Arch Virol (2008) 153:1391–1396
DOI 10.1007/s00705-008-0129-3

BRIEF REPORT

Pichindé virus is trafficked through a dynamin 2 endocytic pathway that is dependent on cellular Rab5- and Rab7-mediated endosomes

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Received: 15 February 2008 / Accepted: 30 April 2008 / Published online: 10 June 2008
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Abstract Pichindé virus (PICV) is a New World arenavirus that has been shown to enter cells through a clathrin-dependent endocytic pathway. In this study, we determined that PICV is trafficked through the cellular dynamin 2 (dyn2) endocytic pathway. Additionally, the data suggest that PICV entry is pH-dependent and that the virus travels through Rab5-mediated early and Rab7-mediated late endosomes. In all, this study characterizes the endocytic pathway utilized by the arenavirus PICV.

The family *Arenaviridae* consists of single-stranded, enveloped RNA viruses that include several members capable of causing hemorrhagic fever in humans. Rodents are the natural host reservoir, and infection of humans generally occurs through inhalation of aerosols or through contact with rodent excreta [13, 19]. The Old World arenaviruses lymphocytic choriomeningitis virus (LCMV) and Lassa virus (LASV) enter cells by binding to cellular α-dystroglycan [2], while the New World arenaviruses Machupo virus (MACV), Junín virus (JUNV), and Guanarito virus (GTOV) bind to the cellular transferrin receptor 1 (TfR 1) [2, 16]. In addition, studies have demonstrated that entry of JUNV and PICV occurs through clathrin-mediated endocytic vesicles that are dependent on cellular cholesterol [11, 21], whereas LCMV is believed not to use clathrin-coated pits [1]. Furthermore, some viruses have been shown to depend on cellular dynamin 2 (dyn2) [4, 10, 15], a GTPase involved in clathrin-mediated endocytosis [14, 17] during viral entry. Rab5 and Rab7 are ubiquitous GTPases involved in intracellular transport and trafficking of endosomes, and entry of several viruses [3, 7, 9, 18]. In this present study, we show that entry of the New World arenavirus PICV occurs through a dyn2-dependent process, with fusion occurring through Rab7-mediated endosomes.

To test whether PICV infection in Vero cells is dependent on cellular dyn2, a dyn2 dominant negative (DN) mutant (K44A) (graciously donated by Drs. Hong Cao and Mark A. McNiven) was used. K44A-transfected Vero cells were infected with PICV. K44A-transfected cells exhibited a reduction in viral glycoprotein 1 (GP1) and nucleoprotein (NP) expression without significantly affecting cellular transferrin receptor (TfR) expression, as observed by western blot analysis (Fig. 1a). Plaque assays revealed a 79% reduction in viral titers produced from K44A-transfected Vero cells when compared to dyn2 wild-type (wt)-transfected Vero cells (Fig. 1b). Additionally, replication-deficient Murine leukemia virus (MLV) pseudotyped viruses expressing the PICV env, MLV gag/pol, and β-galactosidase marker gene were used to determine whether dyn2 was necessary for PICV entry. Entry of the PICV-MLV pseudotype was significantly reduced in Vero cells transfected with K44A when compared to cells

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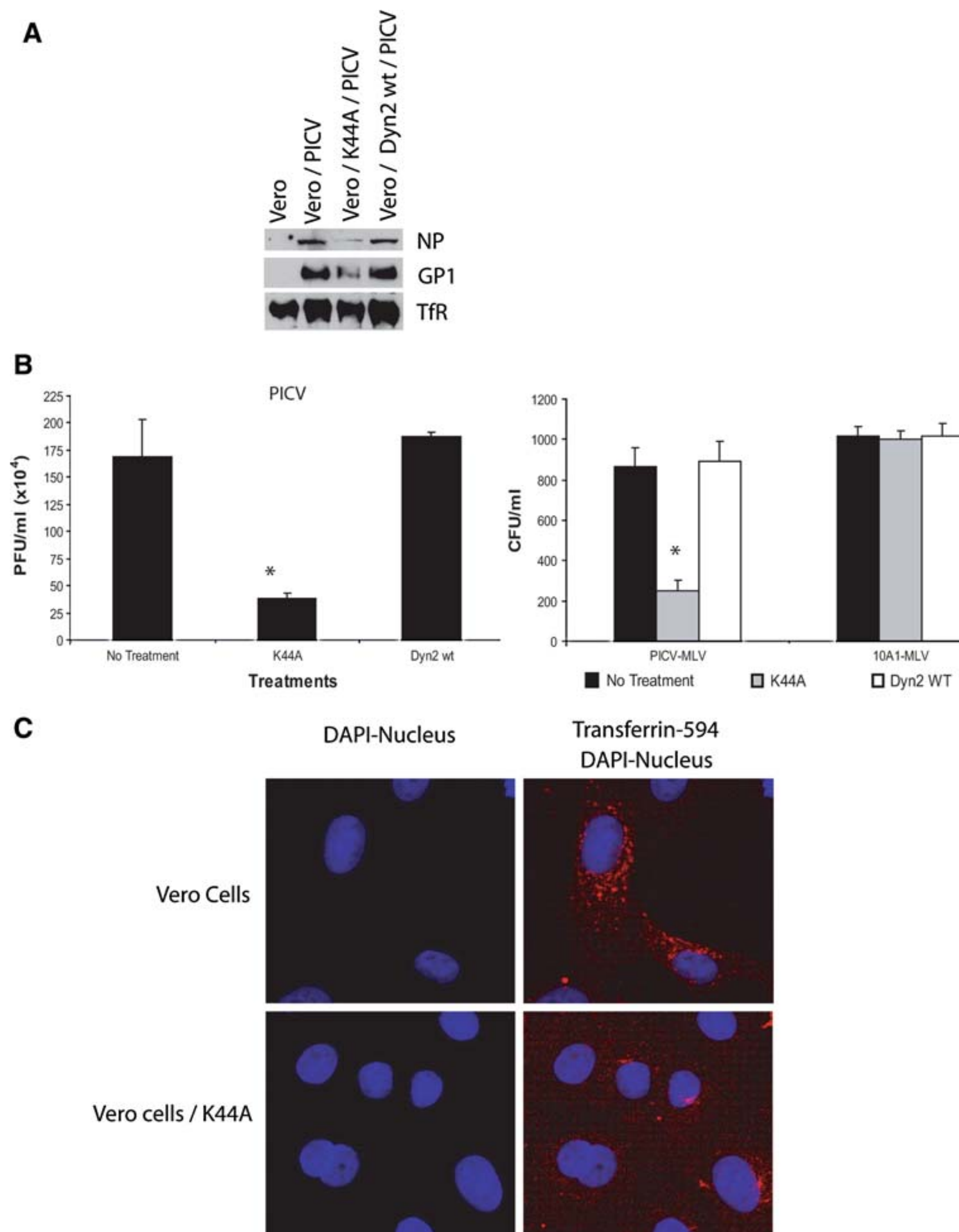

Fig. 1 PICV infection of host cells is dependent on cellular dyn2. **a** Vero cells were transfected with the dyn2 DN mutant K44A or with the dyn2 wt as previously described [21] and infected with PICV (MOI of 1). Western blot analysis was performed using antibodies that recognize PICV NP, 3B3.1 (donated by Dr. M. Buchmeier) and PICV GP1, PICV.GP1-256. The cellular TfR antibody was also used (Zymed Laboratories, Inc., San Francisco, CA, USA). At least three experiments were performed, and the presented western blot data is an accurate representation. **b** Supernatants were harvested from K44A- or dyn2-wt-transfected-Vero cells infected with PICV and titered by plaque assays (*left panel*). K44A- or dyn2-wt-transfected Vero cells were transduced with a PICV-MLV pseudotype or a 10A1-MLV pseudotype and analyzed for β-galactosidase or GFP marker gene expression (*right panel*). These experiments were performed at least three times, and the results are reported as means with standard deviations. *Asterisks* (*) indicate $P < 0.05$ by Student's t test. **c** Trafficking of transferrin is altered in K44A-transfected, serum-starved Vero cells when compared to mock-transfected, serum-starved cells when incubated with labeled transferrin (transferrin-594) for 20 min. Confocal analysis was performed two times on cells seeded on an 8-well chambered slide, and at least 100 cells were visualized. The cells were embedded in 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 and 364 nm) and Transferrin-594 (excitation at 543 nm). Zeiss LSM imaging software was used to analyze the images

transfected with the dyn2 wt (Fig. 1b). However, no reduction of transduction of K44A-transfected Vero cells with the 10A1-MLV pseudotype (containing the MLV envelope, MLV gag/pol, and pFB-GFP marker gene) [3] was observed, demonstrating that K44A-transfected cells are able to undergo cellular processes required for infection

by this non-clathrin-requiring pseudotyped virus. Additionally, no significant difference in cell viability was observed when comparing control-transfected cells to K44A-transfected cells (data not shown). To demonstrate the independent effect of K44A, transferrin uptake into K44A-transfected Vero cells was visualized by confocal microscopy. Serum-starved cells transfected with K44A or mock-transfected were incubated with transferrin-594 (Molecular Probes, Invitrogen) for 20 min. Labeled transferrin was transported and evenly distributed in the cellular cytoplasm in mock-transfected cells (Fig. 1c). However, transferrin-594 uptake into the cytoplasm was disrupted in K44A-transfected cells, demonstrating that K44A inhibits normal endocytosis of transferrin and the TfR. These data suggest that entry of PICV into host cells occurs through an endocytic pathway dependent on cellular dyn2.

We next investigated whether inhibiting endosomal acidification would inhibit viral infection, as has been shown for LCMV [5]. Vero cells were treated with 50 nM bafilomycin A1 (Baf-A1) or 20 mM ammonium chloride (NH_4Cl) for 1 h prior to the addition of PICV to prevent endosomal acidification. PICV NP and GP1 expression were inhibited in Baf-A1- and NH_4Cl-treated Vero cells when compared to untreated cells (Fig. 2a). Inhibition of viral protein expression was not due to the carrier (DMSO). Furthermore, no cellular toxicity was observed in drug-treated cells (data not shown). Additionally, a 76 and 94% reduction of PICV titers was observed in Baf-A1- and NH_4Cl-treated Vero cells, respectively, when compared to carrier-treated Vero cells (Fig. 2b). Similarly, entry of the PICV-MLV pseudotype was also inhibited in Baf-A1- and NH_4Cl-treated Vero cells. However, no reduction of

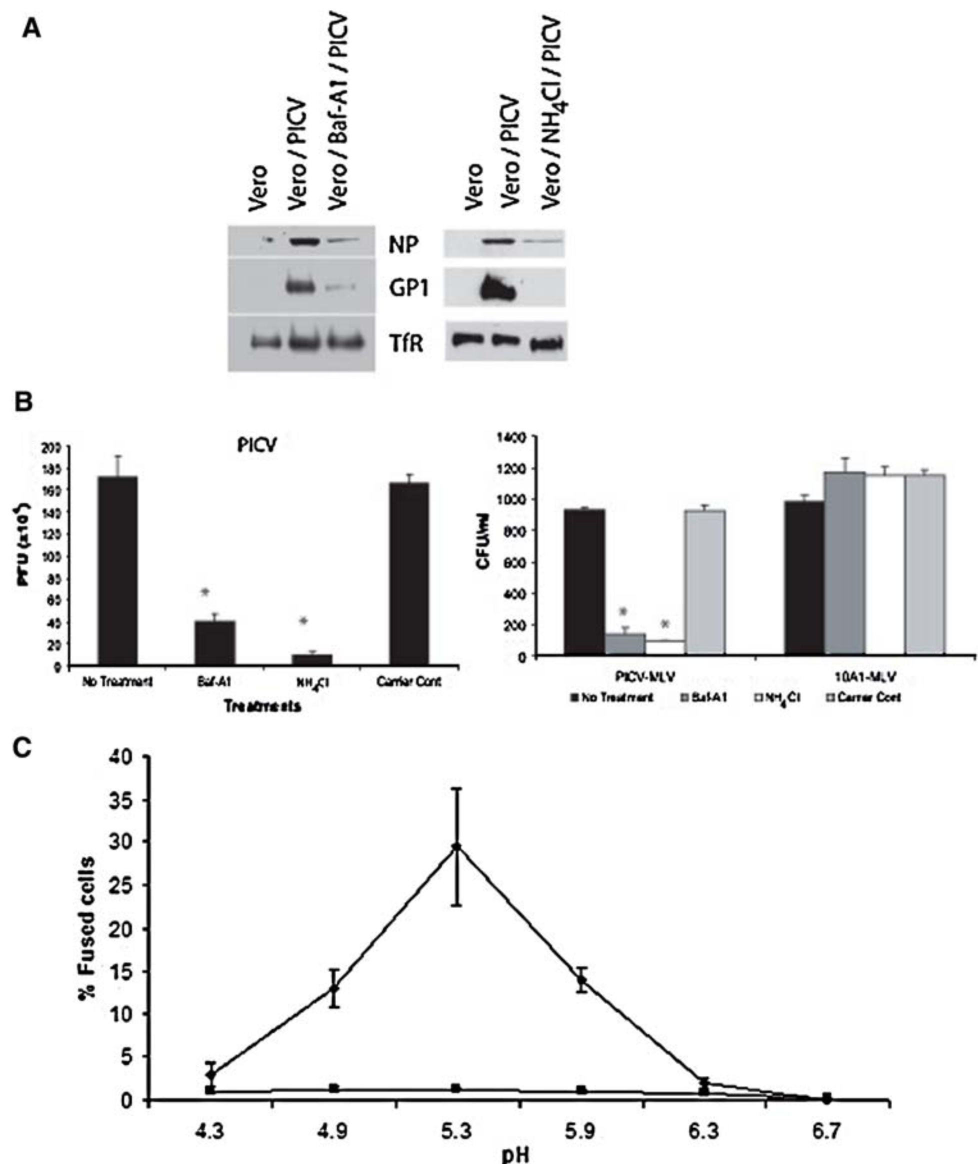

Fig. 2 Treatment of cells with Baf-A1 or NH_4Cl inhibits PICV infection. **a** Vero cells were treated with 50 nM of Baf-A1 or 20 mM of NH_4Cl for 1 h at 37°C prior to PICV incubation. The drugs were removed during viral exposure. At 48 h post-infection, western blots were performed for viral proteins, and TfR was utilized as a loading control. **b** Supernatants harvested from Vero cells treated with Baf-A1, NH_4Cl, or the carrier control prior to PICV infection were analyzed by plaque assays (*left panel*). Drug-treated Vero cells were transduced with PICV-MLV pseudotyped virus or a 10A1-MLV pseudotyped virus and analyzed by marker gene expression (*right panel*). **c** Cell-to-cell fusion assays were performed utilizing PICV-infected Vero cells that express the PICV glycoprotein in media with varying pH values (*filled triangle*) control Vero cells (*filled square*). An *asterisk* (*) indicates a $P < 0.05$ by Student's t test

10A1-MLV pseudotype transduction was observed in Baf-A1- and NH_4Cl-treated Vero cells, which is in agreement with previous studies [3]. Although these data imply that the inhibition of viral protein synthesis and viral production in PICV-infected-BafA1- and NH_4Cl-treated Vero cells is likely due to the inhibition of viral entry, it should be noted that affecting the endosomal pH is also likely to affect post-entry cellular events that may lead to the inhibition of viral replication.

Additionally, a cell-to-cell fusion assay was performed to determine the optimal pH required for PICV GP2-mediated cellular membrane fusion. Briefly, PICV-infected Vero cells (exposed to virus for 1 h) were washed and distributed into flasks for 48 h at 37°C. The cells were then incubated for 2 h in media that contained different amounts of 1 M bicarbonate solution. After the incubation, fresh medium was added to the cells, and the cells were incubated for 15 h. The cells were then fixed in methanol, stained with Diff-Quik, and the percentage of fused cells was determined. Peak cell fusion was observed at a pH of 5.3 (Fig. 2c). These data demonstrate that fusion of the PICV GP2 with the cellular membrane occurs at a low pH that mimics the acidic pH required for fusion of the viral envelope to the endosomal membrane.

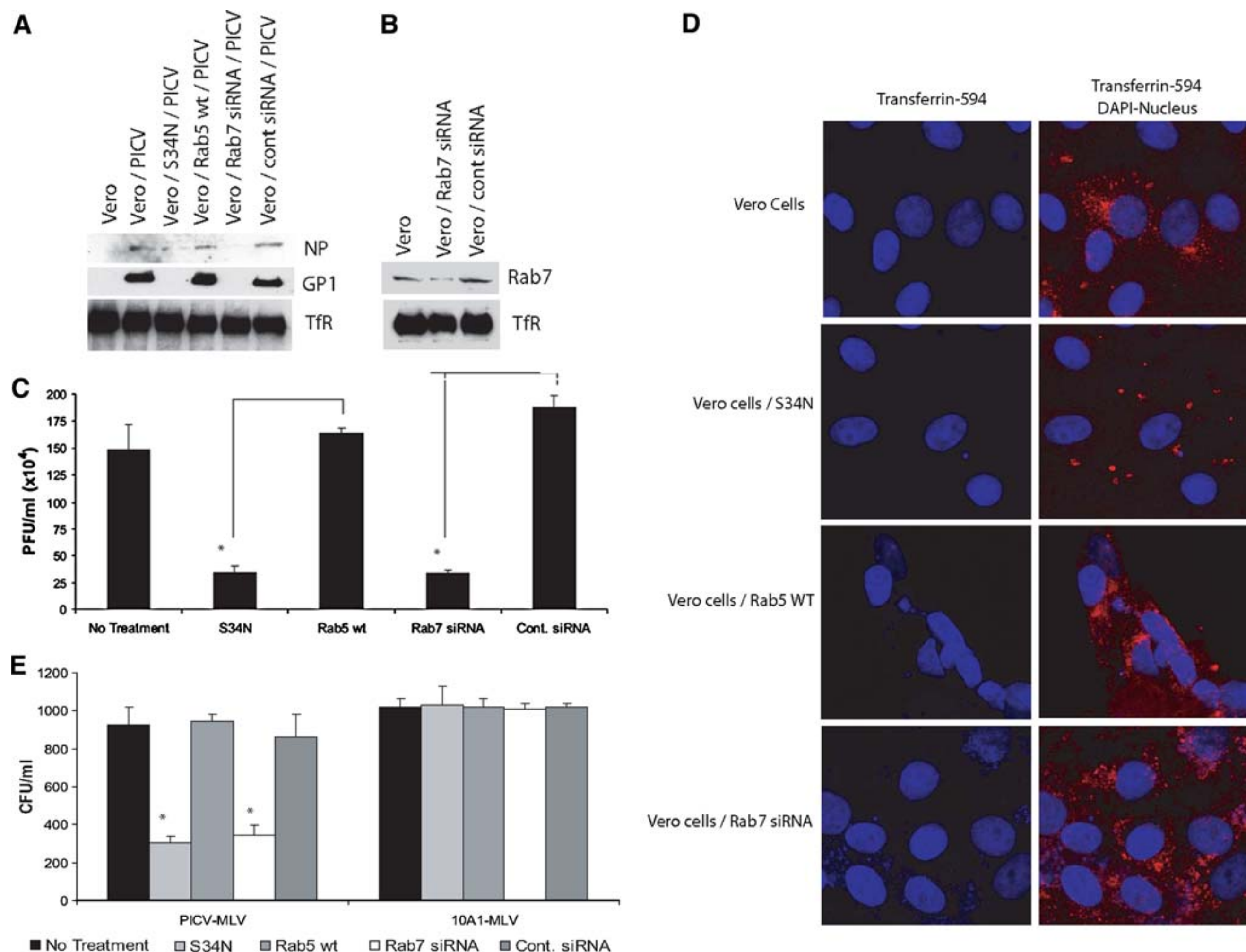

Fig. 3 PICV is trafficked through Rab5 early and Rab7 late endosomes. Vero cells were transfected with S34N, Rab5 wt, Rab7 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or control siRNA (Santa Cruz Biotechnology). **a** Western blot analysis was performed on PICV-infected Vero cells using antibodies recognizing the PICV NP, GP1, and cellular TfR. **b** Western blot analysis for Rab7 was performed on Vero cells transfected with Rab7 siRNA. **c** Plaque assays were performed on supernatants collected from S34N, Rab5 wt, Rab7 siRNA, or control siRNA-transfected Vero cells infected with PICV for 72 h. **d** Transferrin-594 was used to visualize the endocytosis of TfR into mock-transfected, S34N-, Rab5 WT-, and Rab7 siRNA-transfected Vero cells. The cells were stained with SlowFade® Gold antifade reagent with DAPI (Molecular Probes). **e** PICV-MLV and 10A1-MLV pseudotypes were used to transduce Vero cells transfected with S34N, Rab5 wt, Rab7 siRNA, or control siRNA. Transduction of host cells was quantitated by microscopy where colonies of blue-forming units (β-galactosidase expressing) or GFP-forming units (CFU) were assessed. *Brackets* indicate the conditions that were compared, and *asterisks* (*) indicate $P < 0.05$ by Student's t test

These data confirm that entry of PICV into host cells is pH-dependent, which has been reported for other arenaviruses [5].

We next tested whether PICV is trafficked through Rab5 early and/or Rab7 late endosomes. The pH of Rab5 early and Rab7 late endosomes is generally between 6.2–6.5 and 5.0–6.0, respectively [6, 12]. Based on the pH requirement for endosomes and our fusion data, we predicted that PICV enters cells through Rab5 early endosomes and uncoates and fuses with cells through Rab7 late endosomes. We employed S34N (a Rab5 DN mutant) to determine whether PICV utilizes Rab5-mediated early endosomes during the infection process. A reduction in viral proteins and PICV titers was observed in S34N-transfected cells when compared to Rab5 wt control-transfected cells (Fig. 3a, b). Additionally, an 80% reduction in PICV titers was observed when Vero cells were transfected with Rab7 siRNA when compared to cells transfected with a control siRNA. To demonstrate that inhibition of Rab5 early endosomes and Rab7 late endosomes affected cytoplasmic trafficking as expected, labeled transferrin was utilized since uptake of transferrin and the TfR occurs through Rab5 early endosomes but not Rab7 late endosomes. Rab5 DN mutants have been demonstrated to inhibit TfR endocytosis and trafficking to the cytoplasm, while Rab7 DN mutants have not been shown to affect TfR endocytosis [3, 8]. Normal endocytosis and trafficking of transferrin-594 was observed in mock-transfected Vero cells (Fig. 3d). However, endocytosis and trafficking of TfR was inhibited in S34N-transfected Vero cells, but not in Vero cells transfected with Rab7 siRNA (Fig. 3c). These data suggest that cellular trafficking was functioning as expected in cells transfected with S34N or Rab7 siRNA. Altogether, these data suggest that PICV utilizes both Rab5-mediated early endosomes and Rab7-mediated late endosomes.

To determine whether PICV was utilizing Rab5 and Rab7 endosomes during entry, replication-deficient MLV pseudotypes containing the PICV envelope and β-galactosidase marker gene were constructed as previously described [21]. Because these pseudotypes are replication deficient, they allow for the study of viral entry. Transduction of Vero cells with the PICV-MLV pseudotype was reduced by 68 and 60% when cells were transfected with S34N and Rab7 siRNA, respectively (Fig. 3d). As a control, the entry of the 10A1 MLV pseudotype was not affected in Vero cells transfected with the S34N, Rab5 wt control, Rab7 siRNA, or control siRNA (Fig. 3e). These data suggest that PICV is trafficked through Rab5 early and Rab7 late endosomes.

It has previously been demonstrated that the New World arenaviruses JUNV and PICV enter cells through clathrin-mediated endocytosis [11, 21]. Furthermore, the entry of PICV into host cells appears to be dependent on cellular tyrosine kinase activity [20]. In this report, we aimed to delineate the trafficking events required for fusion of PICV with the cellular cytoplasm. In all, the data suggest that PICV entry into host cells occurs through a dyn2-dependent endocytic mechanism and is trafficked through Rab5 early and Rab7 late endosomes.

Acknowledgments We would like to thank Eugene Knutson for his assistance with confocal microscopy. This work was supported by the NIAID Training in Emerging and Reemerging Infectious Diseases T32 AI007536. Robert A. Davey is supported by grants from NIAID 1R01AI063513-01A2 and U54 AI057156.

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