Virology 444 (2013) 301–309

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Glycolytic control of vacuolar-type ATPase activity: A mechanism to regulate influenza viral infection

Hinissan P. Kohio, Amy L. Adamson *

Department of Biology, University of North Carolina at Greensboro, Greensboro, NC 27402, USA

ARTICLE INFO

Article history:
Received 22 March 2013
Returned to author for revisions
24 April 2013
Accepted 24 June 2013
Available online 19 July 2013

Keywords:
Influenza
V-ATPase
Glucose
Glycolysis

ABSTRACT

As new influenza virus strains emerge, finding new mechanisms to control infection is imperative. In this study, we found that we could control influenza infection of mammalian cells by altering the level of glucose given to cells. Higher glucose concentrations induced a dose-specific increase in influenza infection. Linking influenza virus infection with glycolysis, we found that viral replication was significantly reduced after cells were treated with glycolytic inhibitors. Addition of extracellular ATP after glycolytic inhibition restored influenza infection. We also determined that higher levels of glucose promoted the assembly of the vacuolar-type ATPase within cells, and increased vacuolar-type ATPase proton-transport activity. The increase of viral infection via high glucose levels could be reversed by inhibition of the proton pump, linking glucose metabolism, vacuolar-type ATPase activity, and influenza viral infection. Taken together, we propose that altering glucose metabolism may be a potential new approach to inhibit influenza viral infection.

Introduction

Influenza viruses are respiratory viruses, and members of the *orthomyxovirus* family of virus. Influenza viral infection of cells is mediated by the viral proteins hemagglutinin (HA) and matrix protein 2 (M2) (Palese and Shaw, 2007). HA binds to sialic acid residues on cell surfaces, triggering endocytosis of the virus into an endocytic vesicle. This acidic pH within the endosome promotes two actions: (1) HA changes conformation and fuses the viral envelope with the endosome membrane, and (2) the M2 proton channel becomes activated and transports protons into the viral core (Palese and Shaw, 2007). M2 activity lowers the pH within the virion allowing for viral uncoating (Betakova, 2007), and subsequent transport of the viral genome from the virion into the cytosol (Palese and Shaw, 2007).

The cellular vacuolar-type H^+ ATPase (V-ATPase), located within endosomal membranes, other intracellular compartments, as well as the plasma membrane, has been identified to be involved in influenza viral entry by maintaining the acidic pH necessary within endosomes for viral release (Adamson et al., 2011; Guinea and Carrasco, 1995; Jefferies et al., 2008). V-ATPase protein complexes are multi-molecular proton pumps composed of a peripheral V_1 domain consisting of subunits A, B, C, D, E, F, G, and an integral V_0 subcomplex containing subunits a, c, d and e, with subunit c consisting of different isoforms, c′ and c″ (Jefferies et al., 2008). The V-ATPase pumps couple ATP hydrolysis to the transport of protons from the cytoplasm into the lumens of endosomes or organelles. Specifically, ATP binds to catalytic sites on the V_1 domain, (on the A and B subunits). This activity drives proton translocation from the cytoplasm into the lumen, acidifying the interior of intracellular compartments necessary for multiple cellular processes (Jefferies et al., 2008). Previous work has demonstrated that inhibition of the V-ATPase (by the drug bafilomycin A1) inhibits influenza viral infection (Adamson et al., 2011; Guinea and Carrasco, 1995; Ochiai et al., 1995; Perez and Carrasco, 1994). Likewise, overexpression of specific subunits of the pump (V_1A, V_1B, V_1C) enhanced viral infection (Adamson et al., 2011). Therefore, the V-ATPase provides an interesting target in relation to limiting influenza viral infection.

V-ATPase activity is regulated through disassembly of the V_1 and V_0 domains. This process helps regulate the pH levels of endosomes and organelles inside the cell (Kane, 2006). One of the mechanisms that causes the dissociation of V-ATPase is glucose depletion (Beyenbach and Wieczorek, 2006; Kane, 2006). This dissociation mechanism was first observed in yeast. In addition, V-ATPase assembly on membranes can be stimulated by glucose; this has been shown to be mediated by the phosphatidylinositol 3-kinase signaling pathway in renal epithelial cells (Sautin et al., 2005). Stimulation by glucose has also been shown to increase V-ATPase activity in MDCK and A549 cells (Marjuki et al., 2011). Furthermore, studies with yeast and mammalian cells have

* Correspondence to: Room 201, Eberhart Building, Department of Biology, University of North Carolina at Greensboro, Greensboro, NC 27402, USA.
Fax: +1336 334 5839.
E-mail address: aladamso@uncg.edu (A.L. Adamson).

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http://dx.doi.org/10.1016/j.virol.2013.06.026

indicated a direct physical interaction between subunits of the V-ATPases and glycolytic enzymes (Lu et al., 2001, 2004; Su et al., 2003). Lu et al. found an interaction between the V-ATPase a, B, and E subunits and the aldolase enzyme, the glycolytic enzyme that converts fructose-1, 6-biphosphate into glyceraldehyde-3-phosphate (step 4 of glycolysis) (Lu et al., 2001, 2004). Furthermore, Lu et al. have shown that there is an increase in the interaction between aldolase and the subunits of V-ATPases when glucose levels are increased (Lu et al., 2004). Su et al. have shown in human kidney cells that the rate limiting enzyme of the glycolytic pathway, phosphofructokinase 1 (PFK-1), which mediates step 3 of glycolysis, interacts with the V-ATPase a subunit (Su et al., 2003). A study conducted in our lab identified glyceraldehyde-3-phosphate dehydrogenase (GAPDH; catalyzes step 6 of glycolysis) as a genetic modifier of M2 ion channel activity in a *Drosophila* model system (unpublished data).

Since the V-ATPase is ATP-dependent, sufficient ATP levels are needed to maintain the pump's assembly and activity. In fact, pump disassembly has been attributed to a reduction in ATP levels (Parra and Kane, 1998). Taken together, reductions in glucose levels or the inhibition of glycolysis would likely promote V-ATPase disassembly, reduce acidification of endosomes, thus inhibiting influenza M2 and activities, and ultimately inhibit influenza viral replication.

Antiviral drugs for the treatment of influenza infection mainly target viral proteins such as neuraminidase (NA) and M2, however these drugs typically lose their effectiveness as the virus mutates (Betakova, 2007; Ison, 2011; Nguyen et al., 2012). Therefore, it is essential to identify more alternatives to control influenza infection, including targeting the host cellular processes that the virus utilizes to promote infection and replication. In this work, we explored how influenza virus infection is affected by the manipulation of glucose levels and glycolysis. We found that viral infection is dependent upon abundant glucose supplies, and that inhibition of glycolysis limits viral infection; these events occur in accordance with V-ATPase disassembly.

Results

Higher glucose concentrations correlate with higher levels of influenza A H1N1 viral infection

We previously identified several genetic modifiers of influenza M2 activity in our *Drosophila* model system (Adamson et al., 2011). In our genetic screen we also identified that a mutation in the *Drosophila* gene *Gapdh1* suppressed M2 activity (data not shown). The human homolog of *Gapdh1* is the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase, which mediates step six of glycolysis. This suggested to us a link between glucose metabolism and influenza viral infection. To determine if influenza viral infection was dependent upon glucose metabolism, we initially examined the efficiency of influenza viral infection of MDCK cells grown at varying glucose concentrations, which were subsequently infected with influenza A H1N1. We observed that higher glucose levels led to an increase in viral protein accumulation within infected cells, as judged by HA protein levels, as well as an increase in the percentage of infected cells (Fig. 1). We found a significant increase in the percentage of infected cells in the higher glucose level conditions (4–6 mg/ml), compared to the control treatment (1 mg/ml) ($P \leq 0.05$) (Fig. 1E). To ensure that the changes in infection were not due to viability changes across the different glucose concentrations, we performed viability tests on MDCK cells treated with a glucose concentration series, and found no significant changes in cell viability at the different glucose concentrations (Fig. 2).

Inhibition of hexokinase with 2-DG and 3-BrPa lowered influenza viral infection/replication

To determine if the metabolism of glucose via glycolysis was necessary for viral infection, we tested the effect of glycolytic inhibitors upon influenza infection. 2-deoxyglucose (2-DG) is an analog of glucose and a competitive inhibitor of hexokinase, the first enzyme of glycolysis (Pelicano et al., 2006). 3-bromopyruvate (3-BrPa) is an alkylating agent that inhibits hexokinase (Pelicano et al., 2006). Lonidamine (LND) is a molecule that dissociates hexokinase from mitochondria and inhibits hexokinase (Pelicano et al., 2006). MDCK cells were grown for 24 h at high glucose levels containing 2-DG (0, 0.25, 2, or 5 mM), 3-BrPa (0, 30, 300, or 600 μM) or LND (0, 6, 62.5, or 200 μM). Cells were subsequently infected with influenza A H1N1 for 24 h. Cells were immunostained with an anti-HA antibody to detect infected cells. Viral infection was reduced by at least 90% with 2-DG treatment (Fig. 3A), and by at least 80% with 3-BrPa treatment (Fig. 3B) when compared to the control treatments. Unexpectedly, we found that cells treated with LND became significantly more infected as we increased the levels of this inhibitor (Fig. 3C). We subsequently found that LND is reported to be an inhibitor of hexokinase in the cancer cells only, and that LND actually increases glycolysis in the normal cells (such as the ones we used here) (Floridi et al., 1981). To determine how LND would affect influenza viral infection of the cancer cell line, we grew the lung carcinoma cell line A549 at high glucose containing 0, 6, 62.5, or 200 μM LND for 24 h. Cells were infected with influenza A H1N1 for 24 h, then immunostained with anti-HA antibody. The number of infected cells was significantly reduced by LND treatment in a dose-dependent manner, however the viability of the cells was also reduced, especially at the highest dose of LND (data not shown). Therefore we found that LND is not an effective inhibitor in relation to viral infection, as it increased infection in normal cells, but was toxic to cancer cells.

Addition of extracellular ATP restored influenza viral infection

We found that 2-DG and 3-BrPa were effective inhibitors of influenza viral infection; viral infection was completely inhibited with higher levels of 2-DG. As the main purpose of glycolysis and further metabolism is to generate ATP, we investigated whether the addition of extracellular ATP could restore influenza viral infection. Cells grown in high glucose were treated with the inhibitor 2-DG (at 5 mM to ensure complete inhibition) for 24 h, then cells were simultaneously infected with influenza virus and treated with 0, 10, 25, 50 or 100 μM ATP. Immunostaining was performed 24 h later with an anti-HA antibody to detect infected cells. Fig. 4 shows that viral infection was restored when ATP was added, in a dose-dependent manner, seeming to have a maximal effect upon infection at 50 μM.

High glucose levels increased V-ATPase proton pump assembly in mammalian cells

Previous work has demonstrated that V-ATPase pump activity is down-regulated by glucose depletion, through disassembly of the peripheral V_1 and the integral V_0 domains in yeast cells, and stimulated by increased glucose levels (Beyenbach and Wieczorek, 2006; Kane, 2006; Marjuki et al., 2011). Assembly of the pump components onto intracellular compartment membranes was associated with a more punctate localization pattern (Parra and Kane, 1998). To examine pump localization in mammalian cells differing glucose concentrations, we immunostained A549 and HeLa cells that had been grown in either low or high glucose levels (Fig. 5). Human cells were utilized for this analysis because our anti-V-ATPase pump protein antibodies do not recognize canine

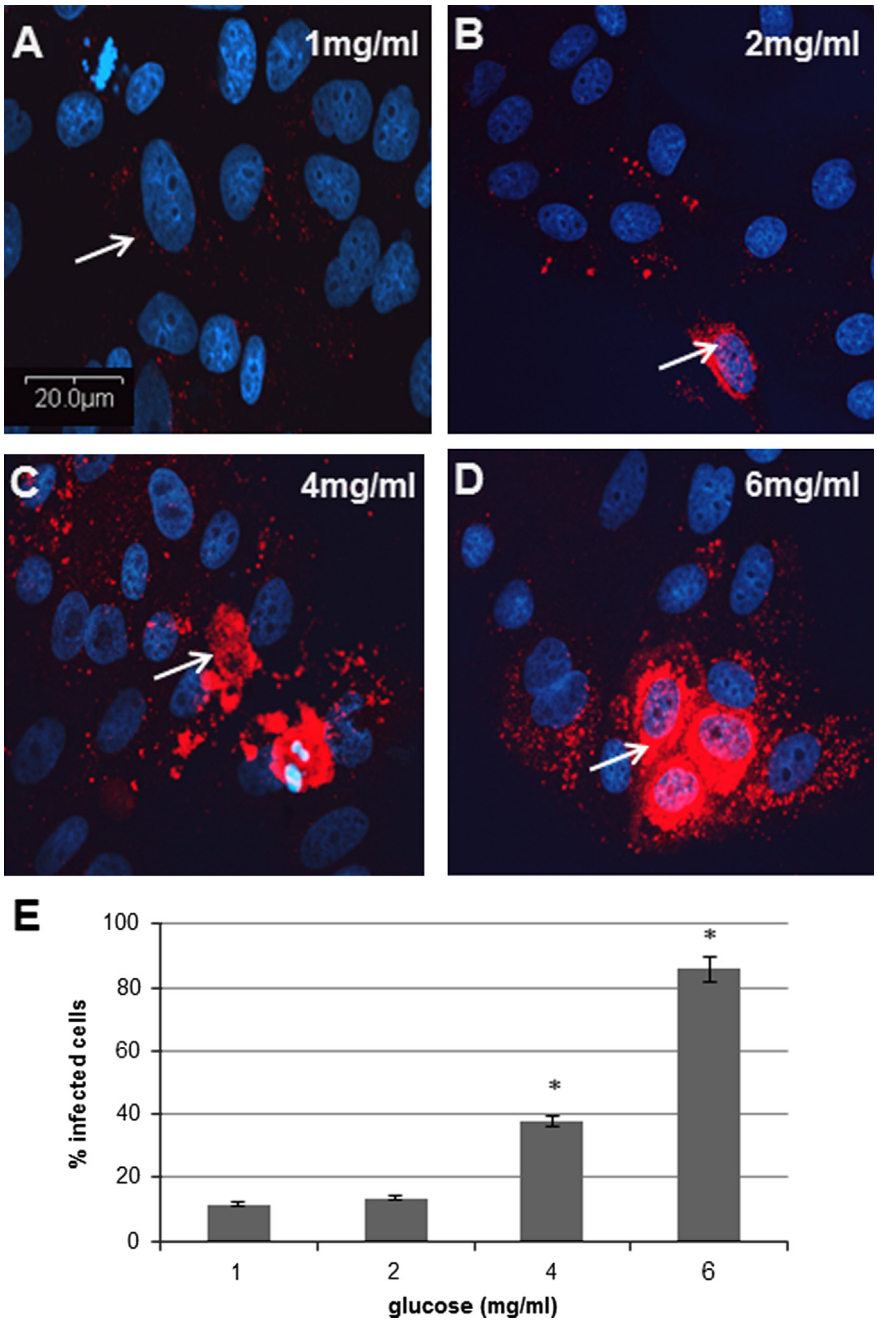

Fig. 1. Increased levels of glucose increased influenza viral infection of MDCK cells. (A–D) MDCK cells were grown in 1–6 mg/ml glucose as indicated then infected with influenza A H1N1 for 24 h. Cells were subsequently fixed and stained with anti-HA-antibody (red). Cells were also stained with Hoescht stain (blue) to identify nuclei. Confocal imaging was performed. (E) Graph representing the percentage of infected cells at the differing glucose levels. * indicates a P-value≤0.05 compared to 1 mg/ml. At least four separate fields of cells were counted for each condition as specified in Materials and Methods.

proteins. With an anti-V_1A antibody, we observed a low level of pump assembly at low glucose (Fig. 5A and C), and an increase in pump assembly at high glucose levels, as determined by the punctate localization pattern seen in Fig. 5B and D. This staining pattern was confirmed by staining for the V_1B subunit (data not shown).

To correlate V-ATPase pump localization with increased proton transport activity, we grew MDCK cells in low or high glucose and examined the relative pH of intracellular compartments with the pH-sensitive dye SNARF-1. This dye emits different wavelengths according to pH (Han and Burgess, 2010). We found that, relative to the low glucose condition, the cells grown in high glucose had a greater number of acidic compartments, as well as a lower pH in these compartments (Fig. 6 and Table 1). Taking the ratios of acidic/basic staining intensities, we found that the cells grown in high glucose had an average 3.7 fold increase of the acidic/basic ratio, suggesting that cells grown in high glucose have more acidic compartments than those grown in low glucose (Table 1), correlating increased assembly in high glucose with increased proton transport activity.

V-ATPase protein synthesis was not induced by high glucose concentrations

To determine if the more punctuate localization pattern or increased activity of the V-ATPase was a result of increased pump

protein synthesis, we examined V_1A_1 and $V_1B_{1/2}$ protein levels via Western blot. A549 cells were grown in 1, 2, 4, and 6 mg/ml glucose, and the V-ATPase V_1A_1 and $V_1B_{1/2}$ protein levels were determined (Fig S1 shows V_1A_1 protein levels within A549 cells). Protein levels of the V-ATPase pump subunits V_1A or V_1B (data not shown) did not increase with increased glucose levels, indicating that the increase we saw in assembly was not due to an increase in pump subunit protein production, but to localization of the pump subunits on intracellular compartment membranes.

V-ATPase disassembly could be reversed by the addition of ATP

As the localization of the V-ATPase on intracellular membranes was found to be dependent upon glucose levels, we investigated whether the need for glucose could be bypassed by the addition of extracellular ATP. A549 cells were grown in low or high glucose for 24 h, and were subsequently treated with 50 μM ATP for 10 min or one hour prior to fixation and staining for the V_1A subunit. Fig. 7 shows that in low glucose, assembly of the pump could be stimulated by ATP treatment within 10 min, with the assembly matching that of high glucose by 1 h of ATP treatment (compare Fig. 7A, C, and E). Addition of ATP to cells grown at high glucose did not appear to have any further effect upon assembly (compare Fig. 7B, D, and F).

To examine whether V-ATPase localization was linked to glycolysis, we treated A549 cells, grown in high glucose, with the glycolytic inhibitor 2-DG. V-ATPase assembly was reduced after 2-DG treatment (Fig. 8B). This disassembly could be reversed, however, after treatment with 50 μM ATP for 1 h (Fig. 8C).

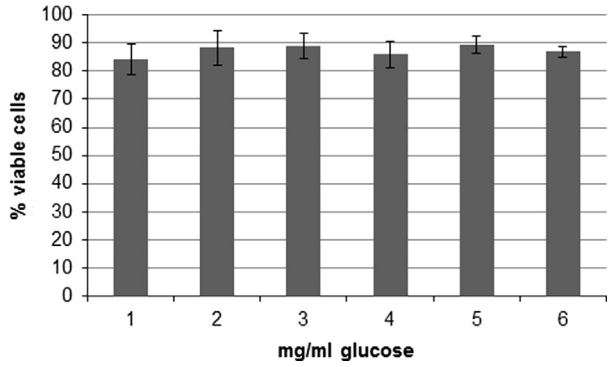

Fig. 2. Differing glucose levels do not affect viability of cells at the time of infection. MDCK cells were grown in 1–6 mg/ml glucose as indicated for 24 h. Cell viability was determined by incubating cells in Guava ViaCount reagent and assayed by flow cytometry. The percentage of viable cells is presented. The graph represents three separate trials; the slight variation in cell viability was not statistically significant.

The increased viral infection associated with high glucose levels is reversed by inhibition of V-ATPase

To confirm that the increased frequency of influenza virus infection that we noted at higher glucose levels was indeed due to the increased activity of the V-ATPase, we grew MDCK cells in low and high glucose levels, then subsequently treated the cells with bafilomycin, a V-ATPase inhibitor (Drose and

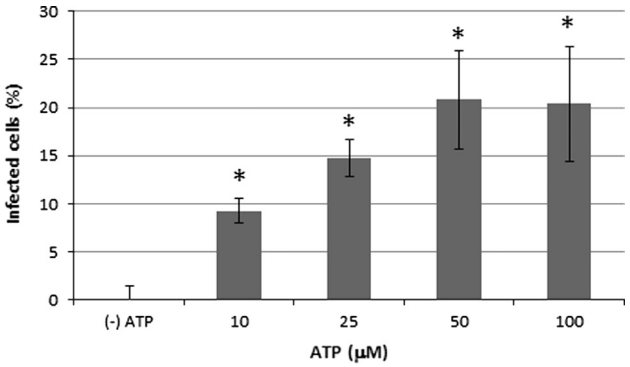

Fig. 4. The addition of ATP reverses the glycolytic inhibitor-mediated inhibition of influenza viral infection. MDCK cells were treated with 5 mM 2-DG for 24 h, then infected with influenza virus in the presence of extracellular ATP (0–100 μM as indicated). Cells were immunostained for HA and the percentage of infected cells was determined. * P-value≤0.05, comparing each ATP treatment group to the control (−) ATP group. At least four separate fields of cells were counted for each condition as specified in Materials and Methods.

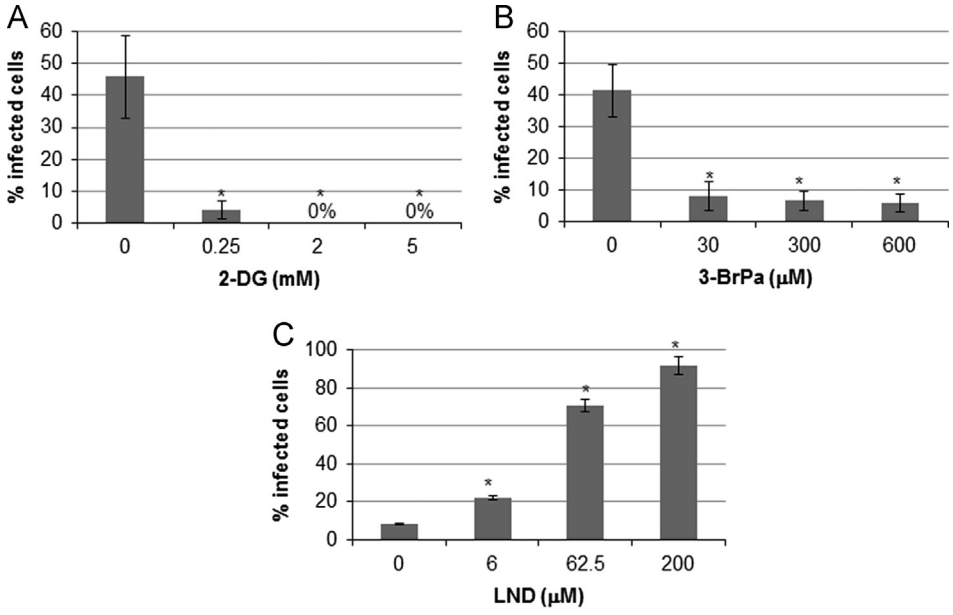

Fig. 3. Influenza viral infection is inhibited by glycolytic inhibitors. MDCK cells were treated for 24 h with the glycolytic inhibitors 2-DG (0, 0.25, 2, and 5 mM) (A), 3-BrPa (0, 30, 300 and 600 μM) (B), or LND (0, 6, 62.5 and 200 μM,) (C) and subsequently infected with influenza A H1N1 for 24 h. Cells were immunostained for HA and the percentage of infected cells was determined. * number of infected cells for each concentration when compared to the control treatment had a P-value≤0.05. At least four separate fields of cells were counted for each condition as specified in Materials and Methods.

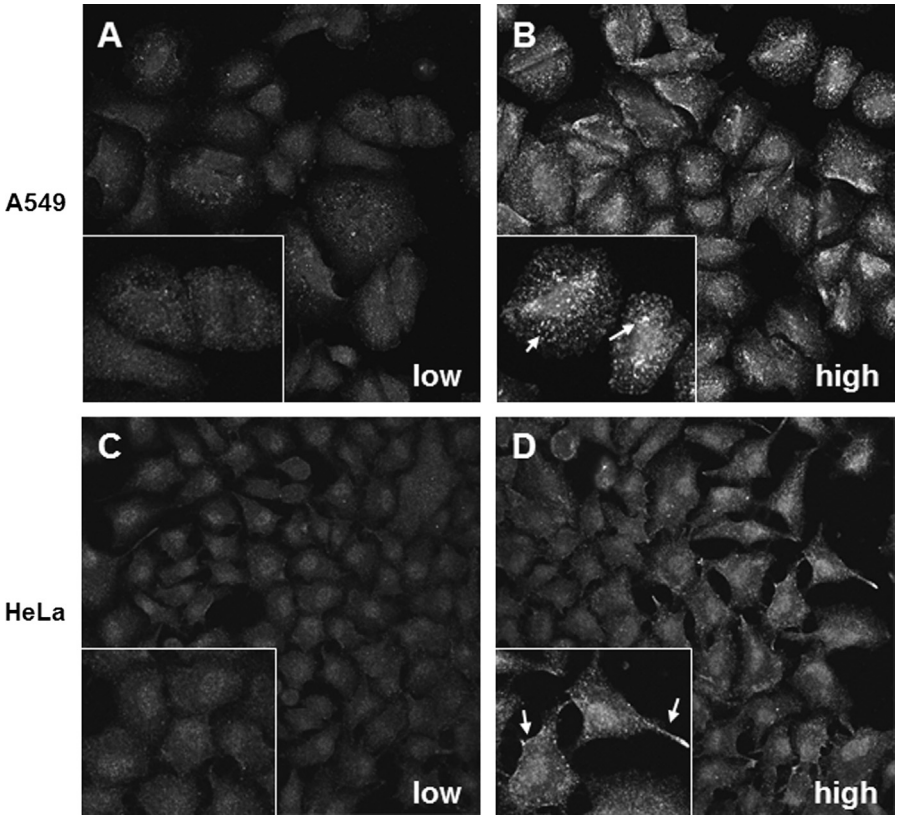

Fig. 5. The V-ATPase pump localizes to intracellular compartments to a greater degree at high glucose levels. A549 (A-B) or HeLa cells (C-D) were grown in low or high glucose for 24 h and immunostained with anti-V_1A_1 antibody. Confocal imaging was performed, each with identical acquisition settings. Insets are zoomed-in images. Arrows refer to the V-ATPase localization; note the more punctate pattern of localization at the higher glucose concentrations (B, D).

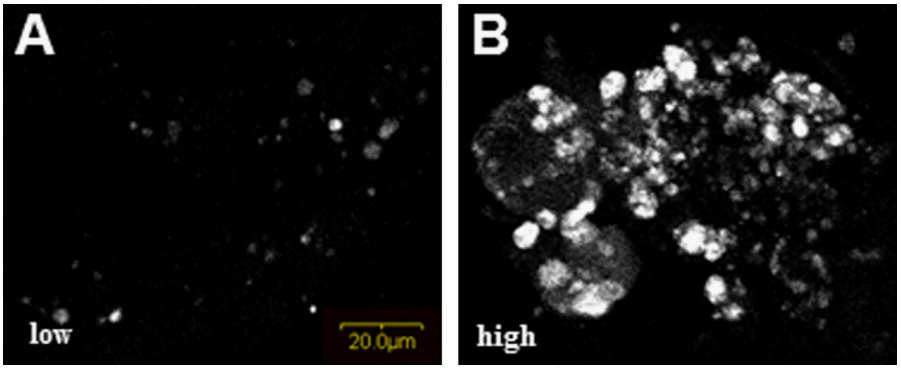

Fig. 6. High glucose leads to more acidic intracellular compartments. MDCK cells were grown in low or high glucose for 24 h then incubated in SNARF-1 dye. Live cells were imaged by exciting the dye with a 488 nm wavelength, then capturing images at 580 nm and 640 nm wavelengths. The images from the 580 nm emission (which denotes acidity) are presented.

Table 1
Intracellular compartments in high glucose-treated cells are more acidic.

Glucose level	n	Ratio of 580 nm/635 nm intensities
Low	57	0.27 ± 0.14
High	67	1.0 ± 0.34 ($P = 5.94 \times 10^{-22}$)

Altendorf, 1997), and infected the cells with influenza A H1N1 virus. Cells were immunostained for the influenza HA protein to identify infected cells. Fig. 9 shows that bafilomycin A1 treatment negated the effect of increased glucose upon infection success, further correlating that the increased infection was mediated by the increased activity of the V-ATPase pump.

Discussion

Influenza infection is a constant and major public health concern. Many current antiviral drugs that target the influenza viral envelope proteins are rendered ineffective over time, as different viral strains emerge. Thus, it is important to identify alternative ways to control influenza viral infection, by not only targeting viral particle proteins, but by also targeting the host cellular processes that the virus may utilize for infection and replication.

Influenza virus is dependent upon many host cellular molecules in order to infect, replicate inside, and egress from a cell [reviewed in (Watanabe et al., 2010)]. An early event important for the viral life cycle is the uncoating of viral particles, and the subsequent release of viral ribonucleoprotein (vRNP) complexes

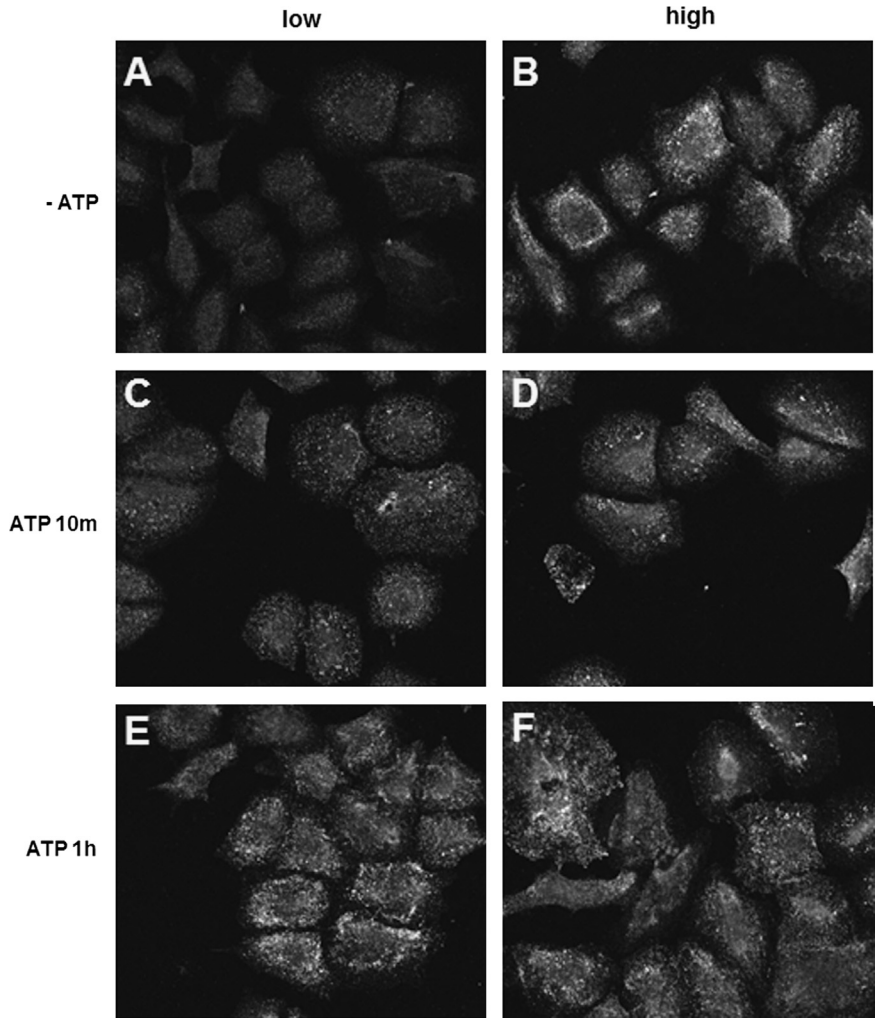

Fig. 7. ATP reverses the disassembly of the V-ATPase due to low glucose. A549 cells were grown in the presence of low glucose (A, C, and E) or high glucose (B, D, and F) for 24 h. Cells were treated with no ATP (A-B), or 50 μM ATP for 10 min (C-D) or 1 h (E-F), then immunostained with anti-V_1A_1 antibody. Confocal imaging was performed, each with identical acquisition settings.

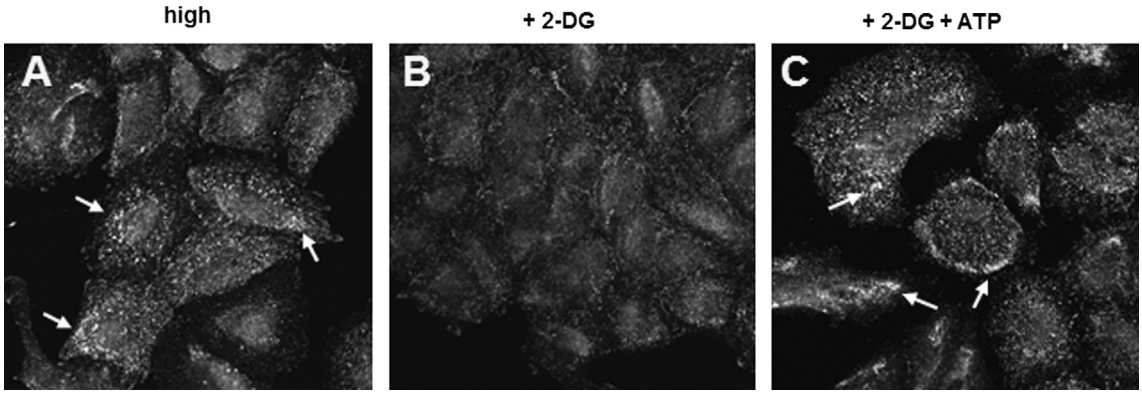

Fig. 8. V-ATPase assembly can be reversed by glycolytic inhibition. A549 cells were grown in high glucose and subsequently treated with 2 mM 2-DG for 24 h (B). A subset of cells were treated with ATP for the last hour prior to fixation and staining with an anti-V_1A antibody (C). Confocal imaging was performed, each with identical acquisition settings.

into the cytosol. This uncoating event is mediated by the low pH within endosomes, which in itself occurs in a multi-step process. Initially the virus-containing endosome is transported to the periphery of the nucleus, the pH is lowered to ~pH 6, then a second acidification event decreases the pH to ~pH 5 (Lakadamyali et al., 2003). This acidic pH is created by the action of the cellular V-ATPase; inhibition of the V-ATPase suppresses viral infection. Specific subunits of the V-ATPase have been identified as particularly important for influenza viral infection, including the V_1 subunits A, B, and C, as well as the V_0 subunits B, C, and D1 (Adamson et al., 2011; Hao et al., 2008; Watanabe et al., 2010). The action of the V_0D1 subunit appears to have some

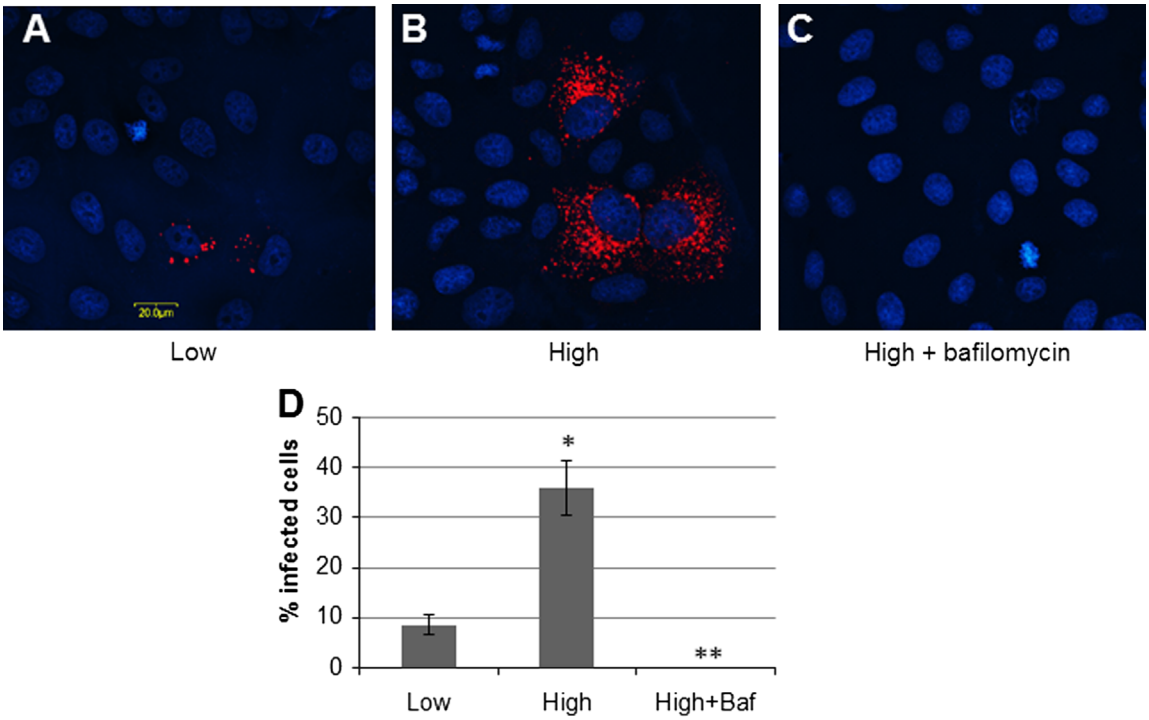

Fig. 9. Inhibition of the V-ATPase reverses the high glucose-mediated increase of infection. MDCK cells were grown in low or high glucose for 24 h, then infected with influenza A H1N1. At the time of infection, cells were also treated with 5 nM bafilomycin A1 (C). Cells were immunostained 48 h post-infection for HA (red) and stained with Hoescht (blue). (D) The percent of infected cells at each condition is presented. * denotes that the difference between high and low glucose has a $P < 0.05$; ** denotes that the high glucose plus bafilomycin A1 data is different from low or high glucose alone, both with a $P < 0.05$. At least four separate fields of cells were counted for each condition as specified in Materials and Methods.

specificity to influenza virus; it has been speculated that influenza virus specifically manipulates this subunit to perhaps trigger endosome acidification at the nuclear periphery (Hao et al., 2008).

In this study, we targeted the V-ATPase proton pumps, which regulate the pH of intracellular compartments, in order to control influenza infection. In this report we demonstrated that regulating the V-ATPase pump activity through glucose metabolism may be a useful approach in controlling and inhibiting influenza replication during infection of mammalian cells. As previously mentioned, the V-ATPase activity can be regulated through glucose depletion leading to disassembly of the V_1 and V_0 domains (Beyenbach and Wieczorek, 2006; Kane, 2006). Additionally, in yeast cells it has been shown that V-ATPase assembly is modulated by the relative concentration of extracellular glucose (Parra and Kane, 1998). Under high glucose conditions in mammalian cells we have provided additional evidence for a higher activity of the V-ATPase (Figs. 5 and 6), which is consistent with the fact that the pumps couple ATP hydrolysis and activity. Furthermore, we have linked glucose levels with V-ATPase activity in relation to influenza viral infection (Fig. 1). Our results are different than those of Marjuki et al., which found that glucose stimulation promoted V-ATPase activity but did not alter influenza viral infection of MDCK or A549 cells (Marjuki et al., 2011). The reason for the discrepancy is not clear; however our treatment conditions (media formulations, glucose concentrations, MOI, and timing) were different, and may have allowed more sensitivity in our assays.

As we found that V-ATPase assembly/activity was based upon available glucose levels, we studied the inhibition of glycolysis upon viral infection levels. Glycolysis is the process by which glucose metabolism occurs to generate a net gain of two ATP, two $NADH^+$ and two molecules of pyruvate. The pyruvate molecules are further metabolized to produce more ATP. Specific enzymes catalyze each stage of glycolysis. Previously, researchers have demonstrated direct links between glycolytic enzymes and specific V-ATPase subunits (Lu et al., 2001, 2004, 2007; Parra and Kane, 1998; Su et al., 2003). We analyzed the effects of inhibition of the hexokinase enzyme, the first enzyme of the glycolytic pathway. With a reduction of glycolysis, the intracellular ATP level would consequently be lowered, leading to a decrease in pump assembly/activity (as we demonstrated in Fig. 8). We showed that inhibition of the glycolytic pathway with the hexokinase inhibitors 2-DG and 3-BrPa reduced influenza infection. A 92.8% decrease in infection was observed with the 5 mM 2-DG treatment, and a reduction of 81.8% with the 600 μM 3-BrPa treatment, when compared to the control cells. Infection was not completely inhibited at our highest dose of 3-BrPa (600 μM); higher 3-BrPa concentrations may be required for complete inhibition of influenza infection. While infection was inhibited with 2-DG and 3-BrPa, "inhibition" of hexokinase with LND significantly increased influenza infection in MDCK cells. These results likely were observed because LND reportedly enhances glycolysis in normal cells, only causing a decrease in glycolysis in cancer cells (Floridi et al., 1981). This selection in activity may be due to LND acting upon the mitochondrially-bound hexokinase, which is usually absent in normal cells (Floridi et al., 1981). This result actually supports our hypothesis that infection is tied to glycolysis, since LND would cause increased glycolysis, and did cause increased infection in MDCK cells.

ATP is considered to activate V-ATPase by binding to the catalytic sites on the A and B subunits of the pump. In the present study, we bypassed glucose metabolism through the addition of extracellular ATP. Addition of extracellular ATP after glycolytic inhibition with 2-DG restored influenza infection in mammalian culture cells, presumably through reassembly of the V_1 and V_0 domains (as shown in Figs. 4 and 8). This suggests that in relation to the influenza viral infection process, glycolysis is not absolutely required to take place in order for the V-ATPase to function in endosomes, as long as additional ATP is provided.

Our work is consistent with what appears to occur clinically in regard to patients and animal models with diabetes. A study that characterized the link between human patients with an active H1N1 infection and diabetes found that patients with diabetes had more severe viral infections and influenza symptoms (Allard et al., 2010). In a mouse model system, it was found that influenza A virus replicated to significantly higher levels in the lungs of diabetic mice than in non-diabetic mice; these higher levels were proportional to higher glucose levels, and could be reversed with insulin treatment (however this effect was only seen with H3N2, not H1N1, virus) (Reading et al., 1998).

Overall, we have found that we can limit influenza viral infection by manipulating the energy available (either in form of glucose or ATP) for the V-ATPase pumps to function. Cells normally grown in a high glucose medium (4–6 mg/ml glucose), when switched to a low glucose medium (1 mg/ml) were much less susceptible to infection by the influenza virus. Inhibition of glucose metabolism yielded the same effect. The infection rate under these varying conditions correlated perfectly with the assembly/disassembly of the V-ATPase pump under the same conditions. Therefore, the lowering of glucose levels and/or inhibition of glycolysis may be an avenue for the reduction of infection in mammals.

Materials and Methods

Cell culture

HeLa and Madin-Darby Canine Kidney (MDCK) cells (ATCC) were cultured in Dulbecco's Modified Eagles Medium (DMEM). A549 cells (lung carcinoma, ATCC) were cultured in F-12 Ham's medium. All media was supplemented with 10% Fetal Bovine Serum (FBS) as well as penicillin/streptomycin and antifungal agents. Cells were maintained at 37 °C in an air jacketed incubator with 5% CO_2.

Influenza viral infection

Cells were plated (8×10^4 cells) in 35 mm cell culture dishes, with coverslips. Infection medium was prepared with influenza A H1N1 virus (A/PR/8/34) (ATCC) with a Multiplicity of Infection (MOI) of 1 (or an MOI of 0.25 in the case of LND), in low (1 mg/ml) glucose DMEM (with no additives), along with 1 μg/ml trypsin, 0.125% BSA, and 1% HEPES. Cells were infected with influenza A H1N1 for 24 h, washed with 1 × PBS, and new medium was added accordingly for 24 h.

Immunostaining

Cells grown on coverslips were rinsed in 1 × PBS and fixed in 4% paraformaldehyde in PBS at room temperature for 15 min. Cells were incubated in incubation mix (1 × PBS, 0.3% BSA, 0.1% Triton X, 5% goat serum) for 10 min. Cells were incubated for 1 h at 37 °C with primary antibody [anti-HA, anti-V_1A, anti- V_1B antibodies (all from Santa Cruz), diluted 1:200 in incubation mix]. Cells were washed 4 times with 1 × PBS followed by incubation for 40 min at 37 °C with secondary antibody [goat-anti-mouse-CY3, donkey-anti-rabbit-CY3 (Jackson Immuno Research Laboratories)] diluted 1:400 in incubation mix. Cells were washed 4 times, including one wash containing Hoescht stain (diluted 1:2000 in 1xPBS). Coverslips were mounted in Fluorescent Mounting Medium (DakoCytomation). Images were taken with an Olympus confocal microscope. Staining intensities were quantified with FluoView software.

Western blotting

Cells were washed in 1 × PBS, resuspended in ELB buffer (0.25 M NaCl, 0.1% NP40, 50 mM HEPES pH 7, 5 mM EDTA, protease inhibitors) and freeze-thawed twice. Equal amounts of protein were loaded onto a 10% SDS-PAGE gel, electrophoresed, and transferred to Immobilon (Millipore). The membrane was blocked in 1 × PBS, 0.1% Tween 20, 5% bovine serum albumin and incubated in primary antibody [anti-V_1A, anti-tubulin (Developmental Studies Hybridoma Bank)] diluted 1:200 in blocking solution, for 1 h at room temperature, and then washed with 1 × PBS, 0.1% Tween 20. The membrane was incubated with goat-anti-mouse-horseradish peroxidase secondary antibody (Jackson ImmunoResearch Laboratories), diluted 1:10,000 in blocking solution, at room temperature for 60 min. The membrane was washed and bound antibody was viewed using SuperSignal (Thermo Scientific Pierce).

Treatments

2-deoxyglucose (2-DG), 3-bromopyruvate (3-BrPa), Lonidamine (LND), adenosine 5'triphosphate disodium salt hydrate (ATP), and bafilomycin A1 were purchased from Sigma.

Glycolytic inhibitors: MDCK or A549 cells were plated in high glucose (4.5–6 mg/ml) DMEM. Cells were treated 24 h later with 2-DG (0, 0.25, 2, and 5 mM), 3-BrPa (0, 30, 300, and 600 μM) or LND (0, 6, 62.5, and 200 μM,) [concentrations were established from (Hulleman et al., 2009)], each for 24 h prior to infection with influenza A H1N1. 24 h post-infection, cells were washed with 1 × PBS and new medium was added, including the inhibitors. Immunostaining was conducted as previously described using an anti-HA primary antibody and a goat-anti-mouse-CY3 secondary antibody (Jackson Immuno Research Laboratories).

ATP: To promote infection: MDCK cells were plated in high (4.5–6 mg/ml) glucose DMEM and treated 24 h later with 2-DG (5 mM). 24 h later, cells were treated with ATP (0, 10, 25, 50 or 100 μM) [concentrations were established from (Probst et al., 1989)] along with influenza A H1N1 and the inhibitor for 24 h. Cells were washed and medium replaced (with no extra ATP nor inhibitor). Twenty four hours later, cells were fixed and stained with an anti-HA antibody as previously described.

ATP: To promote V-ATPase assembly: A549 cells were plated in low (2 mg/ml) or high (6 mg/ml) glucose medium for 24 h. ATP (50 μM) was added for 10 min or 1 h, then the cells were fixed and stained for V_1A as previously described.

Bafilomycin A1: MDCK cells were plated in low (1 mg/ml) or high (6 mg/ml) glucose medium, and 24 h later were treated with 0 or 5 nM bafilomycin A1 [concentrations were established from (Adamson et al., 2011)] and infected with influenza A H1N1. 24 h later the media was replaced with low glucose media (with no bafilomycin A1). Cells were immunostained with anti-HA antibody 24 h later.

Viability

Cells were removed from the culture plates with Cell Stripper (Cellgro), washed with 1 × PBS, and incubated with Guava ViaCount for 5 min. Viability was measured with the ViaCount program of a Guava InCyte flow cytometer.

SNARF-1 staining

MDCK cells were plated in low (1 mg/ml) or high (6 mg/ml) glucose for 24 h. The pH-sensitive SNARF-1 dye (Molecular Probes) was added to cells to a final concentration of 1 μM for 30 min at 37 °C. Cells were rinsed twice with 1 × PBS and mounted in 10%

glycerol in 1xPBS. Live cells were immediately imaged on an Olympus confocal microscope with a single excitation wavelength of 480 nm and the two emission wavelengths of 580 nm (higher fluorescence intensity denotes acidity) and 640 nm (higher fluorescence intensity denotes basicity). Fluorescence intensities were quantified with FluoView software and the ratios of the 580 nm/640 nm intensities calculated.

Quantification and statistics

To determine the percentage of infected cells for a given condition, slides of cells were methodically scanned, and every distinct field that contained infected cells (where possible) were imaged via confocal microscopy. Multiple fields of cells (at least 4 fields for each condition), typically containing at least 100 cells/field, were assessed as to the total number of cells (by counting Hoescht-stained nuclei), and the infected cells (by counting cells with HA staining). P values were determined using the student T-test in Excel.

Acknowledgments

The anti-tubulin antibody developed by M. Klymkowsky was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.06.026.

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