EQUINE INFECTIOUS ANEMIA VIRUS ENTRY OCCURS THROUGH CLATHRIN-MEDIATED ENDOCYTOSIS

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Abstract

Entry of wild-type lentivirus equine infectious anemia virus (EIAV) into cells requires a low-pH step (10, 31). This low-pH constraint implicates endocytosis in EIAV entry. To identify the endocytic pathway involved in EIAV entry, we examined the entry requirements for EIAV into two different cells: equine dermal (ED) cells and primary equine endothelial cells. We investigate the entry mechanism of several strains of EIAV and find that both macrophage tropic and tissue culture adapted strains utilizes clathrin coated pits for entry. In contrast, a superinfecting strain of EIAV, EIAV_{vMA-1c}, utilizes two mechanisms of entry. In cells such as ED cells that EIAV_{vMA-1c} is able to superinfect, viral entry is pH independent and appears to be mediated by plasma membrane fusion, whereas, in cells where no detectable superinfection occurs, EIAV_{vMA-1c} entry that is low-pH-dependent occurs through clathrin coated pits in a manner similar to wild type virus. Regardless of the mechanism of entry being utilized, internalization kinetics of EIAV is rapid with 50% of cell associated virions internalizing within 60-90 minutes. Cathepsin inhibitors did not prevent EIAV entry, suggesting that the low-pH step required by wild-type EIAV is not required to activate cellular cathepsins.
**Introduction**

Enveloped virus infection is initiated by the viral glycoprotein binding to its cellular receptor. The binding event either triggers membrane fusion at the plasma membrane or internalization of the virus into an endosome. For those viruses that are endocytosed, subsequent endosomal events lead to fusion of the viral membrane with the vesicle, releasing the core particle into the cytoplasm. Cells utilize several endocytosis mechanisms to take up nutrients from their environment and viruses usurp these mechanisms for internalization. Defined pathways of endocytosis include clathrin-mediated endocytosis, caveolae-mediated endocytosis, non-clathrin non-caveolae mediated endocytosis, and macropinocytosis (40). Many of these pathways traffic through acidic compartments. Viruses can take advantage of the pH decrease to stimulate events that trigger membrane fusion (1, 10, 65). Two mechanistically diverse examples of viral use of a low-pH step are influenza virus and Ebolavirus. The vesicle associated, low-pH environment initiates conformational changes in the influenza glycoprotein, leading to membrane fusion (65), whereas endosomal low-pH activated proteases cleave the Ebolavirus glycoprotein 1, allowing subsequent fusion events (14, 60, 65).

An evolution in the understanding of retroviral entry has occurred and now incorporates a role for endocytosis in the internalization of many retroviruses. A general model for mammalian retroviral entry was initially proposed 17 years ago (45); however, the specific requirements for internalization of only a handful of viruses had been closely examined at that time. The model proposed that a prototypic retrovirus enters cells at the plasma membrane through a pH-independent fusion event (44, 45). As mechanisms of entry of more retroviruses has been examined, numerous retroviruses have been determined to utilize a low-pH-dependent mechanism of entry (8, 10, 19, 31, 47). In fact, within the family, a low-pH-dependent entry mechanism may be more commonly used than direct fusion with the plasma membrane. Retroviruses such as ecotropic murine leukemia virus, avian leukosis virus, and mouse mammary tumor virus use low-pH-dependent entry mechanisms (19, 47, 58, 59).
With the realization that many retroviruses exploit a low-pH entry mechanism, the method of endocytosis utilized by the viruses has been examined. Avian sarcoma and leukosis virus B (ASLV-B) enters through clathrin-coated pits, whereas ASLV-A entry requires intact lipid rafts for efficient entry (20, 48). While HIV principally enters cells through direct fusion with the plasma membrane (44), HIV has been shown to productively enter CD4 expressing HeLa cells through clathrin-mediated endocytosis, and into polarized trophoblastic cells through a clathrin-, caveolin-, and dynamin-independent endocytosis event (18, 71). The pH-independence of fusion events associated with amphotropic MLV had led investigators to believe that fusion occurred at the plasma membrane, but amphotropic MLV has since been shown to enter cells through caveolae endocytosis (2, 20, 48). Hence, within this single family of viruses, individual family members have evolved to utilize several different cellular mechanisms presumably in order to most effectively take advantage of their targeted cellular receptor.

The lentivirus, equine infectious anemia virus (EIAV), is responsible for the first described retroviral-mediated disease and was one of the first filterable agents described (37, 69). Although the disease that EIAV causes was initially characterized more than 150 years ago, little is known about its mechanism of entry. In vivo EIAV is primarily if not exclusively macrophage tropic; however, in tissue culture the virus is able to adapt to infect additional cell-types including endothelial cells and fibroblasts from not only equine species, but from feline and canine origin as well (52, 61). Changes within the long terminal repeat and envelope are associated with both altered virulence and cell tropism (13, 41, 55). Interaction with the cellular receptor, equine lentiviral receptor 1 (ELR1) has been demonstrated to be responsible for EIAV internalization (74). EIAV entry into both primary cells and tissue culture cell lines has recently been shown to be dependent on a low-pH step (10, 31) implicating a requirement for endocytosis of the virus. However, the mechanism of endocytosis mediating productive entry of EIAV has not been investigated.

In this study we examine the pathway of EIAV internalization using the low-pH-dependent, tissue culture strain of EIAV_{MA-1}. Parallel studies were performed to determine the
internalization pathway used by a variant, superinfecting strain of EIAV that enters cells it
superinfects in a pH-independent manner (10, 42). Entry of EIAV_{MA-1} that is representative of
wild-type strains of EIAV was inhibited by treatments that blocked the formation of clathrin-
coated pits. In contrast, entry of the superinfecting strain EIAV_{vMA-1c} into target cells that it
superinfects was not inhibited by these same treatments; however, inhibition of actin cytoskeletal
rearrangements decreased entry. These latter findings indicate that EIAV_{vMA-1c} superinfection
does not require the same endocytosis pathway as wild-type EIAV and suggests that this variant
virus is able to fuse with the plasma membrane of those target cells.
Materials and Methods

Cells lines used. ED cells, an equine fibroblastic cell line derived from dermal cells (ATCC CCL57), and primary equine umbilical vein endothelial cells (eUVEC) were used to characterize the entry requirements for EIAV. Human embryonic kidney cells 293T (22) cells were used for transfections to produce pseudotyped particles. Cells were maintained in high-glucose Dulbecco’s modified Eagle’s medium with penicillin and streptomycin. Media was supplemented with 10% fetal calf serum for 293T cells, and 15% for ED cells, and 40% for eUVEC cells.

Virus strains. Various tissue culture strains of EIAV were used in this study. EIAV_{Th,1} is a macrophage-tropic strain obtained from the first viremic episode of a horse inoculated with a field isolate from Massachusetts (13). EIAV_{MA-1} is an avirulent, tissue culture-adapted strain of EIAV_{Th,1} (13). EIAV_{vMA-1c} is a cytopathic, superinfecting strain that was derived by serial passages of EIAV_{MA-1} in ED cells, as described in (42). EIAV_{SP19} is a non-virulent molecular clone of EIAV (56). EIAV_{WSU5} is a strain from Washington State University that was derived from the Wyoming strain and continues to be passaged in equine fetal kidney cells and back passaged through ponies to maintain virulence (51). EIAV_{UK} is a mildly virulent molecular clone of EIAV (17).

Generation and titering of viral stocks. Viral stocks of EIAV_{SP19} and EIAV_{UK} were produced by transfecting ED with the molecular clone using Amaxa transfections. The transfected populations were monitored for EIAV antigen and stocks were collected when cells were >95% positive for EIAV antigen as determined by EIAV antigen immunostaining. Viral stocks of EIAV_{MA-1} and EIAV_{vMA-1c} were produced by infecting ED cells and EIAV_{Th,1} was produced by infecting eUVEC cells. Supernatants were harvested from cells that were >95% positive for EIAV antigen as determined by EIAV antigen immunostaining. Supernatants were centrifuged for 5 min at 13,500 x g to remove cell debris, aliquotted, and frozen at -80°C until needed. Viral titers were determined by infection of ED cells with serial dilutions of stock, followed by 75% acetone/25% water fixation at 40 hr following infection and anti-EIAV immunostaining of the
cells as previously described (43). The EIAV antigen-positive cells within the infected cell monolayer were counted and titers were determined. In all experiments, cells were infected with an MOI of 0.005. The titers were determined on cell type used for the experiment.

**Detection of EIAV replication.** EIAV infection was determined by immunostaining of viral antigens as previously described (43). Acetone fixed cells were immunostained with polyclonal horse anti-EIAV antiserum (1:800) from a long-term-infected horse (WSU 2085, a kind gift from Dr. J. Lindsay Oaks, Washington State University). This antiserum primarily recognizes envelope (gp90 and gp46) and Gag proteins, as well as Gag precursor polyproteins. Primary anti-serum was incubated for 3 hr at 37°C, followed by several washes with phosphate-buffered saline. Peroxidase-conjugated goat anti-horse immunoglobulin (1:800) (Jackson Immunoresearch) was incubated as the secondary antibody for 45 min at 37°C. Peroxidase activity was detected with the substrate 3-amino-9-ethylcarbazole (Sigma). Cells were enumerated by microscopic visual inspection.

**Generating VSVG and EBOV pseudotyped particles.** 293T cells were transfected with a total of 75 µg of DNA consisting of vesicular stomatitis virus glycoprotein (VSVG) expression construct, a pONY3.1gag-pol expression plasmid, and pONYψβgal (46) at a ratio of 1:2:3 respectively. The DNA was transfected into 80% confluent 15 cm dishes of 293T cells using calcium-phosphate transfection (32). Ebolavirus glycoprotein lacking the mucin domain (EBOVΔO) pseudotyped onto FIV particles were produced as previously described (9). Supernatants were collected at 24, 36, 48, 60 and 72 hr following transfection and frozen at -80°C. Supernatants were thawed, passed through a 0.45 µm filter, and virus was pelleted by a 16 hr centrifugation step (7000 rpm 4°C in a Sorvall GSA rotor). The pellet was resuspended in 250 µL DMEM for an approximate 200 fold concentration. Pseudotyped particles were aliquoted and stored at -80°C until use.

**Detection of VSVG and EBOV pseudotype transduction.** ED cells were plated in a 48-well format the night before the transduction. On the day of the transduction, media was refreshed with either our standard DMEM or DMEM containing the appropriate concentration of the
endocytosis inhibitors and pseudotyped particles were added. Forty hours following
transduction, the cells were fixed in 3.7% formalin and evaluated for β-galactosidase activity
using the substrate X-gal. The number of β-galactosidase positive cells was enumerated by
microscopic visual inspection.

**Internalization assay.** ED cells were incubated with virions or pseudotyped particles at 4°C for
1 hr to allow virus binding, but not internalization. The virus containing media was replaced
with fresh ED media and shifted to 37°C. At various time points following 37°C shift, cells were
treated with citric acid buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) for 1 min
to inactivate any particles that remained on the surface. The cells were washed 2 times with
media to remove the acidic buffer, and fresh ED media was added. Cells were fixed and stained
for either EIAV antigens or β-galactosidase activity 40 hr following infection and the number of
positive cells was determined. Control values represent the number of positive cells when there
was no citric acid wash.

**Cellular membrane fractionation.** 5 x 10^5 ED cells were trypsized and aliquoted into
microfuge tubes. Media was added and cells were incubated at 37°C for 1 hr to allow re-
expression of plasma membrane proteins. Cells pelleted and resuspended in ice cold media in the
presence or absence of EIAV (EIAV_{MA-1} or EIAV_{vMA-1c}). Cells were held for 1 hr on ice and
washed with ice cold PBS twice to remove unbound virus. Separation of detergent insoluble
membranes was performed as previously described (49). Cells were lysed on ice with lysis
buffer (1% Brij, 150 mM NaCl and 10 mM Tris (pH 7.5)) for 50 min. Nycodenz media (70%
Nycodenz, 20 mM Tris (pH 7.5), 150 mM NaCl) was added to the lysed cells for a final
concentration of 38.75% Nycodenz and this was layered under a Nycodenz step gradient was
generated in a sterile polycarbonate ultracentrifuge tube by adding 200 µl each of 25%, 22.5%,
20%, 18%, 15%, 12% and 8% Nycodenz media to the top of the lysed cell mixture. Tubes were
centrifuged for 4 hr at 200,000 x g at 4°C and stopped without a brake. Two hundred microliter
aliquots were collected from the top of the tube and stored at -80°C until analyzed by
immunoblotting.
**Immunoblotting.** Proteins present in the membrane fractions were separated on a 4-20% Tris/glycine SDS-PAGE gel (Invitrogen), and transferred onto nitrocellulose. ELR1 was detected by incubating the membranes with rabbit anti-ELR1 polyclonal sera (1:1000) (a kind gift of Dr. Baoshan Zhang, University of Pittsburgh) for 3 hr and with secondary peroxidase-conjugated goat anti-rabbit antiserum (1:20,000) (Sigma) for 1 hr. EIAV antigen was detected by incubating the membranes with equine antisera 2085, serum from an EIAV infected horse (1:5000) for 3 hr and with secondary peroxidase-conjugated goat anti-horse antiserum (1:10,000) (Jackson Immunoresearch) for 1 hr. Caveolin 1 was detected by incubating with a monoclonal anti-cav1 (1:1000 from BD Biosciences) overnight at 4°C and with secondary peroxidase conjugated rabbit anti-mouse antiserum (1:20,000). Membranes were visualized by the chemiluminescence method according to manufacture’s instructions (Pierce).

**Drug studies.** All drugs were obtained from Sigma (St. Louis, Mo.) unless noted. The no drug control contained the appropriate dilution of solvent. ED cells were seeded onto 48-well plates and allowed to grow to 90% confluency before treatment and infection. All drug treated cells were fixed and stained for viral antigens or β-gal activity 40 hr following infection.

**Methyl-β-cyclodextrin.** Methyl-β-cyclodextran (MβCD) was resuspended in DMEM with pen/strep and no FCS at 25 mM stock. MβCD was diluted into DMEM pen/strep in the absence of FCS and incubated on cells or with virus for 1 hr. The drug was removed, fresh media was replaced, and the cells were infected.

**Genistein.** Genistein (GEN) was resuspended in DMSO at 20mg/mL. GEN was diluted into media and preincubated with the cells for 1 hr. Cells were infected in the presence of the drug and the GEN containing inoculum was removed 24 hr following infection and fresh media was replaced.

**Chlorpromazine.** Chlorpromazine (CPZ) was resuspended in ethanol at 1 mg/ml. CPZ was diluted into media and preincubated with cells for 1 hr. Cells were infected in the presence of the drug and the CPZ containing inoculum was removed 6 hr following infection and fresh media was replaced.
**Cytochalasin D.** Cytochalasin D (cytoD) was resuspended in DMSO at 25 mM stock. CytoD was diluted in media and incubated with cells for 1 hr. The drug was removed, fresh media was replaced and cells were infected.

**Cathepsin inhibitors.** Cathepsin L inhibitor (CalBiochem #219402) and a pan-cathepsin inhibitor (CalBiochem #219419) were resuspended in DMSO at 10mM stock. Cathepsin inhibitors were diluted into media and preincubated with cells for 1 hr. The cells were infected in the presence of the drug and the cathepsin inhibitor containing inoculum was removed 16 hr following infection and fresh media was replaced.

**Cell viability assay.** ED cells were plated and treated with drugs as described above. Forty hours following treatment cell viability was monitored by ATPLite Assay (Packard Biosciences) per manufacturer’s instructions.

**ED cell transfection with Eps15 constructs.** ED cells were transfected with a control Eps15D3Δ2-GFP fusion protein (7), or a dominant negative Eps15Δ95-295-GFP (5) expression plasmid using Amaza transfection reagents. Briefly, 1.5 million ED cells were resuspended in 100 µL Amaza Solution T containing 5 µg of plasmid DNA. ED cells were electroporated using the Amaza nucleotransfection device using program T-30. The transfected cells were divided into 12 wells of a 48-well plate. Twenty-four hours following transfection the cells were infected with EIAV, and 3 wells were analyzed by flow cytometry to determine transfection efficiency. Forty hours following infection the cells were fixed and evaluated for EIAV antigen expression or β-galactosidase activity.

**Adenovirus DN dynamin expression.** ED cells were plated in a 48-well plate. Adenoviral vectors expressing GFP and K44Adynamin1 (a gift from Dr. Jeff Pessin, SUNY-Stony Brook) were incubated with the cells for 24 hours (33). Adenoviral vector transduction efficiency was determined by the percentage of cells expressing GFP, which was greater than 95%. 24 hrs following transduction, cells were infected with EIAV_{MA-1}, EIAV_{vMA-1c} or transduced with VSVG. Cells were fixed and stained 40 hours following infection.
Statistical analysis. All studies were performed at least three independent times. Means and standard errors of the mean are shown. Student’s t-test was used to evaluate the statistical differences between treatments, utilizing the two-tailed distribution and two-sample equal variance conditions. P-values were accessed by comparing the level of infectivity with treatment to the level of cytoxicity seen with that treatment. A significant difference was determined by a p-value of < 0.05 and significance was identified in each figure. If the p-value was > 0.05 the data were not considered significant.
**Results**

EIAV internalization occurs rapidly. Viral entry kinetics provide insights into the pathway(s) of internalization. Clathrin–mediated endocytosis is known to occur very quickly after ligand binding, whereas caveolae uptake occurs more slowly (16, 39). Documented examples of this are the clathrin-mediated internalization of VSVG pseudotyped particles that occurs in minutes, whereas caveolae-mediated internalization of amphotropic murine leukemia virus requires hours (2). To examine the kinetics of EIAV internalization, a tissue culture derived strain of EIAV, EIAV<sub>MA-1</sub>, was bound to equine dermal cells (ED cells) at 4°C for one hour. The unbound virus and supernatant were removed, replaced with warmed cell media and shifted to 37°C to promote internalization. At various time points following the temperature shift, the cells were washed with citric acid to inactivate virion particles that had not been internalized. Cells were examined for EIAV antigen expression 40 hours after infection. Internalization of EIAV<sub>MA-1</sub> occurred rapidly, with 50% of the virions internalized within 1 hour (**Fig. 1**). VSVG pseudotyped EIAV particle entry occurred at a similar rate. These data are consistent with previously published internalization kinetics for virions and ligands that enter through clathrin-coated pits (2, 16). The variant strain EIAV<sub>vMA-1c</sub> that has been shown to primarily enter ED cells through a pH-independent pathway resulting in superinfection (10) was observed to have a similar, rapid rate of uptake.

Wild-type EIAV, but not EIAV<sub>vMA-1c</sub>, is dynamin dependent. Dynamin is a GTPase required for the cellular membrane to pinch off endosomes from the plasma membrane and is needed for clathrin and caveolae-mediated endocytosis and phagocytosis, but it is not required for macropinocytosis (25, 27, 63). Dominant negative dynamin (DNdyn1(K44A)) has decreased GTPase activity that results in reduced endocytosis (26, 70). While dynamin 2 is ubiquitously expressed in cells, dynamin 1 is specific for neuronal cells (63). However, over expression of the dominant negative K44A mutation of either dynamin 1 or 2 has been shown to impact endocytosis in a variety of cells (21, 53, 62, 63). Utilizing adenoviral transducing particles that express either GFP or DNdyn1(K44A), followed by EIAV infection or VSVG pseudotyped
virion transduction, we were able to test the dynamin dependence of EIAV infection. An
adenoviral MOI of 10 transduced more than 94% of the ED cells as evidenced by GFP
expression (data not shown) and the same MOI was used for all the adenoviral transductions.
VSVG pseudotyped MLV particle entry has been previously shown to be inhibited by DNdyn
thus VSVG pseudotyped EIAV particles were used as a positive control (35). EIAV\textsubscript{MA-1} entry
was significantly inhibited by expression of DN\textit{dyn1} (Fig. 2). Similar trends were seen with
expression of DN\textit{dyn2} (data not shown), but DN\textit{dyn2} expression in ED cells resulted in high
levels of cell toxicity. EIAV\textsubscript{vMA-1c} entry into ED cells was not affected by DN\textit{dyn1} suggesting
that neither clathrin-coated pits nor caveolae are important for entry of EIAV\textsubscript{vMA-1c} into these
cells.

A role for actin rearrangement in EIAV\textsubscript{vMA-1c} entry, but not in EIAV\textsubscript{MA-1} entry. Actin
rearrangement is necessary for some endocytosis mechanisms such as caveolae-mediated uptake
and macropinocytosis as well as direct virus fusion with the plasma membrane (12, 38, 66). In
order to determine if EIAV entry requires an intact actin cytoskeleton we utilized cytochalasin D
(cytoD), a drug that depolymerizes actin (38). Immunofluorescence images of actin staining
demonstrate that the concentrations of cytoD used effectively depolymerized the actin network in
ED cells (data not shown). As with some of the other endocytosis inhibitors used in this study,
cytoD was cytotoxic and caused cells to round-up and detach from the surface of the plate.
Consequently, cell viability assays were performed to measure the impact of the drug on the cells
over the 40 h experiment. The effect of cytoD on EIAV entry was evaluated and results were
compared to the drugs impact on cell viability. VSVG dependent transduction is not affected by
cytoD and served as a negative control whereas EBOV glycoprotein dependent entry is
dependent on actin polymerization and served as a positive control (11, 73). EIAV\textsubscript{MA-1} entry
diminished by cytoD in a dose dependent manner, in parallel a loss of cell viability occurred
(Fig. 3A). The decrease in entry mediated by both EIAV\textsubscript{MA-1} and VSVG glycoproteins could be
accounted for by the loss of cell numbers in the culture dish. EBOV-GP mediated entry, into
SNB-19 cells, was significantly decreased when compared to the cell viability (Fig. 3A). The
EBOV GP transduction studies were performed in SNB-19 cells because ED cells were not permissive for EBOV GP dependent transduction. We concluded that actin depolymerization does not directly impact entry of EIAV\textsubscript{MA-1}. Despite the loss of cells in the presence of cytoD, low concentrations of cytoD appeared to slightly enhance EIAV\textsubscript{vMA-1c} entry into ED cells, but this was not statistically significant.

Signal transduction events mediated by tyrosine kinases induce actin rearrangements and can play a role in caveolae endocytosis (54, 57). These events can be blocked by the tyrosine kinase inhibitor genistein (GEN) (57). Inhibition of tyrosine kinase activity by GEN also inhibits HIV-1 entry into primary macrophages by preventing virus fusion with the plasma membrane (67). EIAV\textsubscript{MA-1} and EIAV\textsubscript{vMA-1c} entry into ED cells was examined in the presence of GEN (Fig. 3B). No cytotoxicity was observed with the concentrations of GEN used (data not shown). EIAV\textsubscript{MA-1} entry was not inhibited by GEN, but EIAV\textsubscript{vMA-1c} infectivity did show a dose dependent decrease. Loss of EIAV\textsubscript{vMA-1c} infectivity by the inhibition of tyrosine kinase activity would suggest that actin rearrangement and/or caveolae-mediated endocytosis is important for EIAV\textsubscript{vMA-1c} entry events into ED cells, and these findings are consistent with the trend of enhanced EIAV\textsubscript{vMA-1c} entry in the presence of the lower concentrations of cytoD.

**Cellular cholesterol is not required for EIAV infection.** Methyl-\(\beta\)-cyclodextrin (M\(\beta\)CD) removes the cholesterol from membranes and sequesters it within the cell (30, 34). Removal of cholesterol disrupts lipid rafts and has been shown to inhibit caveolae-mediated endocytosis as well as lipid raft dependent internalization (63). Treatment of ED cells with M\(\beta\)CD did not inhibit entry of EIAV\textsubscript{MA-1}, EIAV\textsubscript{vMA-1c}, or VSVG pseudotyped EIAV particles (Fig. 4A). Similar treatment of SNB-19 cells inhibited EBOV-GP mediated entry that has previously been shown to be dependent on lipid rafts (23).

To further demonstrate efficacy of M\(\beta\)CD at the concentrations used in this study, we sought to determine if viral particles treated with M\(\beta\)CD to eliminate cholesterol from the viral membrane would reduce infectivity. Other retroviruses such as HIV-1 have been shown to bud through lipid rafts and the cholesterol in the viral membrane is important for viral infectivity (3,
Virus was incubated with MβCD for one hour, diluted approximately 200 fold and added to cells. This treatment effectively reduced the infectivity of HIV virions (more than 90% decrease) known to require cholesterol in the viral membrane (data not shown). MβCD treatment dramatically inhibited the entry of EIAV<sub>MA-1</sub> and EIAV<sub>MA-1c</sub>, but did not significantly inhibit the transduction of VSVG pseudotyped particles (Fig. 4B).

**ELR1 is not present in lipid rafts.** If lipid rafts are not important for EIAV entry, we would anticipate that the cellular receptor for EIAV, Equine lentiviral receptor 1 (ELR1) would not colocalize with detergent resistant membranes. Consequently, we next determined the location of ELR1 within the plasma membrane both before and following virion binding. ED cells incubated in the presence or absence of EIAV<sub>MA-1</sub> virions at 4°C were lysed in a Brij lysis buffer that solubilizes the non-lipid raft membranes but keeps the lipid rafts intact. Cell lysates were subjected to membrane floatation on a nycodenz gradient. Centrifugation caused the lipid raft-containing membranes to float to the top of the gradient whereas the solubilized proteins remain at the bottom (28). The gradient was fractionated into 11 fractions and subjected to Western blot analysis to determine which fraction contained ELR1 and EIAV proteins. Caveolin 1, a lipid raft associated protein, was found in the detergent resistant fractions (Fig. 5A), validating our experimental protocol. EIAV capsid protein was found primarily, but not exclusively, in fractions containing detergent resistant membranes when the virions were examined by themselves or after they were bound to cells, suggesting that EIAV’s lipid membrane contains lipid rafts (Fig. 5B and C). EIAV matrix protein was observed in similar fraction (data not shown). ELR1 was only found in the lower fractions that are not associated with detergent resistant membranes, suggesting the protein was solubulized by the lysis buffer and was not found in lipid rafts even after virus binding (Fig. 5B).

Because membrane fluidity is reduced at 4°C, the experiment was repeated with an additional 37°C incubation. Once virus was bound to cells at 4°C, the cells were shifted to 37°C for 20, 40, or 60 minutes, lysed, and membrane floatation was performed. The ELR1 protein was found in the soluble fractions at each time frame (Fig. 5D), indicating that ELR1 does not
move into lipid rafts after viral glycoprotein/receptor interactions. The absence of ELR1 from lipid raft microdomains is consistent with the location of the human homolog of ELR1, the herpes virus entry mediator, which remains in the soluble membrane fractions even after virus binding (4).

**EIAV\textsubscript{MA-1} enters cells through clathrin-coated pits.** Clathrin-mediated endocytosis has been shown to be inhibited by the drug chlorpromazine (CPZ) (63). CPZ redirects the adapter proteins that bind to the plasma membrane proteins to internal membranes, preventing clathrin-mediated endocytosis from the cell surface (72). VSVG is known to enter through clathrin-mediated endocytosis (68), and therefore was utilized as a positive control. ED cells were treated with increasing amounts of CPZ and examined for their ability to inhibit EIAV entry. EIAV\textsubscript{MA-1} virus was inhibited in a dose dependent manner (Fig. 6A). Although the drug had some cytotoxicity, levels of EIAV\textsubscript{MA-1} entry were significantly reduced as compared to chlorpromazine’s impact on cell viability suggesting that wild type EIAV enters cells through a clathrin-mediated pathway. CPZ did not impact EIAV\textsubscript{vMA-1c} entry into ED cells.

The clathrin pit associated protein Eps15 serves as a linker between the clathrin coat and the receptor being internalized (6, 29). A dominant negative form of Eps15 (DN-Eps15Δ95-295) lacks the Eps homology domains that are necessary for clathrin-coated pit targeting, and thus inhibits clathrin uptake (5). ED cells were transfected with GFP, Eps15D3Δ2-GFP that does not interfere with clathrin uptake (6, 29), or DNEps15Δ95-295-GFP and infected with EIAV\textsubscript{MA-1} or EIAV\textsubscript{vMA-1c} 24 hours after transfection. Expression of the DN-Eps15Δ95-295-GFP fusion protein followed by infection with EIAV\textsubscript{MA-1} confirmed that clathrin endocytosis is required for EIAV entry (Fig. 6B). The transfected cells were 57-65\%GFP positive at 24 h following transfection demonstrating good efficiency of transfection. EIAV\textsubscript{vMA-1c} only showed minor inhibition, similar to the slight inhibition seen when a low-pH entry pathway is blocked during EIAV\textsubscript{vMA-1c} infection (10). These findings are consistent with the CPZ findings that EIAV\textsubscript{vMA-1c} does not require clathrin for entry into ED cells.
To understand whether entry via clathrin-mediated endocytosis occurs with other strains of EIAV, the infectivity of four additional strains of EIAV were examined in the presence of CPZ. These EIAV strains, that have previously been shown to enter cells through a low pH-dependent mechanism, were inhibited by CPZ in a dose dependent manner (Fig. 6C). These strains included EIAV	extsubscript{WSU5} that retains the ability to infected horses, suggesting that both tissue-culture adapted strains and field isolates enter ED cells through clathrin-mediated endocytosis.

**EIAV	extsubscript{MA-1} and EIAV	extsubscript{vMA-1c} enter eUVEC cells through clathrin-mediated endocytosis.**

EIAV	extsubscript{vMA-1c} superinfects ED cells in a pH-independent manner, but does not superinfect equine endothelial (eUVEC) cells and enters eUVEC cells through a low pH-dependent pathway (10). Thus EIAV	extsubscript{vMA-1c} behaves like wild-type strains of EIAV in eUVEC cells. To determine if EIAV enters eUVEC cells through a clathrin-mediated endocytosis event, eUVEC cells were infected in the presence of MβCD or CPZ. As observed in ED cells, entry of neither EIAV	extsubscript{MA-1} nor EIAV	extsubscript{vMA-1c} were significantly inhibited (Fig. 7A). Consistent with low pH-dependent entry of both EIAV	extsubscript{MA-1} and EIAV	extsubscript{vMA-1c} entry into eUVEC cells was inhibited in a dose dependent manner by CPZ (Fig. 7B).

**Cathepsin inhibitors do not inhibit EIAV entry.** Recently, the entry of several low pH-dependent viruses has been shown to require viral glycoprotein processing by cellular, low pH-dependent endosomal proteases such as cathepsins (60, 64). To determine if wild-type EIAV’s requirement for low-pH entry is dependent on glycoprotein processing by cellular cathepsin proteases, we infected ED cells in the presence of a pan-cathepsin inhibitor as well as a specific cathepsin L inhibitor. EBOV pseudotyped particles serve as a positive control for these studies whereas VSVG transduction serves as a negative control. Cathepsin L is a ubiquitously expressed protease and has been shown to be involved in Ebolavirus entry (60). Both the pan-cathepsin and cathepsin L inhibitor had no effect on EIAV	extsubscript{MA-1} or EIAV	extsubscript{vMA-1c} entry (Fig. 8A and B). The cathepsin B inhibitor CA-074Me also did not inhibit EIAV entry into ED cells (data not shown).
To further demonstrate that cathepsin L does not play a role in EIAV entry, we performed an in vitro protease cleavage assay. Viral particles were incubated in a low-pH acetate buffer with purified cathepsin L. Virions were analyzed by immunoblotting to determine if proteolysis of the glycoprotein had occurred. There was no detectable decrease in full length glycoprotein and no cleavage products detected with EIAV_{MA-1} or EIAV_{vMA-1c} but the Ebolavirus glycoprotein was efficiently cleaved (Fig. 8C). These findings indicate that EIAV glycoprotein does not require a low pH step for proteolytic processing, but perhaps for glycoprotein conformational changes that are needed to generate a fusion ready state.
Discussion

In summary, we present data that the wild-type EIAV<sub>MA-1</sub> infects cells through clathrin-mediated endocytosis. The clathrin pathway was important for EIAV<sub>MA-1</sub> entry into both the ED cell line and primary eUVEC cells. We also demonstrated that several other strains of EIAV enter ED cells through clathrin-coated pits. The low-pH requirement for EIAV entry is not required for glycoprotein cleavage by cellular endosomal proteases. Instead, we propose that the acidic conditions may be altering the conformation of the intact surface protein and thereby facilitating conditions that allow fusion to occur. In contrast, the superinfecting EIAV strain, EIAV<sub>vMA-1c</sub>, does not require clathrin-mediated endocytosis, lipid rafts or dynamin, but active actin remodeling enhances entry into ED cells suggesting that EIAV<sub>vMA-1c</sub> may fuse at the plasma membrane. However, in UVECs that EIAV<sub>vMA-1c</sub> can not superinfect, the entry pathway appears to be similar to that found for other EIAV strains.

The importance of actin remodeling in clathrin-mediated endocytosis remains controversial. Some reports demonstrate dynamic actin polymerization is required for clathrin-mediated endocytosis (24), but other reports show that this requirement is cell type specific (24, 73). As others have observed for VSVG dependent entry (73), our study demonstrates a clathrin-dependent and actin remodeling independent entry of VSVG. In a similar manner, we propose that wild-type EIAV also uses a clathrin-dependent and actin remodeling independent mechanism for entry.

Conflicting data is present in the literature about VSVG mediated entry and dynamin dependence. Entry of VSVG pseudotyped MLV particles was shown to be inhibited by expression of dominant negative dynamin (K44A) (35). In contrast, recent report showed that VSVG pseudotyped HIV particles were not inhibited by expression of a different mutant dynamin (G273D) (18). Our results with the dynamin (K44A) are consistent with the earlier study that used this same mutant and suggest that the discrepancy may be due to the different dynamin mutants used or the cell type.
While the presence of cholesterol in target cells is not important for EIAV entry, loss of cholesterol from the virion decreases virus infectivity. Other retroviruses such as HIV and amphotropic MuLV have been shown to bud through lipid raft containing membranes (2, 36, 50). Our data indicate that EIAV also buds through lipid raft domains in the plasma membrane.

In contrast to wild-type EIAV, the variant strain EIAV\textsubscript{vMA-1c} enters cells that it superinfects via a pH-independent pathway (10). The absence of a low-pH requirement for ED cell entry suggested that EIAV\textsubscript{vMA-1c} either fuses at the plasma membrane or enters through a pH-independent endocytosis mechanism. Studies presented here allow us to exclude internalization of EIAV\textsubscript{vMA-1c} into ED cells by endocytosis. Our supporting evidence includes the absence of an affect on EIAV\textsubscript{vMA-1c} entry by DNdyn, MβCD, CPZ, and DNEps15 in ED cells. In contrast, our experiments support the hypothesis that EIAV\textsubscript{vMA-1c} fuses with the plasma membrane of ED cells. A trend for enhanced EIAV\textsubscript{vMA-1c} infection was observed at the lower concentrations of cytoD and virus replication was inhibited by the tyrosine kinase inhibitor genistein. Genistein blocks signaling events that mediate actin cytoskeleton movement whereas cytoD depolymerizes the actin cytoskeleton. HIV virion’s that fuses at the plasma membrane have a similar response to these drugs (12, 67). The prevention of actin reorganization by genistein may impede EIAV\textsubscript{vMA-1c} from passing through the actin network, whereas cytoD removes the actin cytoskeleton facilitating virion delivery from the plasma membrane into the cytoplasm.

In contrast, EIAV\textsubscript{vMA-1c} entry into eUVEC cells, which was previously shown to be pH-dependent, occurs through clathrin-mediated endocytosis. Pathways used by EIAV\textsubscript{vMA-1c} for entry into eUVECs were indistinguishable from other strains of EIAV suggesting that EIAV\textsubscript{vMA-1c} uses ELR1 to gain entry into eUVEC cells. Conversely, the pH-independent plasma membrane fusion event that results in EIAV\textsubscript{vMA-1c} superinfection of ED cells profoundly differs from that observed with other EIAV strains. The different pathways by which EIAV\textsubscript{vMA-1c} enters fibroblasts and endothelial cells suggest the intriguing possibility that EIAV\textsubscript{vMA-1c} may utilize different receptors in these two cell types. Further studies will be needed to explore this possibility.
The low-pH requirement for productive entry of many viruses has been investigated (8, 14, 15, 19, 39, 40, 45, 47, 63). Current evidence indicates that low-pH conditions are required to alter the viral surface glycoprotein conformation and thereby remove its steric hindrance of virus/cell fusion. Two different mechanisms of acid-induced conformational changes have been identified. In the case of influenza virus, the conformation of the intact viral glycoprotein is altered under low-pH conditions (65). In contrast, the viral surface glycoprotein of such viruses as Ebolavirus and SARS is cleaved into small peptides by low-pH-activated cellular protease activities (14, 60, 64). Here we demonstrate that EIAV entry is not inhibited by cathepsin inhibitors and cathepsin L does not cleave the EIAV SU protein in vitro. Cathepsin independent, low-pH-dependent surface glycoprotein alterations are consistent with our previously published findings that a low-pH shock of virus bound cells increases infectivity (10). The increase in infectivity at the cell surface suggests that there are no endosomal specific enzymatic activities required for the fusion event to take place.

A previous study using a sucrose shock to block clathrin-dependent entry did not inhibit entry of EIAV into ED cells leading the authors to conclude that EIAV entry was independent of clathrin-mediated endocytosis (31). We also found that similar sucrose treatment did not affect EIAV or VSVG entry into ED cells (data not shown). The findings from the sucrose studies are inconsistent with our findings using more specific means of inhibiting the formation of clathrin-coated pits such as dominant negative Eps15 or chlorpromazine (63). Therefore, we conclude that under the conditions used the sucrose shock was not impacting clathrin-mediated entry.

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References


Figure legends

Fig. 1. Virion internalization kinetics. EIAV<sub>MA-1</sub>, EIAV<sub>vMA-1c</sub> or VSVG pseudotyped EIAV particles were bound to ED cell at 4°C for 1 hr. The virus was removed and media was refreshed. Cells were washed with citric acid buffer at the time points indicated to inactivate all virions that had yet to be internalized. Cells were stained 40 hr following infection and compared to the number of infected cells when no citric acid wash was performed. Data represent the average and standard error of three experiments performed in triplicate.

Fig. 2. EIAV entry is dynamin dependent. ED cells were transduced with an adenoviral vector that expressed either GFP or DN dynamin 1. Twenty-four hours following transduction cells were infected with EIAV<sub>MA-1</sub> or EIAV<sub>vMA-1c</sub> or transduced with VSVG pseudotyped EIAV particles. Cells were stained 40 hr following infection and compared to the number of infected cells transduced with Ad-GFP. Data represent the average and standard error of three experiments performed in triplicate. *, p < 0.05.

Fig. 3. EIAV<sub>vMA-1c</sub> requires dynamic actin rearrangement for efficient entry. CytoD (A) or GEN (B) treated cells were infected with EIAV<sub>MA-1</sub> or EIAV<sub>vMA-1c</sub> or transduced with VSVG or EBOV pseudotyped particles. Cells were stained 40 hr following infection and compared to the number of infected cells treated with DMSO control. Data represent the average and standard error of three experiments performed in triplicate. *, p < 0.05.
Fig. 4. Cholesterol in the target cell is not required for EIAV infectivity. ED cells were treated with MβCD for 1 hour and infected with EIAV\textsubscript{MA-1} or EIAV\textsubscript{vMA-1c} or transduced with VSVG or EBOV pseudotyped EIAV particles (A). EIAV\textsubscript{MA-1} and EIAV\textsubscript{vMA-1c} viral particles or VSVG transducing particles were incubated with MβCD for 1 hour. The particles were diluted into ED media and used to infect ED cells (B). Cells were stained 40 hr following infection and compared to the number of infected cells when particles were treated with control. Data represent the average and standard error of three experiments performed in triplicate. *, p < 0.05; **, p < 0.001.

Fig. 5. ELR1 is not found in lipid rafts. ED cells (A), ED cells bound with EIAV\textsubscript{MA-1} particles (B) or EIAV\textsubscript{MA-1} viral particles alone (C) were lysed and run on a nycodenz gradient. Detergent resistant membranes (DRM) floated to the top fractions (1-5), whereas solubilized protein can be seen in the bottom fractions (6-11). The fractions were analyzed for ELR1, EIAV capsid, and caveolin 1 by Western blotting. EIAV\textsubscript{MA-1} bound ED cells were shifted to 37°C for 20, 40, or 60 min, and fractions were Western blotted for ELR1 (D).

Fig. 6. EIAV\textsubscript{MA-1} enters ED cells through clathrin-mediated endocytosis. ED cells were treated with CPZ for 1 hour and infected with EIAV\textsubscript{MA-1} or EIAV\textsubscript{vMA-1c} or transduced with VSVG pseudotyped EIAV particles (A). ED cells were transfected with GFP, Eps15D3Δ2-GFP, or DNEps15Δ95-295-GFP. Twenty-four hours following transfection the cells were and infected with EIAV\textsubscript{MA-1} or EIAV\textsubscript{vMA-1c} or transduced with VSVG pseudotyped EIAV particles (B). ED cells were treated with CPZ for 1 hour and infected with EIAV\textsubscript{SP19}, EIAV\textsubscript{Th.1}, EIAV\textsubscript{WSU5}, or EIAV\textsubscript{UK} (C). Cells were stained 40 hr following infection and compared to the number of
infected cells when particles were treated with control. Data represent the average and standard error of three experiments performed in triplicate. *, p < 0.05.

Fig. 7. EIAV_{vMA-1c} enters eUVEC cells through clathrin-mediated endocytosis. eUVEC cells were treated with MβCD for 1 hour and infected with EIAV_{MA-1} or EIAV_{vMA-1c} or transduced with VSVG or EBOV pseudotyped particles (A). eUVEC cells were treated with CPZ for 1 hour and infected with EIAV_{MA-1} or EIAV_{vMA-1c} or transduced with VSVG pseudotyped EIAV particles (B). Cells were stained 40 hr following infection and compared to the number of infected cells when particles were treated with control. Data represent the average and standard error of three experiments performed in triplicate. *, p < 0.05; **, p < 0.001.

Fig. 8. Cathepsin inhibitors do not inhibit EIAV entry. ED cells were treated with a cathepsin L inhibitor (A) or a pan-cathepsin inhibitor (B) and infected with EIAV_{MA-1} or EIAV_{vMA-1c} or transduced with VSVG pseudotyped EIAV particles. Cells were stained 40 hr following infection and compared to the number of infected cells when particles were treated with control. Data represent the average and standard error of three experiments performed in triplicate. Viral particles were incubated with cathepsin L protease for 1 hour and Western analysis was examined for cleavage of the viral glycoprotein (C). **, p < 0.001.
Fig 1

**Graph Description:**

The graph depicts the percent of virions internalized over time of citric acid wash (min). The x-axis represents time in minutes, ranging from 0 to 180, while the y-axis represents percent of virions internalized, ranging from 0 to 100. Three lines are plotted, each representing a different condition:

- **MA1**
- **vMA1c**
- **VSVG**

Each line shows an increasing trend as time progresses, indicating a higher percent of virions internalized with longer wash times.
Fig 2

![Graph showing cell viability comparison between Ad-GFP and Ad-DN dyn1](image-url)

* MA1v
* vMA1c
* VSVG
* cell viability

Percent of control
Fig 3
Fig 4
Fig 5
Fig 6
Fig 7
Fig 8