Evidence for the role of cell protein phosphorylation in human cytomegalovirus/host cell fusion

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The mechanism by which human cytomegalovirus (HCMV) enters cells is unknown. We sought evidence that protein phosphorylation plays a role in HCMV infection in two ways: (1) by determining whether the degree of phosphorylation of a constitutively phosphorylated 92.5 kDa putative cell membrane receptor for HCMV gH is changed following exposure to HCMV or monoclonal anti-idiotype antibodies (MAb2) that antigenically mimic HCMV gH, and (2) by studying the effects of protein kinase inhibition on receptor phosphorylation and HCMV adsorption or fusion. Phosphorylation of the 92.5 kDa cell membrane protein was specifically increased within 10 min of incubation with HCMV or MAb2 that had been crosslinked by goat anti-mouse antibodies. In addition, fusion of viral envelope with the cell membrane was inhibited by certain protein kinase inhibitors which also inhibited receptor phosphorylation, while the adsorption of [3H]HCMV to human embryonic lung cells was not affected. Tyrosine kinase inhibitors inhibited virus/cell fusion to a greater extent than protein kinase C (PKC) inhibitors, and an inhibitor which primarily affects cAMP and cGMP kinases had little effect. In addition, fusion was stimulated by preincubating cells with agents that stimulated receptor phosphorylation including a phorbol ester, tyrosine phosphatase inhibitor and serine/threonine phosphatase inhibitor. These data indicate that increased phosphorylation of a 92.5 kDa putative cell membrane protein receptor for gH is an early event in response to HCMV, and that cell protein phosphorylation by tyrosine kinase(s) and PKC may facilitate HCMV/cell membrane fusion.

Introduction

The mechanism by which human cytomegalovirus (HCMV) enters cells is beginning to be elucidated. HCMV is a member of the herpesvirus family. Herpesviruses enter cells by a series of processes including attachment, fusion of viral envelope with the cell membrane and penetration of the capsid. Direct cell-to-cell spread of virions also occurs between contiguous cells (reviewed by Mocarski, 1993). Like other herpesviruses, HCMV can bind nonspecifically to heparin-like molecules on the cell surface via gC envelope glycoproteins (Kari & Gehrz, 1992; Compton et al., 1993). HCMV virions or purified HCMV envelope glycoproteins also appear to bind specifically to at least two cell membrane components: a 30–34 kDa species (Taylor & Cooper, 1990; Adlish et al., 1990) and a 92-5 kDa glycoprotein (Keay et al., 1989).

Using monoclonal anti-idiotype antibodies (MAb2) that antigenically mimic an HCMV envelope glycoprotein (gH, gp86 or gpUL75) and induce HCMV-neutralizing antibodies in naive mice (Keay et al., 1988), we previously identified the 92-5 kDa protein which is located on the surface of human embryonic lung (HEL) fibroblasts and then determined that both the MAb2 and affinity-purified HCMV gH bind to this cell membrane protein (Keay et al., 1989). These MAb2 which immunoprecipitate the 92.5 kDa cell membrane protein, did not inhibit adsorption of radiolabelled HCMV to HEL cells but blocked fusion of octadecyl rhodamine B chloride-labelled virus with these cells (Keay & Baldwin, 1991), suggesting that this cell membrane protein functions in the fusion of HCMV with HEL cell membranes. Preliminary characterization of this putative HCMV gp86 receptor indicated that it is a glycoprotein which is phosphorylated in the constitutive state (Keay & Baldwin, 1992). This cell membrane protein also appears to function in transmembrane signalling events resulting in an increased cytoplasmic calcium ion concentration in response to HCMV or crosslinked MAb2 (Keay et al., 1995).
Phosphorylation of cell membrane receptors is sometimes associated with receptor internalization (Hoxie et al., 1988; Klausner et al., 1984), as well as binding or penetration by certain enveloped viruses including Epstein–Barr virus (EBV) (Aquino et al., 1993; Cirone et al., 1990) and human immunodeficiency virus type 1 (HIV-1) (Fields et al., 1988). In addition, the phosphorylation of specific cellular components in response to viral infection such as tyrosine kinase phosphorylation of a 30 kDa protein involved in syncitia formation induced by HIV-1 (Cohen et al., 1992), is evidence that cell protein phosphorylation may also be involved in eukaryotic cell membrane fusion and viral pathogenesis.

Because cell protein phosphorylation may be important for infection of cells by enveloped viruses, we studied whether the degree of phosphorylation of the 92.5 kDa putative cell membrane receptor for HCMV gH is changed following exposure to HCMV or MAb2, and whether certain classes of protein kinase inhibitors had any effect on HCMV adsorption or fusion. To inhibit tyrosine kinase activity, cells were treated with genistein or its inactive control genistin; tyrphostin B46 or its inactive control tyrphostin A1; or herbimicin A. Protein kinase C (PKC) activity was inhibited with either H-7 or sphingosine; because H-7 also inhibits cAMP and cGMP kinases (IC₅₀ (concentration that inhibits 50% of enzyme activity) = 3·0 and 5·8 mM). HA1004 [which inhibits cAMP kinase or cGMP kinase (IC₅₀ = 0·2-3·1·3 mM), but has much less activity against PKC (IC₅₀ = 40 mM)] was also used as a control. Trifluoperazine (TFFP) was used to inhibit calmodulin activity. To provide additional evidence for the role of protein kinases in HCMV infection, serine/threonine phosphatases were inhibited with okadaic acid, and tyrosine phosphatases were inhibited with phenylarsine oxide (PAO).

**Methods**

### Cell culture and virus propagation

Human embryonic lung (HEL-299) fibroblasts were obtained from the ATCC (CCL-137) and grown in Eagle’s minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) (Sigma). Human embryonic lung fibroblasts AD169 was passaged in HEL cells and harvested as previously described (Keay et al., 1995). Viral titre was determined by plaque formation in HEL cells (Keay et al., 1989).

### Antibodies

A monoclonal anti-idiotyp antibody (MAb2), 4-3-5, that mimics gH of HCMV was generated, characterized and affinity-purified as previously described (Keay et al., 1988). BALB/c immunoglobulin M (IgM) control antibody was obtained from Southern Biotechnology Associates, Birmingham, Ala., USA, and affinity-purified by the same method as the MAb2. For most of the experiments described below, 4-3-5 or IgM control antibody was crosslinked with purified goat anti-mouse IgM antibody (Southern Biotechnology Associates) (ratio ranging from 10 mg:1 mg to 2 mg:1 mg) for 1 h at 37 °C.

### Radiolabelling of cell proteins

Confluent HEL cell monolayers were preincubated in phosphate-free medium, then labelled with [³²P]orthophosphoric acid (New England Nuclear) in phosphate-free buffer for 3 h at 37 °C as previously described (Keay & Baldwin, 1992); for select experiments, duplicate cultures were labelled with [³⁵S]methionine (100 μCi per 7 x 10⁸ cells) (New England Nuclear) in medium containing 30% unlabelled methionine for 48 h at 37 °C. HCMV, 4-3-5 antibody, control buffer (Hanks') or isotype control antibody was added to the cell culture flasks at the concentrations indicated, for various time-periods as indicated. Cells were then rinsed in phosphate buffer, scraped into the phosphate buffer and pelleted by centrifugation. Membrane proteins from the pellet were solubilized in buffer containing 2% NP40 (Sigma), and nonsolubilized material was pelleted by centrifugation at 20 000 × g for 20 min at 4 °C. Supernatant was removed and stored at -80 °C.

### Immunoprecipitation

³²P-labelled solubilized HEL cell membrane proteins were incubated with affinity-purified 4-3-5 (200 μg per 10⁶ cells) at 37 °C for 2 h followed by rat anti-mouse IgM-Sepharose (200 μl per 10⁷ cells) (Zymed Laboratories) at room temperature for 1·5 h, and receptor was eluted as previously described (Keay et al., 1988). Samples containing immunoprecipitated receptor from 5 x 10⁸ cells were then loaded into each well of two 7·5% polyacrylamide gels and electrophoresed under denaturing conditions, as previously described (Keay et al., 1988; Keay & Baldwin, 1992). One gel was subsequently processed for autoradiography using Kodak X-omat film, and proteins in the second gel were stained with Coomassie blue G250 (Sigma) in ethanol-acetic acid-water (9:2:9) and destained with ethanol–acetic acid–water (6:1:13). The amount of protein phosphorylation for each sample was then determined by transmission densitometry of the autoradiograms using a Bio-Rad 620 Video Densitometer; the amount of receptor protein per sample was determined by transmission densitometry of the Coomassie blue-stained gels.

### Kinase/phosphatase inhibitors

To determine the effect of tyrosine kinase inhibitors on HCMV/cell fusion and HCMV adsorption, HEL cells were preincubated with various amounts (0·1–100 μM) of genistein (IC₅₀ = 2-6 mM) (Akiyama et al., 1987) (Sigma), 0·1–10 μM-herbimicin A (IC₅₀ = 0·9 mM) (Park et al., 1991) (Sigma), 0·1–100 μM-tyrphostin B46 (IC₅₀ = 0·7-2·6 mM for most tyrosine kinases) (Levitski, 1990) (Calbiochem), the inactive controls (0·1–100 μM-genestin) (Sigma) or 0·1–100 μM-tyrphostin A1 (Calbiochem) or the equivalent amount of DMSO carrier in standard culture medium for appropriate time intervals (30 min for tyrphostin, 60 min for genistein and 16 h for herbimicin A); other cells were incubated with an inhibitor of tyrosine phosphatase, PAO (0·1–10 μM) (Sigma) for 60 min. To determine the effect of inhibitors of serine/threonine phosphorylation, cells were preincubated with various concentrations (0·1–10 μM) of sphingosine (IC₅₀ = 4-0 mM) (Hannun et al., 1986) (Sigma), 0·1–100 μM-H-7 [1-(5-isopropinolyl-sulphonyl)-2-methylpiperezine, IC₅₀ = 6·0 mM] (Hidaka et al., 1984) (Sigma), 0·1–100 μM-HA1004 (Calbiochem) or DMSO carrier alone; some cells were incubated with an inhibitor of serine/threonine phosphatase, okadaic acid (1–100 mM) (Sigma), its negative control methyl okadate (Calbiohem), or phorbol myristic acid (PMA) (0·01–0·1 μM) (Sigma). For comparison, some cells were preincubated with 0·1–10 μM of a calmodulin inhibitor, trifluoperazine (TFFP) (IC₅₀ = 5-8 mM) (Uneyama et al., 1993) (Calbiochem) or the equivalent amount of DMSO carrier.

The effect of specific kinase inhibitors on 92.5 kDa receptor phosphorylation was determined by preincubating [³²P]orthophosphoric acid-labelled cell cultures with each kinase inhibitor, then incubating the cells with HCMV (m.o.i. = 1) for 30 min at 37 °C, harvesting the cells and immunoprecipitating the receptor from solubilized membrane proteins. The amount of protein loaded per well was standardized by [³⁵S]-labelling of duplicate cultures.

### HCMV/cell fusion assay

Fusion of HCMV with the cell membrane was determined using octadecyl rhodamine B chloride (R₁₂₅)-labelled...
virus. HCMV (strain AD169) stocks containing 2 x 10^7 p.f.u./ml were labelled with octadecyl rhodamine B chloride (Molecular Probes) and virus/cell fusion measured as previously described (Keay & Baldwin, 1991). HEL cells grown to confluence on Lab-Tek 8-well plastic tissue culture slides (Nunc) were preincubated with protein kinase inhibitors, protein phosphatase inhibitors or their controls at 37 °C for the appropriate time interval as indicated, after which R18-HCMV was added to the cultures. Cells were examined using a Nikon Diaphot inverted phase fluorescence microscope; quantification was achieved using a Zeiss axioplan microscope connected to the image analysis system previously described (Keay & Baldwin, 1991). This system analysed the relative amount of specific fluorescent signal remaining after subtraction of nonspecific background fluorescence in a negative control.

**HCMV adsorption assay.** Following pretreatment of HEL cells with kinase inhibitors, phosphatase inhibitors or their controls, a virus adsorption assay was performed using [3H]HCMV as previously described (Keay & Baldwin, 1991).

**Results**

**Changes in receptor phosphorylation following exposure of cells to HCMV or 4-3-5**

To determine whether the degree of receptor phosphorylation could be influenced by early HCMV infection, confluent cells were incubated with [32P]orthophosphoric acid for 3 h, then exposed to HCMV or equivalent amount of control buffer for 30 min at 37 °C. Receptor protein was then immunoprecipitated from solubilized cell membrane proteins, and the relative amount of phosphorylation of the immunoprecipitated 92.5 kDa receptor was subsequently determined by scanning densitometry of the autoradiograms. As shown in Fig. 1 (a), the degree of 92.5 kDa receptor phosphorylation was dependent on the amount of virus to which the cells were exposed, as determined by m.o.i.; maximal phosphorylation occurred between m.o.i. values of 1 and 10 in repeated experiments. Quantification of this difference by scanning densitometry, which allowed for subtraction of background signal and standardization of the baseline for each lane, indicated a mean maximal increase in phosphorylation signal of approximately 350% (range 340–386%) of the signal present in the constitutively phosphorylated receptor (Fig. 1b).

Confluent [32P]orthophosphoric acid-labelled HEL cells were similarly exposed to affinity-purified crosslinked 4-3-5 or control BALB/c IgM antibody at the concentrations indicated, to determine whether binding of 4-3-5 to the cells could also influence the degree of receptor phosphorylation (Fig. 2). Exposure of cells to relatively low amounts of MAb2 (30–300 μg per 10^7 cells) for 30 min at 37 °C resulted in an increase in receptor phosphorylation in repeated experiments, with 100 μg per 10^7 cells repeatedly giving a peak response.

Experiments in which cells were exposed to optimal concentrations of HCMV (m.o.i. = 1) or 4-3-5 (100 μg per 10^7 cells) for various periods of time prior to solubilization of cell membrane proteins were then performed to determine the amount of time necessary for increased receptor phosphorylation to occur following HCMV or 4-3-5 exposure. Both stimuli resulted in an increase in receptor phosphorylation within 10 min at 37 °C. As shown in Fig. 3, only MAb2 that had been crosslinked prior to incubation with cells was able to stimulate receptor phosphorylation; noncrosslinked 4-3-5 (which is able to bind to the receptor by Western blot and immunoprecipitation) and crosslinked IgM control antibody had no effect. These data suggest that receptor aggregation, or patching, may be necessary for the transmembrane signalling which results in an increase in receptor phosphorylation.

![Fig. 1. Increased receptor phosphorylation in response to HCMV.](image)

(a) Autoradiogram of the gel; (b) quantification of [32P] signal in the 92.5 kDa cell membrane protein determined by transmission densitometry. Bars indicate standard error of the mean for duplicate experiments.
Effects of kinase or phosphatase inhibitors on 92-5 kDa receptor phosphorylation

To determine whether the inhibition of tyrosine or serine/threonine phosphorylation by kinase inhibitors, or its stimulation by phosphatase inhibitors, affected phosphorylation of the 92-5 kDa receptor, 32P-labelled cells were incubated with specific inhibitors at concentrations capable of inhibiting HCMV/cell fusion by > 25% (a minimum percentage at which inhibition was previously determined to be reliably reproducible). The cells were then subsequently incubated with HCMV (m.o.i. = 1), membrane proteins solubilized and the receptor immunoprecipitated. As shown in Fig. 4, the tyrosine kinase inhibitors (genistein and tyrphostin B46) inhibited phosphorylation of the 92-5 kDa receptor by 79% and 90% respectively as compared to their controls, whereas the serine/threonine inhibitor (H7) inhibited by only 43%. In contrast, both tyrosine phosphatase and serine/threonine phosphatase inhibitors stimulated receptor phosphorylation.

Fig. 2. Increased receptor phosphorylation in response to crosslinked MAb2 (4-3-5). 32P-labelled HEL cells were incubated with various amounts of crosslinked MAb2 or glycine-Tris control buffer alone for 30 min at 37 °C. The 92-5 kDa receptor was immunoprecipitated from solubilized cell membrane proteins and electrophoresed on a 7.5% polyacrylamide gel under denaturing conditions. The 32P signal was quantified by transmission densitometry; bars indicate standard error of the mean for triplicate experiments.

Fig. 3. Inability of noncrosslinked 4-3-5 or crosslinked control IgM antibodies to stimulate receptor phosphorylation. 32P-labelled HEL cells were incubated with 100 μg per 10^7 cells of crosslinked 4-3-5 (□), noncrosslinked 4-3-5 (■) or crosslinked IgM control antibody (□) for various periods of time. The 92-5 kDa receptor was immunoprecipitated from solubilized cell membrane proteins and electrophoresed on a 7.5% polyacrylamide gel under denaturing conditions. Autoradiograms were made, and the relative amount of 32P signal was quantified by transmission densitometry. Bars indicate standard error of the mean for triplicate experiments.

Fig. 4. Inhibition of 92-5 kDa protein phosphorylation by kinase inhibitors. 32P-labelled HEL cells were incubated with specific kinase or phosphatase inhibitors prior to incubation with HCMV; putative receptor protein was immunoprecipitated from solubilized membrane proteins, run on an SDS-polyacrylamide gel under denaturing conditions, and the amount of radioactivity incorporated determined using an Ambis image acquisition and analysis system. Data are expressed as percentage change in radioactivity compared to controls.
Inhibition of HCMV/cell fusion by protein kinase inhibitors

Both tyrosine kinase and PKC inhibitors appeared to inhibit HCMV/cell fusion. Tyrosine kinase inhibitors had the greater effect, with relatively low concentrations of all three inhibitors inhibiting the emergence of fluorescent signal as compared to an equivalent amount of DMSO (Fig. 5 a–d); the inactive controls genistein and tyrphostin A1 had no discernible effect (Fig. 5 e, f), whereas the tyrosine phosphatase inhibitor PAO stimulated the rapid emergence of highly fluorescent signal.
Fig. 7. Inhibition of HCMV/cell fusion by inhibitors of serine/threonine phosphorylation. HEL cells were pretreated with various concentrations of PKC inhibitors (sphingosine or H-7), a control for H-7 (HA1004) or an equivalent amount of DMSO carrier, after which they were further incubated with R18-labelled HCMV. Shown in this composite picture are the results obtained 30 min after the addition of R18-HCMV to cells pretreated with (a) DMSO alone, (b) 10 μM-sphingosine, (c) 10 μM-H-7, (d) 10 μM-HA1004, (e) 30 nM-okadaic acid, (f) 30 nM-methyl okadaate, (g) 0.1 μM-PMA or (h) 10 μM-TFP.

Discussion

The data presented indicate that phosphorylation of the 92.5 kDa putative receptor for HCMV gH occurs in response to HCMV or a crosslinked monoclonal anti-idiotype antibody, and suggest that cell protein phosphorylation, including phosphorylation of the 92.5 kDa receptor, facilitates HCMV fusion with HEL cell membranes. The MAb used for these studies has previously been shown to antigenically mimic an HCMV envelope glycoprotein (gH, gp86 or gpUL75), bind to a 92.5 kDa cell membrane protein to which affinity-purified gH also binds, and block HCMV/cell fusion; the same MAb was recently used to clone receptor peptides which have also been shown to specifically inhibit HCMV/cell fusion (Baldwin et al., 1996). Results of the current experiments therefore also suggest that the interaction of gH with the 92.5 kDa cell membrane protein results in cell protein phosphorylation which is important for the fusion process. Both PKC and tyrosine kinase activities appear to be required for HCMV/cell fusion to occur normally. In comparison, cAMP and cGMP kinases appear to have much less of an effect on the fusion process, and calmodulin activity may actually have an inhibitory effect.

The fact that an apparent optimal concentration of either virus or antibody exists for receptor phosphorylation to be stimulated suggests that an optimal ratio of ligand to receptor results in receptor-mediated signalling in response to receptor aggregation. We have previously shown that receptor patching in response to crosslinked, but not noncrosslinked, antibody is indeed evident by immunofluorescence microscopy.
Although the MAb 2 used for these studies (4-3-5) is of the IgM class and is therefore 'crosslinked' to a certain degree, it appears that additional crosslinking of the antibody is necessary for aggregation of receptor molecules to be evident (Keay et al., 1995).

It was also evident from these studies that the virus itself was more effective than antibody for inducing receptor phosphorylation, causing a 3–5-fold greater increase. This difference could result from a relative difference in concentrations of receptor-binding sites on each ligand over a given area, and therefore a difference in the optimal number of receptor molecules that can undergo aggregation. Alternatively, it is possible that ligands on the viral envelope in addition to the pH binding site mimicked by the MAb 2 may influence receptor phosphorylation (i.e. additional epitopes on gH or other viral glycoproteins).

Phosphorylation of the 92-5 kDa cell membrane protein may be important for virus/cell fusion or receptor patching (Keay et al., 1995) and internalization (Keay & Baldwin, 1995). Our findings suggest that both tyrosine and serine/threonine phosphorylation of the 92-5 kDa putative gH receptor are correlated with the HCMV/cell fusion process. However, definitive evidence that phosphorylation of the 92-5 kDa cell membrane protein is necessary for HCMV entry into the cell will require the establishment of a transfected cell model in which expression of recombinant receptor protein that has been mutated at specific phosphorylation sites is compared to expression of nonmutated protein.

Internalization of other cell membrane proteins, such as the transferrin receptor and CD4, has also been linked to receptor phosphorylation (Foxie et al., 1988; Klausner et al., 1984). In addition, fusion of the enveloped viruses HIV-1 and EBV has been associated with cell protein phosphorylation. HIV-1 induces phosphorylation of the CD4 receptor via PKC, and the kinetics of CD4 phosphorylation correlate with the kinetics of virus entry; however, it is unclear whether CD4 phosphorylation is required for HIV-1 penetration (Fields et al., 1988; Cohen et al., 1992; Orloff et al., 1991). Penetration, but not binding, of EBV into Raji cells requires PKC phosphorylation of complement receptor 2 (CR2); both phosphorylation and virus penetration are inhibited by PKC inhibitors (Aquino et al., 1993; Cirone et al., 1990). The mechanism whereby protein phosphorylation influences virus/cell fusion is unknown, but may involve changes in membrane structure and fluidity via the activation of phospholipases (PLA2, PLC or PLD), or changes in protein phosphorylation within the membrane or at the membrane–cytoskeletal interface (Kinunnen, 1991).

The interaction of specific viral components with various cell membrane receptors has also been shown to result in cellular protein tyrosine phosphorylation, such as results from interaction of Friend spleen focus-forming virus gp55 with the erythropoietin receptor (Showers et al., 1992), or the interaction of HIV-1 with CD4 (Cohen et al., 1992). However, the mechanisms whereby viruses stimulate general cellular protein phosphorylation are also unknown.

One mechanism by which protein phosphorylation could facilitate virus/cell fusion is by stimulating inositol phosphate metabolism and a consequent increase in intracellular calcium ion concentration ([Ca2+]i). We previously reported that specific exposure of HEL cells to either crosslinked MAb 2 or HCMV resulted in an increase in [Ca2+]i which is temporally associated with an increase in intracellular inositol triphosphates and therefore appears to be mediated by inositol phosphate metabolism (Keay et al., 1995). The fusion of some enveloped viruses with cell membranes appears to be Ca2+-dependent, and in some cases protein phosphorylation and changes in [Ca2+]i appear interrelated. For example, EBV penetration requires an increase in [Ca2+]i which is associated with translocation of PKC from the cytosol to the cell membrane (Dugas et al., 1988). CD4-independent fusion of simian immunodeficiency virus with lysosomes is also stimulated by Ca2+ (Larson et al., 1990), the fusion of cell membranes mediated by CD4 and HIV-1 envelope glycoprotein (gp120-gp41) requires Ca2+ (Dimitrov et al., 1993), and Ca2+ appears to play a role in Sendai virus-induced membrane fusion (Volsky & Loyter, 1978). Studies to assess the role of Ca2+ in HCMV/cell fusion are in progress.

The authors thank Dr Peter Hornbeck for assistance with the scanning densitometer and helpful discussions. This work was supported by Merit Review Funding from the Veterans Administration.

References


