Focal Adhesion Kinase Plays a Pivotal Role in Herpes Simplex Virus Entry

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Development of strategies to prevent herpes simplex virus (HSV) infection requires knowledge of cellular pathways harnessed by the virus for invasion. This study demonstrates that HSV induces rapid phosphorylation of focal adhesion kinase (FAK) in several human target cells and that phosphorylation is important for entry post-binding. Nuclear transport of the viral tegument protein VP16, transport of viral capsids to the nuclear pore, and downstream events (including expression of immediate-early genes and viral plaque formation) were substantially reduced in cells transfected with dominant-negative mutants of FAK or small interfering RNA designed to inhibit FAK expression. These observations were substantiated using mouse embryonic fibroblast cells derived from embryonic FAK-deficient mice. Infection was reduced by >90% in knockout cells relative to control cells and was further reduced if the knockout cells were transfected with small interfering RNA targeting proline-rich tyrosine kinase-2, which was also phosphorylated in response to HSV. The knock-out cells were permissive for viral binding, and virus triggered an intracellular calcium response, but nuclear transport was inhibited. Together, these results support a novel model for invasion that implicates FAK phosphorylation as important for delivery of viral capsids to the nuclear pore.

Herpes simplex virus (HSV) type 2 is a major global health problem, the leading cause of genital herpes and neonatal herpes encephalitis, and a major cofactor for human immunodeficiency virus (HIV) infection. Novel strategies to prevent HSV-2 and other sexually transmitted infections are urgently needed. Development of new approaches requires an understanding of the molecular and cellular events critical for the establishment of infection. Previous work demonstrates that HSV entry is a complex process requiring the following steps: (i) binding to heparan sulfate receptors; (ii) engagement of glycoprotein D coreceptors; (iii) penetration, which, for human epithelial cells, is mediated primarily by fusion of the viral envelope with the cell membrane; (iv) transport of the viral tegument protein VP16 (trans-inducing factor (αTIF)) to the nucleus; and (v) transport of viral capsids to the nuclear pore with release of viral DNA into the nucleoplasm (1, 2). Glycoprotein B plays the major role in mediating binding of HSV-2 to heparan sulfate receptors, whereas glycoprotein C plays a more dominant role in mediating binding of HSV-1 (3, 4). For both serotypes, glycoproteins B, D, and H–L are required for penetration, but their precise role and the cellular pathways important for viral invasion have not been fully elucidated. The observations that heparan sulfate is the major attachment receptor for HSV and that heparan sulfate moieties also interact with gp120 of HIV fostered the development of sulfated or sulphonated polymers as candidate topical microbicides to prevent viral transmission. These compounds prevent genital herpes in the mouse and simian immunodeficiency virus in the macaque and are currently being evaluated in Phase II/III clinical trials (5, 6). However, competitive antagonists of viral binding are not likely to be sufficient to fully protect against sexual transmission. Experience with systemic therapy indicates that combination therapies that target more than one step in the viral life cycle are optimal. Thus, elucidating the cellular pathways required for viral entry may provide novel targets for microbicide development.

We reported previously that both serotypes of HSV trigger inositol triphosphate-mediated release of endoplasmic reticulum (ER) Ca^{2+} stores and that Ca^{2+} signaling plays a critical role in viral entry (7). Pharmacological inhibition of ER Ca^{2+} release or chelation of intracellular Ca^{2+} reduces nuclear transport of the viral tegument protein VP16, a surrogate marker for capsid transport, and the downstream events in the viral life cycle, including expression of viral immediate-early genes and viral plaque formation. Activation of Ca^{2+} signaling is associated with triggering of phosphorylation signaling pathways. Specifically, we observed previously that HSV-1 and HSV-2 trigger the phosphorylation of focal adhesion kinase (FAK) within 5–10 min following exposure of Vero (monkey kidney) or CaSkI (human cervical epithelial) cells to virus. FAK phosphorylation appears to occur downstream of release of ER Ca^{2+} stores, as treatment of cells with 2-aminoethoxydiphenyl borate, an inositol triphosphate receptor antagonist that prevents release of ER Ca^{2+}, or chelation of intracellular Ca^{2+} blocks the virus-induced phosphorylation of FAK. Activation of both pathways (Ca^{2+} and FAK phosphorylation) requires exposure to entry-competent virus, as HSV-1 deleted in the glycoproteins essential for penetration (glycoproteins B, D, and H–L) fails to induce Ca^{2+} release or to trigger FAK phosphorylation, whereas Ca^{2+} release and phosphorylation are preserved following exposure to the repaired viruses and to inactivated virus, which retain the ability to penetrate cells. These
findings led to the proposal of a new paradigm for HSV entry in which HSV triggers inositol triphosphate-mediated release of ER Ca\(^{2+}\) stores, which may facilitate fusion of the viral envelope and cell membrane, allowing delivery of capsids to the cytoplasm (7). In response to activation of the Ca\(^{2+}\) pathways, we hypothesize that FAK and related signaling pathways are activated, which may promote transport of the viral capsids to the nuclear pores. This study was designed to test this hypothesis and to evaluate whether HSV exploits FAK signaling pathways to promote its nuclear transport. We used complementary molecular techniques, including dominant-negative variants, small interfering RNA (siRNA), and knockout cells, to explore the role played by FAK in HSV invasion. We tested the hypothesis with both serotypes using human and murine cells. Our findings indicate that FAK phosphorylation plays a central role in promoting transport of viral capsids.

**MATERIALS AND METHODS**

**Cells and Viruses**—CaSki (human cervical epithelial), CaCo-2 (human colonic epithelial), and SK-N-SH (human neuroblastoma) cells were obtained from American Type Culture Collection and passaged in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Immortalized human endocervical and ectocervical cells were a generous gift from R. N. Fichorova and D. J. Anderson (8). Mouse embryonic fibroblast (MEF) cells derived from embryonic FAK-deficient mice (FAK\(^{-/-}\)) and control MEF cells (FAK\(^{+/+}\)) were originally described by Ilie et al. (9) and were a generous gift from I. H. Gelman (Mount Sinai School of Medicine). The FAK\(^{-/-}\) cells were derived from the same age embryos (day 8). Cells were cultured overnight in serum-free medium to minimize background tyrosine phosphorylation due to growth factors in serum. HSV-2(G) (provided by B. Roizman, University of Chicago, Chicago, IL) and vesicular stomatitis virus-Indiana (provided by P. Palese, Mount Sinai School of Medicine) were grown on vero cells. HSV-1 containing a green fluorescent protein (GFP)-VP26 fusion protein (signaled K96GFP) was a generous gift from P. Desai (The Johns Hopkins University) (10). Heat-inactivated virus was prepared by heating to 56 °C for 30 min.

**Purification and Quantification of Viruses**—For binding and Ca\(^{2+}\) studies, dextran-purified viruses were used as described previously (4, 11). Titers of the purified virus were determined by plaque assays. Relative viral particle numbers were determined by comparing the amounts of glycoprotein D by optical densitometry after Western blotting with anti-glycoprotein D monoclonal antibody (mAb) 1103 (Runbaugh-Goodwin Institute, Plantation, FL) as described previously (3, 12).

**Reagents**—EGTA and ionomycin were purchased from Calbiochem and diluted in Me\(_2\)SO or phosphate-buffered saline (PBS) following the manufacturer’s instructions. Fura-2 acetoxymethyl ester was purchased from Molecular Probes, Inc. (Eugene, OR). Heparin and cycloheximide were purchased from Sigma.

**Plasmids and Transfections**—pKH3-FAK carrying the gene for wild-type FAK and pKH3-Y397 carrying the FAK gene with a Y397F mutation were gifts from J.-L. Guan (Cornell University, Ithaca, NY). All transfections were done with Effectene transfection reagent (Qiagen Inc.) following the manufacturer’s protocol. CaSki, FAK\(^{-/-}\), and FAK\(^{+/+}\) cells were grown in 12-well plates and transfected with siRNA for FAK and Pyk2 and with control nonspecific siRNA using siRNA/siAB™ assay kits (Upstate Biotechnology Inc.) at a concentration of 100 pmol/well. 24 h post-transfection, the cells were infected with virus and analyzed for expression of phosphorylated FAK[pY397] and phosphorylated Pyk2pY256 by performing Western blotting as detailed below.

**Measurement of [Ca\(^{2+}\)]\(_i\)**—Cells were loaded with 25 mm fura-2 aectoxymethyl ester prepared in PBS for 30–60 min, rinsed with PBS for 30 min at 37 °C, and then exposed sequentially to PBS buffer or HSV diluted in PBS at 37 °C. The viral inoculum was equivalent to a multiplicity of infection (m.o.i.) equal to 1–5 plaque-forming units (pfu/cell). Using a Nikon Eclipse TE300 inverted epifluorescence microscope linked to a cooled Pentamex CCD camera (Princeton Instruments) interfaced with a digital imaging system (MetaFluor, Molecular Devices, Downingtown, PA), [Ca\(^{2+}\)], was measured in individually identified fura-2-loaded cells visualized using a Nikon S Fluor \(\times 40\) objective (numerical aperture of 0.9 and window of 0.3) as described previously (7). Cells were alternately excited at 340 and 380 nm, and the images were digitized for subsequent analysis. Images were acquired every 2–10 s. An intracellular calibration was performed at the conclusion of each experiment as described previously (7). 8–15 cells were monitored for each experiment.

**Plaque Assays**—Briefly, cells in 6-well dishes were exposed to virus for 2 h at 37 °C; the inoculum was removed; and cells were washed three times with PBS. The cells were then overlaid with medium containing 0.5% methylcellulose for 48 h. For HSV-1(KOS) and HSV-2(G), plaques were counted by immunoassay (black plaque) (4). For vesicular stomatitis virus, cells were overlaid with 1% methylcellulose, fixed after 24 h with methanol, and stained with Giemsa stain. Unless indicated otherwise, the m.o.i. was selected to yield 200–500 pfu/well (e.g., m.o.i. 0.005 pfu/cell) on control wells.

**Viral Binding and VP16 Time Course Assays**—To examine which steps in HSV infection are blocked in transfected or knockout cells, viral binding and VP16 transports to the nucleus were compared. For binding studies, cells were exposed to serial 2-fold dilutions of purified HSV-2(G) for 5 h at 4 °C. Unbound virus was removed by washing, and cell-bound virus was analyzed by preparing Western blots of cell lysates and probing with anti-glycoprotein D mAb 1103. To examine transport of VP16 to the nucleus, synchronized infection assays were conducted as described previously (7). Cells were precooled and exposed to HSV at 4 °C for 2 h at a m.o.i. of 0.1, 1, or 10 pfu/cell to allow binding. Unbound virus was removed, and the cells were washed three times with PBS and then transferred to 37 °C for 15 min to allow penetration. Unpenetrated virus was inactivated by washing the cell monolayer with a low pH citrate buffer adjusted to pH 3.0 for 2 min and then by washing three times with PBS. The cells were overlaid with fresh medium, and nuclear extracts were prepared 1 h after citrate treatment. The nuclear proteins were separated on a 10% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (PerkinElmer Life Sciences) using a Trans-Blot system (Bio-Rad), and blocked overnight in 5% milk-containing Tris-buffered saline. Membranes were incubated with mouse anti-VP16 antibody (1:500 dilution; Santa Cruz Biotechnology, Inc.), diluted in 5% milk-containing Tris-buffered saline for 2 h, and rinsed extensively with 50 mm Tris-CI (pH 7.4), 150 mm NaCl, and 0.05% Tween 20. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:1000 dilution; Calbiochem) in 5% milk-containing PBS for 2 h. After rinsing, the mem-
branes were developed using Immuno-Star-AP chemiluminescent kit (Bio-Rad). Blots were scanned and analyzed using the GelDoc 2000 system.

Detection of FAK or Pyk2 Phosphorylation by Immunoblotting—Nearly confluent cell monolayers were preincubated with serum-free medium for 12 h before infection and then exposed to HSVs at a m.o.i. of 1 pfu/cell for the indicated times (5–120 min). Cell lysates were prepared as described above, and proteins were separated, transferred by Western blotting, and incubated with anti-phospho-Tyr397 FAK antibody (FAK[pY397]). Blots were then stripped and reprobed with mAb to total FAK and with antibody to β-actin. Representative blots for FAK+/− (left panel) and FAK−/− (right panel) cells are shown. B, to compare susceptibility to HSV infection, the cells were exposed to serial dilutions of HSV-1(Kos)/VP26-GFP, HSV-2(G), or vesicular stomatitis virus (VSV). Plaques were counted 48 h post-infection, and viral titer (plaque-forming units/ml) were determined. The results are means ± S.D. of two independent experiments conducted in duplicate.

A.

FIG. 2. FAK+/− cells exhibit reduced susceptibility to HSV infection. A, FAK+/− and FAK−/− cells were infected with HSV-2(G) (m.o.i. = 1 pfu/cell). At the indicated times post-infection, cell lysates were prepared, and proteins were separated, transferred by Western blotting, and incubated with anti-phospho-Tyr397 FAK antibody (FAK[pY397]). Blots were then stripped and reprobed with mAb to total FAK and with antibody to β-actin. Representative blots for FAK+/− (left panel) and FAK−/− (right panel) cells are shown. B, to compare susceptibility to HSV infection, the cells were exposed to serial dilutions of purified HSV-2(G) at the m.o.i. indicated for 5 h at 4 °C. The input and cell-bound viral particles were detected by analyzing Western blots of cell lysates for glycoprotein (gD). Blots were also probed with mAb to β-actin to control for cell loading. B, alternatively, cells were exposed to the indicated m.o.i. of HSV-2(G) in a synchronized infection assay; and 1 h post-citrate treatment, nuclear extracts were prepared and analyzed for the presence of the viral tegument protein VP16 by Western blotting. The results shown are representative of three independent experiments. C, to confirm that nuclear VP16 represents transported and not synthesized protein, experiments were conducted in the absence or presence of cycloheximide as indicated. Nuclear extracts (Nuc) and cell lysates (Cell; after separating nuclear pellets) were analyzed for VP16 by Western blotting. The results are representative of two independent experiments.

B.

C.

FAK Phosphorylation Facilitates HSV Entry

Confocal Microscopy—CaSkis, FAK+/−, and FAK−/− cells were grown on glass coverslips in 12-well plates and, if indicated, transfected with siRNA as described above and then infected 24 h post-transfection with K26GFP at the indicated m.o.i. (range of 0.1–10 pfu/cell). At the indicated times post-infection, the infected cell monolayers were washed three times with PBS. To label plasma membranes, the cells were stained for 30 min with EZ-Link sulfosuccinimidobiotin reagent (1:1000 dilution; Pierce), which reacts with primary amines on cell-surface proteins prior to fixation (13), and the biotinylated cells were reacted with Alexa Fluor 647-conjugated streptavidin antibody (Molecular Probes, Inc.). Microtubules were labeled with α-tubulin mAb (1:200 dilution; catalog no. A1126, Molecular Probes, Inc.) and Alexa Fluor 647-conjugated goat anti-mouse secondary antibody (1:200 dilution; Molecular Probes, Inc.). Nuclei were detected by staining with 4,6-diamidino-2-phenylindole nuclear acid stain (Molecular...
RESULTS

We previously showed that HSV induces FAK phosphorylation in CaSki or Vero cells and that phosphorylation requires fusion-competent virus (7). This study was expanded to include multiple cell types known to be susceptible to HSV-2. Cells were serum-starved overnight and exposed to HSV-2 or mock-infected. At the indicated times post-infection, cell lysates were prepared and examined for expression of total and phosphorylated FAKs by Western blotting. HSV-2 induced an increase in phosphorylated FAK within 5 min in CaSki cells (Fig. 1). Similar results were obtained with immortalized human ectocervical cells (Ecto1E6/E7), colonic cells (CaCo-2), neuronal cells (SH-N-SK), and macrophages (Fig. 1).

To determine whether phosphorylated FAK contributes to HSV infection, we used several different strategies. First, we exposed MEF cells derived from embryonic FAK-deficient mice (FAK−/−) (9) and control MEF cells (FAK+/−) to HSV-2 and examined FAK response and susceptibility to infection. HSV-2 induced an ~3-fold increase in the level of phosphorylated FAK (data not shown). Taken together, these results suggest that, in the absence of FAK, viral invasion is blocked at a step post-infection, 100 cells were analyzed and counted.

To more carefully explore which steps in viral infection are impaired in FAK−/− cells, we compared viral binding and nuclear transport of the viral tegument protein VP16 in FAK−/− and FAK+/− cells. The results demonstrate that there was no reduction in binding of virus to cells in FAK−/− cells relative to FAK+/− cells (Fig. 3A). However, nuclear transport of VP16 1 h after citrate treatment was substantially reduced in FAK−/− cells compared with FAK+/− cells (Fig. 3B). The blots were scanned, and optical densitometry corrected for differences in loading. At a m.o.i. of 10 pfu/cell, there was an ~93% reduction in the relative amounts of VP16 detected in the nuclei of FAK−/− cells compared with FAK+/− cells. The amount of nuclear VP16 detected was comparable with that observed if FAK+/− cells were infected with 10-fold less virus (m.o.i. = 1 pfu/cell). To ensure that nuclear VP16 reflects transported and not newly synthesized protein, experiments were also conducted in the presence of cycloheximide, an inhibitor of protein synthesis (Fig. 3C). Comparable amounts of nuclear VP16 were detected 1 h after citrate treatment in FAK+/− cells independent of cycloheximide treatment and accounted for ~75% of total cell-associated VP16. As anticipated, a substantial reduction in viral ICP4 expression was also observed in FAK−/− cells compared with FAK+/− cells, consistent with impaired delivery of viral DNA to the cell nucleus and thus viral gene expression (data not shown). Taken together, these results suggest that, in the absence of FAK, viral invasion is blocked at a step post-binding, leading to reduced nuclear transport of VP16 and inhibition of downstream events, including expression of immediate-early gene products and plaque formation.

Because the embryonic knockout cells are murine and because our goal is to identify the signaling pathways required for HSV infection of human target cells, we adopted a complementary strategy to assess the role of FAK and its phosphorylation in human cervical epithelial cells. CaSki cells were transfected with siRNA specific for FAK, a dominant-negative variant of FAK (Y397F) in which the major autophosphorylation site has been mutated (a gift from J.-L. Guan) (14), or appropriate controls, and the impact on HSV infection was examined. Transfection with FAK sequence-specific siRNA (but not control nonspecific siRNA) reduced FAK protein expression by ~85% (based on optical densitometry scanning of blots) and prevented HSV-induced FAK phosphorylation (Fig. 4A). Similarly, a substantial reduction (~70%) in virus-triggered phosphorylated FAK was also observed in CaSki cells transfected with Y397F compared with cells transfected with wild-type

Fig. 4. Transfection of CaSki cells with FAK-specific siRNA or with the dominant-negative variant Y397F reduces susceptibility to HSV. A, CaSki cells were transfected with FAK-specific or control siRNA and then, 24 h later, mock-infected or infected with HSV-2(G) (m.o.i. = 1 pfu/cell). 5 min post-infection, cell lysates were prepared, and proteins were separated, transferred by Western blotting, and incubated with anti-phospho-Tyr397 FAK antibody (FAK[pY397]). Blots were then stripped and reprobed with mAb to total FAK, or the Y397F variant and, 24 h later, infected with HSV-2 (m.o.i. = 1 pfu/cell). Cell lysates were prepared 5 min post-infection and analyzed for total and phosphorylated FAKs by immunoblotting. C, to assess the impact of the transfections on susceptibility to HSV, cells were transfected and, 24 h later, infected with ~150 pfu of HSV-2/G/H9252 well. Plaques were counted after immunostaining 48 h post-infection, and the results are means ± S.D. of three independent experiments.
FAK (Fig. 4B). Transfection with the FAK-specific siRNA or the Y397F FAK variant also reduced HSV-2 infection (Fig. 4C), supporting the results obtained for FAK−/− cells showing that FAK and its phosphorylation play important roles in HSV infection.

To determine whether FAK inactivation prevents viral capsid transport, confocal microscopy studies were conducted. For these studies, we took advantage of the viral variant, K26GFP, an HSV-1 that expresses a GFP-VP26 fusion protein; VP26 is a major viral capsid protein. K26GFP grows comparably to wild-type virus in cell culture (10), and infection of FAK−/− cells was impaired to a similar extent for K26GFP compared with HSV-2(G) (Fig. 2B). To determine the optimal m.o.i. for confocal experiments, CaSki cells were first infected with K26GFP at a m.o.i. of 0.1, 1, or 10 pfu/cell; at the indicated times post-infection, fixed and stained with EZ-Link sulfosuccinimidobiotin to detect cellular plasma membranes and with 4',6-diamidino-2-phenylindole to detect nuclei; green, viral capsid GFP fusion protein. B, cells were infected in the absence (upper panels) or presence (lower panels) of cycloheximide (Cyclohex; 10 µg/ml). Magnification is ×100. The results are representative of three independent experiments.

FIG. 5. Delivery of viral capsids to the cytoplasm and newly synthesized GFP are readily detected by confocal microscopy following infection of cells with HSV-1(KOS)(GFP-VP26) (m.o.i. = 10 pfu/cell). A, CaSki cells were infected at a m.o.i. of 0.1 (upper panels), 1 (middle panels), or 10 (lower panels) pfu/cell and, at the indicated times post-infection, fixed and stained as described under “Materials and Methods.” Red, plasma membrane; blue, 4',6-diamidino-2-phenylindole staining delineating nuclei; green, viral capsid GFP fusion protein. B, cells were infected in the absence (upper panels) or presence (lower panels) of cycloheximide (Cyclohex; 10 µg/ml). Magnification is ×100. The results are representative of three independent experiments.

To further explore the role of FAK in capsid transport, confocal experiments were modified; and instead of staining the plasma membrane, microtubules were immunostained using anti-α-tubulin mAb. In CaSki and FAK−/− cells, viral capsids were readily detected associated with microtubule structures 1 and 2 h post-infection (Fig. 7, upper and middle panels, respectively). In contrast, no GFP was detected in microtubules in FAK−/− cells (Fig. 7, lower panels). Taken together, these results indicate that, in the absence of FAK, there is a restriction to capsid transport, leading to a reduction in the delivery of viral DNA to the nucleus and subsequent viral gene expression.
FIG. 6. Transport of capsids to the nuclear pore and subsequent synthesis of new viral proteins are impaired in the absence of FAK. A, CaSki cells were transfected with nonspecific (NS; upper panels), FAK-specific (middle panels), or Pyk2-specific (lower panels) siRNA and, 24 h later, infected with HSV-1(KOS)(GFP-VP26) at a m.o.i. of 10 pfu/cell for the indicated times, fixed, and stained as described under "Materials and Methods." B, confocal microscopy studies were also conducted following infection of FAK+/+ (upper panels) and FAK−/− (lower panels) cells with HSV-1(KOS)(GFP-VP26) at a m.o.i. of 10 pfu/cell. C, for each experiment, the relative amount of intracellular GFP and nuclear GFP detected 1 and 8 h post-infection, respectively, was compared by collecting data from ~100 cells from different fields using NIH Image densitometric software. The results are expressed as the intensity/cell in relative units. siNS, nonspecific siRNA; siFAK, FAK-specific siRNA; siPyk2, Pyk2-specific siRNA.
Our previous study suggested that phosphorylation of FAK is triggered downstream of Ca$^{2+}$ signaling because pharmacological agents that block the release of ER Ca$^{2+}$ or chelation of intracellular Ca$^{2+}$ prevent virus-induced phosphorylation of FAK (7). These observations suggest that Ca$^{2+}$ signaling should be preserved in FAK$^{-/-}$ cells. To test this directly, we examined the virus-induced Ca$^{2+}$ signaling response by excitation ratio fluorometry of cells loaded with the Ca$^{2+}$ indicator fura-2. FAK$^{+/+}$ and FAK$^{-/-}$ cells were exposed sequentially to PBS or HSV-2(G) at a m.o.i. of 5 pfu/cell. A rapid increase in the superfusate flow rate of PBS alone, simulating the conditions elicited by the addition of virus to the bathing medium, had no effect on resting Ca$^{2+}$ (mean 101.1 nM, Fig. 8). Exposure of both FAK$^{+/+}$ and FAK$^{-/-}$ cells to HSV resulted in a significant rapid increase in [Ca$^{2+}$], which peaked within 1 min and returned to the base line within ~3 min (p < 0.001, analysis of variance) (Fig. 8), although the mean change in peak [Ca$^{2+}$], was greater for FAK$^{+/+}$ cells compared with FAK$^{-/-}$ cells (181.4 ± 101.1 nM versus 70 ± 45 nM, respectively; mean ± S.E., n = 20 cells), possibly reflecting a reduction in virus-induced Ca$^{2+}$ signaling in the absence of FAK or perturbations of other signal transduction pathways in the knockout cells. The ability of HSV to induce a significant Ca$^{2+}$ response in FAK$^{-/-}$ cells supports the notion that FAK phosphorylation occurs downstream of virus-induced release of ER Ca$^{2+}$.

Pyk2 is a non-receptor tyrosine kinase related to FAK; is activated by various extracellular signals, including an increase in [Ca$^{2+}$], and is overexpressed in embryonic FAK$^{-/-}$ cells (15, 16). We hypothesized that HSV may also trigger phosphorylation of Pyk2, which could contribute to signaling pathways required for capsid transport. CaSki, FAK$^{+/+}$, and FAK$^{-/-}$ cells were serum-starved and then exposed to HSV-2(G) at 1 pfu/cell or mock-infected. Cell lysates were prepared at different times post-infection and analyzed for phosphorylated and total Pyk2 proteins. HSV-2 triggered Pyk2 phosphorylation within 5 min following inoculation of cells (Fig. 9A). The response was substantially greater in FAK$^{-/-}$ cells, presumably reflecting the increase in total Pyk2 in these cells and the absence of any FAK (Fig. 9A, lower panels). The observation that Pyk2 phosphorylation occurs in the absence of FAK suggests that Pyk2 is phosphorylated independent of FAK phosphorylation. To further address the independence of Pyk2 and FAK phosphorylation, we again took advantage of siRNA strategy. CaSki cells were transfected with control nonspecific siRNA or with Pyk2- or FAK-specific siRNA and then mock-infected or infected with HSV-2(G). Transfection with Pyk2-specific siRNA led to an ~70% reduction in Pyk2 expression and virus-induced Pyk2 phosphorylation (Fig. 9B, upper panels). In contrast, virus-induced Pyk2 phosphorylation was preserved in cells transfected with FAK-specific siRNA (Fig. 9B, middle panels), and virus-induced FAK phosphorylation was preserved in cells transfected with Pyk2-specific siRNA (lower panels, which show results with FAK$^{-/-}$ cells; similar results were obtained with CaSki cells).

To evaluate whether Pyk2 phosphorylation contributes to HSV infection, plaque assays were conducted in cells transfected with siRNA specific for FAK or Pyk2 alone and in combination. Compared with cells transfected with control nonspecific siRNA, HSV-2 infection was inhibited by 74 ± 6, 60 ± 5, and 94 ± 7% in CaSki cells transfected with siRNA for FAK, Pyk2, or both, respectively (Fig. 10A). Consistent with the low level of Pyk2 phosphorylation observed in FAK$^{+/+}$ cells (Fig. 9A), transfection of these cells with Pyk2-specific siRNA had little inhibitory effect (22%), whereas transfection with FAK-specific siRNA reduced viral infection by 71%. In contrast, transfection of FAK$^{-/-}$ cells with Pyk2-specific siRNA further reduced viral infection by >99%. To directly
evaluate whether Pyk2 phosphorylation also contributes to nuclear transport, CaSki cells were transfected with nonspecific or Pyk2-specific siRNA, and the effects on viral binding and VP16 transport to the nucleus were evaluated. Transfection with Pyk2-specific siRNA had no effect on viral binding (Fig. 10B), but had a modest inhibitory effect on nuclear transport of VP16 (Fig. 10C). Optical densitometry scanning of the gels demonstrated an ~40% reduction in nuclear VP16 following Pyk2-specific siRNA transfection, consistent with the modest inhibition of viral plaque formation (Fig. 10A). Similarly, transfection with Pyk2-specific siRNA also had a moderate effect on delivery of GFP to the nuclear pore as detected by confocal microscopy (Fig. 6A, lower panels). Together, these results suggest that HSV induces phosphorylation of Pyk2 independent of phosphorylation of FAK and that, although FAK plays the predominant role, activation of both kinases contributes to viral infection.

DISCUSSION

The dependence of viruses on host cell machinery for transcription, translation, and genome replication is well established. However, viruses presumably also must harness host cell pathways for entry and egress (16). The results obtained in this study demonstrate for the first time that HSV triggers phosphorylation of the cellular kinases FAK and Pyk2 to promote successful invasion. HSV independently induces phosphorylation of FAK and Pyk2, and disruption of these pathways impedes delivery of the tegument protein VP16 to the nucleus and delivery of capsids to the nuclear pore, leading to a reduction in downstream events (viral gene expression and plaque formation).

Several studies suggest that HSV uses microtubule-based transport to delivery viral capsids to the nuclear pore, where viral DNA is released into the nucleus (1, 17–19). Immunofluorescence studies demonstrate that HSV-1 capsids co-localize...
with dynein and dynactin. Microtubule-depolymerizing agents, such as nocodazole, block HSV infection in culture and in animal models (1). Moreover, nuclear transport of capsids is reduced by dynein inhibitors (19). Precisely how HSV (or other viruses) engages and regulates the microtubule complexes is not known. It has been suggested that cellular kinases triggered by viral infection or perhaps encoded by viruses themselves may play this regulatory role. The results obtained in this study support this notion and suggest that cellular FAK and the related tyrosine kinase Pyk2 are activated by HSV to promote transport along microtubules.

FAK was originally identified as a tyrosine kinase localized to focal adhesions, which are multiprotein structures that link the extracellular matrix to the actin cytoskeleton through integrin receptors. More recent studies indicate that FAK also participates in the regulation of microtubule function. For example, integrin-mediated activation of FAK is required for microtubule stabilization in mouse fibroblasts (20). Additionally, FAK phosphorylation at Ser732 is important for microtubule organization, nuclear movement, and neuronal migration (21). The latter study suggests that FAK phosphorylation regulates microtubule-based nuclear translocation and neuronal migration. Pyk2 (also called FAK2) belongs to the same family of non-receptor tyrosine kinases as does FAK. In contrast to FAK, which is expressed ubiquitously, Pyk2 expression is more restricted, but is found in hematopoietic, neuronal, and epithelial cells. Pyk2 shares substantial homology with FAK and interacts with intracellular signaling molecules to activate a variety of cellular pathways, including regulation of microtubules (15). Notably, increases in intracellular Ca\(^{2+}\) levels are associated with Pyk2 phosphorylation. We found that HSV also induces Pyk2 phosphorylation independent of FAK. Pyk2 phosphorylation was most evident in FAK\(^{-/-}\) fibroblasts, which are known to overexpress Pyk2 (15), and also contributed to viral infection post-binding. Thus, the observation that HSV capsid nuclear transport occurs via microtubules and the association of FAK and Pyk2 with microtubule regulation suggest that HSV triggers these cellular kinases to harness the microtubule machinery for its nuclear transport.

Several viruses activate FAK or related signaling pathways, but the precise role played by these pathways in viral invasion or pathogenicity has not been delineated previously. FAK is activated by integrin binding and aggregation. Thus, viruses that bind integrin receptors, such as adenovirus and human herpesvirus 8 (HHV-8), trigger FAK phosphorylation. Adenovirus induces tyrosine phosphorylation of FAK within 15 min following infection of human corneal fibroblasts, but its role in viral pathogenicity is not known (22). HHV-8 glycoprotein B, which possesses an RGD motif, binds to integrin receptors on target cells and triggers rapid phosphorylation of FAK and related signaling pathways. The finding that HHV-8 infection of FAK-null cells is substantially reduced suggests that FAK may also be required for HHV-8 entry, but this has not yet been experimentally demonstrated (23–25). Additionally, binding of HIV gp120 to T cells also triggers tyrosine phosphorylation of FAK (26), which may promote apoptosis (27), but has not yet been demonstrated to play any role in HIV entry or nuclear transport.

It is not clear yet how HSV activates the FAK cell signaling pathway. Although glycoprotein B is highly conserved among herpesviruses, only HHV-8 glycoprotein B possesses the RGD motif and interacts with integrin molecules as a receptor (28). Recent studies demonstrate that HSV-1 glycoprotein H binds to \(\alpha_\beta_2\) integrins; however, the functional significance of this interaction is unclear (29). A recombinant virus with the RGD motif in glycoprotein H mutated to RGE shows no impairment in entry or infectivity in vitro (30) and also induces FAK phosphorylation.

The results from our study suggest that FAK phosphorylation occurs downstream of Ca\(^{2+}\) signaling and is triggered either directly by the increase in [Ca\(^{2+}\)], or in response to Ca\(^{2+}\)-dependent viral penetration. This notion is supported by the findings that HSV induces an increase in [Ca\(^{2+}\)], in FAK\(^{-/-}\) cells (Fig. 9) and that treatment of cells with pharmacological agents that block release of ER Ca\(^{2+}\) stores or chelation of
intracellular Ca^{2+} prevents the virus-induced phosphorylation of FAK (7). In contrast to what has been observed for HHV-8 glycoprotein B or HIV gp120, no single HSV glycoprotein-cell interaction appears to be sufficient to activate the requisite cell signaling pathways. Our studies with deletion viruses indicate that penetration-competent virus is required to induce signaling. This follows from the observations that HSV-1 deleted in glycoprotein L or glycoprotein D, both of which bind but do not penetrate, fails to induce the signaling responses, whereas heat-inactivated virus induces phosphorylated FAK (7). Possibly, one of the HSV envelope glycoproteins interacts with a cell-surface receptor to trigger the signaling cascade, but the interaction is dependent on the other glycoproteins being present in the viral envelope. This could occur if, for example, the other glycoproteins induce a conformational change in and/or recruit the requisite glycoprotein(s) to a specific site at the membrane.

Defining precisely how HSV triggers the signaling pathways is critical, as the viral proteins involved are excellent candidate targets for antiviral therapies. Although targeting critical cellular pathways such as FAK phosphorylation will undoubtedly have pleiotropic and potentially deleterious effects as suggested by the reduction in magnitude of virus-induced Ca^{2+} transients in FAK knockout cells (15), targeting the viral proteins that activate the requisite signaling pathways should lead to the development of novel specific antiviral therapies. The results from this study provide an important piece to the puzzle of FAK Phosphorylation Facilitates HSV Entry 

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