Arenavirus entry occurs through a cholesterol-dependent, non-caveolar, clathrin-mediated endocytic mechanism

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Abstract

Arenaviruses are important causes of viral hemorrhagic fevers in humans. Arenavirus infection of cells occurs via a pH-dependent endocytic route, but detailed studies of entry pathways have not been done. We investigated the role of cell membrane cholesterol, caveolae, and clathrin coated pits in infection by Lassa virus (LASV), which utilizes alpha-dystroglycan (α-DG) as a receptor, and Pichindé virus (PICV), which does not. Depletion of cellular cholesterol by treatment with methyl betacyclodextrin (MβCD) or nystatin/progesterone inhibited PICV replication and transfer of packaged marker gene by LASV or PICV pseudotyped retroviral particles. In cells lacking caveolae due to silencing of the caveolin-1 gene, no inhibition of PICV infection or LASV pseudotype transduction was observed. However, PICV infection and LASV and PICV pseudotype transduction was inhibited when an Eps15 dominant negative mutant was used to inhibit clathrin-mediated endocytosis. Altogether, the results indicate that diverse arenaviruses have a common requirement for cell membrane cholesterol and clathrin mediated endocytosis in establishing infection.

Keywords: Pichindé virus; Lassa virus; Arenavirus; Cholesterol; Viral entry; Hemorrhagic fever; Clathrin

Introduction

Arenaviruses are rodent-borne, enveloped RNA viruses, several of which cause hemorrhagic fever syndromes in humans mainly in areas of tropical Africa and Latin America. Of the arenavirus hemorrhagic fevers, Lassa fever is the most prevalent, affecting approximately a quarter of a million individuals in endemic regions of West Africa annually (Fisher-Hoch and McCormick, 2001; McCormick et al., 1987). Because of its capacity for person-to-person spread, the lack of practical and readily available diagnostic tests, and limited therapeutic options, Lassa virus is included in the list of category A priority pathogens for biodefense research (Borio et al., 2002). Like some other highly hazardous viruses, hemorrhagic fever-causing arenaviruses have not been well studied; hence many basic mechanisms of their life cycle in the infected cell have not been elucidated. In particular, significant gaps exist in the knowledge of mechanisms of cellular uptake/viral entry, events which could provide targets for antiviral therapeutic approaches.

Cellular entry is initiated when viruses bind cellular receptors via their surface glycoproteins. Arenaviruses possess surface spikes composed of trimeric GP1 subunits non-covalently attached to a transmembrane stalk comprised of trimeric GP2 (Burns and Buchmeier, 1991; Eschli et al., 2006). GP1 is responsible for receptor binding, whereas GP2 drives fusion of the virion membrane with cellular membranes (Glushakova et al., 1990; Glushakova et al., 1992). The laminin-binding protein alpha-dystroglycan functions as a receptor for Lassa virus as well as the prototype virus, Lymphocytic choriomeningitis virus (LCMV) (Cao et al., 1998). The pathogenic New World arenaviruses Junin, Guanarito, and Machupo have recently been shown to utilize the cellular transferrin 1 receptor (TfR 1) during viral entry (Radoshitzky et al., 2007). Arenavirus infection is pH-dependent (Glushakova and Lukashevich, 1989), and may require GP1 dissociation from GP2 at low pH,
exposing fusogenic domains of GP2, triggering penetration (Di Simone et al., 1994; Di Simone and Buchmeier, 1995). Unlike the majority of viruses that exhibit pH-dependent entry, the Old World arenavirus LCMV does not appear to utilize the clathrin-mediated endocytic route as judged by ultra-structural studies showing LCMV internalization into smooth-walled vesicles (Borrow and Oldstone, 1994). However, recent evidence implicates clathrin-mediated endocytosis for Junin virus entry (Martinez et al., 2007). Whether the other arenaviruses utilize the clathrin-mediated endocytic pathway remains to be determined.

It has recently been shown that depletion of cellular cholesterol inhibits infection by the prototype arenavirus, LCMV (Shah et al., 2006). Among the cholesterol-dependent entry pathways, those that utilize lipid rafts or caveolae are best studied (Marsh and Helenius, 2006; Pelkmans and Helenius, 2003). Caveolae and lipid rafts are cholesterol and glycosphingolipid enriched plasma membrane microdomains that are insoluble in non-ionic detergents and have been implicated in modulating cell adhesion, membrane trafficking, pathogen entry, and signal transduction (Brown and London, 1998; Hakomori, 2000a, 2000b; Simons and Ikonen, 1997). Caveolae are identifiable as stable invaginations of the plasma membrane which are enriched in caveolin, a cholesterol-binding membrane protein. Expression of caveolin 1 (cav-1) has been shown to be sufficient to produce caveolae in cells (Lipardi et al., 1998). An endocytic pathway leading from caveolae at neutral pH to the smooth ER or Golgi is utilized by some viruses such as SV40 (Pietiainen et al., 2005) and Coxackie virus B4 (Triantafilou and Triantafilou, 2004). However, there are also examples of pH-dependent viruses that require caveolae/lipid rafts for entry, such as filoviruses (Bavari et al., 2002; Schomberg et al., 2006; Takada et al., 1997).

Available experimental evidence of entry of the Old World arenavirus LCMV therefore suggests a potentially novel pH-dependent, cholesterol-dependent pathway. The goal of this study was to clarify the roles of cholesterol, and caveolar or clathrin-mediated endocytic pathways in cellular infection of representative Old World and New World arenaviruses or their pseudotyped surrogates. Two arenaviruses were studied: (1) the guinea pig-passaged Pichindé virus (PICV) variant P18, which causes hemorrhagic fever in guinea pigs (Jahrling et al., 1981; Zhang et al., 1999), and does not utilize alpha-dystroglycan as a receptor (Rojek et al., 2006), and (2) the Lassa-pseudotyped murine leukemia virus (LASV-MLV), which enters by an alphadystroglycan-dependent mechanism. We show that cholesterol is required for cellular infection of PICV and the LASV-MLV pseudotype in several relevant cell types in a caveolin-1 independent mechanism and that the effect of cholesterol depletion on infection is at the level of entry. In addition, the data suggests that entry of PICV and the LASV-MLV pseudotype into host cells occurs through the clathrin-mediated endocytic pathway.

Results

To determine whether removal of cholesterol affected the infection of cells with the arenavirus Pichindé (PICV), plaque reduction assays and viral growth curves were performed after treatment of cells with methyl-β-cyclohexetrin (MβCD). MβCD has been shown to remove cell membrane cholesterol (Beer et al., 2005; Carrasco et al., 2004; Choi et al., 2005; Danthi and Chow, 2004; Simons and Ehehalt, 2002). When Vero cells were pretreated with MβCD prior to exposure to PICV, a 90% reduction in plaque numbers was seen (Fig. 1A). The residual plaques on MβCD-treated Vero cell were 29% smaller ($p < 0.01$) than those in untreated Vero cell cultures (data not shown). When MβCD-treated cells were reconstituted with cholesterol prior to the addition of virus, plaque numbers were restored to control levels. MβCD treatment of Vero cells did not have a significant effect on Vesicular stomatitis virus (VSV) plaque formation. Treatment of Vero cells with nystatin/progesterone (nys/prog), which disrupts cholesterol enriched domains by specifically binding to cholesterol and by interfering with cholesterol synthesis, also inhibited PICV infection. Incubating cells with nys/prog resulted in a reduction of plaque numbers similar to that of MβCD-treated Vero cells, without affecting VSV plaque formation (Fig. 1A). VSV infection is known to occur via clathrin-mediated endocytosis (Sun et al., 2005) and is not sensitive to membrane cholesterol depletion (Danthi and Chow, 2004). This control also rules out significant drug-induced cytotoxicity.

We also studied the effect of host cellular cholesterol depletion on PICV yield from infected cells. PICV yield at 48 h post infection for MβCD-treated cells was reduced 70–76% compared to untreated cells (Figs. 1B and C). This inhibition was observed in Vero cells and P388D1 cells (a murine macrophage-like cell line). Reconstituting cholesterol into MβCD-treated cells prior to viral infection restored viral yield to control levels. Pre-treating cells with nys/prog also resulted in inhibition of PICV yield from infected Vero cells and P388D1 cells (Figs. 1B and C). Titers of VSV were unaffected by MβCD or nys/prog treatment of Vero cells. At later time points (72 and 96 h), after infected cells were incubated in the presence of complete media and cholesterol containing serum, no significant difference in PICV titers were observed between MβCD-treated cells and untreated cells, with all cultures generating $10^5$–$10^6$ pfu/ml (data not shown). Approximately 85–90% of cellular cholesterol was removed by MβCD treatment (Fig. 1D). MβCD-treated, cholesterol reconstituted cells had slightly increased cholesterol levels when compared to untreated control cells (Fig. 1D).

The effect of MβCD treatment on the synthesis of viral proteins in cells infected with PICV was tested. In these experiments, viral protein synthesis was assayed by immunoblot analysis using a polyclonal anti-peptide antibody GPC (59–79) that recognizes the glycoprotein precursor GPC (75 kDa) and GP1 (50 kDa) and a monoclonal antibody (3B3.1) that recognizes the nucleoprotein NP (60 kDa) of PICV. The production of the PICV GPC, GP1, and NP in MβCD-treated Vero cells was reduced by 85–95% when compared to untreated Vero cells at 48 h post infection (Figs. 2A and B). The addition of cholesterol into MβCD-treated Vero cells prior to virus infection led to an increase in the synthesis of the viral proteins when compared to MβCD-treated Vero cells. The
levels of the cellular TfR were unaffected by drug treatment or virus infection. These results indicated that the synthesis of viral protein was reduced in cholesterol-depleted cells.

To determine whether cholesterol depletion primarily inhibited early events in the viral life cycle, Vero cells were treated with MβCD at various time points relative to the addition of virus: 30 min prior to the addition of virus (−30); 10 min after the addition of virus (+10); or 30 min after the addition of virus (+30) (Fig. 3A). Immunoblot analysis was performed to assay for viral proteins. The levels of the viral proteins NP, GPC, and GP1 were decreased when cells were treated with MβCD 30 min prior to or 10 min after the addition of virus (+30) (Fig. 3B). However, adding MβCD 30 min after virus addition had little effect on the amount of viral proteins produced. A corresponding reduction in viral titers at 48 h post infection was observed (Fig. 3C). Altogether, these data suggest that cholesterol depletion of target cells affects early event in the virus life cycle.

To determine whether cholesterol depletion affects viral entry per se, replication deficient arenavirus pseudotyped retroviruses were used. We used both PICV and LASV-envelope protein pseudotyped particles for these analyses. Retroviral pseudotypes allowed work to be performed in a BSL-2 laboratory setting and isolated the envelope function from other viral components and functions. To characterize the PICV and LASV-MLV pseudotypes, both the DG control hemizygous murine ES DG(+/−) and the DG knockout murine ES DG(−/−) cell lines (Shah et al., 2006; Zhan et al., 2005) were transduced with the PICV and LASV env-MLV pseudotypes (Fig. 4A). LASV (an Old World arenavirus) has been shown to infect cells in an α-DG-dependent manner, whereas PICV (a New World Clade A arenavirus) infection occurs independently of α-DG (Rojek et al., 2006; Spiropoulou et al., 2002). As expected, the LASV-MLV pseudotypes transduced the ES DG(+/−) cells significantly better than the ES DG(−/−), which is in agreement with other published reports (Rojek et al., 2006; Kunz et al., 2005; Kunz et al., 2001). However, no significant difference was observed among ES DG(+/−) and ES DG(−/−) when transduced with the PICV-MLV pseudotype. This demonstrates that PICV-MLV pseudotypes, similar to the Clade B human pathogenic viruses, do not require α-DG for infection. No difference was observed in infection of the ES DG(+/−) or ES DG(−/−) cell lines by the control VSV-MLV pseudotype.

The PICV and LASV-MLV pseudotypes were then used to infect MβCD-treated and untreated Vero cells (Fig. 4B). MβCD treatment of cells prior to exposure to PICV or LASV-MLV pseudotyped virus resulted in 95.7% and 93.0% inhibition of transduction, respectively, when compared to untreated cells. Furthermore, reconstitution of cholesterol in MβCD-treated
cells restored both the PICV and LASV-MLV pseudotype transduction. However, treated cells with MβCD had a minimal effect on the entry of the pseudotyped virus bearing the VSV-G envelope protein, as previously reported for VSV (Shah et al., 2006; Yonezawa et al., 2005). Therefore, we conclude that the entry of PICV and LASV requires cell membrane cholesterol.

To test whether the entry of PICV and the LASV-MLV pseudotype was mediated by a caveolar pathway, we utilized human hepatoma 7 (Huh7) cells which lack functional caveolae (Damm et al., 2005; Vainio et al., 2002). We observed that both the LASV-MLV pseudotype and PICV infected Huh7 cells (Table 1) as well as they infected Vero cells. In addition, Vero cells transfected with cav-1 siRNA, resulting in the silencing of cellular cav-1 (Fig. 5A), were readily infected by both PICV and the LASV-MLV pseudotype (Figs. 5B and C). This demonstrates that the LASV-MLV and PICV entry into host cells is not primarily dependent on caveolae.

It has recently been shown that the Clade B arenaviruses Junin, Machupo, and Guanarito bind to the cellular TIR1 (Radoshitzky et al., 2007) during viral entry. Uptake of the natural ligand of TIR1, transferrin, occurs via clathrin-coated pits. Therefore, we studied the contribution of clathrin-mediated endocytosis in arenavirus entry. EGFR pathway substrate clone 15 (Eps15) is a constituent of clathrin-coated pits and is required for clathrin-mediated pit assembly and function (Benmerah et al., 1999). We used a dominant negative mutant of Eps15 (Δ95-295) which encodes a mutant Eps15 and a GFP fusion protein to inhibit clathrin-mediated endocytosis (Benmerah et al., 1999). Using the Amaxa NucleoFector II (as described in the Materials and methods section), we measured 79.5±1.3% of Vero cells that expressed GFP after transfection with the Δ95-295 (data not shown). We observed a significant reduction of PICV replication in Vero cells transfected with Δ95-295 when compared to untreated-infected-Vero cells or infected-Vero cells transfected with the empty vector control plasmid (Fig. 6). VSV was used as a positive control and showed the expected inhibition of infection in Δ95-295-transfected cells. Immunoblot analysis demonstrated a reduction of PICV GPC and GP1 in Vero cells transfected with Δ95-295 when compared to PICV-infected cells transfected with the empty vector control plasmid (Fig. 6B). Expression of a marker gene after transduction of cells with the LASV-MLV or PICV-MLV pseudotypes was also reduced in Δ95-295 transfected cells by approximately 71% and 69.8%, respectively (Fig. 6C). Examination by fluorescent microscopy revealed that the vast majority (98.3%) of PICV-MLV pseudotype-transduced Vero cells (β-gal +) did not express GFP from the Δ95-295 plasmid suggesting that in the presence of the Eps15 DN mutant, viral pseudotype uptake was inhibited.

We considered the possibility that the inhibitory effect of cholesterol depletion related to inhibition of clathrin-mediated endocytosis and transferrin-receptor uptake, although the use of TIR as receptor by PICV has not yet been described. To determine whether the cholesterol depletion affected clathrin-mediated uptake of transferrin, MβCD treated cells were exposed to fluorescent-labeled transferrin, and viewed by confocal microscopy after 20 min. Uptake of labeled transferrin was qualitatively diminished in MβCD-treated cells (Figs. 6D–F), as has been previously demonstrated (Subtil et al., 1999).

**Discussion**

In this study, we demonstrated that cell membrane cholesterol was required for infection of Vero and P388D1 cells by the New World arenavirus PICV, given that viral protein synthesis and viral production were inhibited when cells were treated with MβCD or nys/prog. These effects were not due to cytoxicity because replication of a control virus, VSV, was unaffected by drug treatments. When cholesterol was restored in target cells, virus replication increased to levels seen in untreated cells. The cholesterol-depleting drug MβCD has been shown to have additional effects on cells that can influence events downstream of virus entry, such as inhibition of calcium channels (Liu et al., 2006). Two lines of evidence in our study, suggest that cellular cholesterol is required for early events in the arenavirus life cycle: (1) when cellular cholesterol was depleted 30 min after exposure of virus to cells, there was no inhibition of infection and (2) cholesterol depletion inhibited transfer of marker gene by replication defective, PICV and LASV-MLV pseudotyped particles. Altogether, these results demonstrate that arenavirus envelope glycoprotein-mediated entry into host cells is dependent on the presence of cholesterol in cellular membranes. This effect was seen in both primate fibroblast and murine macrophage type cells. These results are in agreement with a previously published study that reported that cellular infection by the prototype arenavirus LCMV could...
Fig. 3. Effect of timing of cholesterol depletion on PICV infection. (A) A schematic representation of the experimental layout for addition of MβCD to host cells. Cells were treated with MβCD 30 min prior, 10 min after, or 30 min after PICV was added to host cells. (B) 48 h after infection, immunoblot analysis of the viral proteins was performed for each of the indicated experimental conditions. Antibodies that recognize the PICV NP, GPC and GP1, and the cellular TfR were utilized. (C) Viral titers in supernatants of MβCD-treated cells 48 h after infection are indicated. Asterisks (*) represent $p<0.05$, using Student’s $t$-test analysis.
be inhibited by MβCD treatment (Shah et al., 2006). We have extended this observation to arenaviruses that are macrophage-tropic and cause primarily hemorrhagic fever syndromes in susceptible hosts. The effect was similar for LASV-MLV pseudotypes, which utilize α-DG as receptor, and PICV (virus and pseudotype), which does not (Rojek et al., 2006). We have also shown for the first time that PICV and LASV pseudotypes were capable of infecting cells devoid of caveolin-1, indicating that arenaviruses enter cells by a non-caveolar pathway. This is consistent with previous ultra-structural studies of LCMV entry which suggested that virus-containing smooth walled endocytic vesicles were 150–300 nm in diameter (Borrow and Oldstone, 1994), which is typically bigger than caveolae (Raznani et al., 2002). We cannot rule out the possibility that multiple entry pathways may be used simultaneously by arenaviruses, as has been previously shown for SV40 (Damm et al., 2005).

Mechanisms by which cholesterol may be involved during viral entry include virus binding to cellular receptor(s) that are localized within lipid raft microdomains, which includes caveolae and non-caveolar cholesterol-enriched domains. For example, the HIV-1 CD4 primary receptor is constitutively associated with lipid rafts and can be detected within detergent insoluble fractions of lysed cells (Kozak et al., 2002; Millan et al., 1999). The disruption of lipid rafts by cholesterol removal
results in the dispersal of the cellular receptor(s) throughout the cellular membrane and reduces the opportunity for multiple receptor interactions that enhance virus uptake. Alternatively, viral receptors may transiently associate with lipid rafts/caveolae after being engaged by viral ligand and this may be important for viral endocytosis (Triantafilou and Triantafilou, 2004). Although not well studied, there is currently no evidence that arenavirus receptors are localized to lipid rafts. GPI-linked proteins, which tend to be localized and concentrated in lipid rafts, do not serve as viral receptors for the New World arenaviruses (Rojek et al., 2006). The TfR 1, which has been recently identified as the receptor for some New World arenaviruses, is not constitutively localized to lipid rafts (Harder et al., 1998). α-DG (LCMV receptor) or its cellular partner β-DG are not present in detergent insoluble domains of susceptible cells, even after LCMV binds to cells (Shah et al., 2006). This conclusion is weakened by the lack of studies using labeled LCMV to show translocation to lipid raft or non-lipid raft fractions. Additional limitations of the study include insufficient sensitivity of the assay to detect small but relevant amounts of α-DG, variations of protein-lipid raft association depending on the state of the cells (Kusumi and Suzuki, 2006), and the detergent utilized to isolate detergent insoluble domains (Schuck et al., 2006). However, our observation that arenavirus infection did not require caveolae supports the conclusions of Shah et al. in that caveolae are normally associated with lipid raft domains.

Since the New World arenaviruses Guanarito, Junin, and Machupo utilize the TfR 1 as their receptor (Radoshitzky et al., 2007), and since the TfR 1 is known to translocate from the cellular surface to endosomes via clathrin-coated pits, we
investigated the role of clathrin-mediated endocytosis in virus entry. We observed substantial inhibition of PICV infection in cells that were transfected with an Eps15 DN mutant (EΔ95/295). Infection with VSV, used as a control to ensure the effective inhibition of the clathrin-mediated uptake by this dominant negative mutant protein, was also diminished in transfected cells. We also observed that transduction of cells by the LASV-MLV or PICV-MLV pseudotypes was significantly reduced in cells which expressed the Eps15 DN mutant. This was demonstrated both by substantially reduced titers of β-gal expressing pseudotypes in EΔ95/295-transfected Vero cell monolayers, and by the observation that those few cells that did express the marker gene β-gal generally did not express the mutant Eps15-GFP fusion protein. Therefore, we believe that reduced infection/transduction in EΔ95/295 transfected cells is largely occurring in cells that maintain functional Eps15. The finding of small number of double positive (β-gal/GFP) cells suggests that an alternate, non-clathrin pathway may support viral entry to a limited degree.

Cellular uptake of labeled transferrin, a marker for clathrin-mediated endocytosis and for the trafficking of a putative New World arenavirus receptor, was diminished after cholesterol depletion of cells. However, drug treatment in host cells did not affect total TfR levels as determined by immunoblot analysis. Classically, viruses that utilize the clathrin-mediated endosomal entry pathway are less sensitive to cholesterol depletion than viruses that utilize caveolea or lipid rafts during entry. However, studies have demonstrated that cholesterol depletion of cells affects the morphology of clathrin-coated pits and inhibits the detachment of coated pits from the plasma membrane during clathrin-mediated endocytosis (Subtil et al., 1999). Overall, this suggests that PICV enters cells through clathrin-coated pits and that cholesterol depletion may inhibit entry by inhibiting clathrin-coated pit internalization. While this manuscript was in review, a new study demonstrated that the New World arenavirus Junin enters cells through clathrin-mediated endocytosis, in agreement with our findings (Martinez et al., 2007). However, this study concluded that Junin entry is only moderately inhibited in the presence of nystatin and MβCD (20–40%). It is possible for Junin virus, a Clade B arenavirus, and PICV, a Clade A arenavirus, to have similar routes of entry but differ in their sensitivity to cholesterol depletion.

Our findings of clathrin-mediated entry is at variance with studies of different Old World arenaviruses (Zhan et al., 2005). The results from that study suggest a role for dystroglycan in regulating the clathrin-mediated endocytosis of transferrin. However, LCMV binding and infection of cells is not dependent on β-DG (Kunz et al., 2003). These results suggest that although β-DG is not required for arenavirus entry, clathrin-mediated endocytosis is required. Intermediary events initiated by TFR1 and α-DG binding by different arenaviruses need to be studied. Altogether, our data demonstrate that PICV and the LASV-MLV pseudotype enter host cells through a cholesterol-dependent, caveolin-independent, clathrin-mediated endocytic pathway. The alphavirus Semliki Forest virus (SFV) entry into host cells is mediated by the clathrin-dependent endocytosis and has been shown to be sensitive to cholesterol depletion (Kolokoltsov et al., 2006) and it has been reported that cholesterol and sphingolipid is required for SFV fusion with host cells independently of lipid rafts (Ahn et al., 2002; Waarts et al., 2002). We propose that the entry of PICV and the LASV-MLV pseudotype is similar to SFV in that entry of all are sensitive to cholesterol depletion and mediated by clathrin-dependent endocytosis. These findings may result in the design of therapeutics aimed at blocking the early stages of arenavirus entry.

Materials and methods

Cell lines, reagents, and viruses

Vero cells were maintained in minimal essential medium (Cellgro, Mediatech, Inc., Herndon, VA) supplemented with 5% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 10 mM of HEPES, and 100 μg/ml of streptomycin (Gibco BRL, Invitrogen, Carlsbad, CA). The Vero cells were used between passages 10 and 40. Huh7 cells were maintained in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 10 mM of HEPES, and 100 μg/ml of penicillin and 100 μg/ml of streptomycin. The murine macrophage-like P388D1 cells were maintained in RPMI (Gibco BRL) supplemented with 5% heat-inactivated fetal bovine serum and 100 μg/ml of penicillin and 100 μg/ml of streptomycin. 293FT cells were maintained in DMEM supplemented with 10% FBS and 100 μg/ml of penicillin and 100 μg/ml of streptomycin. Mouse embryonic stem cells deficient in α-DG (ES DG −/−) and hemizygous for α-DG (ES DG +/+ ) were a gift from S. Carbonetto (McGill University Health Centre, Montreal, Quebec) and were maintained in DMEM with 10% FBS, 100 μg/ml of penicillin and 100 μg/ml of streptomycin, 1% nonessential amino acids, 2 mM l-glutamine, 0.1 mM β-mercaptoethanol, and 1000 U/ml of ESGRO, a serum-free culture medium that contains BMP4 and the cytokine leukemia inhibitory factor (LIF) obtained from Chemicon (Temecula, CA).

Guinea pig-passaged variants of PICV strain CoAn 4763 were developed by Peter Jahrling (Jahrling et al., 1981). The high-passage virulent PICV-P18 (Zhang et al., 1999) was utilized at an MOI of 1 for all infection experiments. Vesicular stomatitis virus-Indiana strain (VSVIndiana) was a gift from T. Jerrells (University of Nebraska Medical Center, NE).

Chemical treatment of cells

Cells were treated with 5 mM methyl-β-cyclodextran (MβCD) (Sigma-Aldrich, St. Louis, MO) diluted in serum-
free DMEM for 30 min at 37 °C, respectively. The cells were vigorously washed 3× with PBS to remove any chemicals prior to incubation with virus. In some experiments, cells were treated with 0.03 mM of both nystatin and progesterone (Sigma-Aldrich) overnight prior to infection, washed, and infected with virus for 20 min. After the virus incubation stage, media containing nystatin/progesterone was added. Nystatin/progesterone was present for the duration of the experiment post-viral binding. For cholesterol reconstitution experiments, cholesterol (Sigma-Aldrich) diluted to a final concentration of 0.25 mM in DMEM with 5% FBS, was added to cholesterol-deficient cells for 1 hr at 37 °C. The cells were washed 3× prior to the addition of virus.

**Cholesterol assay**

Cells were harvested and washed 3x with PBS prior to measuring the amount of cellular cholesterol. The cholesterol content in 10^5 cells was measured using the Amplex Red Cholesterol Assay (Molecular Probes, Invitrogen) as directed by the manufacturer. Fluorescence was measured with a BioTek Synergy HT fluorescent platter reader at 530–560 nm and equipped with BioTek KC4 software and the amount of cellular cholesterol was calculated by comparing the fluorescence obtained from the samples to the fluorescence obtained from the standard curve.

**Expression plasmid vector constructs**

pGAG-POL which encodes the MLV gag and polymerase (Kolokoltsov et al., 2005), the Eps15 DN mutant (Eps15-Δgal; Invitrogen) which encodes the LASV(Josiah) GPC CMV immediate-early promoter was purchased from Clonetch and the empty vector control plasmid (pLenti6/V5-D-Topo, (Kolokoltsov et al., 2005), the Eps15 DN mutant (Δgal) and the ψβ-galactosidase flanked by the MLV-LTR and packaging sequences. pVSV-G encoding VSV-G under the control of the CMV immediate-early promoter was purchased from Clontech (Palo Alto, CA), pLGP which encodes the LASV(GPS) GPC envelope ORF in the pCMV-β vector was a gift from M. Buchmeier (The Scripps Research Institute, CA).

Constructs encoding the PICV P18 envelope were created by replacing the β-gal ORF in pCMV-β (Clontech, Mountain View, CA) with the PICV glycoprotein precursor (GPC) ORF, hence this construct was analogous to pLGP. The P18 GPC sequences were first cloned into pcDNA3.1/V5-His Topo (Invitrogen). PICV P18 GPC insert flanked by NotI (5′) and SalI (3′) restriction sites was generated by PCR using the following primers: forward (5′ CGTACTCCGCGCCATGCGACAAAGTGTGACTTTGATCCAG 3′) and reverse (5′ CTAGGCGTCCGACTTTATTTACCAGTCTCACC 3′) (restriction sites underlined). The gel purified PICV-GPC PCR products were digested with NotI and SalI to generate NotI hanger at the 5′ end and the SalI hanger at the 3′ end. The PCR fragment was purified (Qiagen, Valencia, CA), and ligated into pCMV-β, in which β-gal ORF was previously removed by NotI and SalI digestion. After transformation of Topo competent cells (Invitrogen), plasmids were isolated and inserts were confirmed by DNA sequencing.

**Generation of arenavirus envelope pseudotyped MLV particles**

Replication incompetent retroviral pseudotypes expressing either the LASV envelope glycoproteins, the PICV envelope glycoproteins, or the VSV-G envelope were produced following methods similar to those published elsewhere (Kolokoltsov et al., 2005). 293FT cells were plated 1 day prior to transfection at a concentration of 3×10^5 cells per well (in a 6 well plate). The next day, 6 μl of TransIT-LT1 transfection reagent (Mirus Corporation) was added to 200 μl of serum-free media (DMEM), gently mixed, and incubated for 20 min at room temperature. Plasmid DNA, purified by cesium gradient or Qiagen kit, was added to TransIT-LT1 mixture (total 2 μg DNA, 1:1:1 ratio pGAG-POL: ψβ-gal: envelope plasmid) and incubated for 20 min at room temperature. The TransIT-LT1/DNA complex was then added to the 293FT cells, gently mixed, and incubated for 48 h at 37 °C. The supernatant was harvested and filtered through a 0.45 μm filter (GE Osmonics Laboratory) to remove cellular debris. The pseudotype titer was determined by incubating Vero cells with virus-containing culture supernatants and staining cells after 48 h with X-gal solution (0.05 M potassium ferrocyanide and potassium ferricyanide, 2 mM X-gal, and PBS) for 2 h. β-Galactosidase positive foci were counted and expressed as colony forming units (CFU). As a control, transfections were also performed by omitting envelope plasmid.

**Immunoblot blot analysis of PICV-infected cells**

Immunoblot assays for viral protein synthesis were performed using standard methods on PICV-infected and uninfected cells. As primary antibodies, the following reagents were used: (1) mouse monoclonal antibody 3B3.1 recognizing the PICV nucleoprotein (NP) (Buchmeier et al., 1981) (donated by Dr. M. Buchmeier, The Scripps Research Institute, CA); (2) polyclonal rabbit anti-peptide antibody GPC(S59–79) raised against the LCMV-ARM GPC sequence recognizing the PICV and LASV GPC/GP1 (donated by Dr. M. Buchmeier, The Scripps Research Institute, CA) (Buchmeier et al., 1987); and (3) anti-transferrin receptor (TfR) antibody (H68.4) (Zymed Laboratories, Inc., San Francisco, CA). After washing steps and incubation with appropriate HRP-conjugated secondary antibody, signal was developed using the SuperSignal West Femto (Pierce Biotechnology, Inc., Rockford, IL) as the substrate. Densitometry was performed on at least three immunoblot experiments using the OneD-scan program (Scانalytics, a division of CSPI, Billerica, MA). The viral protein band intensities were normalized to TfR expression levels.

**Transfection of Vero cells with siRNA or dominant negative mutants**

Vero cells were transfected using the Nucleofector II gene transfer device (Amaxa, Gaithersburg, MD). Cav-1 siRNA,
control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA), EA95/295, or the empty vector control plasmid was added to the Nucleofector siRNA transfection solution or transfection kit V solution and transferred to 1.0 × 10^6 Vero cells as instructed by the company. The cells were then transferred to the Nucleofector II gene transfer device and electroporation occurred using the program V-001. The cells were then removed and transferred to 6-well plates and incubated for 24 h at 37 °C prior to any infection or protein expression analysis. Cav-1 expression was assayed by Immunoblot analysis using rabbit polyclonal Cav-1 antibody (N-20) (Santa Cruz Biotechnology). We observed GFP expression in 84.6% of transfected Vero cells using an eGFP plasmid provided by the company. Transfections of Vero cells with the EA95/295–GFP fusion plasmid resulted in GFP expression in 79.5% of cells.

**Plaque assay**

Drug-treated and untreated Vero cell monolayers were infected with PICV for 30 min at 37 °C in a 5% CO_2 humidified atmosphere in serum-free MEM, followed by 3 washes with PBS to remove any unbound virus. Fresh complete MEM containing 5% FBS was added and the cells were incubated for 48 h at 37 °C. The virus-containing supernatant was collected at 48 h, 72 h, or 96 h, filter sterilized to remove any cellular debris, and a standard plaque assay was performed on Vero cells to measure virus production. Briefly, monolayers were exposed to virus for 30 min, after which monolayers were overlaid with 3 ml of methylcellulose containing 2x Eagle’s minimal essential media (EME), 2% FBS, and 1% methylcellulose for 96 h. Neutral Red overlay containing 0.12 mM of Neutral Red (Sigma Aldrich) was added to the monolayers for 24 h and the plaques were counted.

For the plaque reduction assay, drug-treated or untreated Vero cells were infected with 100 µl of PICV stock in a 6 well plate for 45 min at 37 °C. After inoculum was removed, monolayers were overlayed with methyl-cellulose-containing overlay and plaque assays were performed directly as described. Plaque numbers and sizes were recorded.

**Confocal microscopy**

Vero cells were incubated with Tf-594 (Molecular Probes) for 20 min prior to 4% paraformaldehyde fixation. The cells were washed 3× to remove any paraformaldehyde and slides were prepared using SlowFade® Gold antifade reagent with DAPI (Molecular Probes) for nuclear staining. The Zeiss LSM 510 Meta Confocal microscope with ×63 objective was utilized. To visualize DAPI, excitation at 351 nm and 364 nm was utilized and excitation at 543 nm was used to visualize Tf-594. Zeiss LSM imaging software was used to analyze the images.

**Statistical analysis**

The results were expressed as averages ± standard deviation. Statistical significance was assessed by Student’s t-test and statistical significance was ascribed when p<0.05.

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**References**


