

Human cytomegalovirus infection inhibits epidermal growth factor (EGF) signalling by targeting EGF receptors

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Infection with human cytomegalovirus (HCMV) is known to involve complex interactions between viral and cellular factors resulting in perturbation of a number of cellular functions. Specifically, HCMV infection targets control of the cell cycle, cellular transcription and immunoregulation, presumably to optimize the cellular environment for virus persistence and productive infection. Here, we show that HCMV infection also prevents external signalling to the cell by disrupting the function of epidermal growth factor receptor (EGFR). Infection with HCMV resulted in a decrease in cell-surface expression of EGFR. This decrease was correlated with a concomitant decrease in steady-state levels of EGFR protein. Consistent with this, HCMV inhibited EGF-mediated receptor autophosphorylation. Infection with a mutant HCMV deleted of all viral gene products known to be involved in down-regulation of MHC Class I receptors still resulted in this down-regulation, implying that EGFR down-regulation by HCMV is mediated by a novel virus function. We suggest that a primary goal of HCMV is to 'isolate' the infected cell from host-mediated signals so that the cell responds solely to an array of virus-specific signals which optimize the cell for virus production.

Introduction

HCMV is a ubiquitous human herpesvirus which, like all herpesviruses, can establish life-long persistence after primary infection, with reactivation occurring often as a result of immunosuppression. In contrast to primary infection, which is generally asymptomatic, reactivation of the virus, particularly in the immunocompromised, often results in life-threatening disease (Griffiths & Grundy, 1988). During productive infection, HCMV undergoes a regulated three-phase cascade of gene expression, with the phases operationally defined as immediate early (IE), early (E) and late. Generally IE gene products, the major transcripts of which map to the major IE region of the genome and generate the major IE72 and IE86 family of proteins, play a pivotal role in regulating expression of early and late viral genes as well as regulating cellular gene expression (Stenberg, 1996).

Like other DNA viruses, the ability of HCMV to perturb normal cellular control mechanisms is well established. Virus-induced changes in cellular gene expression occur immediately on binding of virus to the cell (Boldogh *et al.*, 1990; Simmen *et al.*, 2001; Yurochko & Huang, 1999). Similarly, expression

of the viral IE and E genes also results in physical and functional interactions between the viral gene products and cellular factors, resulting in perturbation of cellular transcription, cell cycle and expression of secreted chemokines and cytokines (Fortunato *et al.*, 2000). Perturbation of specific cellular transcription factors has been related to transcriptional activation of viral and cellular genes required during infection, and disruption of cell cycle control is believed to optimize the cellular environment for viral DNA replication.

HCMV infection is also known to inhibit killing by cytotoxic T cells, by down-regulating cell-surface expression of MHC Class I receptors by a variety of specific mechanisms (Alcami & Koszinowski, 2000; Barnes & Grundy, 1992; Warren *et al.*, 1994). Similarly, other cell surface proteins associated with peptide processing have also been shown to be down-regulated during HCMV infection, although the relevance of this is not yet known (Phillips *et al.*, 1998).

Here, we now show that down-regulation of cell receptors that mediate a variety of cell signals, resulting in the abrogation of receptor-mediated cell signalling, may be a common occurrence during HCMV infection in that HCMV infection also results in the perturbation of the receptor for EGF.

EGF is a key mediator of lung maturation in the foetus (Klein *et al.*, 2000) and is known to stimulate production of surfactant proteins such as SP-A (Klein *et al.*, 1995) which play

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an important role in host innate defence in the lung (Crouch & Wright, 2001) – a major site of HCMV-mediated disease.

EGF is also known to be a general mitogenic stimulator of fibroblast and monocytic cell types (Carpenter & Cohen, 1976; Higashiyama *et al.*, 1991), but it can also inhibit growth in cells with high levels of EGFRs and has been suggested to be important in differential cell cycle control (Baker & Yu, 2001; Bromberg *et al.*, 1998). Cellular response to EGF is mediated via EGFR and EGF ligand binding to the receptor results in receptor dimerization and stimulation of intrinsic tyrosine kinase activity which, in turn, results in receptor autophosphorylation (Prigent & Lemoine, 1992; Lemmon & Schlessinger, 1994) and initiation of an intracellular signal transduction cascade involving MAPK and ERK (Daub *et al.*, 1996; Holt *et al.*, 1996). EGF has also been shown to act as an inhibitor of cytokine mediated apoptosis in some cell types (Garcia-Lloret *et al.*, 1996) and to affect cell motility through the MAP kinase pathway (Glading *et al.*, 2000).

Clearly, EGF has profound effects on cells. We believe that in order for a virus to efficiently 'hi-jack' normal cellular functions it is essential for the virus to prevent the cell from responding to external cellular signals which might conflict with the virally induced signals required to optimize the cellular milieu for productive infection.

Methods

■ **Cell culture and virus.** MRC 5 (primary lung fibroblasts) cells were maintained in Eagle's MEM + 10% foetal calf serum at 37 °C and in 5% CO₂. Routinely, cells were infected with HCMV at 5 p.f.u. per cell for 24 h. Infection with HCMV (AD169), UV-inactivated virus or a recombinant deletion virus missing IRS1-US9 and US11 (Jones *et al.*, 1995) has been described previously (Sinclair *et al.*, 2000).

■ **FACS analysis.** For FACS analyses, unfixed infected or unfixed control cells were stained with a mouse monoclonal anti-EGFR antibody directly conjugated to PE (R + D Systems) or isotype-matched control antibody (Ig2B) directly conjugated to PE (R + D Systems). Similarly, MHC Class I expression was determined using a FITC-conjugated mouse anti-HLA Class I A, B and C or FITC-conjugated isotype matched control antibody (IgG1) (R + D Systems). Alternatively, ethanol-fixed infected or control cells were stained with a goat anti-EGFR (Santa Cruz) antibody or goat immunoglobulins as control antibody and detected with PE-conjugated donkey anti-goat immunoglobulins. An aliquot of each cell population was also fixed, as above, and stained with an FITC-conjugated mouse anti-IE72/IE86 antibody (Biosys) to determine levels of HCMV infection. Cells were analysed using a Becton Dickinson FACSort.

EGFR expression was also analysed at specific times of infection as follows. For IE expression, cells were pre-treated with cycloheximide (50 µg/ml) for 3 h prior to, and for 3 h during, virus adherence. After this adherence period, virus and cycloheximide were washed off the cells and infection was allowed to progress for 3 h in the absence of cycloheximide. After this time, actinomycin D (20 µg/ml) was added for a further 14 h. Cells were then harvested and stained for EGFR as above. To inhibit viral late gene expression, cells were infected in the continual presence of the viral DNA polymerase inhibitor phosphonoformate (100 µg/ml).

■ **Detection of EGFR and its autophosphorylation.** Cells (1×10^7) were infected with HCMV at approximately 5 p.f.u. per cell. Control or HCMV-infected cells were labelled with [³⁵S]methionine,

harvested 24 h post-infection, lysed in EBC buffer and immunoprecipitated with anti-EGFR or anti-IE72 antibody as described previously (Hagemeyer *et al.*, 1994). Immunoprecipitates were analysed by SDS-PAGE and autoradiography.

To detect EGF-mediated phosphorylation of the EGFR, cells were mock infected or infected with HCMV at 5 p.f.u. per cell. At various times after infection, recombinant EGF (10 ng/ml) was added for 1 min and cells were harvested and immunoprecipitated using an anti-EGFR antibody as described above. Immunoprecipitated complexes were then analysed by SDS-PAGE and Western blotting using an HRP-conjugated anti-phosphotyrosine antibody (Santa Cruz), followed by detection using the ECL system (Amersham).

Results

EGFR cell-surface expression is down-regulated during HCMV infection

Recently, it has been shown that infection of cells with DNA viruses can often result in specific inhibition of cell surface receptors and these include EGFR (Stewart *et al.*, 1995). Consequently, we asked if HCMV infection also perturbed EGFR expression. Fig. 1 shows that HCMV infection leads to a rapid decrease in cell-surface expression of EGFR in primary fibroblast cells (compare panels a and c). Interestingly, UV-inactivated virus showed no such down-regulation (panel e), implying that viral gene expression was required for HCMV-mediated EGFR perturbation.

Down-regulation of EGFR is associated with viral early gene expression

In order to determine which viral genes may be responsible for EGFR down-regulation during infection, we carried out infections in the presence of cycloheximide and actinomycin D under conditions which prevented early and late gene expression. We also treated cells with phosphonoformate to prevent viral DNA replication and hence viral late gene expression. As can be seen in Fig. 2, infection of untreated cells down-regulated EGFR expression as expected (panel b). Similarly, treatment of cells with phosphonoformate still resulted in down-regulation of EGFR (panel h). In contrast, no such down-regulation of EGFR was observed on infection of cells treated with cycloheximide and actinomycin D to allow only IE gene expression (panel e). As expected, treatment with cycloheximide/actinomycin D or phosphonoformate had no effect on viral IE72 expression (panels f and i, respectively). Cycloheximide/actinomycin D-treated cells did not express UL44 and UL56 (viral early gene products) as determined by indirect immunofluorescence (data not shown), confirming a lack of early gene expression in these infected cells.

Viral gene products associated with perturbation of MHC Class I expression are not involved in down-regulation of EGFR expression

HCMV infection of fibroblasts has already been shown to result in retention in the Golgi and a down-regulation in cell-

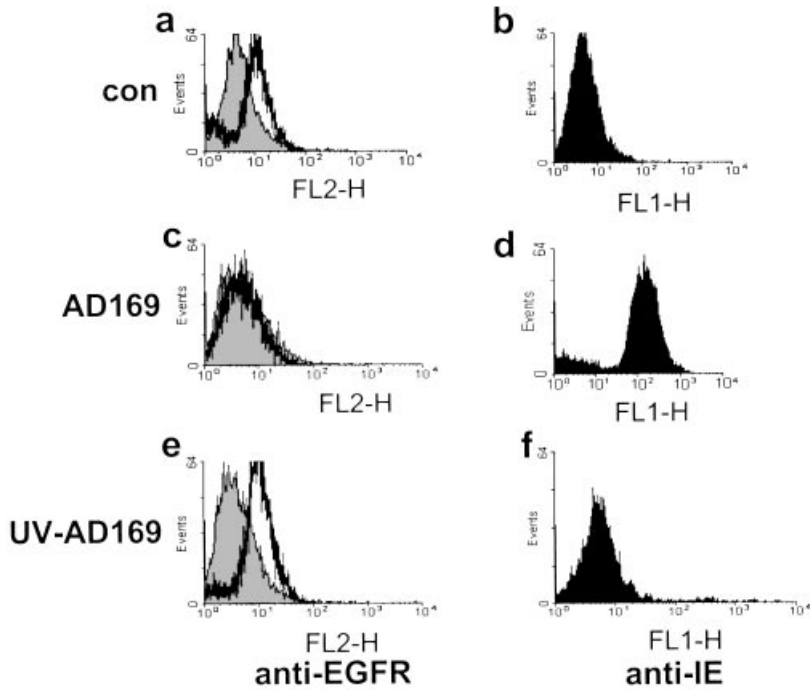


Fig. 1. HCMV infection reduces cell-surface expression of EGFR. Unfixed, uninfected fibroblasts (a), fibroblasts infected with HCMV strain AD169 at 5 p.f.u per cell for 24 h (c) or UV-inactivated AD169 (e) were stained with anti-EGFR antibody (open profile) or an isotype-matched control antibody (dark profile) and analysed by FACS. Aliquots of control uninfected cells (b), cells infected with AD169 (d) or cells infected with UV-inactivated virus (f) were also fixed and permeabilized and then stained with an anti-IE72/IE86 antibody to determine levels of HCMV IE expression.

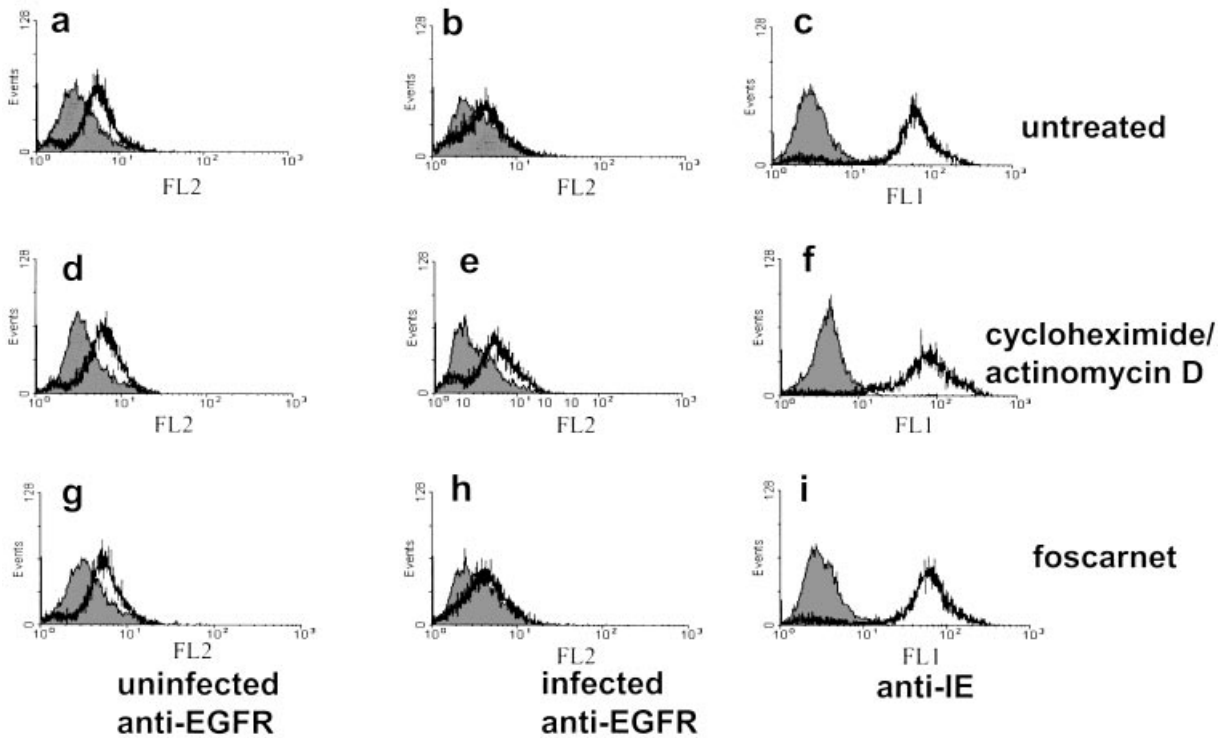


Fig. 2. Viral early but not IE or late gene expression is required for EGFR down-regulation. Uninfected (a) or infected (b) untreated fibroblasts or uninfected (d) or infected (e) fibroblasts treated with cycloheximide and actinomycin D or uninfected (g) or infected (h) fibroblasts treated with phosphonoformate (foscarnet) were stained with a PE-conjugated anti-EGFR antibody (open profile) or a PE-conjugated isotype-matched control (Ig2B) antibody (dark profile) and analysed by FACS. An aliquot of these cells was also fixed and untreated infected cells (c) or infected cells treated with cycloheximide and actinomycin D (f) or infected cells treated with phosphonoformate (foscarnet) (i) were stained with a FITC-conjugated anti-IE antibody (open profile) or a FITC-conjugated isotype-matched (IgG1) control (closed profile) and analysed by FACS.

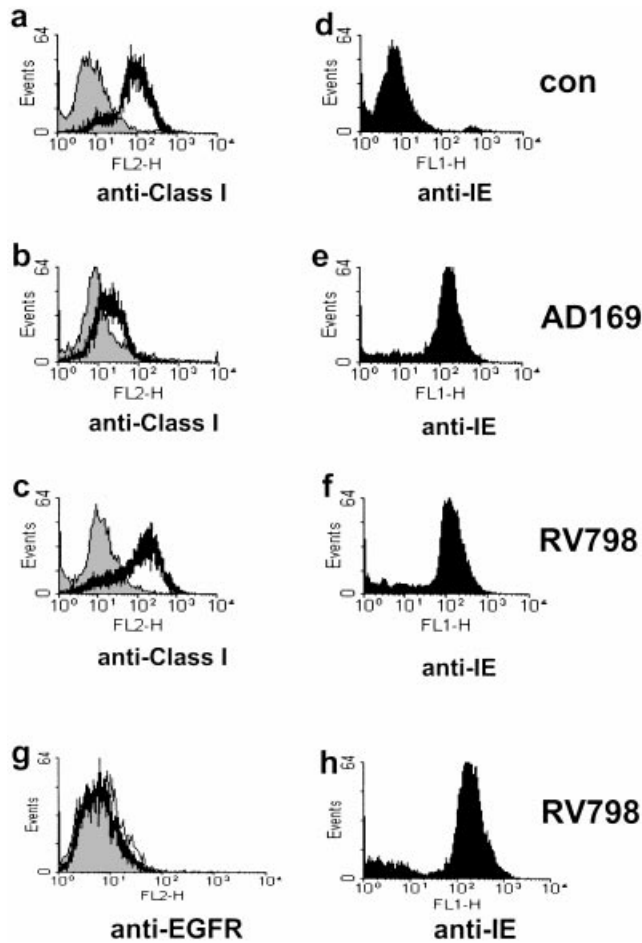


Fig. 3. Viral genes associated with MHC Class I down-regulation are not involved in EGFR down-regulation. Control uninfected fibroblast cells (a), cells infected for 24 h at 5 p.f.u per cell with AD169 (b) or cells infected with RV798 (c) were stained with an anti-MHC Class I PE-conjugated antibody (open profile) or an isotype-matched control (IgG1) PE-conjugated antibody (shaded profile) and analysed by FACS. Aliquots of control (d), AD169-infected (e) or RV798-infected (f) cells were also fixed and stained with an anti-IE1 FITC-conjugated antibody to assess levels of infection. Fibroblasts infected with RV798 (g) were also stained directly with a PE-conjugated anti-EGFR antibody (open profile) or a PE-conjugated isotype-matched control (Ig2B) antibody (shaded profile) and analysed by FACS. An aliquot of these cells was also fixed and stained with a FITC-conjugated anti-IE antibody (h).

surface expression of at least one other cellular protein, MHC class I (Ahn *et al.*, 1996; Jones *et al.*, 1996a; Jun *et al.*, 2000). These elegant experiments have shown that the US3 gene product of HCMV, a viral gene expressed at IE times of infection, encodes a Golgi retention signal, preventing trafficking of Class I to the cell surface. Along with other viral products that target Class I and which localize to the US2-11 region of HCMV, US3 is believed to play an important role in immune avoidance of infected cells by cytotoxic T cells (CTLs) (Jones & Sun, 1997; Jones *et al.*, 1996b; Jun *et al.*, 2000; Lehner *et al.*, 1997; Reusch *et al.*, 1999).

Consequently, we also tested whether RV798 (Jones *et al.*, 1995), which is deleted for all gene products involved in MHC

Class I down-regulation, was still capable of EGFR re-localization. Firstly, we confirmed that, in our hands, RV798 was incapable of down-regulating MHC Class I cell-surface expression, as has previously been shown (Jones *et al.*, 1995). Fig. 3 shows that, as expected, Class I expression on fibroblasts infected with HCMV AD169 is extensively down-regulated (panel b) whereas fibroblasts infected with RV798 show no such Class I down-regulation (panel c). Fig. 3 also shows that RV798 infection still resulted in down-regulation of EGFR (panel g). Consequently, it appears that inhibition of EGFR expression involves a novel function of HCMV not associated with MHC Class I down-regulation.

Down-regulation of EGFR results from an overall reduction in levels of EGFR expression

As our experiments clearly pointed to virus-mediated down-regulation of cell-surface expression of EGFR by HCMV, we asked, specifically, whether EGFR down-regulation on the cell surface was reflected at the intracellular level. Fig. 4(A) shows that the lack of cell-surface expression of EGFR appears to be due to an overall reduction in steady state levels of EGFR expression, as analysis of EGFR by immunoprecipitation assay (Fig. 4A) clearly showed that HCMV infection resulted in a major reduction in total cellular levels of receptor. Similarly, FACS analysis of fixed and permeabilized cells using anti-EGFR antibodies also showed a decrease in total levels of cellular EGFR on infection (Fig. 4B). This is consistent with a recent report showing a decrease in EGFR mRNA on long-term infection of human fibroblasts with HCMV (Prosch *et al.*, *Abstracts of the 26th International Herpesvirus Workshop*, abstr. 12.22, 2001).

HCMV infection inhibits EGF-mediated receptor autophosphorylation

We next confirmed that the observed HCMV-induced reduction of EGFR expression also results in a lack of functional surface receptor. As EGF binding to EGFR results in autophosphorylation of the latter, we analysed the levels of EGF-induced EGFR phosphorylation that occurs on HCMV infection. Fig. 5(A) clearly shows that, as expected, treatment of fibroblast cells with EGF results in extensive phosphorylation of EGFR as determined by immunoprecipitation of EGFR followed by subsequent detection of levels of phosphorylation of the receptor using Western blot analysis with a phosphotyrosine-specific antibody. In contrast, infection with HCMV resulted in a lack of detectable phosphorylated EGFR after EGF stimulation, consistent with a lack of functional EGFR on the surface of these infected cells.

We also carried out a time-course analysis of inhibition of EGFR autophosphorylation by HCMV (Fig. 5B). This showed that EGFR inhibition does not occur earlier than 6 h post-infection and that viral gene products are required for this effect, as UV-inactivated virus does not inhibit receptor autophosphorylation.

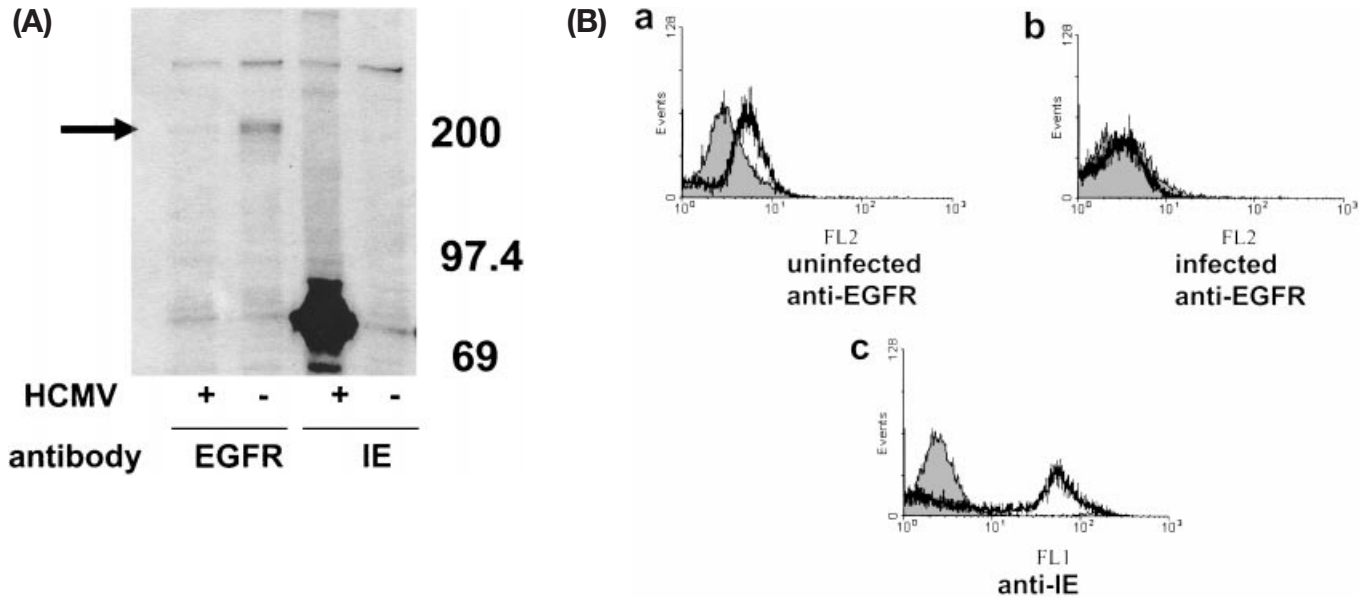


Fig. 4. HCMV infection reduces total cellular expression of EGFR. (A) Uninfected (–) or infected (+) fibroblast cells were labelled with [³⁵S]methionine and immunoprecipitated with an anti-EGFR antibody (EGFR) or an anti-IE72/IE86 (IE) antibody. Immunoprecipitated complexes were separated by SDS–PAGE and gels were autoradiographed. Molecular mass markers (kDa) are shown and the position of EGFR is marked with an arrow. (B) Control uninfected fibroblast cells (a) or cells infected with AD169 at 5 p.f.u per cell for 24 h (b) were fixed and stained with a goat polyclonal antibody to EGFR (open profile) or goat control antibodies (shaded profile) and antibodies were detected using PE-conjugated donkey anti-goat immunoglobulins. Cells were then analysed by FACS. An aliquot of these cells was also stained with a control antibody (shaded profile) or anti-IE antibody (open profile) (C).

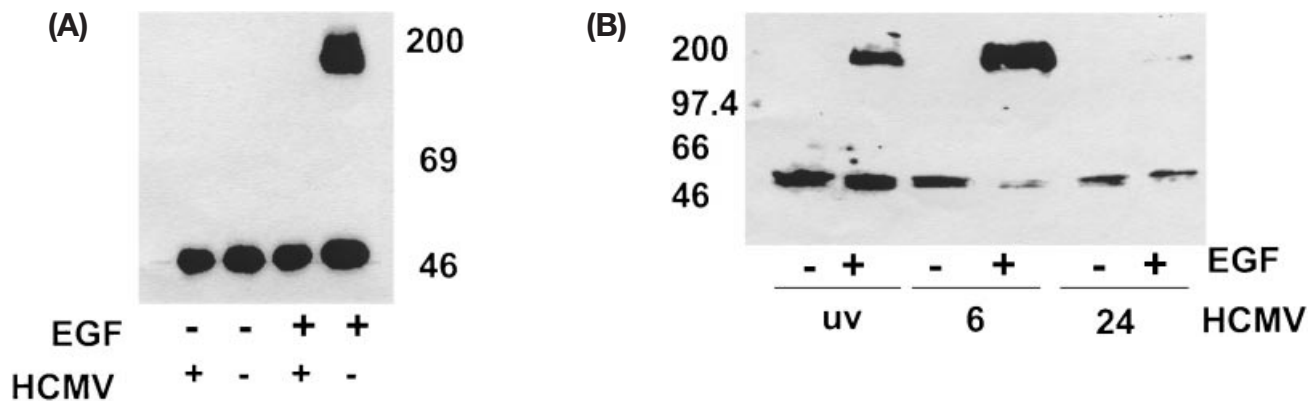


Fig. 5. HCMV infection results in a reduction in EGF-mediated EGFR autophosphorylation. (A) Uninfected fibroblasts (–) or fibroblasts infected with AD169 at 5 p.f.u per cell for 24 h (+) were left untreated (EGF–) or treated with EGF (EGF+) and then immunoprecipitated with an anti-EGFR specific antibody. Immunoprecipitated complexes were separated by SDS–PAGE and analysed by Western blot with a phosphotyrosine-specific monoclonal antibody. Molecular mass markers (kDa) are shown. (B) Fibroblasts were infected with 5 p.f.u per cell AD169 for 6 h (6), 24 h (24) or with UV-inactivated HCMV for 24 h (UV). Infected cells were then left untreated (EGF–) or were treated with EGF (EGF+) and then immunoprecipitated with an anti-EGFR-specific antibody. Immunoprecipitated complexes were separated by SDS–PAGE and then analysed by Western blot with a phosphotyrosine-specific monoclonal antibody. Molecular mass markers (kDa) are shown.

Discussion

Perturbation of cellular functions appears essential for viruses to optimize the cell for productive infection. In HCMV, a number of virus-encoded gene products have been identified which interdict control of functions such as cellular transcription, cell cycle, immunomodulation and regulation of

chemokine and cytokine gene expression. Often this is accomplished by direct physical and functional interactions between viral proteins and key cellular regulatory proteins (Fortunato *et al.*, 2000).

A number of DNA viruses also perturb cellular receptor function (Stewart *et al.*, 1995) and, at least in certain cell types, this may result from receptor inhibition (Tollefson *et al.*, 2001).

As HCMV is known to perturb cell-surface MHC Class I expression, we also looked for the ability of HCMV to perturb additional receptors. HCMV infection clearly resulted in a reduction of cell-surface EGFR as determined by FACS analysis and this virus-induced reduction in cell-surface expression of EGFR was also reflected in a lack of detectable total cellular EGFR as determined by protein immunoprecipitation and FACS.

Interestingly, similar down-regulation of EGFR is observed as a result of adenovirus infection (Stewart *et al.*, 1995) and may be mediated at least in part by adenovirus E1A (Prudenziati *et al.*, 2000).

We also determined which HCMV gene products may be responsible for this effect on the EGF receptor. Firstly, UV-inactivated virus appeared not to have any effect on cell-surface expression of EGFR, implying that viral gene expression and not virus binding per se was required for this phenomenon. Secondly, restriction of virus infection to IE events also did not result in EGFR down-regulation. In contrast, treatment of cells with phosphonoformate, which prevents viral DNA replication and hence viral late gene expression, still resulted in receptor down-regulation. Consequently, we believe that HCMV early gene products are likely to be responsible for this virus-mediated EGFR perturbation.

As HCMV infection of fibroblasts has already been shown to result in down-regulation of cell-surface expression of at least one other cellular protein, MHC class I (Ahn *et al.*, 1996; Jones *et al.*, 1996a; Jun *et al.*, 2000), and all the viral gene products associated with this phenomenon have been shown to be encoded in the US2-11 region of HCMV (Jones & Sun, 1997; Jones *et al.*, 1996b; Jun *et al.*, 2000; Lehner *et al.*, 1997; Reusch *et al.*, 1999), we also tested a virus deleted of US2-11, RV798 (Jones *et al.*, 1995), for EGFR down-regulation. The observation that infection with RV798 still resulted in down-regulation of EGFR cell-surface expression suggests that a novel viral function, not associated with MHC Class I down-regulation, is responsible for this effect. Interestingly, the down-regulation of EGFR was also observed with myelotropic strains of HCMV such as TB40E (Sinzger *et al.*, 2000) as well as with other laboratory strains of HCMV such as Towne (data not shown).

Consistent with a lack of surface EGFR, HCMV-infected cells showed no EGF-mediated autophosphorylation of EGFR. Time-course analysis suggested that virus functions associated with viral gene expression were required for EGFR down-regulation as UV-inactivated virus showed no such receptor inhibition and this down-regulation did not occur prior to 6 h post-infection. This is, again, consistent with early and not IE viral gene products being responsible for EGFR perturbation.

EGF is known to be a general mitogenic stimulator of fibroblast cells (Carpenter & Cohen, 1976) but can also inhibit growth in cells expressing high levels of receptor (Gardner & Shimizu, 1994). Consequently, it is possible that HCMV

infection lowers EGFR on the infected cell to prevent the cell from responding to host EGF signalling, which might drive the cell into a state that conflicts with an optimal state for virus production. Also, more recently, it has been suggested that EGFR inactivity is required for progression through G_1 into S phase (Baker & Yu, 2001). Whilst conflicting data exist as to just how far through the cell cycle HCMV advances the infected cell (Bresnahan *et al.*, 1996; Dittmer & Mocarski, 1997; Jault *et al.*, 1995; Lu & Shenk, 1996; Murphy *et al.*, 2000; Salvant *et al.*, 1998; Sinclair *et al.*, 2000), it is clear that virus does induce cell functions associated with progression through G_0/G_1 into early S phase. Consequently, it is possible that the observed down-regulation of EGFR function by HCMV may be required in order for virus to mediate cell cycle advance in resting cells. However, it must be said that, at present, we are not certain of the specific effects of EGFR down-regulation on the infected cell.

Whilst we have not directly addressed whether the inhibition of steady-state levels of EGFR expression shown here result from transcriptional or post-transcriptional events, interestingly, inhibition of EGFR mRNA expression has also been observed on HCMV infection in human lung fibroblasts (Prosch *et al.*, Abstracts of the 26th International Herpesvirus Workshop, abstr.12.22, 2001).

It is interesting to note that HCMV, as well as apparently inhibiting EGFR-mediated signalling in the cell, has been shown to activate certain arms of the extracellular signal-regulated kinase cascade (Johnson *et al.*, 2000; Rodems & Spector, 1998; Chen & Stinski, 2002). It is therefore possible that HCMV infection results in the expression of viral factors that activate some, but not all, aspects of normal cellular EGF-mediated signalling as well as concomitantly inducing down-regulation of cellular EGFR to ensure that viral signals are dominant.

It is clear that HCMV infection results in the expression of a number of viral gene products which optimize the cell for virus productive infection by interdicting control of a number of normal cellular functions. We believe that an integral part of this is the ability of the virus to isolate the infected cell from host-specific signals forcing the cell to respond solely to virus signals and these specifically optimize the cellular environment for productive infection. An understanding of what mechanisms are used by the virus to 'hi-jack' the cell and modify its response to cell signals will be important in fully understanding the biology and pathogenesis of HCMV.

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