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Host cell autophagy promotes BK virus infection

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ABSTRACT

Autophagy is important for a variety for virus life cycles. We sought to determine the role of autophagy in human BK polyomavirus (BKPyV) infection. The addition excess amino acids during viral infection reduced BKPyV infection. Perturbing autophagy levels using inhibitors, 3-MA, bafilomycin A1, and spautin-1, also reduced infection, while rapamycin treatment of host cells increased infection. siRNA knockdown of autophagy genes, ATG7 and Beclin-1, corresponded to a decrease in BKPyV infection. BKPyV infection not only correlated with autophagosome formation, but also virus particles localized to autophagy-specific compartments early in infection. These data support a novel role for autophagy in the promotion of BKPyV infection.

Introduction

Autophagy is a eukaryotic degradation process that is activated during nutrient deprivation, metabolic stress, developmental processes, and in response to some microbial infections (Wirawan et al., 2012). Early in autophagy a fold of membrane, known as the isolation membrane, curves around cytoplasmic cargo destined for degradation. The ends of the isolation membrane join together to form a double membranous vesicle. This new structure, known as an autophagosome, fuses with the lysosome forming the autolysosome, which is rich in hydrolases that act to enzymatically catabolize the vesicular contents (Juhasz and Neufeld, 2006). By isolating and then directing organelles and large biomolecules toward the lysosome, autophagy enables the cell to break down and redistribute molecules to pathways that are more critical for cell survival.

For some intracellular pathogens autophagy acts as antimicrobial defense by helping to deliver invading microorganisms to the lysosome for degradation, antigen presentation, or toll-like receptor engagement (Lee et al., 2007; Gutierrez et al., 2004; Talloczy et al., 2006; Liang et al., 1998; Virgin and Levine, 2009; Dengjel et al., 2005). Autophagy degrades some intracellular pathogens, such as *Mycoplasma tuberculosis* and herpes simplex virus-1 (HSV-1) (Gutierrez et al., 2004; Jiang et al., 2011; Talloczy et al., 2006). Interestingly, some viruses utilize the autophagosome to promote viral replication, translation, and regulate cell lysis (Hussein et al., 2012; Meng et al., 2012; Meng et al., 2012; Dreux et al., 2009; Sir et al., 2012; Zhou and Munger, 2009; O'Donnell et al., 2011). Many RNA virus including poliovirus, coxsackievirus, coronavirus, and hepatitis C virus induce autophagy levels in the infected host cell to benefit the viral life cycle (Dreux et al., 2009; Wong et al., 2008; Taylor and Kirkegaard, 2008; Maier and Britton, 2012). RNA viruses are thought to utilize the double membranous autophagosome structure as a platform for promoting viral transcription or replication (Maier and Britton, 2012).

The human BK polyomavirus, (BKPyV), is a small double-stranded DNA virus known to cause tumors in rodents. In humans BKPyV is the causative agent of polyomavirus associated nephropathy, a viral complication that affects approximately 5–10% of kidney transplant recipients (Drachenberg et al., 2007; Schaub et al., 2010). As BKPyV is an intracellular pathogen, its life cycle is intimately connected with its host cell. Previous studies have shown the BKPyV requires vesicular acidification during the infectious process, but the virus does not seem to use the canonical endosome–lysosome pathway (Eash and Atwood, 2005; Eash et al., 2004; Jiang et al., 2009). In this study, we explored the importance of autophagy in BKPyV infection.

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Results

Excess amino acids reduce BKPyV infection

To test the affect of nutrient availability on BKPyV infection levels, Vero cells, a green monkey renal epithelial cell line, were incubated with media containing differing concentrations of essential amino acids before and after being challenged with BKPyV (Fig. 1A). Sodium hydroxide was added to amino acid-supplemented media so that media had identical pH levels. Increasing the concentration of amino acids led to a decrease in BKPyV infection in host cells indicating that higher levels of amino acids hinder BKPyV infectivity. Addition of amino acids did not lead to a change in Vero cell death or proliferation (Fig. 1B). One hypothesis is that cells treated with amino acids have lower autophagy levels, as nutrient deprivation is a major activator of cellular autophagy. To evaluate the level of autophagy in amino acid supplemented cells, a plasmid encoding microtubule-associated protein light chain 3 fused to green fluorescent protein (LC3-GFP) was transfected into cells. LC3 is distributed in a diffuse pattern throughout the cytoplasm in the presence of low levels of autophagy (LC3-I), and acquires a distinct punctate distribution during autophagy (LC3-II) (Mizushima, 2004; Kabeya et al., 2000). LC3-GFP transfected cells were treated with 100 nM rapamycin, a drug known to activate autophagy, in media with different concentrations of amino acids. 24 h later the number of LC3-GFP+ punctae per cell was scored for 80 cells. Addition of amino acids led to a decrease in the number of LC3-GFP+punctae per cell suggesting that amino acid supplementation suppressed autophagy (Fig. 1C).

Modulating autophagy levels affects BKPyV infection

To determine whether autophagy is important in BKPyV infection, host cells were treated with pharmacological agents that affect autophagy levels, infected with BKPyV, and stained for VP1 72 h post infection. Rapamycin is a drug that inactivates the protein mammalian target of rapamycin (mTOR), a kinase that promotes cell growth and protein synthesis. Rapamycin also elevates autophagy levels (Meng et al., 2012; Noda and Ohsumi, 1998). Vero cells treated with 100 nM rapamycin showed elevated BKPyV infection levels compared to DMSO-treated cells indicating that inhibition of mTOR elevates infection (Fig. 2A and F). 3-MA is an inhibitor of autophagy that blocks phosphatidylinositol 3-kinase (PI3K) activation, an event that is necessary for membrane trafficking during autophagy (Blommaart et al., 1997; Petiot et al., 2000). Cells treated with 3-MA showed a dose-dependent decrease in infection (Fig. 2B and F) suggesting that autophagy promotes BKPyV infection. Rapamycin was able to restore infection to 3-MA-treated Vero cells (Fig. 2C). Another autophagy inhibitor, spautin-1, degrades class III PI3K by inhibiting the activity of the ubiquitin specific peptidases, USP10 and USP13 (Mateo et al., 2013; Liu et al., 2011). Spautin-1 also reduced BKPyV infection in a dose-dependent manner (Fig. 2D and F). Vero cells treated with bafilomycin A1, a vacuolar H+ ATPase inhibitor that blocks lysosomal acidification that is necessary for both autophagic degradation and endosome maturation, were also less susceptible to BKPyV infection suggesting that vesicular acidification is important for BKPyV infection (Fig. 2E and F) (Tanigaki et al., 2003). Taken together these data suggest that autophagy may be important in BKPyV infection. A trypan blue exclusion assay verified that the pharmacological agents used in this study no statistically significant difference in cytotoxicity compared to no drug control (Supplemental Fig. 1). BKPyV-infected HeLa cells, which are permissive to BKPyV and have similar infection kinetics to Vero cells, showed nearly identical infection levels after drug treatments (Supplemental Fig. 2). When VP1 staining was compared in untreated cells infected with BKPyV for 3 h versus 72 h, VP1 nuclear staining was only observed in the 72 h condition

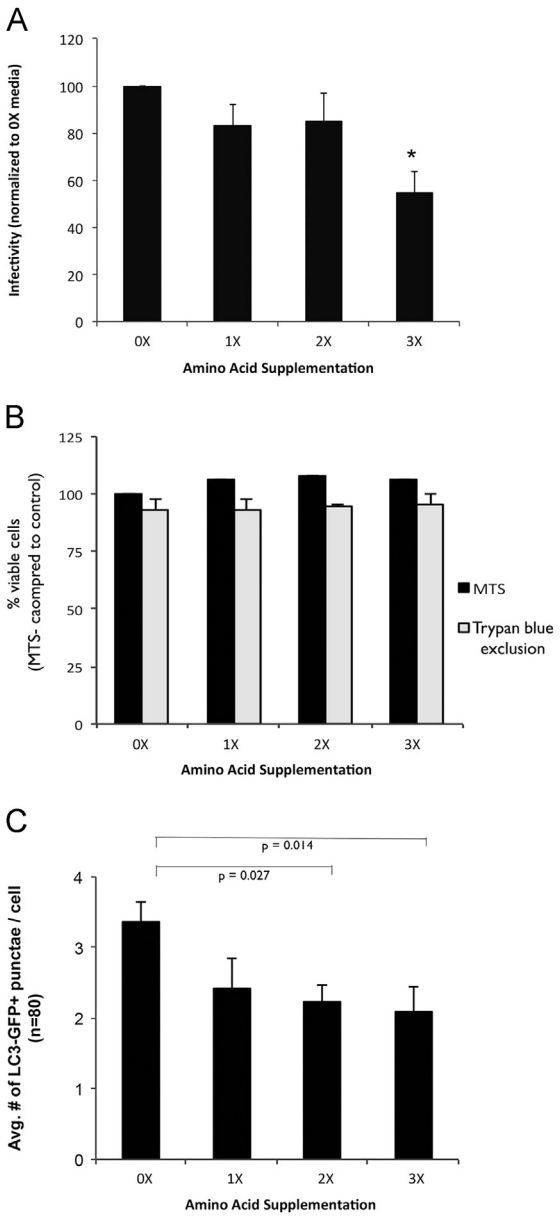

Fig. 1. Amino acid supplementation decreases BKPyV Infection. (A) Vero cells were challenged with BKPyV in EMEM media with 5% fetal bovine serum with and without additional essential amino acids. EMEM without additional supplementation is labeled 0 ×. After infection, the cells were replaced with EMEM media with 5% fetal bovine serum with or without the addition of amino acids and left for duration of infection. Cells were fixed at 72 h post infection using paraformaldehyde and permeabilized with Triton X-100. Infection was determined by using an antibody (PAB597) specific to the viral protein VP1 and then scoring the number of VP1+ cells using indirect immunofluorescence. (B) Cell death was evaluated 24 h following amino acid supplementation by scoring the number of cells excluding trypan blue and graphing the percentage of cells that excluded the trypan blue dye. To measure cell proliferation a MTS assay was used. Vero cells in 96 well plate were incubated with amino acids for 24 h after which 20 μl of CellTiter 96 AQueous One Solution Reagent – MTS (Promega) reagent was added directly to cells and media for 2 h, and absorbance was measure at 450 nm. The absorbance of 0 × was used as a control for cell viability. (C) Vero cells were transfected with a plasmid expressing LC3-GFP and incubated for 24 h. Cells were treated with different concentrations of amino acid for 24 h in the presence of 100 nM rapamycin. LC3-GFP distribution was observed using immunofluorescence. The average number of LC3-GFP+ punctae per cell for 80 cells was scored. Each graph represents the average of three or more experiments. Error bars on bar graphs represent the SEM. $^{**}p$-value < 0.02.

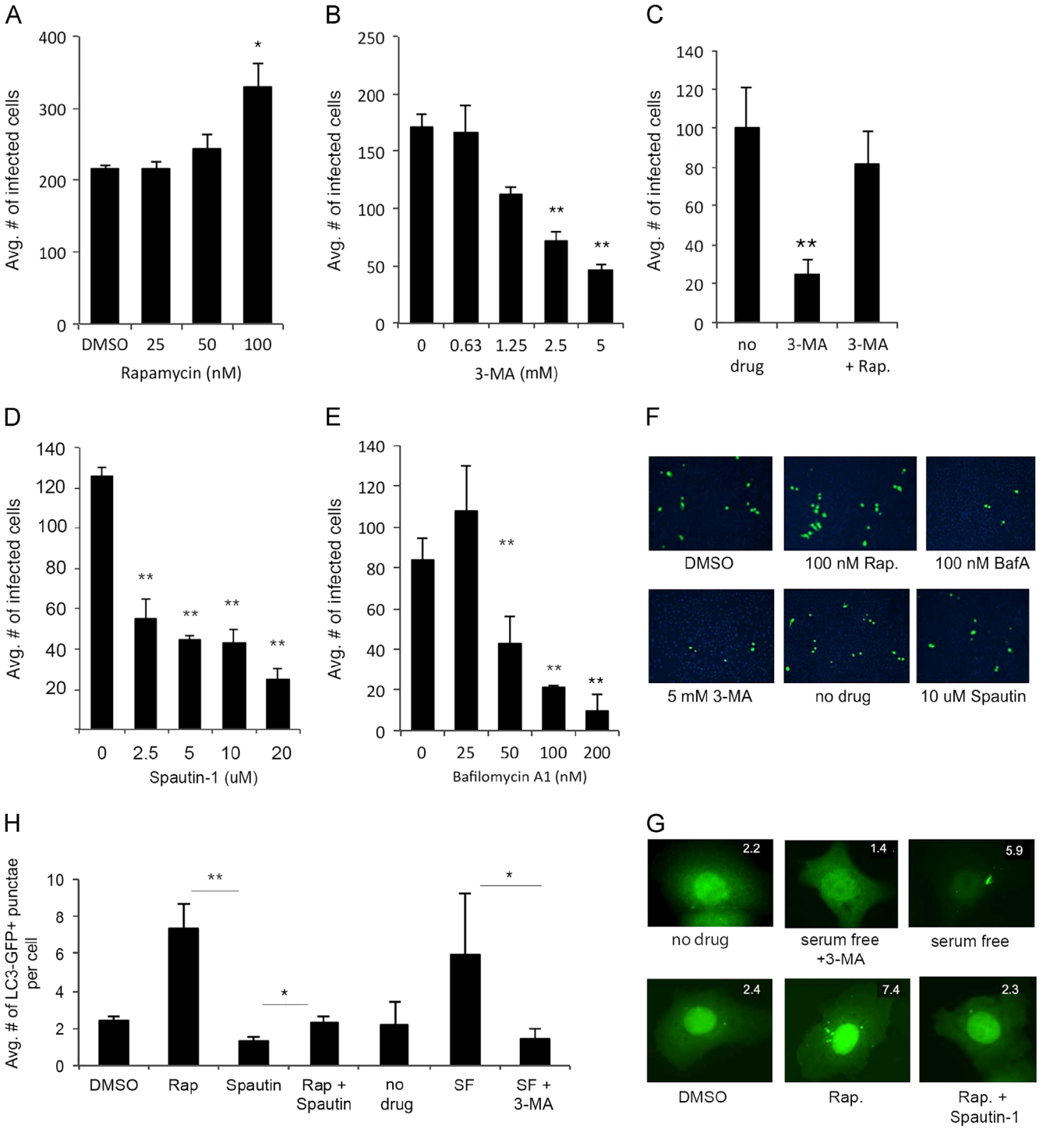

Fig. 2. Autophagy affects BKPyV infection. (A) Vero cells were pretreated with rapamycin or an equal volume of DMSO for 24 h, infected with BKPyV, and rapamycin added back for 24 h. Cells were fixed and stained for VP1 at 72 h post infection as in Fig. 1. (B) Vero cells were pretreated with 3-MA for 3 h, infected with BKPyV in the absence of 3-MA, and then media containing 3-MA was added back to cells for 24 h. Cells were fixed and stained for VP1 at 72 h. (C) Vero cells were pretreated with 100 nM of rapamycin for 24 h and 5 mM of 3-MA for 3 h where indicated, infected with BKPyV, and drugs added back for 24 h. After 72 h cells were stained for VP1 expression. (D) After Vero cells were infected with BKPyV, cells were treated with spautin-1 or DMSO for 72 h after which cells were fixed and stained from VP1 protein. (E) Vero cells were infected with BKPyV in the absence of drug. After infection, cells were treated the indicated concentrations of bafilomycin A1 or the DMSO control for 24 h. Cells were fixed and stained for VP1 at 72 h (F) Representative images of indirect immunofluorescence staining of VP1 (BKPyV infected cells) in Vero cells treated with drugs from 0 to 24 h at 72 h post infection. (G) Vero cells were transfected with a plasmid expressing LC3-GFP and incubated for 24 h. Cells were treated with media alone (no drug), serum free media for 3 h, 100 nM rapamycin for 24 h, 5 uM spautin-1 for 24 h, 5 mM 3-MA for 3 h, DMSO for 24 h and fixed then with paraformaldehyde. LC3-GFP distribution was observed using immunofluorescence. A representative image of an LC3-GFP+ expressing cell after treatment shows the change in LC3 localization (magnification 1000 ×). The number in the top right corner is the average number of punctae scored for 80 cells. (H) The average number of LC3-GFP+ punctae per cell for 80 cells was scored. The treatments were as follows: 100 nM rapamycin, DMSO, and 5 uM spautin-1 for 24 h, and serum free media and 5 mM 3-MA for 3 h. Graphs represent data from three or more experiments. Error bars on bar graphs represent the SEM. *p-value = 0.02–0.05 and **p-value < 0.02, or p-value was included.

indicating that input virus canot cause the strong nuclear signal dize in Fig. 2F (data not shown).

Pharmacological agents influence LC3 distribution

To test the effect of these drugs on autophagy levels in Vero cells, cells were transfected with a plasmid encoding LC3-GFP. In LC3-GFP-expressing Vero cells, we found rapamycin and serum starvation increased autophagy levels as determined by a change in cellular LC3 localization and an increase in LC3-GFP+ punctae, while 3-MA and spautin-1 inhibited serum starvation and rapamycin-induced autophagy, respectively (Fig. 2G and H). Our results were consistent with previous findings showing these drugs influence autophagy levels (Hussein et al., 2012; Meng et al., 2012; Noda and Ohsumi, 1998; Blommaart et al., 1997; Mateo et al., 2013; Tanigaki et al., 2003; Seglen and Gordon, 1982; Blazquez et al., 2013).

Autophagy inhibitors reduce BKPyV infection early in the viral life cycle

To elucidate the kinetics of autophagy-specific regulation of BKPyV infection, Vero cells were treated with autophagy inhibitors for various intervals before and following viral challenge. A significant decrease in infection was observed when cells were treated within the first 24 h following infection or for the 72 h duration of infection (Fig. 3). BKPyV was significantly inhibited by the addition of 3-MA, spautin-1 and bafilomycin A1 during the first 8 h of infection suggesting that autophagy is important early in the BKPyV infectious process (Fig. 3). Autophagy may play an antiviral role at later stages in infection as addition of autophagy inhibitors at time intervals later in the viral life cycle increases infection.

Knockdown of autophagy genes decrease BKPyV infection

To further test the effect of restricting autophagy on BKPyV infection, siRNA molecular silencing was used to reduce levels of LC3, Beclin-1, and ATG7, genes that regulate autophagy. In HeLa cells, a mixture of 3–5 siRNA molecules directed against Beclin-1 or ATG7 reduced BKPyV infection. Interestingly, LC3 siRNA treatment did not decrease BKPyV infection (Fig. 4B), suggesting that greater knockdown of the autophagic LC3-II form is required to effectively inhibit BKPyV (Fig. 4A). Taken together, these data suggest autophagy-specific proteins promote BKPyV infection in HeLa cells.

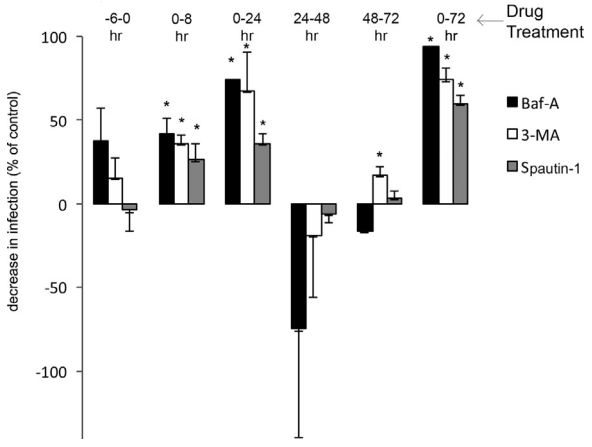

Fig. 3. Autophagy Inhibitors decrease BKPyV infection early in the viral life cycle. Cells were treated for the indicated times before or after BKPyV infection with 5 mM 3-MA, 5 uM Spautin-1, 100 nM bafilomycin-A, and the media- or DMSO-control. Cells were fixed at 72 h post infection, and infection was determined by scoring the number of VP1 expressing cells. The effect of the drugs on infection levels was calculated by comparing the average number of infected cells in control-treated conditions to the average number of infected cells in inhibitor-treated cells. A positive value indicates the autophagy-inhibitor reduced BKPyV infection. Each bar graph represent the average of three experiments *p-value < 0.02. Error bars on bar graphs represent the SEM.

BKPyV associates with autophagosome

We next asked whether BKPyV physically interacts with the autophagosome. To address this the localization of BKPyV and LC3-GPF vesicles was analyzed using confocal microscopy. LC3-GFP-expressing Vero cells were infected with infectious AlexFluor-633-labeled BKPyV (BKPyV-633). Of the 60 cells that were analyzed, 27% ($n=16$) of cells showed viral particles had penetrated the cell membrane and entered the host cell. Analysis of the infected cells revealed that 38% of these cells (6/16 cells) showed strong colocalization of BKPyV and LC3-GFP+ in small or large autophagosomes. This differed from the 0% of cells (0/16 cells) that showed BKPyV colocalized with the diffusely expressed, non-autophagic LC3-GFP+. Our data suggest that BKPyV is more likely to colocalize with LC3-GFP+ when it is found in an autophagosome. This colocalization of BKPyV with the LC3-GFP+ autophagosomes was observed at 3 h post infection, indicating that the virus associates with an autophagic vesicle at a time point consistent with endocytic trafficking (Fig. 5A–D).

In LC3-GFP+ transfected Vero cells, localization of LC3 was found in one of three patterns: diffuse (Fig. 2G), small and filled-in autophagosomes (Fig. 5D), or in larger donut-shaped autophagosomes (Fig. 5C). After establishing that BKPyV localized to LC3-GFP autophagosome (Fig. 5A and B), we next examined if the cellular location of BKPyV correlated with autophagosome formation. LC3-GFP expressing Vero cells were challenged with BKPyV-633 and then location of BKPyV-633 and type of LC3-GFP pattern recorded. In Vero cells that showed no internalized BKPyV-633, LC3-GFP was found to be primarily diffuse or in small punctae (Fig. 5E, left). In cells infected with BKPyV, as determined by the presence of intracellular BKPyV-633, LC3-GFP localization changed and it localized to small punctae and larger autophagosomes (Fig. 5E, middle and right). Cells that showed colocalization of BKV-AF633 and LC3-GFP were scored for the pattern of LC3-GFP distribution. When BKV-AF633 and LC3-GFP showed overlapping localization, LC3-GFP was distributed to small or larger autophagosomes (Fig. 5E, right). These data suggest that BKPyV infection may not only rely on autophagy for viral infection, but may also play an active role in promoting cellular autophagy.

Discussion

Autophagy functions as an antimicrobial defense against some viruses and promotes the replication and infection of other viruses (Gutierrez et al., 2004; Liang et al., 1998; Talloczy et al., 2006; Hussein et al., 2012; Meng et al., 2012; O'Donnell et al., 2011; Sir and Ou, 2010). Autophagy may have evolved as an antimicrobial defense process because its degradative function within the cell eliminates some invading microbes. For example, autophagy is believed to control and degrade both Sindbis virus and herpes simplex virus-1 (HSV-1) (Liang et al., 1998; Talloczy et al., 2006). HSV-1 is equipped with a viral protein called ICP34.5, which inactivates autophagy. HSV-1 mutants missing ICP34.5 show greater autophagic degradation and an attenuated neurovirulence phenotype in mice (Talloczy et al., 2006; Orvedahl et al., 2007). Autophagy has also been shown to stimulate the innate immune system and link it to the adaptive immune system. Autophagy can

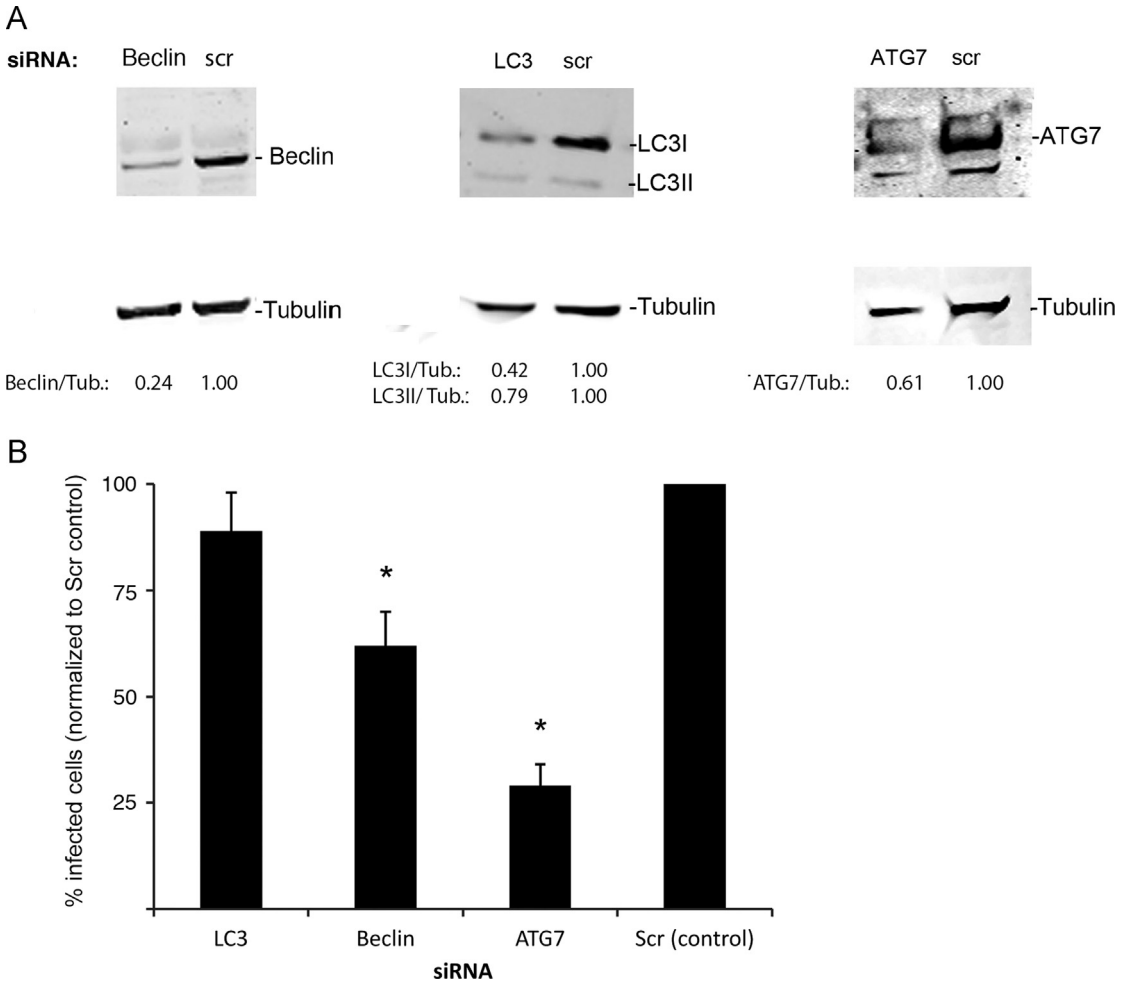

Fig. 4. Knockdown of autophagy proteins, Beclin-1 and ATG7, reduced in BKPYV infection. (A) HeLa cells were transfected with 70 nM siRNA. After 48 h, cells were harvested, protein lysates were resolved by SDS-PAGE gel, and immunoblot analysis for LC3, Beclin-1, ATG7, and tubulin was performed. Tubulin expression was used as a loading control. Numeric values represent the ratio of immunoblot band intensity of indicated gene/tubulin. (B) HeLa cells were transfected with 70 nM siRNA, which contained a mixture of 3–5 siRNA molecules directed to different regions of a gene, and incubated for 48 h. Cells were then infected with BKPyV and fixed at 72 h post infection. Infected cells were by scored by VP1 expression. The graph represents the percentage of infected cells from three or more experiments, each which have been normalized to scrambled siRNA control sequence (Scr). *p-value of siRNA treatment compared to Scr control is < 0.02. Error bars on bar graphs represent the SEM.

promote antigen processing and presentation on major histocompatibility (MHC) molecules (Dengjel et al., 2005). Autophagosomes have been reported to help transport microbial cargo towards the lysosome where toll like receptor (TLR) engagement leads to Type I interferon production and other innate immune responses (Lee et al., 2007). Moreover, surface TLR engagement and signaling can activate autophagy (Xu et al., 2007). Mutations and deletions in autophagy genes are also associated with intestinal inflammation, disrupted Paneth cell secretion, and altered gut microflora (Virgin and Levine, 2009).

Paradoxically, autophagy promotes the replication and infection of a number of viruses (Hussein et al., 2012; Meng et al., 2012; O'Donnell et al., 2011; Sir and Ou, 2010). There are multiple ways that autophagy promotes viral infection. RNA viruses require intracellular membranes to serve as scaffolds for viral replication within the cytoplasm (Salonen et al., 2005). Autophagy orchestrates the trafficking of vesicles to new locations within the cell thereby providing new membranous structures for viral replication to occur. Autophagy proteins have also been shown to aid in the translation of Hepatitis C virus proteins as well as viral replication (Sir et al., 2012; Dreux et al., 2009). Some DNA viruses also utilize autophagy. For example, adenovirus and the E7 protein of human papillomavirus 16 (HPV16) induce autophagy, which researchers hypothesize stimulates host cell death resulting in greater release of viral progeny (Zhou and Munger, 2009; Rodriguez-Rocha et al., 2011).

Recently, autophagy has been demonstrated to occur in human JC polyomavirus infected cells. JC polyomavirus T-antigen (T-Ag) was found to suppress expression of a host autophagy gene called Bag3. Overexpression of Bag3 in host cells, results in elevated autophagy levels and decreases JC infection. These data suggest that autophagy acts as an anti-viral process in JC polyomavirus infection (Sariyer et al., 2012). In contrast, elevation of host autophagy occurs when cells were made to express small t-antigen of simian virus 40 (SV40), a polyomavirus family member related to BKPyV (Kumar and Rangarajan, 2009). The small t antigen seems to help the cells to survive and respond to low nutrient conditions by stimulating host AMP-activated protein kinase (AMPK). When AMPK is activated it inactivates mTOR and thus increases the amount of autophagy occurring within the cell. These steps are thought to block premature host cell death during the viral life cycle, a state that would maximize viral production (Kumar and Rangarajan, 2009). Whether polyomavirus large and small t-antigen have opposing roles in controlling autophagy or if autophagy influences individual polyomaviruses members differently has yet to be determined.

In this report, we show that induction of host autophagy early in the viral life cycle promotes BKPyV infection. Pharmacological

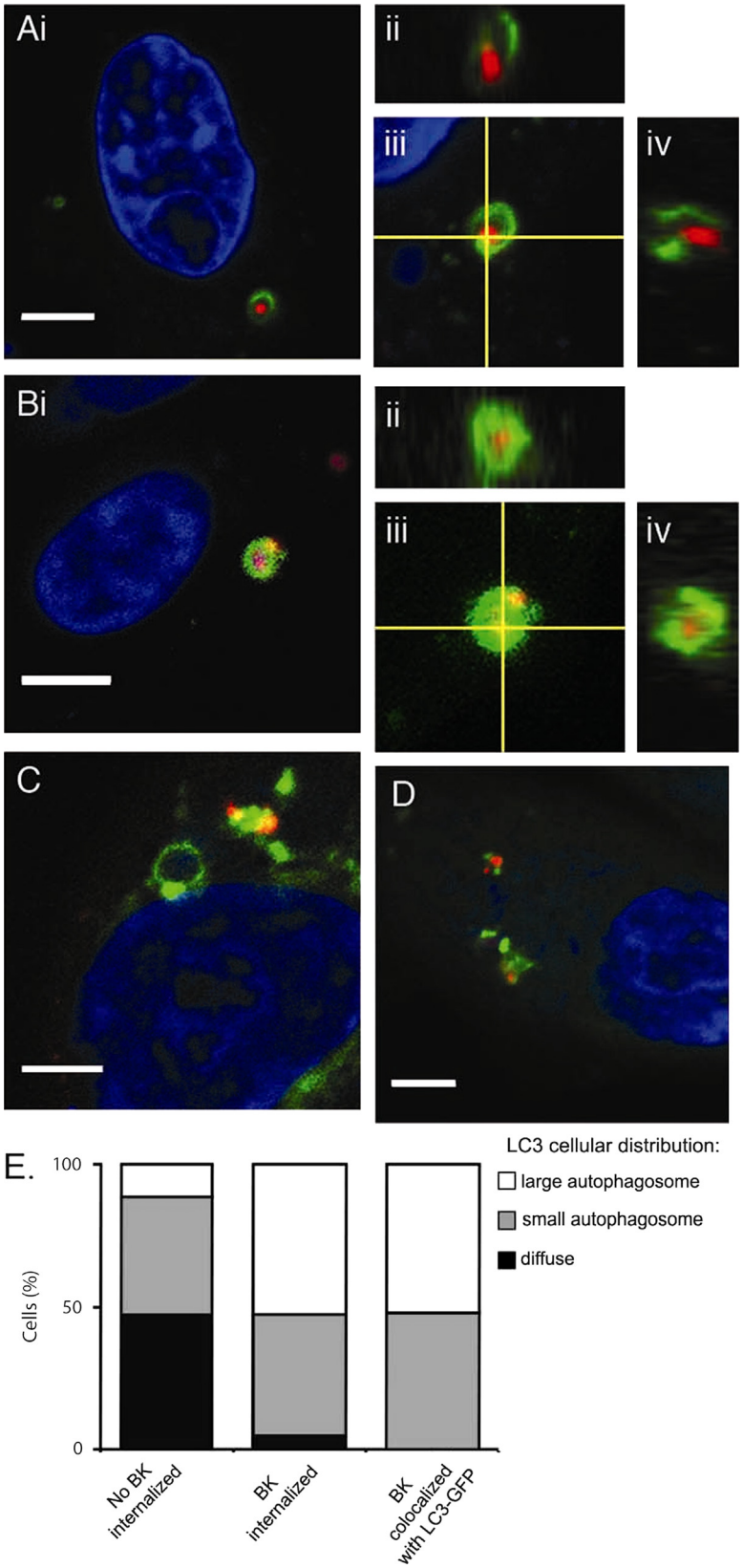

Fig. 5. BKPyV localizes to the autophagosome. (A–D) Vero cells were plated on coverslips and transfected with 0.5 μg LC3-GFP plasmid and incubated for 24 h. Cells were then infected with BKPyV-AF633 at 37 °C for 3 h, fixed and mounted onto slides using DAPI mounting media. Cells were analyzed for appearance of LC3-positive autophagosomes (green) and infection of labeled BKPyV (red) use a Zeiss 710 confocal microscope and Zen imaging software. Images analyzed using ImageJ image analysis software. Four representative cells show that BKPyV-Af633 resides within the LC3-GFP+ autophagosome and with smaller LC3-GFP+ punctae (magnification 630X). (A and B) (ii) An orthogonal Z slice of (iii) (along yellow *x*-axis). (iii) A magnified image of BKPyV and LC3-GFP+ as shown in (i). (iv) An orthogonal Z slice of (iii) (along yellow *y*-axis). (E) LC3-GFP transfected cells were scored for the pattern of LC3-GFP distribution ($n=60$, left). BKPyV-633 infected/LC3-GFP transfected cells were scored for LC3-GFP distribution ($n=60$, middle). BKPyV-633 infected that showed colocalization with LC3-GFO were scored for pattern of LC3-GFP distribution ($n=60$, right).

autophagy inhibitors, high amino acid concentrations, and knockdown of autophagy genes reduced BKPyV infection, while rapamycin treatment led to an increase in BKPyV infection. BKPyV is sensitive to autophagy inhibitors during the first 8 h of infection, a time period consistent with viral entry and intracellular trafficking (Eash and Atwood, 2005; Eash et al., 2004; Jiang et al., 2009). Interestingly we did note that addition of autophagy inhibitors from 24 to 48 h post infection increases BKPyV infection of Vero cells (Fig. 3). We also observed a similar increase in infection levels when autophagy inhibitors were added 16–24 h following infection (data not shown). From 16 to 48 h post infection, T-Ag transcription and protein production, viral replication, and V-Ag protein production occur in this sequential order (Jiang et al., 2009). Therefore it is possible that at this time frame, autophagy acts has an antiviral mechanism by degrading newly translated viral proteins. Addition of autophagy inhibitors midway through the viral lifecycle may allow more viral protein synthesis and virion assembly to occur. Eukaryotic cells respond to stressful stimuli using autophagy and the unfolded protein response. Recent evidence suggests that Rab1 protein may have important roles in both pathways (Chua and Tang, 2013). Therefore pharmacologically blocking autophagy between 16 h and 48 h post viral challenge may elevate BKPyV infection by reducing the unfolded protein response allowing translation to proceed despite ER-stress.

Using confocal immunofluorescence, our data also shows that BKPyV was found within LC3-positive structures 3 h following infection (Fig. 5A and B). Strikingly, BKPyV infected cells correlated with a change in the localization of LC3-GFP from a diffuse pattern to a small and large punctate distribution pattern suggesting that BKPyV has a mechanism to stimulate host autophagy (Fig. 5E).

Some of the early steps in the BKPyV life cycle have been elucidated in recent years. BKPyV attaches to its host cell by binding to a disialic acid-containing b-series ganglioside receptor (GD3, GD2,GD1b, GT1b) (Neu et al., 2013; Low et al., 2006). Upon caveolae-mediated endocytosis, BKPyV enters the cell and requires some acidification event in the first few hours following infection (Eash et al., 2004; Jiang et al., 2009). Dynamic microtubules are thought to help shuttle viral particles to the ER where virus localizes at 8–12 h following adsorption (Jiang et al., 2009). Within the ER, capsid disassembly occurs. BKPyV also utilizes the proteasome and ER-associated degradation pathway (ERAD) to help BKPyV exit from the ER and for viral DNA accumulate in the cytoplasm (Jiang et al., 2009; Bennett et al., 2013). Nuclear transport of viral DNA then occurs.

This is the first report indicating that autophagy plays a role in BKPyV infection of host cells. Our study indicates that BKPyV localizes to the autophagosome 3 h post infection and that autophagy aids in productive BKPyV invasion of host cells. While the exact reason for this BKPyV-autophagosome interaction has yet to be elucidated, there are many possible explanations. First, an autophagosome may facilitate trafficking the virus towards the ER for viral disassembly. Autophagosomes use microtubules to shuttle themselves towards the lysosome to and group together with other autophagosomes in perinuclear regions (Jahreiss et al., 2008; Mackeh et al., 2013; Bouzas-Rodriguez et al., 2012). Drug studies show that microtubule assemble and disassembly is necessary for BKPyV infection (Eash and Atwood, 2005; Jiang et al., 2009). Therefore BKPyV may utilize the coordinated interaction between the autophagosome and microtubules to be shuttle closer to the ER. BKPyV does not seem to use the canonical early to late endosomal pathway to reach the ER, so this alternative trafficking strategy is a possibility ((Eash and Atwood, 2005; Eash et al., 2004; Jiang et al., 2009) personal communication with Mengxi Jiang and Michael J. Imperiale).

Another second possibility is that BKPyV interacts with the autophagosome to utilize the low pH and hydrolase-rich environment during the autophagosome–lysosome fusion process. Other studies have shown that acidification is necessary for viral infection (Mateo et al., 2013; Mackeh et al., 2013) Moreover, BKPyV infection requires an acidification event to occur within 2 h following infection (Eash et al., 2004; Jiang et al., 2009). BKPyV may utilize this low pH environment or specific enzymes that become activated in low pH to modify its capsid or partially degraded structural proteins. A third possibility is that stimulating autophagy may promote a pro-survival cell response that primes the cell to deal with the stress of a viral infection. In some cells types, autophagy is reported to inhibit cell death, and this block is cell death has been associated with greater viral yield (Kumar and Rangarajan, 2009; Tovilovic et al., 2013; Tovilovic et al., 2013; Joubert et al., 2012). Conversely for some viruses, activation of autophagy leads to apoptosis, which increases the number of viral particles for same virus species (Xi et al., 2013; Shai et al., 2013).

Autophagy is a complex process that affects viruses differently depending on cell type, viral species, and viral life cycle stage. Our observations suggest that autophagy plays a positive role in BKPyV infection process. How exactly autophagy aids BKPyV and whether these observations extend to other polyomavirus members, including the wide array of recent discovered polyomaviruses, warrant further investigation.

Methods

Cell and virus

Vero and HeLa cells (American Type Culture Collection; ATCC) were maintained in a humidified 37 °C CO_2 chamber in Eagle's minimal essential medium (EMEM) (Mediatech) supplemented with 1% penicillin/streptomycin (HyClone) and 5% and 10% heat inactivated fetal bovine serum (Mediatech), respectively. The Dunlop strain of BKPyV Gardner (ATCC) were propagated in Vero cells and purified using CsCl as described previously (Raptis, 2001; Eash et al., 2004). To label BKPyV, fluorochrome conjugation with 10 μg/mL of AlexaFluor 633 carboxylic acid–succinimidyl ester was added to BKPyV according to the manufacturer's labeling procedure (MP00143; Molecular Probes). The infectivity of CsCl purified BKV before and after labeling (BKPyV-633) was nearly identical.

Infection of host cells

5×10^3 cell were plated in 96-well culture plates. The following day, cells were treated with multiple drugs that influence autophagy levels, rapamycin, 3-methyladenine, bafilomycin-A, DMSO, and spautin-1 (Sigma) before and after being infected with BKPyV (MOI:5) for 1 h at 37 °C. 72 h post infection, cells were fixed using 2% paraformaldehyde (EMB) and permeabilized using 0.5% Triton X-100 (Shelton Scientific). To detect the number of infected cells expressing viral protein, cells were incubated with the anti-V antigen (V-Ag) monoclonal antibody PAB597, secondary Alexa-Fluor-488-labeled goat anti-mouse antibody (Molecular Probes), and then counterstained with DAPI-mounting media (Vector Labs). The PAB597 hybridoma produced a monoclonal antibody against the SV40 major capsid protein VP1 that cross-reacts with BKPyV VP1. Infected cells were scored using a Nikon epifluorescence microscope (Eclipse E800; Nikon, Inc.). Approximately 20,000 cells, the entire surface of the 96 well, were screened for V-Ag expression.

Amino acid supplementation

EMEM media was supplemented with additional the MEM essential amino acids found in MEM media (50 × MEM Amino

Acid Solution from Life Technologies). The concentration of amino acids found in 1× supplementation experiments is as follows: 1.2 mM L-arginine hydrochloride, 200 nM L-cysteine, 400 nM L-histidine hrdrochloride-H20, 800 nM L-isoleucine, 3.6 mM L-leucine, 790 nM L-lysine hydrochloride, 200 nM L-methionine, 400 nM L-phenylalanine, 800 nM L-threonine, 100 nM L-tryptophan, 400 nM L-tyrosine, and 800 nM L-valine. A 1X supplementation corresponded to the manufacturer's suggested supplementation, while 2× was twice this amount. NaOH was added to restore supplemented media to pH 7.2. Cells were treated with these media starting 24 h prior to infection and remained present for the 72 h following infection. During infection, BKPyV in EMEM with 2% FBS and no amino acid supplementation was added to cells.

Cell death assays

Vero cells were plated at 1×10^4 in 96 well plates. Media containing excessive amino acids or drugs were added to cells and incubated for 24 h. To evaluate cell death using trypan blue exclusion, cells were lifted from plate using trypsin for 5 min. Media was added to inactivate trypsin. A 1:1 mixture of the cell suspension was mixed with trypan blue. The total cell number versus cells that appeared blue was scored using a hemacytometer. To evaluate cell proliferation, 20 μl of a tertazolium compound from CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) (Promega) was added to media and cells after incubation with amino acids or drugs for 24 h. The plate was incubated for 2 h at 37 °C and then absorbance read at 450 nm using plate reader (BioRad).

LC3 cellular distribution

Vero cells were plated at 1×10^5 in a 6 well plate with a glass coverslip and transfected with 1 μg of human LC3-EGFP (Addgene) using 5 μl Fugene-HD (Jackson et al., 2005). The next day, transfected cells were treated with complete media, serum starved for 3 h with or without 5 mM 3-MA and the number of LC3+ punctae in 80 cells was scored using immunofluorescence and oil immersion. The number of punctae per cell was also determined for LC3-GFP+ transfected Vero cells treated with DMSO, and 100 nM of rapamycin with or without 5 μM spautin-1 for 24 h.

siRNA knockdown

1×10^5 HeLa cells were plated onto a 12 well dish. The next day, cells were transfected with 70 nM siRNA using 4 μl Lipofectamine 2000 and antibiotic free media (Invitrogen). The following siRNA molecules used in this study consist of a mixture of three to five different sequences that specifically target different regions of the same gene: ATG7 (Hs_APG7L, SI02655373, Qiagen) negative control sequence (1027310, Qiagen), Beclin-1 (sc29797, Santa Cruz), LC3B (sc43390, Santa Cruz) and negative control siRNA-A (sc37007, Santa Cruz). 48 h following transfection with the siRNA molecular, cells were either infected with BKPyV at an MOI:10 and scored for VP1 expression using indirect immunofluorescence or protein lysates was collected.

Immunoblot analysis

Cells were plated at 1×10^5 in 12 well dishes and transfected with siRNA. Two days later, protein lysates were collected from adherent cells by removing media and 150 μl of 2× Laemmli buffer with 5% β-mercaptoethanol was added. After 5 min, cell were scraped from the well surface, collected in an eppendorf tube and, boiled for 10 min. Half of this sample was applied to a 4–15% SDS-PAGE acrylamide gel (Biorad) and gel electrophoresis conducted at 100 V. The protein on the gel was then transferred to an equilibrated PVDF membrane (Biorad) using a semi-dry transfer apparatus (Biorad) at 15 V for 15 min. The membrane was blocked using 5% milk in Tris buffer saline with 1% Tween (TBST) for 1 h. Antibodies in 1% milk in TBST were added to the blots and incubated overnight at 4 °C. Antibodies used in this study were: rabbit anti-ATG7 (2631, Cell Signaling), rabbit anti-Beclin-1 (sc-11427, Santa Cruz), rabbit anti-GAPDH (14C10, Cell Signaling), mouse anti-tubulin (sc-5286, Santa Cruz), goat anti-mouse AlexaFluor 790 (A-11375, Technologies), and goat anti-rabbit AlexaFluor 680 (A-21076, Life Technologies). The TBST washed blots were incubated with the secondary antibody in 1% milk in TBSTS for 1 h at room temperature and developed using Li-Cor blot scanner and densitometry measured using ImageJ.

Confocal microscopy

1×10^5 Vero cells were plated onto a 6-well dish containing a glass coverslip and transfected with 1 μg of LC3-GFP using 5 μl of Fugene-HD. 24 h after transfection, cells were challenged with BKPyV-633 (MOI 20) in 2% FBS EMEM media for 3 h. Cells were then washed 2× with EMEM and fixed using 2% paraformaldehyde for 15 min. Cells were counterstained with DAPI-mounting media (Vector Labs) and applied to a microscope slide and visualized using Zeiss LSM 510 Meta laser-scanning confocal microscope. Images were analyzed using ImageJ image analysis software. Colocalization of BKPyV-633 and LC3-GFP+ vesicles was investigated in 60 cells. Cells were also scored for their predominate type of LC3 distribution in (1) all cells, (2) only cells where BKPyV had entered, and (3) only cells where colocalization of BKPyV-633 and LC3-GFP was observed.

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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.2014.03.009.

References

Blommaart, E.F., Krause, U., Schellens, J.P., Vreeling-Sindelarova, H., Meijer, A.J., 1997. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. Eur. J. Biochem. 243 (1-2), 240–246.

Blazquez, A.B., Escribano-Romero, E., Merino-Ramos, T., Saiz, J.C., Martin-Acebes, M.A., 2013. Infection with usutu virus induces an autophagic response in mammalian cells. PLoS Negl. Trop. Dis. 7 (10), e2509.

Bennett, S.M., Jiang, M., Imperiale, M.J., 2013. Role of cell-type-specific endoplasmic reticulum-associated degradation in polyomavirus trafficking. J. Virol. 87 (16), 8843–8852.

Bouzas-Rodriguez, J., Zarraga-Granados, G., Sanchez-Carbente Mdel, R., et al., 2012. The nuclear receptor NR4A1 induces a form of cell death dependent on autophagy in mammalian cells. PLoS One 7 (10), e46422.

Chua, C.E., Tang, B.L., 2013. Linking membrane dynamics and trafficking to autophagy and the unfolded protein response. J. Cell Physiol. 228 (8), 1638–1640.

Dengjel, J., Schoor, O., Fischer, R., et al., 2005. Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. Proc. Natl. Acad. Sci. USA 102 (22), 7922–7927, http://dx.doi.org/10.1073/pnas.0501190102.

Dreux, M., Gastaminza, P., Wieland, S.F., Chisari, F.V., 2009. The autophagy machinery is required to initiate hepatitis C virus replication. Proc. Natl. Acad. Sci. USA 106 (33), 14046–14051, http://dx.doi.org/10.1073/pnas.0907344106.

Drachenberg, C.B., Hirsch, H.H., Papadimitriou, J.C., et al., 2007. Polyomavirus BK versus JC replication and nephropathy in renal transplant recipients: a prospective evaluation. Transplantation 84 (3), 323–330, http://dx.doi.org/10.1097/01.tp.0000269706.59977.a5.
Eash, S., Atwood, W.J., 2005. Involvement of cytoskeletal components in BK virus infectious entry. J. Virol. 79 (18), 11734–11741, http://dx.doi.org/10.1128/JVI.79.18.11734-11741.2005.
Eash, S., Querbes, W., Atwood, W.J., 2004. Infection of vero cells by BK virus is dependent on caveolae. J. Virol. 78 (21), 11583–11590, http://dx.doi.org/10.1128/JVI.78.21.11583-11590.2004.
Gutierrez, M.G., Master, S.S., Singh, S.B., Taylor, G.A., Colombo, M.I., Deretic, V., 2004. Autophagy is a defense mechanism inhibiting BCG and mycobacterium tuberculosis survival in infected macrophages. Cell 119 (6), 753–766, http://dx.doi.org/10.1016/j.cell.2004.11.038.
Hussein, I.T., Cheng, E., Ganaie, S.S., et al., 2012. Autophagic clearance of sin nombre hantavirus glycoprotein gn promotes virus replication in cells. J. Virol. , http://dx.doi.org/10.1128/JVI.07204-11
Juhasz, G., Neufeld, T.P., 2006. Autophagy: a forty-year search for a missing membrane source. PLoS Biol. 4 (2), e36, http://dx.doi.org/10.1371/journal.pbio.0040036.
Jiang, H., White, E.J., Rios-Vicil, C.I., Xu, J., Gomez-Manzano, C., Fueyo, J., 2011. Human adenovirus type 5 induces cell lysis through autophagy and autophagy-triggered caspase activity. J. Virol. 85 (10), 4720–4729, http://dx.doi.org/10.1128/JVI.02032-10.
Jiang, M., Abend, J.R., Tsai, B., Imperiale, M.J., 2009. Early events during BK virus entry and disassembly. J. Virol. 83 (3), 1350–1358, http://dx.doi.org/10.1128/JVI.02169-08.
Jackson, W.T., Giddings Jr, T.H., Taylor, M.P., et al., 2005. Subversion of cellular autophagosomal machinery by RNA viruses. PLoS Biol. 3 (5), e156.
Jahreiss, L., Menzies, F.M., Rubinsztein, D.C., 2008. The itinerary of autophagosomes: From peripheral formation to kiss-and-run fusion with lysosomes. Traffic 9 (4), 574–587.
Joubert, P.E., Werneke, S.W., de la Calle, C., et al., 2012. Chikungunya virus-induced autophagy delays caspase-dependent cell death. J. Exp. Med. 209 (5), 1029–1047.
Kabeya, Y., Mizushima, N., Ueno, T., et al., 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 19 (21), 5720–5728, http://dx.doi.org/10.1093/emboj/19.21.5720.
Kumar, S.H., Rangarajan, A., 2009. Simian virus 40 small T antigen activates AMPK and triggers autophagy to protect cancer cells from nutrient deprivation. J. Virol. 83 (17), 8565–8574, http://dx.doi.org/10.1128/JVI.00603-09.
Lee, H.K., Lund, J.M., Ramanathan, B., Mizushima, N., Iwasaki, A., 2007. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science 315 (5817), 1398–1401, http://dx.doi.org/10.1126/science.1136880.
Liang, X.H., Kleeman, L.K., Jiang, H.H., et al., 1998. Protection against fatal sindbis virus encephalitis by beclin, a novel bcl-2-interacting protein. J. Virol. 72 (11), 8586–8596.
Liu, J., Xia, H., Kim, M., et al., 2011. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. Cell 147 (1), 223–234.
Low, J.A., Magnuson, B., Tsai, B., Imperiale, M.J., 2006. Identification of gangliosides GD1b and GT1b as receptors for BK virus. J. Virol. 80 (3), 1361–1366, http://dx.doi.org/10.1128/JVI.80.3.1361-1366.2006.
Meng, C., Zhou, Z., Jiang, K., et al., 2012. Newcastle disease virus triggers autophagy in U251 glioma cells to enhance virus replication. Arch. Virol. 157 (6), 1011–1018, http://dx.doi.org/10.1007/s00705-012-1270-6.
Meng, S., Jiang, K., Zhang, X., et al., 2012. Avian reovirus triggers autophagy in primary chicken fibroblast cells and vero cells to promote virus production. Arch. Virol. 157 (4), 661–668, http://dx.doi.org/10.1007/s00705-012-1226-x.
Maier, H.J., Britton, P., 2012. Involvement of autophagy in coronavirus replication. Viruses 4 (12), 3440–3451.
Mizushima, N., 2004. Methods for monitoring autophagy. Int. J. Biochem. Cell Biol. 36 (12), 2491–2502, http://dx.doi.org/10.1016/j.biocel.2004.02.005.
Mateo, R., Nagamine, C.M., Spagnolo, J., et al., 2013. Inhibition of cellular autophagy deranges dengue virion maturation. J. Virol. 87 (3), 1312–1321.
Mackeh, R., Perdiz, D., Lorin, S., Codogno, P., Pous, C., 2013. Autophagy and microtubules – new story, old players. J. Cell Sci. 126 (Pt 5), 1071–1080.
Noda, T., Ohsumi, Y., 1998. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J. Biol. Chem. 273 (7), 3963–3966.
Neu, U., Allen, S.A., Blaum, B.S., et al., 2013. A structure-guided mutation in the major capsid protein retargets BK polyomavirus. PLoS Pathog. 9 (10), e1003688.
O'Donnell, V., Pacheco, J.M., LaRocco, M., et al., 2011. Foot-and-mouth disease virus utilizes an autophagic pathway during viral replication. Virology 410 (1), 142–150, http://dx.doi.org/10.1016/j.virol.2010.10.042.
Orvedahl, A., Alexander, D., Talloczy, Z., et al., 2007. HSV-1 ICP34.5 confers neurovirulence by targeting the beclin 1 autophagy protein. Cell Host Microbe 1 (1), 23–35, http://dx.doi.org/10.1016/j.chom.2006.12.001.
Petiot, A., Ogier-Denis, E., Blommaart, E.F., Meijer, A.J., Codogno, P., 2000. Distinct classes of phosphatidylinositol 3′-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. J. Biol. Chem. 275 (2), 992–998.
Raptis, L. (Ed.), 2001. SV40 Protocols. Humana Press Ic, Totowa, New Jersey.
Rodriguez-Rocha, H., Gomez-Gutierrez, J.G., Garcia-Garcia, A., et al., 2011. Adenoviruses induce autophagy to promote virus replication and oncolysis. Virology 416 (1–2), 9–15.
Sir, D., Kuo, C.F., Tian, Y., et al., 2012. Replication of hepatitis C virus RNA on autophagosomal membranes. J. Biol. Chem. 287 (22), 18036–18043, http://dx.doi.org/10.1074/jbc.M111.320085.
Schaub, S., Hirsch, H.H., Dickenmann, M., et al., 2010. Reducing immunosuppression preserves allograft function in presumptive and definitive polyomavirus-associated nephropathy. Am. J. Transplant. 10 (12), 2615–2623, http://dx.doi.org/10.1111/j.1600-6143.2010.03310.x.
Seglen, P.O., Gordon, P.B., 1982. 3-methyladenine: Specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc. Natl. Acad. Sci. USA 79 (6), 1889–1892.
Sir, D., Ou, J.H., 2010. Autophagy in viral replication and pathogenesis. Mol. Cells 29 (1), 1–7, http://dx.doi.org/10.1007/s10059-010-0014-2.
Salonen, A., Ahola, T., Kaariainen, L., 2005. Viral RNA replication in association with cellular membranes. Curr. Top. Microbiol. Immunol. 285, 139–173.
Sariyer, I.K., Merabova, N., Patel, P.K., et al., 2012. Bag3-induced autophagy is associated with degradation of JCV oncoprotein, T-Ag. PLoS One 7 (9), e45000, http://dx.doi.org/10.1371/journal.pone.0045000 (10.1371/journal.pone.0045000).
Shai, B., Schmukler, E., Yaniv, R., et al., 2013. Epizootic hemorrhagic disease virus induces and benefits from cell stress, autophagy, and apoptosis. J. Virol. 87 (24), 13397–13408.
Talloczy, Z., Virgin 4th, H.W., Levine, B., 2006. PKR-dependent autophagic degradation of herpes simplex virus type 1. Autophagy 2 (1), 24–29.
Taylor, M.P., Kirkegaard, K., 2008. Potential subversion of autophagosomal pathway by picornaviruses. Autophagy 4 (3), 286–289.
Tanigaki, K., Sasaki, S., Ohkuma, S., 2003. In bafilomycin A1-resistant cells, bafilomycin A1 raised lysosomal pH and both prodigiosins and concanamycin A inhibited growth through apoptosis. FEBS Lett. 537 (1–3), 79–84.
Tovilovic, G., Ristic, B., Milenkovic, M., Stanojevic, M., Trajkovic, V., 2013. The role and therapeutic potential of autophagy modulation in controlling virus-induced cell death. Med. Res. Rev.
Tovilovic, G., Ristic, B., Siljic, M., et al., 2013. mTOR-independent autophagy counteracts apoptosis in herpes simplex virus type 1-infected U251 glioma cells. Microbes Infect. 15 (8-9), 615–624.
Virgin, H.W., Levine, B., 2009. Autophagy genes in immunity. Nat Immunol. 10 (5), 461–470, http://dx.doi.org/10.1038/ni.1726.
Wirawan, E., Vanden Berghe, T., Lippens, S., Agostinis, P., Vandenabeele, P., 2012. Autophagy: For better or for worse. Cell Res. 22 (1), 43–61, http://dx.doi.org/10.1038/cr.2011.152 (10.1038/cr.2011.152).
Wong, J., Zhang, J., Si, X., et al., 2008. Autophagosome supports coxsackievirus B3 replication in host cells. J. Virol. 82 (18), 9143–9153, http://dx.doi.org/10.1128/JVI.00641-08.
Xu, Y., Jagannath, C., Liu, X.D., Sharafkhaneh, A., Kolodziejska, K.E., Eissa, N.T., 2007. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity 27 (1), 135–144, http://dx.doi.org/10.1016/j.immuni.2007.05.022.
Xi, X., Zhang, X., Wang, B., et al., 2013. The interplays between autophagy and apoptosis induced by enterovirus 71. PLoS One 8 (2), e56966.
Zhou, X., Munger, K., 2009. Expression of the human papillomavirus type 16 E7 oncoprotein induces an autophagy-related process and sensitizes normal human keratinocytes to cell death in response to growth factor deprivation. Virology 385 (1), 192–197, http://dx.doi.org/10.1016/j.virol.2008.12.003.