Human cytomegalovirus infection downregulates the expression of glial fibrillary acidic protein in human glioblastoma U373MG cells: identification of viral genes and protein domains involved

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Human cytomegalovirus (HCMV) has tropism for glial cells, among many other cell types. It was reported previously that the stable expression of HCMV immediate-early protein 1 (IE1) could dramatically reduce the RNA level of glial fibrillary acidic protein (GFAP), an astroglial cell-specific intermediate filament protein, which is progressively lost with an increase in glioma malignancy. To understand this phenomenon in the context of virus infection, a human glioblastoma cell line, U373MG, was infected with HCMV (strain AD169 or Towne). The RNA level of GFAP was reduced by more than 10-fold at an m.o.i. of 3 at 48 h post-infection, whilst virus treated with neutralizing antibody C23 or with UV light had a much-reduced effect. Treatment of infected cells with ganciclovir did not prevent HCMV-mediated downregulation of GFAP. Although the expression of GFAP RNA is downregulated in IE1-expressing cells, a mutant HCMV strain lacking IE1 still suppressed GFAP, indicating that other IE proteins may be involved. IE2 is also proposed to be involved in GFAP downregulation, as an adenoviral vector expressing IE2 could also reduce the RNA level of GFAP. Data from the mutational analysis indicated that HCMV infection might affect the expression of this structural protein significantly, primarily through the C-terminal acidic region of the IE1 protein.

INTRODUCTION

Human cytomegalovirus (HCMV), a member of the subfamily Betaherpesvirinae, persistently infects the majority of the world’s population. Endothelial, astroglial and neuronal cells in the human brain have been shown to be infected by HCMV in vitro (Plachter et al., 1996; Poland et al., 1990; Schmidbauer et al., 1989). Among the many different cell types that the virus infects in vivo, HCMV has tropism for brain cells and is associated with serious disorders of the central nervous system in congenitally infected infants and HCMV-infected AIDS patients (Cinque et al., 1997). Recently, the presence of the HCMV genome and gene products, including the immediate-early protein 1 (IE1), has been detected in a high percentage of human malignant glioma samples, suggesting that HCMV might contribute to the malignant phenotype of glioma (Cobbs et al., 2002; Mitchell et al., 2008; Sabatier et al., 2005; Scheurer et al., 2008).

The major IE genes encode the first de novo virus-encoded proteins synthesized following infection, some of which function to regulate viral and cellular gene expression during virus replication. The 72 kDa IE1 and 86 kDa IE2 proteins are the most abundantly expressed IE gene products. RNA transcripts originating from the major IE gene region span five major exons that are alternatively spliced to produce IE1 (exons 1–4) and IE2 (exons 1–3 and 5) (Stenberg et al., 1984, 1985). Thus, exon 5 is unique to IE2, whilst exon 4 is unique to IE1 (Mocarski, 2001). The translation of IE1 and IE2 initiates from the ATG codon in exon 2, and these two proteins share 85 identical residues at their N termini. Many data have indicated that both IE1 and IE2 stimulate transcription from a variety of cellular and viral genes involved in viral replication and cell survival, alone or in concert with other transcriptional regulators, including the Rb family members, the E2F family members, p53, SP1, TAFII130/TAF4 and histone modification proteins (Bonin & McDougall, 1997; Bryant et al., 2000; Fortunato et al., 1997; Hayhurst et al., 1995; Lukac et al., 1997; Margolis et al., 1995; Nevels et al., 2004b; Pajovic et al., 1997; Poma et al., 1996; Schwartz et al., 1996). The interplay between the IE proteins of HCMV and cellular factors has been implicated in disrupting normal cell-cycle progression and impeding apoptosis (Castillo...
et al., 2000; Kim et al., 2003; Lukac & Alwine, 1999; Murphy et al., 2000; Song & Stinski, 2002; Tanaka et al., 1999; Yu & Alwine, 2002; Zhu et al., 1995).

We reported previously that the IE1 protein could modulate the expression of various cellular genes, including those implicated in carcinogenesis, in the human glioblastoma cell line U373MG (Lee et al., 2005). The gene most affected by IE1 was glial fibrillary acidic protein (GFAP), an intermediate filament protein that has been widely used as a marker for cells of astroglial lineage under pathological as well as normal conditions (de Armond et al., 1980; Deck et al., 1978). The expression of GFAP is enhanced following astrocyte differentiation and certain pathological conditions such as epilepsy, trauma, demyelination and degeneration (Eng & Rubinstein, 1978; Steward et al., 1991; Tetzlaff et al., 1988). The transition from GFAP-positive to GFAP-negative cells has often been interpreted as the transformation of less cancerous cells to a blastic, less mature, more aggressive state (Duffy, 1982; Eng & Rubinstein, 1978; Hobilkova et al., 2007). Proteomics analysis from glioma samples of different grades has shown that the GFAP protein is decreased in grade IV glioblastomas, but not in grade III tumours (Chumbalkar et al., 2005). Reduced GFAP expression in glioma cell lines was also reported to be associated with cell motility, invasiveness and proliferation, implicating the pathogenesis of glial tumours (Elodeid et al., 2000; Murphy et al., 1998; Rutka & Smith, 1993; Rutka et al., 1994, 1997; Toda et al., 1999; Zhou & Skalli, 2000).

In this study, we extended our previous work demonstrating that IE1 expression greatly reduced the level of GFAP, to test whether GFAP expression is also affected in the context of actual virus infection and also to determine the IE region involved in the downregulation of GFAP. It was found that virus infection could also diminish the level of GFAP expression by a similar magnitude, as in the case of the stable cell line expressing IE1 alone. Furthermore, it was discovered that IE2 also suppressed GFAP expression and that the presence of the acidic domain in the IE1 protein appeared to be crucial for the downregulation of GFAP expression.

METHODS

Cell cultures. Human glioblastoma cell line U373MG (HTB 17) cells were obtained from ATCC. The 293T cell line, provided by Stephen Smale (University of California, Los Angeles, CA, USA), is a subline of the adenovirus (Ad) type 5-transformed human embryonic kidney cell line 293 (ATCC CRL1573) that expresses the simian virus 40 T antigen. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL) supplemented with 10% fetal bovine serum (Gibco-BRL), 100 penicillin U ml⁻¹ and 100 mg streptomycin ml⁻¹ in a 5% CO₂ humidified incubator at 37 °C.

Virus infection. The IE1-defective mutant virus HCMV (CR208) and its parental wild-type HCMV (strain Towne) have been described elsewhere (Greaves & Mocarski, 1998). The virus stocks used in this study were grown and titrated in HFF cells (strains Towne and AD169) or IE1-expressing HFF cells (CR208) as described previously (Lafemina et al., 1989). Infectious virus was neutralized with human monoclonal antibody (mAb) C23 as detailed elsewhere (Klein et al., 1999). To inactivate virus with UV, stocks were placed in 1.5 ml Eppendorf reaction tubes and exposed to 254 nm UV light (40 W) for 1 h. Replication-defective EIA⁺, EIB⁺ and E3⁻ Ad vectors expressing the ‘set-off’ transactivator (Ad-Trans), the IE1 protein (Ad-IE1) or the IE2 protein (Ad-IE2) were provided by Daniel N. Streblov (Oregon Health Science University, OR, USA) and grown in 293 cells as described previously (Murphy et al., 2000). The titres of the various recombinant adenoviral vectors were determined by plaque assay on 293 cells. For transduction experiments, an equal m.o.i. of recombinant Ad vectors in DMEM containing 3 μl Lipofectamine ml⁻¹ (Invitrogen) was always used. After 1 h at 37 °C, the inoculum was removed and the cells were maintained in medium.

Plasmids. Retroviral vector MIN-IE1 was constructed by inserting the cDNA sequence of IE1 into retroviral vector MIN (Yu et al., 2000a). All deletion mutants were constructed by PCR-driven mutagenesis with plasmid MIN-IE1 as template.

Measuring GFAP RNA level by real-time quantitative RT-PCR. CDNAs were synthesized from 1 μg of each RNA sample by reverse transcription. Real-time quantitative RT-PCR analysis was performed using SYBR Green dye and primers specific for GFAP or β-actin (Lee et al., 2005). From each PCR, the CT value was obtained from the amplification curve. The difference in mRNA levels of cellular genes between IE1- or IE1 mutant-expressing and control cell lines was calculated from the ΔCT value as described previously (Martell et al., 1999; Yu et al., 2000b).

Western blot analysis. To confirm the expression of IE proteins, cells were washed in cold PBS and lysed in buffer containing 10 mM Tris/HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and Protease Inhibitor Cocktail (Roche) by several cycles of freezing and thawing. Total cell lysates were centrifuged at 12 000 g for 15 min at 4 °C. Protein concentration was determined by a Bradford assay. Protein concentration was determined by a Bradford assay (Bio-Rad). Equal amounts of protein (40–80 μg) were resolved by SDS-PAGE (15% gel) and transferred to nitrocellulose membranes (Amersham). Immunoblotting was performed with anti-IE1 (MAB810; Chemicon), anti-GFP (NeoMarkers), anti-β-actin (Sigma) or anti-mouse horseradish peroxidase (Sigma)-conjugated mAbs. For quantification of the protein bands, the films were scanned and densitometric analysis was performed using MultiGauge image analysis software (version 3.0; Fuji Film). Band intensities were measured by taking the mean pixel intensity. All band measurements had background levels subtracted.

RESULTS AND DISCUSSION

HCMV downregulates GFAP expression in a dose- and time-dependent manner

To examine the effects of virus infection on GFAP expression, U373MG cells were infected with HCMV (strain AD169) at increasing m.o.i. as shown in Fig. 1. After 48 h, total RNAs were prepared from HCMV-infected cells. The change in the level of GFAP RNA, rather than its protein, was measured by real-time quantitative RT-PCR, because the GFAP protein is known to be very stable, with a half-life of 7–8 days (Chiu & Goldman, 1984; Morrison et al., 1985). HCMV infection resulted in a decrease in the level of GFAP RNA in a
dose-dependent manner (Fig. 1). The level of GFAP RNA was decreased by almost 10-fold at an m.o.i. of 3.

To investigate the time-course effect of HCMV infection on GFAP expression, U373MG cells were infected with HCMV at an m.o.i. of 3, based on the above results. Total RNA and whole-cell lysates were harvested every 24 h over a 5-day period. The level of GFAP RNA was decreased by more than 10-fold at 48 h post-infection (p.i.) (Fig. 2a). Additional experiments have demonstrated that GFAP downregulation can be detected as early as 6 h p.i. (data not shown). The sharp decrease in GFAP RNA coincided with the time point when IE proteins began to be expressed at high levels in these cells (Fig. 2b). These data suggested that HCMV infection can greatly affect the expression of GFAP at the RNA level during a relatively early stage of infection.

Downregulation of GFAP by HCMV infection occurs after virus penetration into the cell, but before virus DNA replication

A series of experiments was set up to identify in which step of the virus life cycle GFAP downregulation occurs. First, whether the downregulation of GFAP resulted from the physical interaction between HCMV virions and U373MG cells was investigated by using an anti-HCMV mAb, C23. Neutralization with this antibody is known to inhibit the penetration of virions into target cells, but allows the attachment of virions to cells (Ohizumi et al., 1992). A total of 10⁶ p.f.u. HCMV virions was incubated with mAb C23 for 4 h at 37 °C and the mixture was used to infect U373MG cells. This treatment is known to fully neutralize virus infectivity and it was indeed confirmed that IE expression was completely inhibited under this condition (data not shown). Total RNAs were harvested every 24 h over a 5 day period to measure the level of GFAP RNA by real-time quantitative RT-PCR analysis. As shown in Fig. 3(a), the neutralized virus reduced the level of GFAP RNA only modestly, whilst untreated virus dramatically decreased the expression level of GFAP RNA. These data suggested that the attachment of HCMV virions was not sufficient to cause the efficient downregulation of GFAP and that an event following virus adsorption was primarily responsible for the change in the level of this structural protein.

Next, whether penetration of HCMV virions is responsible for GFAP downregulation was tested by using UV-inactivated virus. UV-inactivated virus is taken up into cells, but does not support the expression of viral proteins from the damaged viral DNA (Maisch et al., 2002). U373MG cells were infected with UV-inactivated HCMV or intact virus at an m.o.i. of 3. As shown in Fig. 3(a), infection of U373MG cells by UV-inactivated HCMV produced only a 15–30 % reduction in the level of GFAP RNA, indicating that virus penetration of HCMV virions alone was also not sufficient to induce efficient down-regulation of GFAP.

The dramatic decrease in the level of GFAP RNA at 48 h p.i. was uniformly consistent among experiments. Based on this observation, the effect of ganciclovir (GCV) on HCMV-mediated downregulation of GFAP was tested. GCV is a potent inhibitor of viral DNA synthesis (Noble & Faulds, 1998). U373MG cells were infected with HCMV at
Downregulation of GFAP by HCMV IE proteins

IE1-deficient mutant virus (CR208) also downregulates GFAP expression

We have demonstrated previously that IE1 downregulates the expression of GFAP RNA in U373MG cells constitutively expressing IE1 (Lee et al., 2005). To test whether virus-mediated downregulation of GFAP RNA is also dependent on the presence of IE1, a mutant deletion virus lacking IE1 exon 4 was examined. CR208 is an IE1-deficient mutant HCMV derived from Towne strain, lacking exon 4 of the major IE region, a 406 aa coding region unique to IE1 (Greaves & Mocarski, 1998). U373MG cells were infected with HCMV CR208 and Towne at an m.o.i. of 3. Although CR208 cannot express the IE1 protein, it still reduced the level of GFAP RNA by 11-fold at 48 h p.i. The magnitude of decrease was similar to that of wild-type in a reproducible manner (Fig. 4). Because other IE proteins, including IE2, are still expressed in CR208-infected cells, this result suggested that some proteins expressed during the IE stage may also be involved in downregulating GFAP.

IE2 can decrease the level of GFAP RNA

To test whether IE2 is also capable of suppressing GFAP RNA levels, an Ad vector capable of inducing high-level expression of functional IE2 protein was employed (Murphy et al., 2000). This particular vector was chosen because, as yet, it has not proved possible to construct an HCMV deletion mutant or produce a cell line constitutively expressing IE2 (Marchini et al., 2001). The expression of IE1 and IE2 proteins was activated by coinfection with Ad-Trans. Ad-Trans is an Ad vector that encodes a TetR-VP16 fusion protein that binds the tetracycline repressor element to activate a minimal HCMV major IE promoter, driving the expression of IE1 or IE2 (Streblow et al., 1999). U373MG cells were transduced with Ad-Trans (m.o.i. of 10), Ad-Trans (m.o.i. of 5) plus Ad-IE1 (m.o.i. of 5), or Ad-Trans (m.o.i. of 5) plus Ad-IE2 (m.o.i. of 5). After 48 h, total RNA and cell lysates were prepared from transduced cells. The expression of IE1 and IE2 from the adenoviral system was confirmed by Western blot analysis (Fig. 5a). The level of GFAP RNA was measured by real-time quantitative RT-PCR analysis and the result is shown in Fig. 5(b).
Consistent with the previous results from experiments involving a retroviral vector (Lee et al., 2005), transduction of an adenoviral vector expressing IE1 decreased the level of GFAP RNA by almost 10-fold. Expression of IE2 by an adenoviral vector also decreased the level of GFAP RNA, but only by 1.7-fold.

To analyse this effect further, U373MG cells were transduced with Ad-IE1 and Ad-IE2 at various m.o.i. and the changes in GFAP RNA levels were determined by real-time quantitative RT-PCR (Fig. 5c). Both Ad-IE1 and Ad-IE2 decreased the level of GFAP RNA in a dose-dependent manner, but Ad-IE1 downregulated the expression of GFAP RNA at a much lower m.o.i. For example, at an m.o.i. of 5, a 10-fold reduction in the level of GFAP RNA was achieved with Ad-IE1, whilst only a 2-fold reduction was found with Ad-IE2. At m.o.i. higher than 10, Ad-IE2 could also downregulate GFAP expression almost completely. This differential effect could reflect the relative expression levels of the IE proteins in the cell or more efficient downregulation of GFAP expression by IE1. Whatever the cause, these data indicated that IE1 and IE2, independently, exert a negative influence on GFAP expression.

### The C-terminal acidic domain of IE1 is required for GFAP downregulation

To identify the particular domain of IE1 involved in GFAP downregulation, a mutational analysis was carried out. A series of IE1 mutant proteins was constructed as summarized in Fig. 6(a). The N-terminal 24 aa encoded by exon 2 contain a nuclear-localization signal (Lee et al., 2007; Wilkinson et al., 1998) and were included in all constructs. IE1Δex3 lacks exon 3, which is required for IE1-mediated disruption of PML bodies (Wilkinson et al., 1998) and interactions between IE1 and p107 (Johnson et al., 1999; Zhang et al., 2003). This region also appears to contain powerful transactivational activity, and deletion of this region from IE2 completely eliminates its ability to transactivate the HCMV early promoter (Stenberg et al., 1990). The smallest IE1 mutant protein, lacking the entire exon 4 (IE1Δex4), was constructed to evaluate the involvement of this IE1-unique region in GFAP downregulation. Within this region, the amino acid sequences from position 173 to 197, missing in mutant IE1Δ173–197, have homology to the ATP-binding sites found on many kinases (Pajovic et al., 1997). Mutant IE1Δ267–286 lacked the region containing a zinc-finger domain, which has been shown to bind to CTF-1 (Hayhurst et al., 1995). IE1 with a deletion from aa 290 to 320 is defective in PML desumoylation, disruption of PML oncogenic domains, transactivation and p107 binding (Lee et al., 2004; Zhang et al., 2003). A recent study showed that the C-terminal region from aa 421 to 475 is necessary for full trans-complementation of the growth of IE1-defective virus HCMV CR208 (Reinhardt et al., 2005).

The respective fragments were cloned by PCR and inserted into the MIN retroviral vector. Cell-free retroviral vectors were prepared by a three-plasmid transfection method and used to transduce U373MG cells followed by G418 selection. The expression of respective mutant proteins was confirmed by Western blot analysis. IE1 and six deletion mutants were expressed to similar levels (Fig. 6c, lanes 2–8). The level of GFAP RNA was measured by real-time quantitative RT-PCR analysis (Fig. 6b). The level of GFAP RNA from IE1Δex4 was moderately decreased, by 1.6-fold, whilst IE1Δex3 failed to downregulate this
structural gene, suggesting that exon 4 is required for IE1 to downregulate GFAP expression. IE1-mediated suppression of GFAP expression was somewhat inhibited in the three mutant proteins IE1\(^{D_{173–197}}\), IE1\(^{D_{267–286}}\) and IE1\(^{D_{290–320}}\); the magnitude of the decrease in the GFAP RNA level was 1.9-fold, 2.9-fold and 2.6-fold, respectively, compared with 8-fold in the case of full-length IE1 protein. In contrast, IE1\(^{D_{421–475}}\) failed to downregulate GFAP expression.

Because the U373MG cells had been selected by G418 in this case, the level of GFAP protein was also determined by Western blot analysis (Fig. 6c). Consistent with the RNA data, IE1\(^{D_{173–197}}\), IE1\(^{D_{267–286}}\) and IE1\(^{D_{290–320}}\) resulted in an approximate 2-fold decrease in GFAP protein expression, whilst the deletion from aa 421 to 475 abolished the IE1 activity of downregulating GFAP expression. These data showed that the region from aa 421 to 475 is essential for the IE1-mediated downregulation of GFAP.

This C-terminal region (aa 421–475) is considered to be an acidic domain (Lafemina et al., 1989; Stenberg et al., 1984). Among the 55 aa missing in IE1\(^{D_{421–475}}\), 26 residues are glutamic or aspartic acid, and there are seven serine residues that can be phosphorylated (Fig. 6a). This acidic domain is also highly conserved in primate cytomegaloviruses (Reinhardt et al., 2005). Although HCMV IE1 and murine cytomegalovirus IE1 appear to have little in common, they share amino acid similarity in the highly acidic C-terminal region, emphasizing the importance of IE1 activity in general (Keil et al., 1987; Tang & Maul, 2006).

A number of studies have shown that acidic domains can play direct roles in trans-activating transcription or replication by many cellular and viral regulatory proteins (He et al., 1993; Li & Botchan, 1993; Pathakamuri & Theilmann, 2002; Pizzorno et al., 1991; Yeung et al., 1993). We examined whether an IE1 protein lacking the acidic domain could function as a dominant-negative inhibitor of GFAP downregulation. However, additional expression of IE1\(^{D_{421–475}}\) in cells already expressing IE1 did not have any...
effect on the level of GFAP that had been lowered by wild-type IE1. Thus, it is unlikely that the acidic domain of IE1 acts as a classic activation domain of trans-activators for GFAP downregulation.

It is possible that IE1 interacts directly with other cellular protein(s) via its acidic domain. An HCMV mutant defective in sumoylation of IE1 residue K450 exhibited reduced expression of IE2 mRNA, suggesting that the SUMO moiety may mediate interaction with components of RNA-processing complexes (Nevels et al., 2004a). However, our result from the site-directed mutagenesis analysis suggested that GFAP downregulation appeared to be independent of IE1 sumoylation (data not shown). The role of this acidic domain remains to be elucidated. A possible involvement of this region in intramolecular or intermolecular electrostatic interaction is under investigation. In this regard, it is interesting to note that this acidic region is followed by a 16 aa terminus that contains a significant number of basic amino acids.

The presence of HCMV is correlated with glioma tumour malignancy, whilst GFAP is an important biomarker used in predicting the change in cancerous cells to the more aggressive state. We demonstrated that HCMV infection efficiently suppressed GFAP expression and that IE1 and IE2 are major viral factors involved in the downregulation. GFAP suppression was dependent on the amount of IE1 or IE2 expressed in the cell. The IE1-mediated inhibition of GFAP needed the C-terminal acidic domain. Investigation into the exact mechanism of how IE1 downregulates GFAP expression at the structural, biochemical and physiological level should be useful in unravelling the role(s) of HCMV in tumours with poor prognosis.

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