Equine Herpesvirus 1 Enters Cells by Two Different Pathways, and Infection Requires the Activation of the Cellular Kinase ROCK1

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Equine herpesvirus type 1 (EHV-1), a member of the Alphaherpesviridae, displays a broad host range in vitro, allowing for detailed study of the mechanisms of productive infection, including attachment and entry, in various cell culture systems. Previously, we showed that EHV-1 infects Chinese hamster ovary (CHO-K1) cells even though these cells do not express a known alphaherpesvirus entry receptor. In this report, we show by electron microscopy and an infectious recovery assay that entry into CHO-K1 cells occurs via an endocytic or phagocytic mechanism, while entry into equine dermal (ED) or rabbit kidney (RK13) cells occurs by direct fusion at the cell surface. In both cases (endocytic/phagocytic or direct fusion), entry leads to productive infection. Using drugs that inhibit clathrin-dependent or caveola-dependent endocytosis, we showed that EHV-1 entry into CHO-K1 cells does not require clathrin or caveolae. We also show that EHV-1 infection requires the activation of cell signaling molecules. In particular, we demonstrate that activation of the serine/threonine Rho kinase ROCK1 is critical for infection. Inhibition of this kinase by drugs or overexpression of a negative regulator of ROCK1 significantly blocked EHV-1 infection. These results show that EHV-1 can enter disparate cell types by at least two distinct mechanisms and that productive infection is dependent upon the activation of ROCK1.

Alphaherpesviruses enter cells by at least four different pathways. For each mode of entry, a subset of viral glycoproteins (glycoprotein D [gD], gB, gH, and gL) (4, 11, 23, 29) and a cellular entry receptor (HveA, nectin-1, or 3-O-sulfated heparan sulfate) (21, 37, 55) are required for productive infection. Herpes simplex virus (HSV) enters many cell types, including primary neurons and Vero and Hep-2 cells (18, 19, 39, 50, 68), via fusion of the viral envelope with the plasma membrane of the cell. Consequent to fusion between the two lipid membranes, the capsid is released into the cytoplasm of the infected cell, where it is then transported along microtubules to the nucleus of the cell (for a review, see reference 14). Nicola et al. initially described the use of an endocytic pathway by HSV type 1 (HSV-1) for productive infection of CHO-nectin-1 cells (40, 41). In these cells, initial virus entry is not dependent on an interaction of the virus with nectin-1 at the cell surface, but successful fusion of the viral envelope with an endosomal membrane requires both an interaction between gD and nectin-1 and a decrease in endosomal pH. In a subsequent study, it was shown that entry into primary keratinocytes, but not neurons, also occurred via a pH-dependent endocytic mechanism (39). In addition, Milne et al. described a pH-independent endocytic pathway for HSV-1 entry into B78H1 murine melanoma cells that express nectin-1 (C10 cells) (36). In contrast to entry into CHO-nectin-1 cells, entry into C10 cells requires an interaction of gD with nectin-1 at the cell surface to initiate endocytosis, and virus fusion with the endosomal membrane occurs independent of a decrease in pH.

HSV entry via any of the described endocytic routes is not mediated by clathrin-coated pits or caveolae (22), suggesting that virus entry in these instances does not follow a typical or classical endocytic pathway. Recently, Clement et al. reported on a phagocytic mode of HSV-1 entry accompanied by activation of RhoA (8), a member of the Rho subfamily of small guanine nucleotide-binding (G) and GTP-hydrolyzing (GTPase) proteins. Consistent with this study, others have shown that nectin-1 signals via Rho family members (16, 17), and a recent study implied that the alphaherpesvirus pseudorabies virus activates the Rho protein Cdc42 as well as p38 mitogen-activated protein kinase (MAPK), a downstream component of Rho signaling, through gD binding to nectin-1 on neuronal cells (10). These data suggest that binding of a cell surface receptor by gD coupled with the activation of cell signaling pathways may be required for infection.

Additional studies have revealed roles for cellular kinases and Rho family members in alphaherpesvirus infections at early times postentry. Nicola et al. showed that inhibition of phosphatidylinositol 3-kinase in HeLa, CHO-nectin-1 (41), and keratinocyte (39) cells blocked transport of HSV-1 to the nucleus. Activation of the tyrosine kinases focal adhesion kinase (FAK) and pyruvate kinase 2 (Pyk2) was also shown to be required for efficient transport of HSV capsids to the nucleus (6). RhoA was activated at early times postinfection (p.i.) in CHO-nectin-1 cells and was postulated to be involved in virion transit via vesicles (8).

The importance of cell signaling in herpesvirus infection has also been shown for beta- and gammaherpesviruses, including human cytomegalovirus (HCMV) and Kaposi’s sarcoma-assoc-
associated herpesvirus (KSHV; also called human herpesvirus 8) (54). In at least some cases, the initial triggering of these signaling pathways occurs after viral attachment to cell surface integrins. The gammaherpesvirus KSHV induces FAK phosphorylation and activates RhoGTPases in cells after gB binds to α5β1 integrin, and this activation may be a critical event for productive KSHV infection, based on studies that revealed a reduced infection of FAK-null cells by KSHV (54). Binding of HCMV to the epidermal growth factor receptor and αvβ3 integrins activates the phosphorylation of focal adhesion kinase (96, 114), and other integrins, such as α1β1 and α2β1, also mediate productive KSHV infection (51). Binding of CHO-K1 cells to α5β1 integrins activates the phosphatidylinositol 3-kinase and Src pathways, both of which are essential for HCMV entry (66). Other studies have revealed that gH of HSV-1 also binds to αvβ3 integrins (45), but the ability of this binding to activate specific signaling molecules has not been elucidated.

The ability of equine herpesvirus type 1 (EHV-1) to attach to and enter susceptible cells is an active area of investigation. Like the case with related alphaherpesviruses, EHV-1 attachment to cells is mediated via an interaction between virus glycoproteins gB (58) and gC (43) and cellular heparan sulfate. After attachment, an interaction between gD and a putative entry receptor is required to complete the fusion between the viral envelope and a cellular membrane (9, 13). While EHV-1 has been studied in multiple tissue culture and animal systems, a detailed examination of the early cellular events that occur postbinding has not been undertaken. In this study, we combined ultrastructural, biochemical, and pharmacological assays to capture the process of EHV-1 entry at early times postattachment in disparate cell lines. Our data show that EHV-1 can enter cells via fusion at the plasma membrane or by non-classical endocytosis/phagocytosis and that successful infection through either mechanism requires the activation of Rho-associated coiled-coil kinase 1 (ROCK1).

MATERIALS AND METHODS

Cells, viruses, and plasmids. J1.1-2 cells (generously provided by Gabriella Campadelli-Fiume, University of Bologna, Bologna, Italy) and RK13 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA). J-nectin-1 cells were derived from J1.1-2 cells by transfection with a full-length nectin-1 cDNA expression plasmid (61) and cloning of hygromycin B (Invitrogen)-resistant cells. J-HveA cells were derived from J1.1-2 cells by transfection with a full-length HveA cDNA expression plasmid and cloning of neomycin-resistant cells (43). Chinese hamster ovary (CHO-K1) cells were kindly provided by Patricia Spear (Northwestern University, Chicago, IL) and grown in F12-K medium (Invitrogen) supplemented with 10% fetal bovine serum. Equine dermal (ED) cells, a gift from Ron Montelaro (University of Pittsburgh, Pittsburgh, PA), were maintained in minimal essential medium. All cells were maintained at 37°C in 5% CO2. EHV-1 strain L11agLagE contains a lacZ reporter cassette in place of the gI and gE genes (15). VSV-G, coated with vesicular stomatitis virus glycoprotein (VSV-G) and expressing enhanced green fluorescent protein (EGFP) (3), was generously provided by OriGene Technologies Inc. (Rockville, MD).

Electron microscopy (EM). CHO-K1, RK13, and ED cells were seeded to confluence in 35-mm dishes. Cells were washed once with ice-cold DMEM and then placed on ice for 5 min. The medium was removed from the cells, and EHV-1 strain L11agLagE was added to the cells at a multiplicity of infection (MOI) of 100. Virus was allowed to attach to the cells for 1 h at 4°C. Virus was removed from the cells, and DMEM, prewarmed to 37°C, was added. At 0 and 15 min post-temperature shift, medium was removed and the cells were fixed with 2.5% glutaraldehyde (Sigma, St. Louis, MO). The specimens were rinsed in 0.1 M phosphate-buffered saline and then postfixed in 1% OsO4 with 0.1% potassium ferricyanide. Samples were dehydrated stepwise for 15 min each with 30%, 50%, 70%, and 90% ethanol and then embedded in Epon (dodecenyl succinic anhydride, nadic methyl anhydride, scyophy 812 resin, and dimethylaminomethyl; Energy Beam Sciences, East Granby, CT). Semithin sections were cut on a Reichart Ultracut microtome, stained with 0.5% toludine blue (Fisher Scientific, Pittsburgh, PA), and examined under a light microscope. Ultrathin sections were stained with 2% uranyl acetate and Reynold's lead citrate and examined on a transmission electron microscope. Images were captured using transmission at a magnification of ×60,000.

RESULTS

Ultrastructural analysis of EHV-1 entry. Entry of EHV-1 into CHO-K1, ED, or RK13 cells was examined by EM. The virus was allowed to attach to the cells for 1 h at 4°C, and the temperature was then raised to 37°C by the addition of warm medium. Cells were fixed and processed for EM analysis at 0 and 15 min post-temperature shift. At time zero, virions were bound to the surface of each cell type, and representative images are shown in Fig. 1A and B. At 15 min postshift, fully enveloped virions were found in vesicles in CHO-K1 cells (Fig. 1C and D), and fusion between the virus envelope and the vesicle membrane was also observed (Fig. 1D). In ED and RK13 cells, virus was not seen in vesicles, but rather, naked or de-enveloped particles were observed in the cytosol (Fig. 1F to H). In addition, fusion initiation events between a virus particle and the plasma membrane were also captured with ED cells (Fig. 1E). While the EM assay was not quantitative, we observed only enveloped particles in CHO-K1 cells and only de-enveloped particles in RK13 and ED cells. These EM data show that EHV-1 can enter permissive cells by two distinct mechanisms.

Recovery of infectious virus from CHO-K1 cells, but not ED or RK13 cells, at early times p.i. The ability of EHV-1 to enter cells via an endocytic/phagocytic mechanism or via penetration at the cell surface was also analyzed using an infectious virus recovery assay. In this assay, virus was allowed to attach to cells...
at 4°C for 2 hours and the temperature was then raised to 37°C to allow the virus to enter the cells. At various times post-temperature shift, cells were treated with an acidic buffer to inactivate virus remaining on the cell surface, and the cells were washed and harvested. Virus isolated from these cells by freeze-thawing and sonication was then titrated on RK13 cells. Since a virus that enters cells via endocytosis or phagocytosis initially retains its envelope, this virus will infect RK13 cells. However, virus that enters cells via penetration at the plasma membrane will not be infectious, since only naked capsids should be recovered.

RK13, ED, and CHO-K1 cells were assessed for endocytic/phagocytic entry or direct penetration by using the infectious virus recovery assay. All three cell lines are fully permissive for EHV-1 entry (13, 48). As shown in Fig. 2, EHV-1 recovered from CHO-K1 cells reached a titer of 2.3 × 10^4 PFU/ml at 15 min post-temperature shift, with steadily declining yields thereafter. In contrast, little or no infectious EHV-1 was recovered from RK13 and ED cells. These results show that EHV-1 is rapidly endocytosed/phagocytosed in CHO-K1 cells but not in RK13 or ED cells.

BFLA, an inhibitor of the vacuolar ATPase, is routinely used to inhibit infection by viruses, such as VSV, that require a lowering of endosomal pH for virus release into the cytosol (3, 47). To assess whether EHV-1 entry into CHO-K1 cells required a similar drop in pH, cells were incubated with 200 nM of BFLA for 30 min and then infected with EHV-1 expressing a lacZ reporter gene for 6 h in the continuous presence of the drug (Fig. 3A). As controls, ED and RK13 cells were similarly treated with BFLA and infected with EHV-1. VSV infection of CHO-K1 cells in the presence or absence of the drug was included as a positive control of BFLA activity. The results showed a reduction in the number of CHO-K1 cells infected with EHV-1 in the presence of BFLA compared to that for cells that were not treated with the drug. No difference was observed in the number of infected ED or RK13 cells in the presence or absence of BFLA, while VSV infection was completely inhibited in the presence of BFLA.
To quantify the reduction of EHV-1 infection on CHO-K1 cells after BFLA treatment, an ONPG assay was employed. CHO-K1 cells plated in triplicate were treated with BFLA and infected with EHV-1 as described above, and β-galactosidase expression was quantitated 6 h later (Fig. 3B). The results showed that β-galactosidase expression decreased with increasing concentrations of BFLA added to the cells. At the highest concentration tested, EHV-1 infection of CHO-K1 cells was inhibited by 55%. While complete inhibition was not observed in this assay, these data suggest that efficient EHV-1 infection of CHO-K1 cells requires a decrease in pH.

EHV-1 entry into CHO-K1 cells does not require clathrin or caveolae. Many viruses enter cells through either clathrin-mediated (31, 33, 34) or caveola-dependent (46) endocytosis. To investigate which, if either, of these pathways is utilized by EHV-1 for infection of CHO-K1 cells, specific inhibitors of these pathways were used. Chlorpromazine, which prevents the assembly of clathrin-coated pits (65), has been used extensively to inhibit clathrin-mediated uptake of viruses (26, 27, 35, 49), and nystatin, a cholesterol-sequestering drug, is commonly employed to block caveola-mediated endocytosis (52).

CHO-K1 cells were incubated with increasing amounts of inhibitor and infected with EHV-1 or VSV (Fig. 4), which enters cells via clathrin-mediated endocytosis (34, 59, 60). Nystatin at concentrations of up to 100 μg/ml had no effect on EHV-1 (Fig. 4A and B) or VSV (Fig. 4A) entry. Chlorpromazine also did not inhibit EHV-1 infection (Fig. 4A and B), but it did decrease VSV infection in a dose-dependent manner (Fig. 4A). The toxicity of each drug on CHO-K1 cells was assessed by an MTS cell viability assay. Nystatin was not toxic at all concentrations tested, but chlorpromazine was toxic to cells at concentrations above 12.5 μg/ml (Fig. 4B). The inability of either drug to inhibit EHV-1 infection suggests that entry of EHV-1 into CHO-K1 cells does not require clathrin or caveolae.

The tyrosine kinase inhibitor genistein inhibits EHV-1 infection. Previous studies showed a role for cellular tyrosine kinase activity in alphaherpesvirus infection (39). Using the tyrosine kinase inhibitor genistein, we examined whether EHV-1 infection similarly requires cellular tyrosine kinase activation. CHO-K1 or ED cells were incubated for 30 min with genistein and then infected with EHV-1 for 6 h in the continuous presence of the inhibitor (Fig. 5). EHV-1 infection was reduced 84% on CHO-K1 cells and 70% on ED cells at the highest concentration of genistein tested (Fig. 5). The inhibitory effect of genistein was greater on CHO-K1 cells, as evidenced by a 56% reduction of infection in the presence of 6.25 μg/ml genistein. The observed inhibition of infection was not due to drug toxicity, as significant inhibition occurred at doses that did not affect cell viability (Fig. 5). These data suggest that productive EHV-1 infection requires cellular tyrosine kinase activity.

EHV-1 infection requires the Rho kinase ROCK1. Previous studies have identified specific cell signaling molecules and pathways that are critical for virus entry. Many viruses, including human immunodeficiency virus (30), vaccinia virus (2), Epstein-Barr virus (EBV) (20), and Kaposi’s sarcoma-associated herpesvirus (44), activate one or more of the MAPK pathways in order to enter and/or replicate within cells. In addition, Rho GTPases and their upstream and downstream
FIG. 3. Effect of BFLA on EHV-1 entry. (A) CHO-K1, ED, or RK13 cells were mock treated (left panels) or treated with 200 nM of BFLA (right panels) for 30 min at 37°C and then infected with EHV-1 (L11ΔgIΔgE) or VSV-GFP (CHO-K1 cells; bottom panels) at an MOI of 5 for 6 h in the presence of BFLA. At 6 h p.i., cells were fixed with 0.5% glutaraldehyde and stained with X-Gal, or GFP expression was recorded for cells infected with VSV-GFP. (B) CHO-K1 cells were mock treated or treated with increasing amounts of BFLA for 30 min at 37°C and infected with EHV-1 (L11ΔgIΔgE) for 6 h in the continued presence of the drug. At 6 h p.i., ONPG was added and β-galactosidase activity was measured. Triplicate samples were measured at each time point.
mediators and effectors are activated by viruses, including HSV-1 (8), upon infection. One downstream protein of Rho is the Rho kinase ROCK1. ROCK1 is a serine/threonine kinase that is involved in stress fiber formation and contraction (1, 7, 28, 62), cell adhesion (53, 63, 64, 69), cell shape (57), and cell migration (56, 70). Upon activation, ROCK1 induces FAK (24), which leads to the formation of focal adhesions and stress fibers, resulting in cytoskeletal rearrangements.

**FIG. 4.** EHV-1 endocytic entry into CHO-K1 cells does not require clathrin or caveolae. (A) CHO-K1 cells were mock treated or treated with increasing amounts of nystatin or chlorpromazine for 30 min at 37°C and infected with EHV-1 (L11ΔgIΔgE) or VSV-GFP at an MOI of 3 for 6 h in the presence of the drugs. At 6 h p.i., cells were fixed with 0.5% glutaraldehyde and stained with X-Gal, or GFP expression was analyzed in cells infected with VSV-GFP. (B) Triplicate cultures of CHO-K1 cells were mock treated or treated with increasing amounts of nystatin or chlorpromazine for 30 min at 37°C and infected with EHV-1 (L11ΔgIΔgE) at an MOI of 3 for 6 h in the presence of the drug. β-Galactosidase expression was measured by ONPG assay (bars). Cell viability at each concentration of drug was measured by MTS assay (lines).
To determine if EHV-1 infection requires the activation of a MAPK pathway or ROCK1, specific inhibitors of cell signaling molecules were utilized in an infection assay. CHO-K1 cells were incubated with increasing amounts of inhibitors against ROCK1 (Y-27632), p38 MAPK (SB203580), extracellular signal-regulated kinase (ERK) (UO126), and JNK (JNKVIII) for 30 min and then infected with EHV-1. Entry was assayed at 6 h p.i. by measuring β-galactosidase activity in an ONPG assay.

FIG. 5. Inhibition of host cell tyrosine kinase activity blocks EHV-1 infection. Triplicate cultures of CHO-K1 or ED cells were mock treated or treated with increasing amounts of genistein for 30 min at 37°C and then infected with EHV-1 (L11ΔgIΔgE) at an MOI of 3 for 6 h in the presence of the drug. β-Galactosidase expression was measured by ONPG assay (bars). Cell viability at each concentration of drug was measured by MTS assay (lines).

FIG. 6. Inhibition of ROCK1 reduces EHV-1 infection on CHO-K1 cells. Triplicate cultures of CHO-K1 cells were mock treated or treated with increasing amounts of inhibitory drugs for 30 min at 37°C and then infected with EHV-1 (L11ΔgIΔgE) at an MOI of 5 for 6 h in the presence of the drugs. β-Galactosidase expression was measured by ONPG assay (bars). Cell viability (black line) at each concentration of Y-27632 was measured by MTS assay.
EHV-1 infection was significantly inhibited in a dose-dependent fashion in the presence of the ROCK1 inhibitor but not in the presence of the other kinase inhibitors. Cell viability measured by an MTS assay (Fig. 6) showed that the inhibition was not due to any toxicity associated with the ROCK1 inhibitor.

The inhibitors were further assayed on ED cells (Fig. 7). Similar to EHV-1 infection on CHO-K1 cells, infection was decreased in the presence of the ROCK1 inhibitor but not the p38 MAPK, ERK, or JNK inhibitor, and there was no toxicity associated with the ROCK1 inhibitor. These data indicate that ROCK1 inhibition decreases infection of EHV-1 in permissive cell lines, regardless of whether the virus gains entry via endocytosis/phagocytosis (CHO-K1 cells) or fusion at the cell surface (ED cells), and suggest that the inhibition occurs not at the initial penetration step but at an early step p.i.

Since ROCK1 is an upstream activator of the tyrosine kinase FAK (24) and FAK is critical for infection with the homologous alphaherpesvirus family member HSV-1 (6), we hypothesized that inhibition of ROCK1 would also reduce HSV-1 infection of permissive cells. To test this possibility, J-nectin-1 and J-HveA cells were incubated with increasing amounts of the ROCK1 inhibitor Y-27632 for 30 min and then infected with HSV-1 at an MOI of 5 (Fig. 8). X-Gal staining of infected cells showed that there was a dose-dependent inhibition of infection in the presence of the ROCK1 inhibitor. These results suggest that a signaling pathway that leads to the induction of focal adhesions, which are critical for HSV-1 entry, is mediated through ROCK1 activation of FAK.

ROCK1 activity is inhibited by small G proteins, including Gem and RhoE. Gem directly binds ROCK1, and one of the effects of this interaction is stress fiber and focal adhesion

![Fig. 7](image-url)

**FIG. 7.** Inhibition of ROCK1 reduces EHV-1 infection on ED cells. Triplicate cultures of ED cells were mock treated or treated with increasing amounts of inhibitory drugs for 30 min at 37°C and then infected with EHV-1 (L11ΔpΔgE) at an MOI of 5 for 6 h in the presence of the drugs. β-Galactosidase expression was measured by ONPG assay (bars). Cell viability (black line) at each concentration of Y-27632 was measured by MTS assay.

![Fig. 8](image-url)

**FIG. 8.** Inhibition of ROCK1 reduces HSV-1 infection. J-nectin-1 or J-HveA cells were treated with increasing amounts of the ROCK1 inhibitor Y-27632 for 30 min at 37°C and then infected with QOZH at an MOI of 5 for 6 h in the presence of the drug. At 6 h p.i., cells were fixed with 0.5% glutaraldehyde and stained with X-Gal.
disassembly. To analyze the effect of Gem expression on EHV-1 infection, CHO-K1 cells were transfected with a Gem expression plasmid or empty vector and then infected with EHV-1 (Fig. 9). The expression of Gem in CHO-K1 cells significantly inhibited EHV-1 infection. These results add to the drug inhibition assay results and indicate that ROCK1 activation is critical for productive EHV-1 infection.

**DISCUSSION**

The results presented in this study elucidate some of the mechanisms utilized by EHV-1 for successful penetration and infection of permissive cells. An important finding from this work is the ability of EHV-1 to initiate entry into cells by at least two distinct mechanisms. In a previous study, we reported on the ability of EHV-1 to productively infect CHO-K1 cells (13). In this report, we show that EHV-1 utilizes a nonclassical endocytic or phagocytic entry pathway in CHO-K1 cells, while it directly fuses with the plasma membrane to gain entry into other cell types, such as RK13 and ED cells.

The ability of alphaherpesviruses to utilize an entry pathway other than direct fusion was initially described by Nicola et al. (40). This group showed that HSV-1 enters CHO-K1 cells by endocytosis. In addition, productive infection required an interaction between HSV-1 gD and nectin-1 along with a decrease in pH for fusion of the viral and endosomal membranes and subsequent release of the capsid into the cytosol. Similar to the findings with HSV-1, our data revealed that a lowering of pH is also important for efficient EHV-1 infection of CHO-K1 cells, based on the inhibition of infection in the presence of the vacuolar ATPase inhibitor BFLA.

EHV-1 entry into CHO-K1 cells does not require clathrin or caveolae, indicating that EHV-1 employs an entry pathway that is distinct from the classical endocytic pathways (32). The results of Gianni et al. (22) suggest that HSV entry via endocytosis may also occur independent of clathrin or caveolae. Following these studies, Clement et al. described a phagocytic uptake of HSV-1 that resulted in productive infection (8). In common with previous reports, phagocytic HSV-1 infection required a lowering of pH, but not clathrin or caveolae. Coupled with the observations reported here, these studies suggest that alphaherpesviruses may utilize a common pathway for entry into certain cell types, such as CHO-K1, that more closely resembles phagocytosis, or nonclassical endocytosis.

Many viruses, including alphaherpesviruses, activate specific signaling molecules upon attachment or entry or at early times p.i. In this study, we show that EHV-1 infection is significantly inhibited in the presence of the tyrosine kinase inhibitor genistein, indicating a role for cellular kinases in productive infection. The ability of this inhibitor to block entry into CHO-K1 and ED cells suggests that the block to infection most likely occurs postentry, since initial entry into these two cell types occurs by endocytosis/phagocytosis and direct fusion, respectively. This finding is consistent with those of other studies, which showed that genistein blocked the transport of HSV capsids to the nucleus (39). In agreement with this work, another study showed that inhibition of the tyrosine kinase FAK blocked HSV infection, and it was suggested that the inhibition occurred at the stage of viral transport along microtubules (6).

The involvement of FAK in alphaherpesvirus infection, coupled with the inhibitory effect of genistein on EHV-1 infection, led us to investigate the impact that other cell signaling molecules may have on infection. Specifically, since FAK is activated by ROCK1, we determined whether inhibition of this upstream activator of FAK would block EHV-1 infection. Using the ROCK1 inhibitor Y-27632, we observed a significant inhibition of EHV-1 infection in both CHO-K1 and ED cells. In addition to the inhibition of EHV-1 infection, Y-27632 also inhibited HSV-1 infection in J cells expressing either HveA or nectin-1. These findings show that ROCK1 activation is critical for both EHV-1 and HSV-1 infections.

ROCK1 is a 160-kDa serine/threonine protein kinase that is expressed in many tissue types (28, 38). ROCK1 contains an amino-terminal kinase domain, a central coiled-coil region, a Rho-binding domain, a pleckstrin homology domain, and a carboxy-terminal cysteine-rich domain. ROCK1 is activated by binding to RhoA-GTP (25) and, in some cases, by lipids such as arachidonic acid (12), and this activation leads to multiple cell changes. Some of the ROCK1-mediated events that may contribute to successful alphaherpesvirus infection include the formation of focal adhesions from actin stress fibers (28), the regulation of cell-to-cell adhesions (53, 63, 69), and phagocytosis (42). ROCK1 activity is negatively regulated by a different set of small GTP-binding proteins belonging to the Ras super-

**FIG. 9.** Expression of the ROCK1 inhibitor Gem reduces EHV-1 infection. CHO-K1 cells were transfected with a GEM expression plasmid (left) or pcDNA3.1 empty vector (right). At 24 h posttransfection, cells were infected with L11apLagE at an MOI of 1 for 1 h at 37°C. Cells were washed for 30 s with acidic buffer and then washed twice with medium. Cells were fixed at 7 h p.i. and stained with X-Gal.
family. Two of these proteins, Gem and Rad, directly bind to ROCK1, and this binding blocks or counteracts various ROCK1 functions, including ROCK1-mediated phosphorylation of the myosin light chain, ROCK1-mediated cell rounding, and ROCK1-mediated stress fiber assembly and focal adhesion formation (67). In addition to Gem and Rad, RhoE also inhibits the kinase activity of ROCK1, leading to a disruption of ROCK1-mediated downstream signaling events (51).

While the present study shows that ROCK1 activation is critical for EHV-1 infection, it does not address how the virus initiates the cell signaling cascade. One interesting possibility is that attachment of the virus to cell surface molecules such as integrins may trigger the signaling pathway. Many viruses, including herpesviruses, have been shown to bind to integrins and induce cell signaling. Kaposis’s sarcoma-associated herpesvirus binds to αvβ3 integrins via an RGD domain in gB (54), and this binding triggers FAK activation. In addition, αv integrins also bind to αvβ3 integrins (45), but the contribution, if any, of this interaction to the activation of cell signaling has not been shown. Interestingly, an RGD motif is present within the ectodomain of EHV-1 gB, raising the intriguing possibility that EHV-1 gB may bind to integrins to activate signaling pathways.

In conclusion, the data presented in this report show for the first time that EHV-1 can enter and productively infect disparate cell types by at least two distinct mechanisms. EHV-1 activates specific cellular kinases regardless of the mode of initial entry, and this activation is required to complete the infection. Future studies will investigate which components of EHV-1 are required to interact with and activate specific cellular proteins and at what specific stage of infection these interactions occur. A detailed understanding of these mechanisms may lead to the development of better therapeutics or antivirals to combat EHV-1 infection.

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