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Herpes Simplex Virus Entry Is Associated with Tyrosine Phosphorylation of Cellular Proteins

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The initial step in herpes simplex virus (HSV) entry is binding of virion glycoprotein (g)C and/or gB to cell surface heparan sulfate. After this initial attachment, gD interacts with cell surface receptor or receptors, and the virion envelope fuses with the cell membrane. Fusion requires viral glycoproteins gB, gD, gL, and gH, but the cellular factors that participate in or the pathways activated by viral entry have not been defined. To determine whether signal transduction pathways are triggered by viral–cell fusion, we examined the association of viral entry with tyrosine phosphorylation of cellular proteins. Using immunoprecipitation and Western blotting, we found that at least three cytoplasmic host cell proteins, designated p80, p104, and p140, become tyrosine phosphorylated within 5–10 min after exposure to HSV-1 or HSV-2. However, no phosphorylation is detected when cells are exposed to a mutant virus deleted in gL that binds but fails to penetrate. Phosphorylation is restored when the gL-deletion virus is grown on a complementing cell line. Viral entry and the phosphorylation of p80, p104, and p140 are inhibited when cells are infected with virus in the presence of protein tyrosine kinase inhibitors. Taken together, these studies suggest that tyrosine phosphorylation of host cellular proteins is triggered by viral entry.

INTRODUCTION

For herpes simplex virus (HSV), entry into cells is a complex process involving binding of virus to cell surface receptors followed by direct fusion of the virion envelope with the cell plasma membrane (Spear, 1993). Binding is mediated by an interaction between cell surface heparan sulfate (HS) proteoglycans and viral glycoproteins (g)C and/or gB (Gerber *et al.,* 1995; Herold *et al.,* 1991, 1994; Shieh *et al.,* 1992; Williams and Strauss, 1997; WuDunn *et al.,* 1989). After the initial attachment of virus to cell surface HS, it has been proposed that gD binds to specific coreceptor or coreceptors (Johnson *et al.,* 1990; Johnson and Ligas, 1988; Lee and Fuller, 1993). Recent data suggest that there may be several redundant receptors for gD on cell surfaces (Geraghty *et al.,* 1998; Montgomery*et al.,* 1996; Warner *et al.,* 1998; Whitbeck *et al.,* 1997). These include a novel member of the NGF/TNF receptor family called herpesvirus entry mediator A (HveA), which may be the principal coreceptor for infection of human T lymphocytes (Montgomery *et al.,* 1996; Whitbeck *et al.,* 1997); HveB, a member of the immunoglobulin superfamily (Warner *et al.,* 1996); and HveC, another member of the immunoglobulin superfamily (Geraghty *et al.,* 1998). The relative role of each of these coreceptors in HSV entry is yet to be determined. Moreover, whether or how the binding of gC and/or gB to HS and the binding of gD to Hve coreceptors promote viral–cell fusion is also unknown. It has been shown that viral–cell fusion occurs at physiological pH and can be blocked by neutralizing antibodies and enhanced by the chemical fusogen polyethylene glycol (Fuller *et al.,* 1989; Fuller and Spear, 1987; Spear, 1993; Wittels and Spear, 1991). Several virion glycoproteins, including gD, gB, and hetero-oligomers of gH and gL, are required for viral penetration. The precise role of each of these glycoproteins and the cellular factors involved remains unknown.

Many cell surface receptor–ligand interactions trigger protein tyrosine phosphorylation and activation of signal transduction pathways. For example, the activities of fibroblast growth factors (FGFs) are mediated by a family of tyrosine kinase transmembrane receptors, the FGF receptors (FGFRs) (Jaye *et al.,* 1992). Differences in the ectodomain sequence dictate the ligand specificity for each receptor, whereas differences in intracytoplasmic sequence determine the biochemical and functional responses mediated by each receptor. Notably, the interaction of FGFR with FGF requires HS or soluble heparin as a cofactor (Aviezer *et al.,* 1994; Krufka *et al.,* 1996; Rapraeger *et al.,* 1991; Spivak-Kroizman *et al.,* 1994; Yayon *et al.,* 1991). FGF cannot efficiently bind and activate FGFR on cells that fail to express HS (Rapraeger *et al.,* 1991).

Interestingly, several studies have shown that bacterial invasion of epithelial cells may be associated with tyrosine phosphorylation and activation of signal transduction pathways. For example, it has been shown that entry of *Salmonella typhimurium* into some epithelial cell lines stimulates tyrosine phosphorylation of the receptor for epidermal growth factor (Galan *et al.,* 1992; Pace *et al.,* 1993). Similarly, entry of *Yersinia pseudotuberculosis*

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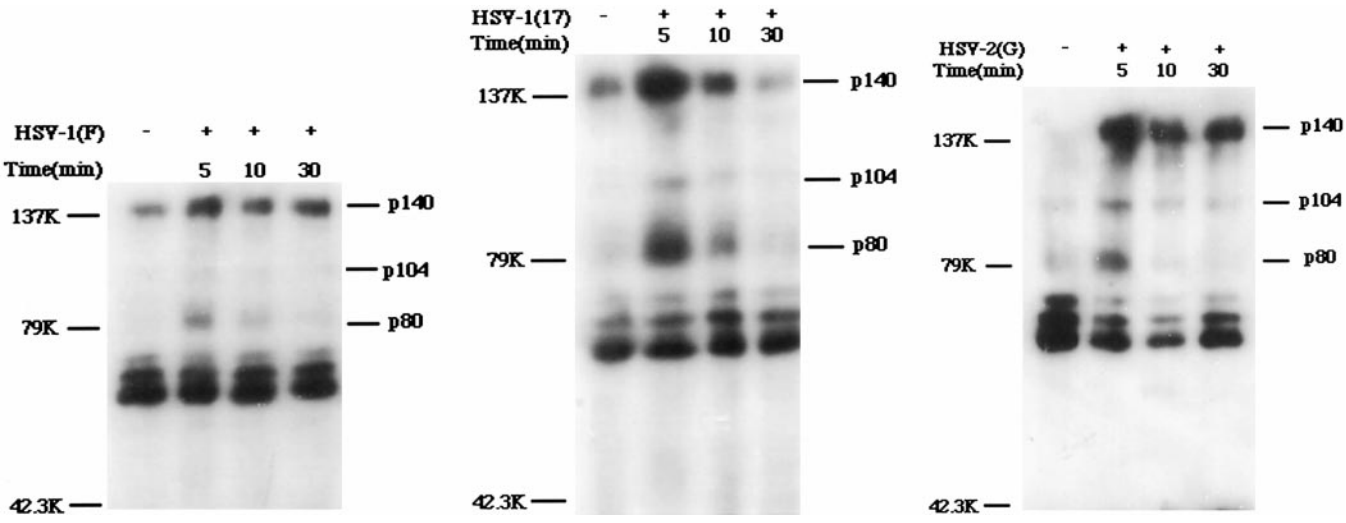

FIG. 1. Stimulation of cellular protein tyrosine phosphorylation after exposure to HSV-1 or HSV-2. Vero cells were mock infected or exposed to 10 PFU/cell of HSV-1(F) (left), HSV-1(17) (middle), or HSV-2(G) (right) for 5, 10, or 30 min. Cell lysates were prepared and analyzed by immunoprecipitation and Western blots as described in Materials and Methods. Molecular weight standards are indicated on the far left. The arrows indicate the three proteins designated p80, p104, and p140 that are tyrosine phosphorylated after exposure to HSV-1 or HSV-2.

into nonphagocytic cells is associated with a signaling process involving tyrosine phosphorylation (Bliska *et al.,* 1993). *Listeria monocytogenes* activates mitogen-activated protein kinase during invasion, and a mitogen-activated protein kinase signal transduction pathway may be involved in mediating bacterial uptake (Mengaud *et al.,* 1996; Tang *et al.,* 1994; Velge *et al.,* 1994). More recently, studies have suggested that tyrosine phosphorylation may play a role in cytomegalovirus (CMV) and human immunodeficiency virus entry (Briand *et al.,* 1997; Keay and Baldwin, 1996).

Taken together, the observation that microbial receptor-ligand interactions can trigger signal transduction pathways, candidate coreceptors for gD are linked to these pathways, and the integral role HS plays in HSV attachment and FGF signaling, suggest that HSV entry might be associated with activation of signal transduction pathways. Therefore, these studies were undertaken to determine whether HSV-1 and HSV-2 entry is linked with tyrosine phosphorylation of cellular proteins and whether phosphorylation facilitates viral entry.

RESULTS

Induction of tyrosine phosphorylation after the exposure of cells to HSV

To determine whether the exposure of susceptible epithelial cells to HSV triggered protein-tyrosine phosphorylation, we used a combination of immunoprecipitation and immunoblotting techniques. Nearly confluent monolayers of cells were exposed to HSV or control buffer for 5, 10, or 30 min. Cell lysates were prepared, and the cellular proteins were immunoprecipitated and subsequently immunoblotted using anti-protein tyrosine antibodies. The results for HSV-1(F) are shown in Fig. 1 (left). Within 5 min of infection, increased tyrosine phosphorylation of two proteins designated p140 and p80 was observed. The increase in tyrosine phosphorylation of p80 was transient and could no longer be observed after 10 min of incubation. In contrast, the increased phosphorylation of p140 persisted but was most intense 5 min postinfection.

To determine whether the observed induction of tyrosine phosphorylation was serotype or strain specific, we also examined the effects of exposure of cells to HSV-1(17) and HSV-2(G) on the tyrosine phosphorylation pattern. Results are shown in Fig. 1 (middle and right, respectively). The brief exposure of Vero cells to HSV-1(17) and HSV-2(G) resulted in a rapid increase in the level of tyrosine phosphorylation of the same two proteins, p80 and p140. Increased tyrosine phosphorylation was also observed for an additional protein with an estimated molecular mass of 104 kDa (p104). The increased tyrosine phosphorylation of p104 was most evident 5 min postinfection.

Next, we examined whether the tyrosine phosphorylated cellular proteins being detected were membrane associated or cytosolic. Vero cells were infected with HSV-1(F) at an m.o.i. of 10 pfu/cell for 5 min followed by cellular fractionation into three fractions: cytosolic, membrane, and Triton X-100 insoluble. All three fractions were analyzed by immunoprecipitation followed by immunoblotting with anti-phosphotyrosine antibodies. As a control, we also examined the distribution of the membrane-associated fibronectin receptor integrin with anti-integrin antibody. The tyrosine phosphorylated proteins p140, p104, and p80 localized predominantly to the cytosolic fraction (Fig. 2), whereas the fibronectin receptor integrin

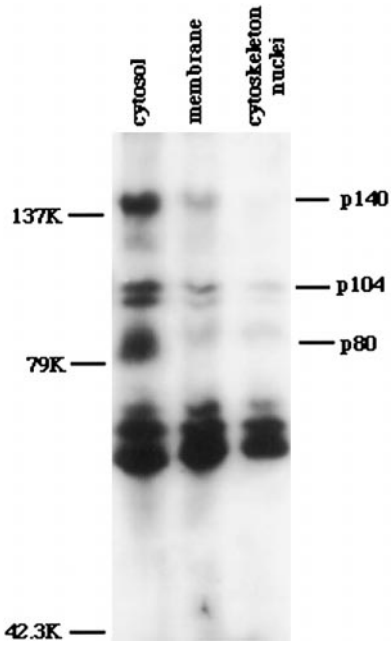

FIG. 2. Localization of tyrosine phosphorylated proteins. Vero cells were exposed to HSV-1(F) for 5 min, harvested, and fractionated into cytosolic, membrane, and Triton X-100-insoluble fractions. All three fractions were analyzed by immunoblotting with anti-phosphotyrosine antibodies.

localized to the membrane fraction (data not shown). An additional band with an apparent molecular mass of 98 kDa was also observed after cellular fractionation and localized predominantly to the cytosolic fraction. The observation that the phosphorylated proteins detected after exposure to virus are cytosolic suggests that these proteins are not themselves viral receptors but rather are being phosphorylated in association with or in response to viral entry.

To determine whether the induction of tyrosine phosphorylation of these cellular cytosolic proteins is triggered by the physical binding of HSV to cells alone or requires viral entry, we compared the ability of a gL-deletion virus grown on noncomplementing or complementing cells to induce tyrosine phosphorylation. The deletion of gL results in mutant virions that can bind to cells but fail to penetrate (Roop *et al.,* 1993; M. Novotny and P. G. Spear, unpublished data). For these studies, stocks of KOS-gL86 harvested from complementing or noncomplementing cells were prepared. The stock harvested from complementing 79VB4 cells had a titer of 8×10^8 pfu/ml on 79VB4 cells; no plaques were seen on Vero cells. In contrast, a stock of KOS-gL86 harvested from noncomplementing Vero cells had a titer of 4×10^3 pfu/ml on 79VB4 cells and 1.5×10^3 pfu/ml on Vero cells. The relative concentration of viral particles was determined by comparing the optical densitometry of the gD band after Western blotting as described in Materials and Methods.

Vero cells were exposed to equivalent concentrations of KOS-gL86 particles grown on complementing or noncomplementing cells for 5, 10, or 30 min; cell lysates were prepared; and immunoprecipitation and immunoblotting were performed as previously described. The results are shown in Fig. 3. Exposure of cells to KOS-gL86 gown on complementing cells resulted in the anticipated increase in tyrosine phosphorylation of p140 and p80. p104 was not detected in these gels. In contrast, exposure of cells to KOS-gL86 grown on noncomplementing cells failed to induce any discernible increase in protein tyrosine phosphorylation. These results suggest that binding of virus to cell surface is not sufficient to induce tyrosine phosphorylation but that rather, phosphorylation occurs in association with or is triggered by viral entry.

Effects of kinase or phosphatase inhibitors on tyrosine phosphorylation of proteins

To determine whether inhibition by kinase inhibitors or stimulation by phosphatase inhibitors of tyrosine phosphorylation affects the observed phosphorylation of p140, p104, and p80, Vero cells were incubated with specific inhibitors, their respective controls, or an equivalent concentration of DMSO carrier and then exposed to HSV-1(F) for 5 min. Cell lysates were prepared and analyzed by immunoprecipitation followed by immunoblotting with anti-phosphotyrosine antibodies. The results are shown in Fig. 4. The tyrosine

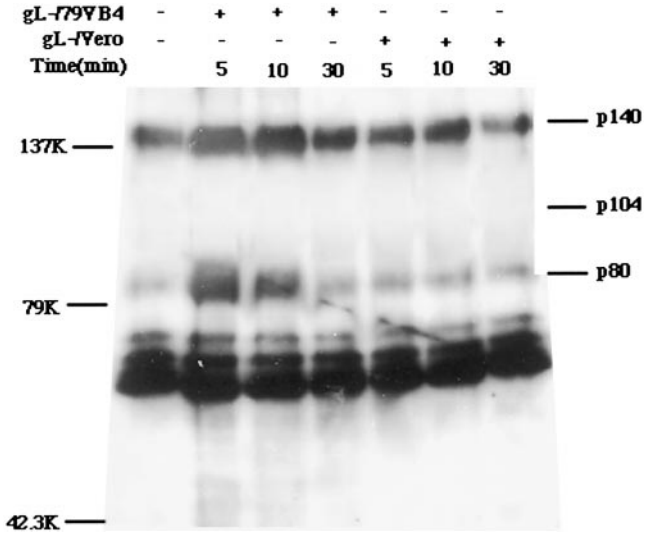

FIG. 3. Tyrosine phosphorylation of cellular proteins in the presence of a gL-deletion virus. Vero cells were mock infected (lane 1) or exposed to relatively equal particle numbers of KOS-gL86 virus grown on complementing cells (gL^-/79VB4) (lanes 2–4) or noncomplementing Vero cells (gL^-/Vero) (lanes 5–7) for 5, 10, or 30 min as indicated. Cell lysates were prepared and analyzed by immunoprecipitation and Western blotting. The complemented virus induces tyrosine phosphorylation of the p80 and p140 cellular proteins as indicated by the arrows, whereas exposure of cells to the noncomplemented virus fails to induce any detectable cellular phosphorylation of proteins.

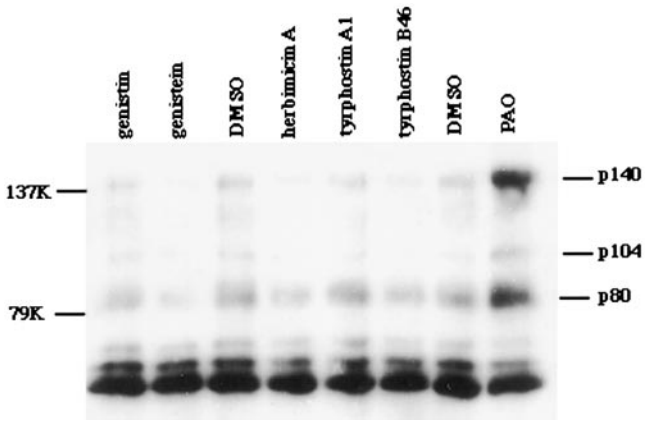

FIG. 4. Effects of kinase or phosphatase inhibitors on cellular protein tyrosine phosphorylation. Vero cells were preincubated with the following kinase inhibitors, phosphatase inhibitor, control compounds, or DMSO vehicle alone for the times indicated in Materials and Methods and then exposed to HSV-1(F) for 5 min: genistein (100 μM), herbimycin A (10 μM), tyrphostin B46 (100 μM), PAO (10 μM), and genistin (100 μM) as control for genistein and tyrphostin A1 (100 μM) as control for tyrphostin B46. Cell lysates were prepared and analyzed by immunoprecipitation followed by immunoblotting with anti-phosphotyrosine antibodies.

kinase inhibitors genistein (100 μM), herbimycin A (10 μM), and tyrphostin B46 (100 μM) inhibited phosphorylation of p140, p104, and p80 by ~50% by densitometry compared with their respective controls. In contrast, the tyrosine phosphatase inhibitor PAO (10 μM) stimulated the phosphorylation of p140, p104, and p80 (2-fold increase by densitometry).

Effects of kinase inhibitors on viral infection

Next, we examined whether viral infection is affected by pretreatment of cells with inhibitors of protein tyrosine kinases (PTKs). Vero cells were pretreated with various concentrations of PTK inhibitors or controls at 37°C for appropriate time intervals. Subsequently, the cells were exposed to virus, and infection was monitored using a standard plaque assay. Results are shown in Fig. 5. The pretreatment of cells with tyrphostin B46, but not the inactive control compound tyrphostin A1, resulted in a significant reduction in viral plaque formation ($P = .0018$, Student's unpaired t test). Notably, pretreatment of the viral particles, but not the cells, had no inhibitory effect on subsequent infectivity (data not shown). Herbimycin A did not inhibit plaque formation at concentrations of 10 mM or less; higher concentrations were cytotoxic. Similarly, genistein had little or no effect on viral plaque formation. Serine/threonine kinase inhibitors also had no effect on HSV infection (data not shown).

To evaluate whether the inhibitory effect of tyrphostin B46 on viral plaque formation could be explained by inhibition of steps early in viral infection, we also examined viral immediate-early gene expression. Taking advantage of an HSV-1 strain derived from HSV-1(17) that contains the *Escherichia coli* β-galactosidase gene under the control of the viral dUTPase gene promoter, we compared viral infection of Vero cells pretreated with the same concentrations of inhibitors or controls. Cultures were assayed for β-galactosidase activity at 5 h after infection as previously described (Roller and Herold, 1997). In preliminary studies, to control for any effects of PTK inhibitors on β-galactosidase expression itself, uninfected cells were transfected with the same β-galactosidase cassette and exposed to PTK inhibitors. The PTK inhibitors did not inhibit β-galactosidase expression (data not shown). As shown in Fig. 6, pretreatment of cells with tyrphostin B46, but not the control compound

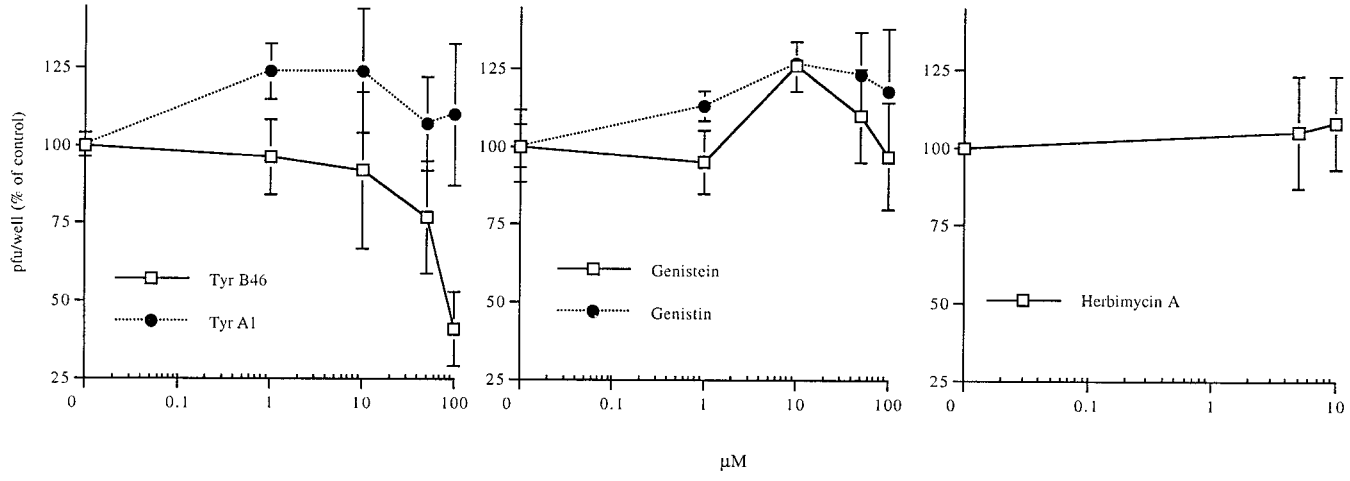

FIG. 5. Effects of kinase inhibitors on viral plaque formation. Vero cells were grown to near confluence in 6-well dishes and then were preincubated with the indicated kinase inhibitors, control compounds, or DMSO vehicle for the times indicated in Materials and Methods. Cells were subsequently infected with HSV-1(17)(dUTPase/LAT at an m.o.i. to yield 100–500 plaques/well on control (DMSO) dishes. The results are presented as PFU/well in the presence of compound as a percentage of PFU formed in the presence of DMSO vehicle alone. Each point is the mean of two independent experiments performed in duplicate, and the error bars indicate standard deviation.

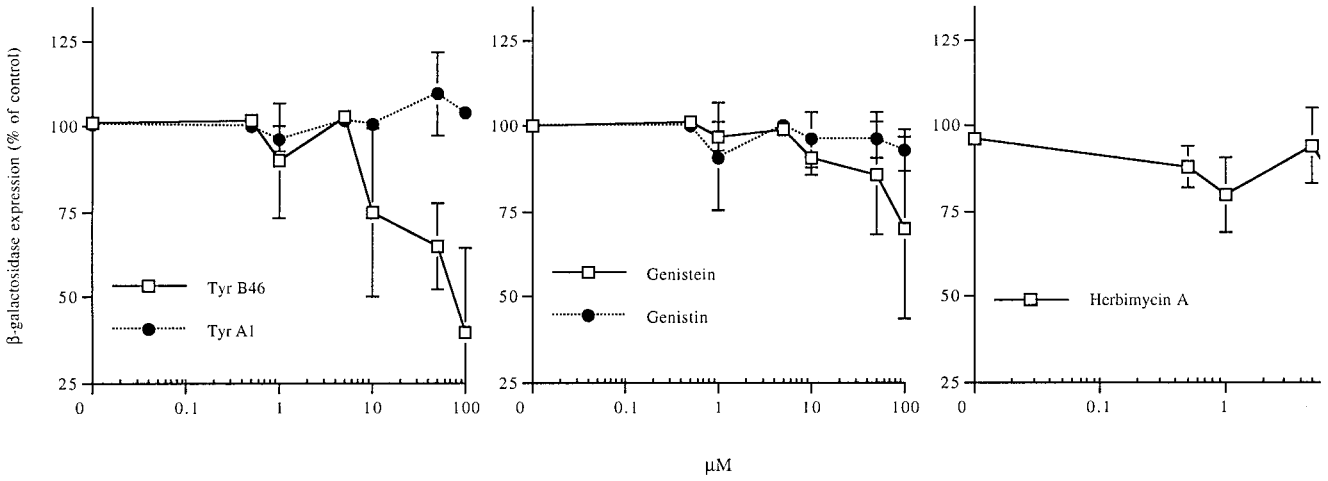

FIG. 6. Effects of kinase inhibitors on viral entry. Cells were preincubated with the indicated kinase inhibitors, control compounds, or DMSO vehicle for the times indicated in Materials and Methods and then infected with HSV-1(17)(dUTPase/LAT). At 5 h after infection, viral entry was quantified by measuring β-galactosidase activity. Results are presented as the absorbance in the presence of kinase inhibitor or control compound as a percentage of the absorbance in the presence of DMSO carrier alone. Each point is the average of three independent experiments performed in duplicate or triplicate, and the error bars indicate standard deviation.

tyrphostin A1, reduced viral entry parallel to its effects on viral plaque formation ($P = .0002$, Student's unpaired t test). Again, neither genistein nor herbimycin A significantly inhibited viral immediate-early gene expression.

DISCUSSION

The data presented here support the notion that HSV-1 and HSV-2 entry is associated with phosphorylation of cellular proteins and, presumably, with activation of signal transduction pathways. Phosphorylation of proteins appears to be important for viral infection because the pretreatment of cells with tyrphostin B46, a known PTK inhibitor, reduced viral immediate-early gene expression and plaque formation in parallel. Notably, the kinetics of phosphorylation of cellular proteins parallel the kinetics of viral entry. In previous studies, we and others have demonstrated that it takes 5–15 min for 50% of wild-type HSV-1 or HSV-2 particles to penetrate Vero cells and penetration plateaus within ~30 min (Gerber *et al.,* 1995; Herold *et al.,* 1991; McClain and Fuller, 1994). Similarly, we found that the increased phosphorylation of p80, p104, and p140 could be detected within 5 min and diminished by 30 min postinfection.

Interestingly, the exposure of cells to a gL-deletion virus grown on noncomplementing cells, which binds but fails to penetrate, did not induce any detectable protein phosphorylation. This observation suggests that tyrosine phosphorylation of p80, p104, and p140 requires more than a physical binding of viral particles to cell surface receptors and, given the time course, suggests that phosphorylation is associated with fusion of the viral envelope with the cellular plasma membrane.

The nature of the signal transduction pathway or pathways being activated and whether activation facilitates viral entry or subsequent early steps in infection are not yet known. The observation that tyrphostin B46, a synthetic PTK inhibitor, blocks viral infection suggests that viral invasion may involve pathways that include cyclin-dependent kinase (cdk)2 activation. Tyrphostin B46 is a potent inhibitor of cdk2. The finding that the naturally occurring PTK inhibitors genistein and herbimycin A block phosphorylation of p80, p104, and p140 but fail to inhibit viral infection may reflect differences in selectivity, potency, and assay conditions. For immunoprecipitation studies, cell lysates are prepared 5 min after the exposure to virus. In contrast, for infectivity studies, infection is assessed 5 h (β-galactosidase expression) or 24 h (plaque assays) postinfection. Possibly, cells begin to recover from the effects of genistein and herbimycin A.

It may be that the signal transduction pathway being activated in association with HSV entry relates to those activated by ligands binding to the recently identified Hve coreceptors. The coreceptors identified include a novel member of the TNF/NGF receptor family, HveA, and two members of the immunoglobulin superfamily, HveB and HveC (Geraghty *et al.,* 1998; Montgomery*et al.,* 1996; Warner *et al.,* 1998; Whitbeck *et al.,* 1997). The physiological role of these coreceptors has not yet been fully elucidated. HveA binds to several members of the TNFR-associated factor (TRAF) family (Marsters *et al.,* 1997). Transient transfection of HveA into human 293 cells causes marked activation of nuclear factor-κB, JunN-terminal kinase, and Jun containing transcription factor AP-1, suggesting that HveA is associated with signal transduction pathways that activate the immune response (Marsters *et al.,* 1997). Whether similar pathways are activated during viral entry remains to be determined.

It is also possible that the pathway being activated during viral entry is similar to pathway activated by FGF or other growth factors. This follows from the observations that there is a requirement for HS by FGF and HSV. FGF binds to cell surfaces at both low- and high-affinity sites. The low-affinity site represents binding of FGF to HS, whereas the high-affinity site represents binding to a specific tyrosine kinase receptor (Rapraeger *et al.,* 1991; Yayon *et al.,* 1991). Several models have been proposed to explain the interaction of HS, FGF, and FGFR. For example, it has been suggested that HS–FGF interactions induce a necessary conformational change such that FGF can be recognized by its specific tyrosine kinase receptor. One could envision a similar model for the interactions of HSV with cell surface. For example, the binding of HSV to cell surface HS might induce a conformational change in either the virus, cell, or both, which facilitates secondary interactions at the cell surface such as the interaction of gD with Hve coreceptors and/or the interaction of other envelope glycoproteins with cell surface components. These interactions might culminate in activation of a signal transduction pathway and trigger viral–cell fusion.

At a biochemical level, there are several mechanisms that could explain how activation of signal transduction pathways might facilitate viral entry. Activation of signal transduction pathways can induce rearrangements of the host cytoskeleton and thus facilitate viral entry. Such a mechanism has been shown to be important in the invasion of HeLa cells by *Shigella* (Dehio *et al.,* 1995). Alternatively, activation of signal transduction pathways may facilitate viral entry by stimulating inositol phosphate metabolism and thus increasing calcium ion concentration. Calcium ions are required for fusion of a wide variety of biological membranes, including the fusion of some enveloped viruses with cell membranes (Papahadjopoulos*et al.,* 1990). For example, binding of human immunodeficiency virus type 1 to the CD4 molecule induces phosphatidylinositol-3-kinase (Briand *et al.,* 1997; Dimitrov *et al.,* 1993). Ca^{2+} also appears to play a role in Epstein–Barr virus penetration and HCMV cell fusion (Dugas *et al.,* 1988; Keay *et al.,* 1995).

Notably, protein phosphorylation has also been suggested to be important for HCMV entry. HCMV initiates infection by binding to HS moieties on the cell surface. Subsequently, HCMV envelope glycoprotein gH interacts with a 92.5-kDa cell membrane receptor. The 92.5- kDa cell membrane protein appears to be tyrosine phosphorylated after exposure of the cells to HCMV (Keay and Baldwin, 1996). Pretreatment of the cells with PTK inhibitors blocks phosphorylation of this cellular membrane receptor and impedes viral entry.

Further studies are needed to identify the cytoplasmic proteins p80, p104, and p140; to identify the signal transduction pathway or pathways being activated by HSV infection; and to determine how this activation facilitates viral infection. Viral entry is a complex process that has not yet been been defined at a molecular or biochemical level. The results reported in these studies suggest a novel approach to address the question of how HSV invades cells.

MATERIAL AND METHODS

Cells and viruses

Vero cells were obtained from the American Type Culture Collection and passaged in medium 199 supplemented with 5% FBS. The cell line 79VB4 (provided by P. Spear, Northwestern University) is a gL-expressing Vero cell line and was passaged in medium 199 supplemented with 5% FBS and 200 μg/ml G418 (Montgomery *et al.,* 1996). Wild-type viral strains were HSV-1(KOS), HSV-1(F), and HSV-2(G). The recombinant HSV-1(17)(dUTPase/LAT) contains the *E. coli* β-galactosidase gene under the control of the viral dUTPase promoter in place of both copies of the LAT genes and has been previously described (gift of Ed Wagner, University of California, Irvine) (Singh and Wagner, 1995). KOS-gL86 is a mutant in which the *E. coli* β-galactosidase gene replaces part of the gL open reading frame (gift of P. Spear, Northwestern University) (Montgomery *et al.,* 1996).

Preparation of cell lysates

Nearly confluent Vero cell monolayers in 25-cm^2 flasks were preincubated with serum-free medium for 24 h before infection. Cells were exposed to HSV at an m.o.i. of 5 PFU/cell for 5, 10, or 30 min (or mock infected as controls). The inoculum was removed, and the cells were washed once with ice-cold PBS (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, pH 7.4) and harvested by scraping. Cells were pelleted by centrifugation and resuspended in lysis buffer (1% Triton X-100, 50 mM Tris–HCl, pH 7.6, 40 mM β-glycerophosphate, 0.4 mM $NaVO_4$, 0.1 mg/ml phenylmethylsulfonyl fluoride, 8 μg/ml aprotinin, and 10 μg/ml leupeptin). The supernatant (soluble fraction) and pellet (insoluble fraction) were separated by centrifugation at 16,000*g*, and the protein concentration of each fraction was determined using the DC Protein Assay (Bio-Rad).

Alternatively, for fractionation into membrane, cytosol, and insoluble fractions, the scraped infected cells were suspended in sonication buffer (50 mM Tris–HCl, pH 7.6, 40 mM β-glycerophosphate, 0.4 mM $NaVO_4$, 0.1 mg/ml phenylmethylsulfonyl fluoride, 8 μg/ml aprotinin, and 10 μg/ml leupeptin) instead of lysis buffer. Cells were then sonicated for two 1-s bursts. The sonicate was then spun (15 min, 100,000*g*), the supernatant containing the cytosolic proteins was removed, and the pellet containing cell membrane and cell nuclei was washed once with sonication buffer. The pellet was then suspended in lysis buffer, the lysate was spun for 5 min at 100,000*g*, and the

supernatant containing membrane proteins was harvested. The remaining pellet containing cytoskeletal, proteins, and nuclei was dissolved in a buffer containing 1% SDS and 10 mM Tris–HCl, pH 7.4.

Immunoprecipitation and immunoblot analyses

Equal concentrations of protein from each sample were mixed with 5 μl of a monoclonal anti-phosphotyrosine antibody coupled to agarose (0.5 mg IgG/0.25 ml agarose, PY20-agarose; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40). Samples were incubated for 1 h or overnight at 4°C with end-over-end rotation. Unbound proteins were removed by washing the agarose three times with lysis buffer, followed by brief spins in an Eppendorf centrifuge. The pellet was suspended in 40 μl of SDS sample buffer (100 mM Tris–HCl, pH 6.8, 1% β-mercaptoethanol, 4% SDS, 0.2% bromphenal blue, 20% glycerol), boiled for 2 min, and then loaded onto a 7.5% SDS–polyacrylamide slab gel. Proteins were transferred to nitrocellulose (Schleicher & Schuell) by Western transfer using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The nitrocellulose was incubated overnight at 4°C in TBS (150 mM NaCl and 20 mM Tris–HCl, pH 7.5) containing 5% skim milk and then for 2 h at room temperature in TBS–5% milk containing monoclonal anti-phosphotyrosine antibody (PY20; Transduction Laboratories, Lexington, KY) (1:500 dilution). After several washes in TBS, the nitrocellulose blot was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:1000 in TBS for 1 h at room temperature. The blot was rinsed several times and developed using the ECL kit (DuPont, Boston, MA).

Preparation and quantification of gL-deletion virus.

Vero and 79VB4 cells were inoculated with KOS-gL86 at an m.o.i. of ~10 PFU/cell, and the virus was harvested after 2 days. The inoculum was a virus stock produced on complementing cells (79VB4) and had a titer of $\sim 10^{10}$ PFU/ml on 79VB4 cells and 10^4 PFU/ml on Vero cells. The relative concentration of viral particles was determined by comparing the relative amounts of gD band by optical densitometry after Western blotting. Several dilutions of samples of each harvest were solubilized, and polypeptides were fractionated by SDS–PAGE. After SDS–PAGE, the proteins were transferred to nitrocellulose by Western transfer. Western blots were performed as described above. The antiserum used was a polyclonal rabbit anti-HSV gD (R72) (obtained from P. Spear, Northwestern University).

Kinase/phosphatase inhibitors

To determine the effect of tyrosine kinase or phosphatase inhibitors on protein phosphorylation, Vero cells were preincubated with the following inhibitors and control compounds diluted in DMSO or preincubated with an equivalent concentration of DMSO alone at 37°C for the appropriate time intervals before exposure of the cells to virus: 0.1–100 μM genistein or genistin (Sigma) × 60 min; 0.1–10 μM herbimycin A (Sigma) × 16 h; 0.1–100 μM tyrphostin B46 or tyrphostin A1 × 30 min (Calbiochem); and 0.1–10 μM PAO (Sigma), an inhibitor of tyrosine phosphatase, × 60 min. The cells were then exposed to virus as described, and immunoprecipitation and immunoblotting were performed.

To assay the effect of the kinase/phosphatase inhibitors on viral infection, plaque assays or β-galactosidase expression assays were conducted as previously described (Roller and Herold, 1997). For plaque assays, cells in 6-well dishes were treated with the appropriate inhibitor or control compound at the concentrations indicated and for the time interval described above and then exposed to HSV-1(17)(dUTPase/LAT) (200–500 PFU/well) for 15 min at 37°C. Subsequently, the inoculum was removed, and cells were treated with citrate buffer (50 mM sodium citrate, 4 mM KCl, adjusted to pH 3.0) to inactivate residual extracellular virus and washed three times. Plaques were counted after 24 h of incubation using a modified blue-plaque assay. Infected cells are fixed with 0.5% glutaraldehyde and permeabilized with 0. 5% Nonidet P-40. and plaques were visualized by X-gal overlay (0.5 mg/ml X-gal dissolved in ferric cyanide solution). For the β-galactosidase expression assays, cells in 96 well-dishes (5×10^4 cells/well) were treated with the appropriate inhibitor or control compound at the concentrations indicated and for the time interval described above and then exposed to HSV-1(17)(dUTPase/LAT) at an m.o.i. of 5 PFU/cell at 37°C. After a 1-h absorption period, cells were treated with low pH citrate buffer to inactivate most of the residual extracellular virus. After a 5-h period, the inoculum was removed, and cells were washed with PBS, fixed with glutaraldehyde, and permeabilized with 0.1% Nonidet P-40 in PBS. To quantify the amount of β-galactosidase activity (reflecting viral entry), cells were incubated with *o*-nitrophenyl-β-D-galactopyranoside (1 mg/ml in 0.1% Nonidet P-40) for 1 h at 37°C, and the absorbance (410 nm) of the soluble dye product was determined.

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