one set was untreated (lanes 1 to 4) and one set was treated with 200 μM deferoxamine (DFO, lanes 5 to 8). After a further 2 h, cycloheximide (100 μg/ml) was added to all monolayers and extracts were made immediately (lanes 1 and 5), or after 10 min (lanes 2 and 6), 30 min (lanes 3 and 7) or 60 min (lanes 4 and 8). HIF-1α levels were analyzed. D. HFFF2 monolayers were infected with uv-HCMV and extracts were analyzed for the presence of HIF-1α at various times. E. HFFF2 monolayers were infected with uv-HCMV (lanes 1 to 3), mock-infected (lanes 4 to 6) or infected with HCMV (lanes 7 to 9), with DF2 replenished each day. Extracts were made at various times and analyzed for the presence of HIF-1α.

controlled by alterations in the degradation rate of the protein, the stability of HIF-1α in uv-HCMV-infected cells was investigated. Cycloheximide was added to cultures at 24 h pi, to inhibit synthesis of the protein, and samples were analyzed at various times (Fig. 1C, lanes 1 to 4). As a control, infected cells were treated with deferoxamine, which stabilizes HIF-1α due to chelation of iron, for 2 h prior to and during analysis of protein stability (Fig. 1C, lanes 5 to 8). HIF-1α in uv-HCMV-infected cells was degraded with a half life of less than 10 min, comparable to that reported for the protein in other cell types (Frede et al., 2006; Laughner et al., 2001; Shin et al., 2008), whereas it was more stable in deferoxamine-treated cells, as expected. The increased HIF-1α level in uv-HCMV-infected cells was therefore not due to stabilization of the protein. Since the response did not require viral gene expression and was therefore initiated early in infection, the timing of HIF-1α induction was investigated (Fig. 1D). Surprisingly, an increase in the HIF-1α level was not detected until 9 h pi, after which time the amount of the protein continued to rise until 24 h pi. Continuing incubation for longer times revealed that HIF-1α levels were greater than those in control cells at 48 h and 72 h pi, irrespective of whether the virus was uv-irradiated, but that the degree of induction declined with time (Fig. 1E).

The delayed appearance of HIF-1α until 9 h pi suggested that it was induced through an indirect process rather than as a response to the initial attachment or entry of the virus particle, and therefore the possible involvement of interferon beta (IFN-β), which is known to be induced and released into the cell culture medium after infection with uv-HCMV, was investigated (Fig. 2). The efficacy of a commercially available anti-IFN-β antibody was tested by adding various amounts to supernatant from uv-HCMV-infected HFFF2 cells, then applying this to fresh cells and analyzing interferon-stimulated gene 15 protein (ISG15) levels after incubation for a further 24 h (Fig. 2A). Addition of 450 units of antibody to 0.5 ml of supernatant completely eliminated production of ISG15. This quantity of antibody, when added to cultures infected with uv-HCMV, did not prevent the induction of HIF-1α, although the extent of the response was decreased by approximately two-fold (Fig. 2B). In addition, incubation of HFFF2 cultures with medium taken from uv-HCMV-infected monolayers resulted in only a marginal induction of HIF-1α after 24 h, and this effect was neutralized by anti-IFN-β serum (Fig. 2C). The experiments shown in Fig. 2 demonstrate that neither IFN-β produced in response to uv-HCMV, nor any other factor released into culture medium, made a major contribution to the induction of HIF-1α.

Previous studies have demonstrated that non-hypoxic signals can induce HIF-1α by increasing the level of the cognate mRNA and/or stimulating translation via the PI3K/Akt/mTOR signaling pathway (Laughner et al., 2001; Page et al., 2002). The amount of HIF-1α-specific RNA was analyzed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), taking samples at 7 h and 24 h after infection with uv-HCMV or unirradiated virus (Table 1). At 7 h pi, a small increase (1.9-fold) was observed in samples from uv-HCMV-treated cultures, but at 24 h pi this rose to 10.2-fold. In samples from cells infected with unirradiated HCMV, there was no significant change at 7 h pi and a 3.5-fold increase at 24 h pi. These results demonstrate that induction of HIF-1α-specific RNA contributes to the greater protein levels observed at 24 h pi. The lesser effect of unirradiated virus is compatible with the lower amounts of HIF-1α accumulated and suggests that virus-specified products made after infection control the magnitude of the response, as found for the induction of IFN-β (Browne et al., 2001; Taylor and Bresnahan, 2005).

The possibility that induction of HIF-1α required the pathway initiated by activation of PI3K was investigated. HFFF2 monolayers were mock infected or infected with uv-HCMV, and phosphorylation of Akt was monitored by probing samples with an antibody specific for the presence of phosphoserine at S473, in conjunction with an antibody that recognized total Akt (Fig. 3A). An increase in phosphorylation, relative to the total amount of Akt, was first detected at 9 h pi, with the extent of modification greater at 13 h and 24 h pi.

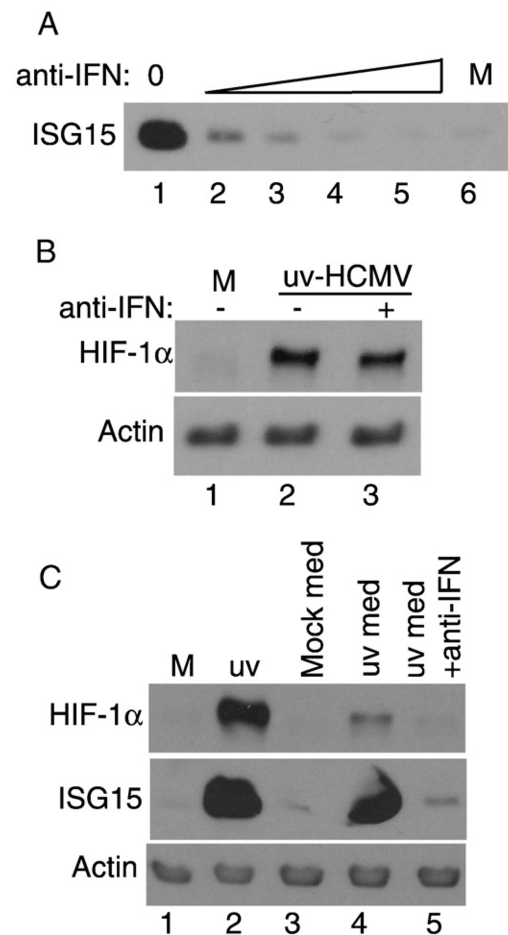

Fig. 2. Effect of IFN-β on induction of HIF-1α. A. HFFF2 monolayers were infected with uv-HCMV. At 24 h pi, the culture medium was harvested, centrifuged at 13,000*g* for 2 min, and the supernatant was added to fresh HFFF2 monolayers, together with anti-IFN-β antibody. The amount of antibody added was none (lane 1), 45 units (lane 2), 150 units (lane 3), 450 units (lane 4) or 1500 units (lane 5). Medium from uninfected cells was also tested (lane 6). After incubation for 24 h, extracts were analyzed for the presence of ISG15. B. HFFF2 monolayers were mock-infected (lane 1) or infected with uv-HCMV. Either no antibody (lane 2) or 450 units of anti-IFN-β antibody (lane 3) was added at 1 h pi. Extracts were made at 24 h pi and analyzed for the presence of HIF-1α. C. HFFF2 monolayers were mock-infected (lane 1), infected with uv-HCMV (lane 2), incubated with medium from mock-infected HFFF2 cells (lane 3), incubated with medium from uv-HCMV-infected HFFF2 cells (lane 4), or incubated with medium from uv-HCMV-infected HFFF2 cells plus 450 units of anti-IFN-β antiserum (lane 5). After 24 h, extracts were made and analyzed for the presence of HIF-1α and ISG15.

This time course mirrors the induction of HIF-1α (Fig. 1D). The effect of the compound LY294002, a general inhibitor of PI3K and PIKKs (Knight et al., 2006), was tested. Accumulation of HIF-1α was severely reduced when the compound was added at the low concentration of 10 μM 1 h after infection with uv-HCMV (Fig. 3B). The stimulation of Akt phosphorylation at S473 in samples from cultures infected with uv-

Table 1
Quantification of HIF-1α-specific RNA.

Infection	Relative HIF-1α-specific RNA
uv-HCMV, 7 h pi	1.9 (0.11)
uv-HCMV, 24 h pi	10.2 (1.82)
HCMV, 7 h pi	1.5 (0.10)
HCMV, 24 h pi	3.5 (0.58)

HFFF2 monolayers were mock-infected or infected with uv-HCMV or unirradiated HCMV. RNA was prepared at 7 h and 24 h pi and analyzed by qRT-PCR. The amount of HIF-1α-specific RNA compared to the value from mock-infected cells is presented, after normalization to the levels of β-actin-specific RNA. The mean values from three experiments are presented, with standard errors in brackets.

HCMV, and also basal phosphorylation in mock-infected cells, was abolished by the presence of LY294002. The results in Fig. 3A and B therefore demonstrate that infection with uv-HCMV results in activation of Akt, and that this is important for the induction of HIF-1α.

The effect of inhibitor KU55933, which is highly specific for ATM kinase (Hickson et al., 2004), was also investigated (Fig. 3C). Addition of this compound at 1 μM and 3 μM resulted in increasing inhibition of HIF-1α accumulation (Fig. 3C, lanes 3 and 4). Phosphorylation of Akt at S473 was also reduced over the same concentration range. This finding raised the possibility that ATM, a PIKK that is distinct from PI3K, transmits a signal initiated by infection with uv-HCMV. Phosphorylation of T308 is also an important step in the activation of Akt, therefore the status of this residue was examined (Fig. 3D). Infection with uv-HCMV resulted in increased phosphorylation at T308 (Fig. 3D, lanes 2 and 3), and this response was inhibited by LY294002 (Fig. 3D, lane 4) and KU55933 (Fig. 3D, lane 1). To ensure that the inhibitors LY294002 and KU55933 were acting as expected, their effects on induction of Akt phosphorylation in response to insulin-like growth factor 1 (IGF-1) were tested, since it is well established that IGF-1 is an effector of Akt phosphorylation through the activities of PI3K and PDK1 (Alessi and Cohen, 1998) (Fig. 3E and F). Phosphorylation of Akt at S473 was detected 2 h after addition of 50 nM IGF-1 (Fig. 3E, lane 3), and was inhibited by 10 μM LY294002 (Fig. 3E, lane 4) but not by 3 μM KU55933 (Fig. 3E, lane 1), demonstrating that the latter compound did not affect the pathway initiated through PI3K. Equivalent results were obtained by analysis of phosphorylation of Akt at T308 (Fig. 3F). Addition of IGF-1 did not elevate HIF-1α levels at either 2 h or 24 h after addition to HFFF2 cells (results not shown).

Further tests to address the specificity of inhibition by LY294002 and KU55933 were carried out. The compounds did not affect production of ISG15 after infection with uv-HCMV (Fig. 3G). In addition, induction of virus-encoded ICP36, an early protein that is synthesized with a time course similar to that of HIF-1α, was not greatly altered after infection with unirradiated HCMV (Fig. 3H). The latter control is relevant, since a number of other compounds that we tested (PI3K inhibitors wortmannin, PI-103, PI3K inhibitor VII and Akt inhibitors IV, VIII and X) blocked HIF-1α induction but also severely reduced ICP36 synthesis, suggesting that they caused additional nonspecific or indirect effects (results not shown).

It was somewhat surprising that induction of HIF-1α had not been reported previously, and one possible reason derives from the fact that the experiments described here were carried out with fetal calf serum present in the culture medium throughout the experiments, whereas many studies on activation of signaling pathways by HCMV have utilized cells that were serum-starved prior to and after infection. The possible influence of serum concentration was therefore investigated (Fig. 4). Induction of HIF-1α was not detectable when fetal calf serum was absent from medium added after virus adsorption (Fig. 4A, lanes 1 and 2), although a full response occurred when the serum concentration was 1% or greater (Fig. 4A, lanes 5 and 6). Increased phosphorylation of Akt at S473 was observed at all serum concentrations, but the absolute levels of phosphorylated protein were greater as the serum level increased to 1%. Synthesis of ISG15 (Fig. 4B), or ICP36 after infection with unirradiated virus (Fig. 4C), was similar whether the culture medium had no serum or 5% serum.

The observation of S473 phosphorylation at 24 h pi, irrespective of the presence of serum, is in apparent contrast with a previous report in which no change in Akt phosphorylation at this residue was detected after at 24 h after infection with uv-HCMV (Johnson et al.,

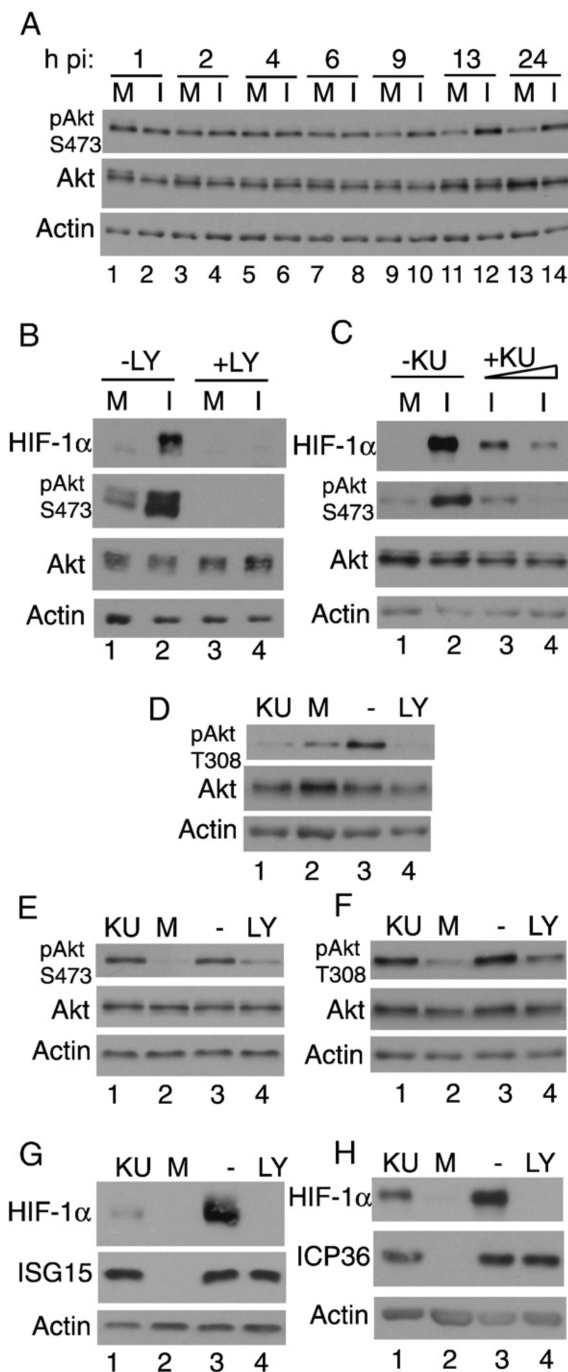

Fig. 3. Effect of LY294002 and KU55933 on induction of HIF-1α. A. HFFF2 monolayers were mock-infected (M) or infected with uv-HCMV (I), and samples were analyzed at various times for the presence of Akt phosphorylated at S473 (pAktS473) or for total Akt (Akt). B. HFFF2 monolayers were mock-infected (M, lanes 1 and 3) or infected with uv-HCMV (I, lanes 2 and 4). LY294002 (10 μM) was added at 1 h pi, and extracts were made at 24 h pi. Extracts were analyzed for the presence of HIF-1α, Akt phosphorylated at S473 or for total Akt. C. HFFF2 monolayers were mock-infected (lane 1) or infected with uv-HCMV (lanes 2 to 4). KU55933 was added at 1 μM (lane 3) or 3 μM (lane 4) at 1 h pi and extracts were made at 24 h pi. Extracts were analyzed for HIF-1α, Akt phosphorylated at S473 or for total Akt. D. HFFF2 monolayers were mock-infected (lane 2) or infected with uv-HCMV and untreated (lane 3) or treated with 3 μM KU55933 (lane 1) or 10 μM LY294002 (lane 4) at 1 h pi. Extracts were made at 24 h pi and analyzed for Akt phosphorylated at T308 (pAktT308) or for total Akt. E. HFFF2 monolayers were untreated (lane 2) or preincubated for 2 h with 3 μM KU55933 (lane 1), no additions (lane 3) or 10 μM LY294002 (lane 4). IGF-1 (50 nM) was added and incubation continued for a further 2 h. Extracts were made and analyzed for Akt phosphorylated at S473 or for total Akt. F. HFFF2 monolayers were treated as described in the legend to E and extracts were analyzed for Akt phosphorylated at T308 or for total Akt. G. HFFF2 monolayers were mock-infected (lane 2) or infected with uv-HCMV and untreated (lane 3) or treated with 3 μM KU55933 (lane 1) or 10 μM Ly294002 (lane 4) at 1 h pi. Extracts were made at 24 h pi and analyzed for the presence of HIF-1α and ISG15. H. HFFF2 monolayers were mock-infected (lane 2) or infected with HCMV and untreated (lane 3) or treated with 3 μM KU55933 (lane 1) or 10 μM LY294002 (lane 4). Extracts were made at 24 h pi and analyzed for the presence of HIF-1α and ICP36.

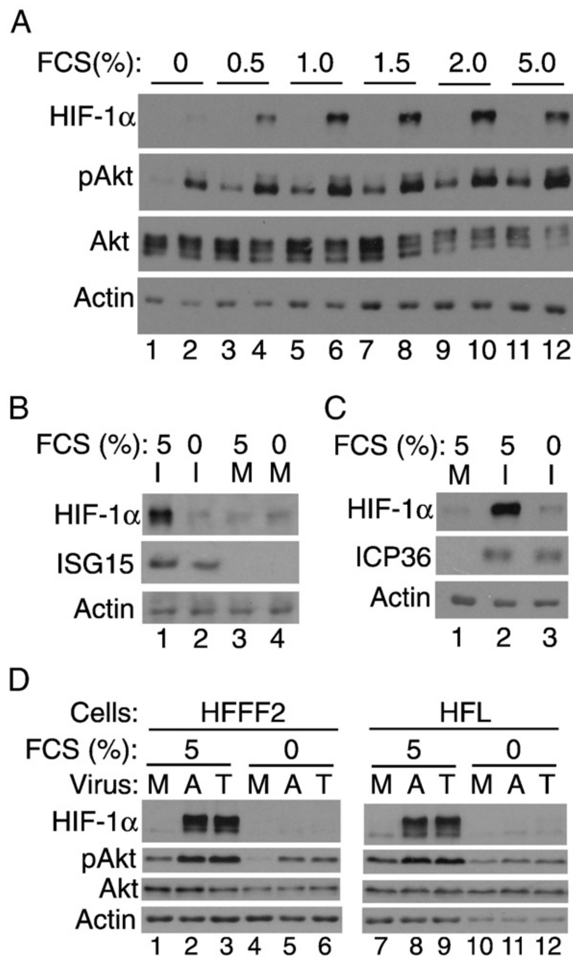

Fig. 4. Effect of serum concentration and rapamycin on induction of HIF-1α. A. HFFF2 monolayers were mock-infected (odd numbered lanes) or infected with uv-HCMV (even numbered lanes). After adsorption of virus for 1 h, monolayers were washed twice with serum-free medium and overlaid with medium containing the indicated concentrations of fetal calf serum. Extracts were made at 24 h pi and analyzed for the presence of HIF-1α, Akt phosphorylated at S473 (pAkt), or total Akt (Akt). B. HFFF2 cultures were infected with uv-HCMV (lanes 1 and 2) or mock-infected (lanes 3 and 4). After adsorption of virus for 1 h, monolayers were washed twice with serum-free medium and overlaid with medium containing 5% serum (lanes 1 and 3) or no serum (lanes 2 and 4). Extracts were made at 24 h pi and analyzed for the presence of HIF-1α and ISG15. C. HFFF2 monolayers were mock-infected (lane 1) or infected with HCMV (lanes 2 and 3). After adsorption of virus for 1 h, monolayers were washed twice with serum-free medium and overlaid with medium containing 5% serum (lanes 1 and 2) or no serum (lane 3). Extracts were made at 24 h pi and analyzed for the presence of HIF-1α and ICP36. D. Monolayers of HFFF2 (lanes 1 to 6) or HFL (lanes 7 to 12) cells were mock-infected (M) or infected with uv-irradiated HCMV strain AD169 (A) or Towne (T). After adsorption of virus for 1 h, monolayers were washed twice with serum-free medium and overlaid with medium containing 5% serum (lanes 1 to 3 and 7 to 9) or no serum (lanes 4 to 6 and 10 to 12). Extracts were made at 24 h pi and analyzed for the presence of HIF-1α, Akt phosphorylated at S473 (pAkt), or for total Akt (Akt).

2001). The protocol used by Johnson et al. (2001) differs from that used in the experiments reported here in a number of respects. The previous study used cells that were serum-starved for 48 h prior to and after infection, and in addition the cell type (human embryonic lung fibroblasts) and HCMV strain (Towne) were different. Induction of HIF-1α and S473 phosphorylation were compared in human fetal foreskin and fetal lung fibroblasts, after infection with uv-irradiated HCMV AD169 or Towne strains (Fig. 4D). In foreskin fibroblasts, infection with either AD169 (A) or Towne (T) induced HIF-1α in the presence, but not the absence, of serum added after adsorption, and Akt phosphorylation occurred irrespective of the presence of serum (Fig. 4D, lanes 1 to 6). Similarly, HIF-1α was induced by both virus strains in the presence of serum in lung fibroblasts, and S473 phosphorylation occurred after infection with either virus strain (Fig. 4D, lanes 7 to 12). In relative terms, however, the degrees of HIF-1α induction and Akt phosphorylation were lower in lung fibroblasts.

HIF-1α-specific RNA levels were determined by qRT-PCR at 24 h pi in cultures treated with LY294002, KU55933 or medium without serum (Table 2). KU55933 had no effect, whereas LY294002 and absence of serum reduced the stimulation by uv-HCMV from 8.6-fold to 4.8- and 3.5-fold, respectively.

Discussion

The results presented here reveal a new aspect of interaction of HCMV with the host cell. The “master” transcription factor HIF was induced upon infection with HCMV, and the response was greater when the virus was irradiated with a dose of uv light sufficient to prevent detectable viral gene expression, indicating that components of the initial infecting virion are responsible. It is surprising that the interaction of virus particle with the host cell can provoke a response that is delayed to such an extent, since by 9 h pi at the relatively high MOI of 3 the viral transcription program had progressed through the stages at which the genome reaches the nucleus, is uncoated and becomes available for transcription. Indeed, observing the time course of HIF-1α induction in isolation would favor the conclusion that viral IE, or even early, proteins play major roles in the response. Presumably the initial events in infection trigger a series of cellular changes that culminate in the production of HIF-1α.

Two elements of the response to HCMV are necessary for induction of HIF-1α, neither of which is sufficient alone. Increased transcription, or an effect on processing of the primary transcript, is required in combination with the maintenance of Akt phosphorylation. The cellular factor NF-κB, when activated, strongly stimulates *HIF-1α* transcription (Frede et al., 2006; Rius et al., 2008; van Uden et al., 2008). Activation and increases in the amount of this factor also occur after HCMV infection, and these events are important for the efficient transcription of IE genes (Caposio et al., 2004; DeMeritt et al., 2004; Kowalik et al., 1993; Sambucetti et al., 1989; Yurochko et al., 1995). It is possible, therefore, that utilization of NF-κB for viral IE transcription brings the additional consequence of HIF-1α induction.

It is not clear from our data whether the uv-HCMV-mediated stimulation of Akt phosphorylation is vital for the induction of HIF-1α, or if the constitutive activity of the enzyme suffices. Nonetheless, the finding that increased Akt phosphorylation coincides with HIF-1α induction after infection with uv-HCMV highlights a delayed response to the initial virus–cell interaction that may have profound effects on cell physiology, since Akt influences apoptosis, protein synthesis, cell proliferation and glucose metabolism. Indeed, we have detected increased phosphorylation of glycogen synthase kinase, a substrate of Akt, at 24 h after infection with uv-HCMV (C.M. Preston, unpublished observations). Activation of the PI3K/Akt pathway has been reported previously (Johnson et al., 2001). That study demonstrated rapid

Table 2
Effect of inhibitors on HIF-1α-specific RNA accumulation.

Treatment	Relative HIF-1α-specific RNA
uv-HCMV	8.6 (7.1, 10.1)
uv-HCMV + KU55933	10.8 (10.7, 10.9)
uv-HCMV + LY294002	4.8 (5.0, 4.5)
uv-HCMV, no serum	3.7 (4.5, 2.8)

HFFF2 cells were mock-infected or infected with uv-HCMV. At 1 h pi, no additions, 3 μM KU55933, 10 μM LY294002, or serum-free medium (after two washes) were added. RNA was prepared at 24 h pi and analyzed for the presence of HIF-1α-specific RNA. The amount of HIF-1α-specific RNA compared to the value from mock-infected cells is presented, after normalization to the levels of β-actin-specific RNA. The means of two experiments are presented, with the individual determinations in brackets.

phosphorylation of Akt within the first 30 min of infection, which subsided at 2 h pi and re-emerged from 4 h onwards after infection with untreated HCMV. The initial activation was observed after infection with uv-HCMV but the second wave was not, in contrast to our finding that Akt phosphorylation was increased by uv-HCMV at 9 h and 24 h pi. We eliminated differences in virus strain as a significant variable, and observed Akt phosphorylation in lung fibroblasts at 24 h pi, albeit at lower efficiency than in foreskin cells. The other major experimental difference between the studies is that Johnson et al. (2001) used cells that were serum-starved for 48 h prior to infection and during the experiment, whereas in our work serum was present throughout. We have not tested cultures preincubated in serum-free medium, but we found increased Akt phosphorylation at 24 h after infection with uv-HCMV when serum was omitted from 1 h pi onwards (Fig. 4).

The inhibitory action of LY294002 merely indicates the involvement of PI3K or a PIKK in induction of HIF-1α, since this inhibitor is active against many classes of these enzymes (Knight et al., 2006). The compound KU55933, however, is very specific for ATM protein kinase, and its inhibitory effect strongly suggests a role for this enzyme in activation of Akt and induction of HIF-1α. The ATM kinase is an early sensor of DNA damage, localizing with DNA-PKcs to double strand breaks and initiating responses that stall cell cycle progression and enable repair of the lesion. It is conceivable that the two free ends of the incoming HCMV genome mimic a DNA lesion which is recognized by ATM. In turn, this may result in phosphorylation of Akt either directly or, more likely, through the action of DNA-PKcs by in response to phosphorylation by ATM (Chen et al., 2007). The ability of DNA-PKcs to promote phosphorylation of Akt at both S473 and T308 in response to damage has been described (Bozulic et al., 2008). In preliminary experiments, we found that the DNA-PKcs inhibitor NU7026 was almost as potent as KU55933 in inhibiting induction of HIF-1α (C.M. Preston, unpublished observations). The possibility that ATM or DNA-PKcs are involved in induction of HIF-1α receives support from a study of the DNA damage response in HCMV-infected fibroblasts (Luo et al., 2007). Increased phosphorylation of Nijmegen break syndrome protein (Nbs1), an ATM substrate, was detected from 4 h pi and maintained throughout infection, suggesting a role for ATM at an early time. The usual relocalization of Nbs1 and most other proteins associated with DNA repair to discrete foci was not observed, however, leading to the conclusion that input viral DNA initiates some aspects of a damage response but that the migration of the associated proteins to the appropriate sites within the nucleus is inhibited (Luo et al., 2007). This is compatible with the hypothesis that ATM integrates a response to the viral genome via DNA-PKcs and activation of Akt, leading to increased translation of HIF-1α mRNA.

Akt activation occurs in the absence of serum but induction of HIF-1α does not, suggesting that the cell components responsive to serum act downstream of Akt. Although there are many candidates, due to the central role of Akt in cell proliferation, there is evidence from other systems that mTOR is important for translation of HIF-1α mRNA (Laughner et al., 2001; Page et al., 2002). Indeed, we have found that rapamycin, which acts specifically on mTOR, strongly depresses HIF-1α induction (M.J. Nicholl and C.M. Preston, unpublished observations). Although mTOR is stimulated by Akt, its activity is known to be reduced by a number of pathways when nutrients are limited or cells are stressed, therefore translation of HIF-1α mRNA may be inhibited in serum-free conditions by one or more mechanisms (Bai and Jiang, 2010; Hotamisligil and Erbay, 2008; Li et al., 2003; Reiling and Sabatini, 2006). Speculation on the possible significance of mTOR in the requirement for serum is complicated by the fact that HCMV infection alters the properties of this enzyme. The substrate specificities and rapamycin sensitivities of the mTOR-associated complexes mTORC1 and mTORC2 are altered by a mechanism that is currently unclear (Kudchodkar et al., 2004; Kudchodkar et al., 2006; Moorman and Shenk, 2010; Walsh et al., 2005). In addition, a viral protein (UL38) interacts with the tuberous sclerosis complex, which regulates mTOR activity, to render it insensitive to stress signals (Moorman et al., 2008). A hypothesis that links mTOR with the requirement for serum in HIF-1α induction requires a resolution of whether the documented changes in the properties of mTOR also occur after infection with uv-HCMV. Its absence in serum-free conditions suggests that HIF-1α is not of major significance for HCMV replication in cultured fibroblasts, because the omission of serum from medium only marginally reduced virus yields (Kudchodkar et al., 2004). The existence and significance of the response in other cell types remain to be elucidated.

The induction of HIF-1α has important effects on cell physiology, since the protein stimulates expression of a plethora of cellular gene products, including those involved in glucose uptake, glycolysis, angiogenesis and inflammation. Future studies should investigate the extent to which the infecting HCMV particle can provoke these HIF-1-mediated changes. In humans, HCMV infects a range of cell types and in many cases the outcome is latency or abortive infection. In these situations, where productive virus replication does not ensue, it is nonetheless possible that activation of Akt and induction of HIF-1α have profound effects on the host cell, and analysis of these possibilities may yield important new insights into HCMV pathogenesis.

Materials and methods

Cells, viruses and inhibitors

Human fetal foreskin fibroblasts (HFFF2, European Collection of Cell Cultures) and human fetal lung cells (HFL, ICN Flow) were propagated in Dulbecco's modified Eagle medium supplemented with 5% v/v fetal calf serum, 5% v/v newborn calf serum, nonessential amino acids, plus 100 units of penicillin and 100 μg of streptomycin per milliliter (D5 + 5). After infection, Dulbecco's modified Eagle medium supplemented with 2% v/v fetal calf serum, nonessential amino acids, plus 100 units of penicillin and 100 μg of streptomycin per milliliter was used. HCMV, strain AD169, was propagated in HFFF2 cells and stocks were prepared by centrifugation of medium harvested from infected monolayers, first at 1000*g* for 15 min to remove cellular debris followed by 23,000*g* for 2 h. Pellets from the latter centrifugation were resuspended in D5 + 5 and purified further by negative viscosity: positive density gradient centrifugation (Talbot and Almeida, 1977). The band identified as virions was removed from the gradient, pelleted by centrifugation at 23,000*g* for 2 h and resuspended in D5 + 5. HCMV strain Towne, prepared from infected cell culture medium, was kindly provided by Dr. D. Dargan (MRC—University of Glasgow Centre for Virus Research, Glasgow, UK). HCMV was uv-irradiated in a strata-linker (Stratagene), as described previously (Lukashchuk et al., 2008). HFFF2 monolayers, consisting of 1.5×10^5 cells, were routinely infected with 3 pfu of HCMV per cell, or an equivalent amount of virus after uv-irradiation. Cycloheximide, IGF-1 and deferoxamine mesylate were obtained from Sigma Aldrich. LY294002, KU55933 and other inhibitors were purchased from Calbiochem Merck.

Protein blotting

Cell extracts were prepared and analyzed by blotting as described previously (Marshall et al., 2002). Human β-actin was used as a loading control. Sources of primary antibodies were: Anti-HIF-1α, BD Transduction Laboratories; anti-phosphoAkt (S473), anti-phosphoAkt (T308), and anti-Akt, Cell Signaling Technology; anti-β-actin, Sigma Aldrich; anti-ISG15, Santa Cruz; anti-HCMV ICP36, Abcam; anti-HCMV IE1/IE2, AbD Serotec.

Quantitative reverse transcription and polymerase chain reaction

Total RNA was isolated using RNA-Bee reagent (AMS Biotechnology) according to the manufacturer's protocol, and quantified by uv spectrometry. RNA was converted to complementary DNA using oligo

(dT) primers and Moloney murine leukemia virus reverse transcriptase. Quantitative polymerase chain reaction analysis was performed using TaqMan probes for HIF-1α (Hs00936366, Applied Biosystems Inc.) and β-actin (4333762, ABI). Reactions were performed under conditions specified in the ABI TaqMan Universal Master Mix II protocol, on an ABI 7500 Real Time PCR machine. Data were quantified by the relative standard curve method.

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