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Bovine Herpesvirus Type 4 Infection Modulates Autophagy in A Permissive Cell Line

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ABSTRACT

Bovine herpesvirus type 4 (BoHV-4), like other herpesviruses, induces a series of alterations in the host cell that modify the intracellular environment in favor of viral replication, survival and spread. This research examined the impact of BoHV-4 infection on autophagy in BoHV-4 infected Madin Darby bovine kidney (MDBK) cells. Protein extracts of BoHV-4 infected and control MDBK cells were subjected to Western blot. The concentrations of the autophagy and apoptosis-related proteins Beclin 1, p21, PI3 kinase, Akt1/2, mTOR, phospho mTOR, p62 and the light chain three (LC3) were normalized to the actin level and expressed as the densitometric ratio. Western blot analysis of virus-infected cells revealed that autophagic degradation pathway was induced in the late phase of BoHV-4 infection. After 48 h post-infection the protein LC3II, which is essential for autophagy was found to be markedly increased, while infection of MDBK cells with BoHV-4 resulted in a depletion of p62 levels. Becline 1, PI3 kinase, Akt1/2 and p21 expression increased between 24 and 48 h post-infection. Surprisingly, mTOR and its phosphorylated form, which are negative regulators of autophagy, also increased after 24 h post-infection. In conclusion, our findings suggest that BoHV-4 has developed mechanisms for modulation of autophagy that are probably part of a strategy designed to enhance viral replication and to evade the immune system. Additional studies on the relationship between autophagy and BoHV-4 replication and survival, in both lytic and latent replication phases, are needed to understand the role of autophagy in BoHV-4 pathogenesis. J. Cell. Biochem. 114: 1529–1535, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: BOVINE HERPESVIRUS 4; AUTOPHAGY; AUTOPHAGIC FLUX; WESTERN BLOT ASSAY

Bovine herpesvirus 4 (BoHV-4) belongs to the family herpesviridae, subfamily gamma-herpesvirinae and genus rhadinovirus. BoHV-4 has no close biological or virological relationship to other known herpesviruses of the family Bovidae [Fabian et al., 2008]. Cattle are the natural hosts of the virus. However, several ruminant and non-ruminant species are also susceptible to BoHV-4 [Egyed et al., 1997]. The replication of most γ-herpesviruses is restricted to their natural host species. BoHV-4 is one of the few exceptions to this rule. Indeed, it has been shown that BoHV-4 is able to replicate in broad range of host species both in vivo and in vitro. BoHV-4 is able to replicate in primary cell cultures or cell lines from a broad spectrum of host species, recently, several

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studies have shown that some human normal and neoplastic cell lines support BoHV-4 infection [Egyed, 1998; Donofrio et al., 2002; Gillet et al., 2005] suggesting its potential use as oncolytic virus for the treatment of cancers.

In comparison to other γ-herpesviruses, BoHV-4 encodes a relatively small number of genes susceptible to affect the biology of the infected cells [Zimmermann et al., 2001]. However, it has been demonstrated that BoHV-4 could interfere with the cell apoptotic machinery through at least two different mechanisms. Indeed, BoHV-4 possesses two genes that could protect the infected cells from apoptosis: ORF16 and ORF71 encoding a v-Bcl-2 and a v-FLIP, respectively [Zimmermann et al., 2001]. Products of these two genes have been previously shown to inhibit apoptosis when over expressed transiently [Wang et al., 1997; Bellows et al., 2000]. At the same time, despite the presence of these antiapoptotic genes, previous studies showed that BoHV-4 replication cycle in permissive cells leads to apoptosis of infected cell [Sciortino et al., 2000; Pagnini et al., 2004].

Macroautophagy (hereafter, referred to as autophagy) is a degradation pathway in which bulk amounts of cytoplasm are sequestered by double-membrane vesicles [Espert et al., 2007]. Such vesicles, termed autophagosomes, ultimately fuse with lysosomes, resulting in formation of autophagic compartments, in which the engulfed cytoplasmic material is degraded [Reggiori and Klionsky, 2002]. At the molecular level, a number of evolutionarily conserved proteins, called autophagy-related (Atg) proteins, are necessary for autophagosome formation [Klionsky et al., 2012]. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a soluble protein with a molecular mass of approximately 17 kDa that is distributed ubiquitously in mammalian tissues and cultured cells. During autophagy, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes. Autophagosomes fuse with lysosomes to form autolysosomes, and intra-autophagosomal components are degraded by lysosomal hydrolases. At the same time, LC3-II in autolysosomal lumen is degraded. Autophagy was first reported as a response to starvation and has subsequently been associated with a variety of phenotypes, including neurodegenerative diseases, aging, cardiomyopathies, and cancer [Shintani and Klionsky, 2004]. With regard to cell survival, it has been proposed that a physiological level of autophagy may contribute to cell homeostasis by allowing for turnover of long lived proteins and disposal of damaged organelles and of aggregate-prone proteins; on the other hand, if induced beyond physiological ranges, autophagy may result in type II programmed cell death [Pattingre et al., 2005].

Various publications have recently begun to address the relationship between virus infection and authophagy [Levine, 2005; Espert et al., 2007]. For example, it has been demonstrated that over expression of the autophagy gene Beclin1, that encoded for a protein required for the formation of the autophagosome membrane [Liang et al., 1999; Leib et al., 2009], blocked Sindbis virus-induced apoptosis of neurons [Liang et al., 1998], tobacco mosaic virus replicated to higher levels in plants in which expression of beclin1 had been suppressed [Liu et al., 2005], and hepatitis C virus infection induced formation of autophagosomes in hepatocytes [Ait-Goughoulte et al., 2008]. In the case of herpesviruses, different viruses appear to differentially modulate autophagy; in fact, herpes simplex virus type 1 (HSV-1), human cytomegalovirus, and murine gamma-herpesvirus 68 have been shown to block completion of the autophagic pathway [Alexander et al., 2007; Orvedahl et al., 2007; Chaumorcel et al., 2008], while Epstein–Barr virus latent membrane protein-1 has been reported to induce autophagy in Epstein–Barr virus-infected cells [Lee and Sugden, 2008]. Takahashi et al. [2009] showed that Varicella–Zoster virus (VZV) induces autophagy in cultured cells, in this study they report that the autophagosome-associated form of LC3B accumulated late in VZV infection of cultured cells, concomitantly with degradation of p62/SQSTM1 and depletion of cytoplasmic material [Takahashi et al., 2009].

No data investigating whether autophagy is involved in BoHV-4 infection have been reported so far. Thus, in this study we investigated autophagy-related molecules, Beclin-1, PI3 Kinase, Akt1/2, mTOR, phospho mTOR, p21, p62, and LC3 in a BoHV-4 infected permissive cell line by Western blot assay.

MATERIALS AND METHODS

CELLS AND VIRUS

Madin Darby bovine kidney (MDBK) cells (CCL-22, American Type Culture Collection) were grown in Dulbecco's modified minimal essential medium (DMEM), supplemented with 100 IU/ml of penicillin, 100 mg/ml of streptomycin, and 5% pestivirus free fetal calf serum (FBS). This cell line was maintained free of mycoplasma and of bovine viral diarrhea virus.

BoHV-4 FI strain, kindly provided by Prof. Buonavoglia (Faculty of Veterinary Medicine, Bari, Italy) was used. It was multiplied on MDBK, and cell extracts, obtained by three cycles of freezing and thawing, were pooled, collected, and stored in aliquots at −80°C. Infectivity titers were expressed as median tissue culture infectious doses (TCID50)/ml.

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis were performed on BoHV-4 infected MDBK cells and on mock-infected MDBK cells as previously described with some modification [Longo et al., 2009]. MDBK cells in 75 cm^2 flask, at confluency of 90%, were infected with BoHV-4 at multiplicity of infection (MOI) 1. At 12, 24, 48, and 72 h post-infection (p.i.), adherent cells were washed twice with PBS and scraped.

Cells were then mixed with cells previously collected by centrifugation from supernatant of the same flask and resuspended in PBS. The pellets, obtained by centrifugation, were stored at −20°C. Cells were homogenized directly into lysis buffer 50 mM Tris pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.25% deoxycholic acid, 1% Triton X-100) with 20 mM sodium pyrophosphate, 0.1 mg/ml aprotinin, 2 mM phenylmethylsulfony fluoride (PMSF), 10 mM sodium orthovanadate (Na_2VO_3) and 50 mM sodium fluoride (NaF). Protein concentrations were determined by use of a protein assay kit (Bio-Rad Laboratories).

Equal amounts of lysate samples were boiled and loaded on bis/acrylamide gels, separated by electrophoresis and proteins were

blotted from the gel onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) in Tris buffered saline (TBS: 12.5 mM Tris–HCl pH 7.4; 125 mM NaCl) at room temperature, washed with TBS-0.1% Tween and incubated with primary antibody.

The primary antibody used were rabbit anti-PI3 kinase class III (Cell Signaling Technologies catalog no. 4263; 1:1,000 dilution), rabbit anti-Beclin-1 (Cell Signaling Technologies catalog no. 3738; 1:1,000 dilution), rabbit anti-Akt1/2 (Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. sc-1619; 1:200 dilution), rabbit anti-mTOR (Cell Signaling Technologies catalog no. 2,972; 1:1,000 dilution), rabbit anti-phospho-mTOR (Cell Signaling Technologies catalog no. 2,971; 1:1,000 dilution), rabbit anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. sc-397; 1:200 dilution), mouse anti-nucleoporin p62 (Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. sc-48389; 1:200 dilution) and rabbit anti-LC3 (MBL International Corporation catalog no. PM036; 1:1,000 dilution).

After appropriate washing steps, peroxidase-conjugated anti-rabbit IgG (GE Healthcare, UK, catalog no. NA934) or anti-mouse IgG (GE Healthcare, catalog no. NA931) was applied for 1 h at a 1:1,000 dilution. The blots were stripped and reprobed against mouse anti-actin antibody (Calbiochem, San Diego, CA; catalog no. CP10) at 1:5,000 dilution to confirm equal loading of proteins in each lane. Protein expression levels were quantitatively estimated by densitometry using a Gel Doc scanner (BioRad) equipped with a densitometric workstation. The protein concentrations were normalized to the actin level and expressed as the densitometric ratio.

STATISTICAL ANALYSIS

The results are presented as mean ± SD of three experiments. One-way ANOVA with Turkey's post-test was performed using GraphPad InStat Version 3.00 for Windows 95 (GraphPad Software, San Diego, CA). *P*-value <0.05 was considered statistically significant.

RESULTS

AUTOPHAGY IS TRIGGERED AT LATE STAGES OF BoHV-4 INFECTION

The purpose of this set of experiments was to determine whether the autophagic pathway was induced in BoHV-4 infections. To this end, replicate cultures of MDBK cells were incubated with an inoculums consisting of criolyzed cells from a BoHV-4/MDBK-infected monolayer exhibiting advanced cytophatic effect (CPE). The multiplicity of infection chosen was 1. At increasing intervals (12, 24, 48, and 72 h) after infection, cells were harvested, solubilized, and subjected to immunoblotting with an antibody against LC3. Moreover, to provide a positive autophagic control MDBK cells were treated with lithium chloride (LiCl Sigma). Treatment with lithium chloride was carried out for 2 h before lysis of cells for Western blot assay. As shown in Figure 1A, a faster-migrating band, of 16 kDa, corresponding to the expected molecolar weight of the autophagosome-associated form LC3-II, was detectable in lysates of mock-infected cells. In uninfected cells, treatment with lithium chloride, significantly increased the levels of LC3-II compared to control cells (Fig. 1A). Importantly, in BoHV-4-infected cells, LC3-II accumulated starting at 12 h p.i. in a time-dependent manner (Fig. 1B) with acme at 48 h post-infection. These data

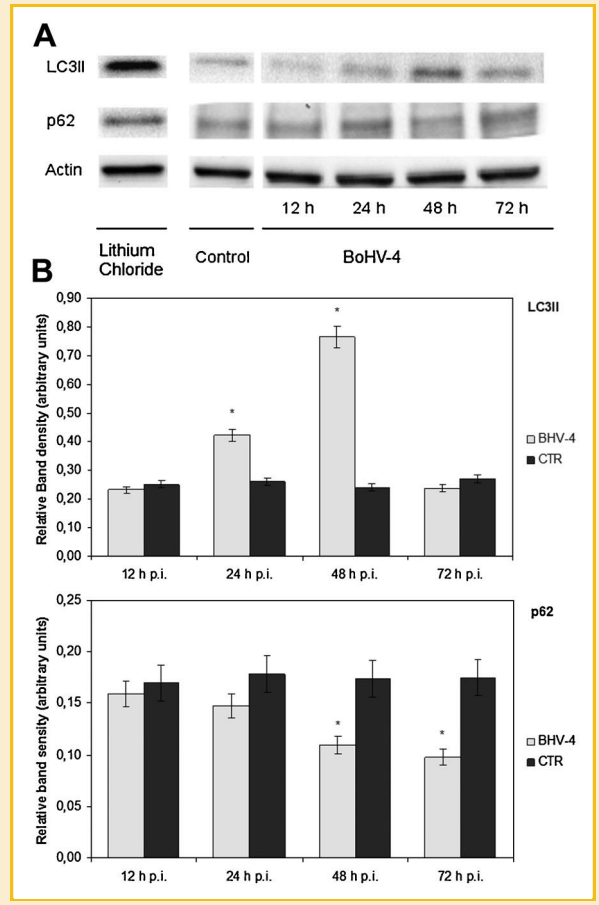

Fig. 1. Autophagy is induced in MDBK cells infected with BoHV-4. A: Replicate cultures of MDBK cells were mock infected or infected with BoHV-4 at MOI 1. At different time points after infection, cells were scraped, harvested, lysed and subjected to Western blot analysis with antibodies against actin, LC3II, and nucleoporin p62. Actin protein levels were detected to ensure equal protein loading. B: Densitometric analysis of blots relative to LC3II and nucleoporin p62. Results are expressed as the mean ± SD of three independent experiments.

indicates that autophagy was induced at late time points in BoHV-4-infected cells.

INDUCTION OF AUTOPHAGY IN BoHV-4-INFECTED CULTURES RESULTS IN CYTOPLASMIC DEPLETION AND DEGRADATION OF p62/SQSTM1

In order to confirm the pro-autophagic potential of BoHV-4, the levels of polyubiquitin-binding protein p62/SQSTM1 (sequestosome 1), a multifunctional protein that interacts with LC3B and is specifically degraded by the autophagic-lysosome pathway, were assayed (Fig. 1A). Levels of p62/SQSTM1 are commonly measured to detect autophagic flux [Klionsky et al., 2012]. As shown in Figure 1B, upon densitometry analysis, infection with BoHV-4 resulted in a decrease of p62/SQSTM1 levels. Importantly, levels of p62/SQSTM1 were higher in the lysates of mock-infected cells. Therefore, these data indicated that in BoHV-4-infected MDBK cells, the p62/SQSTM1 protein was degraded via the autophagic-lysosome

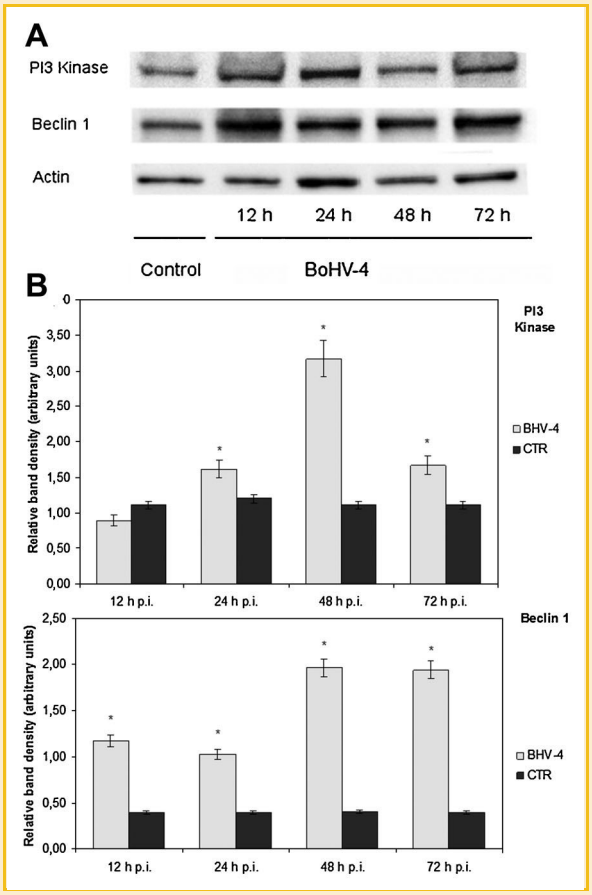

Fig. 2. BoHV-4 upregulates PI3 Kinase pathway. A: Replicate cultures of MDBK cells were mock infected or infected with BoHV-4 at MOI 1. At different time points after infection, cells were scraped, harvested, lysed and subjected to Western blot analysis with antibodies against actin, PI3 kinase and Beclin 1. Actin protein levels were detected to ensure equal protein loading. B: Densitometric analysis of blots relative to PI3 kinase and Beclin 1. Results are expressed as the mean ± SD of three independent experiments.

pathway and that no p62/SQSTM1 protein degradation was observed in mock infected MDBK cells.

BoHV-4 UPREGULATES PI3 KINASE PATHWAY AND ACTIVATES mTOR-SIGNALING PATHWAY

In order to assess the pathway of the autophagic response during BoHV-4 infection, Beclin-1, PI3 Kinase, Akt1/2, mTOR, and phospho mTOR expression was monitored by Western blot assay. To this end, replicate cultures of MDBK cells were infected with BoHV-4, at m.o.i. one for different hours post-infection, then were harvested, solubilized, and subjected to immunoblotting as described above, and band intensity was measured by densitometry analysis. As shown in Figure 2A, the 60 kDa Beclin 1 and the 100 kDa PI3 Kinase proteins were over-expressed in BoHV-4 infected cells starting from 12 to 24 h post-infection respectively with an acme at 48 h post-infection (Fig. 2B). Moreover, BoHV-4 infection in MDBK cells resulted in an increase of AKT1/2, mTOR and phospo-mTOR proteins from 24 h post-infection (Fig. 3A) with an acme at 48 h post-

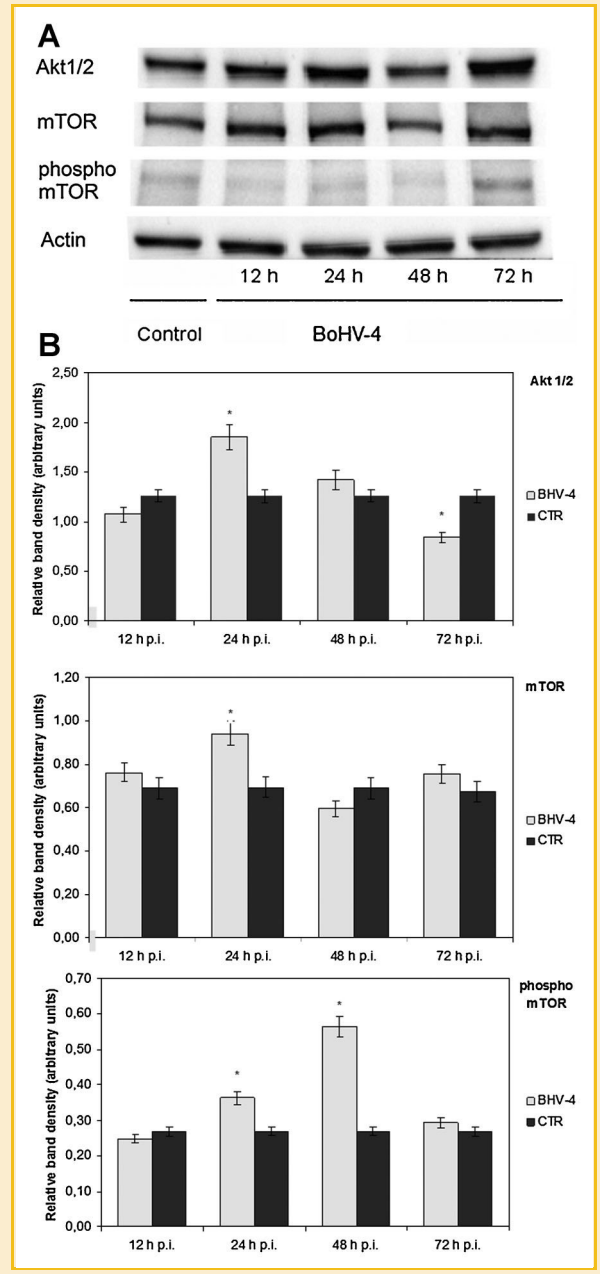

Fig. 3. BoHV-4 upregulates Akt1/2 and activates mTor-signaling pathway. A: Replicate cultures of MDBK cells were mock infected or infected with BoHV-4 at MOI 1. At different time points after infection, cells were scraped, harvested, lysed, and subjected to Western blot analysis with antibodies against actin, Akt1/2, mTOR and phospho mTOR. Actin protein levels were detected to ensure equal protein loading. B: Densitometric analysis of blots relative to Akt1/2, mTOR, and phospho mTOR. Results are expressed as the mean ± SD of three independent experiments.

infection for phospo-mTOR protein, while AKT protein expression decreased in a time-dependent manner (Fig. 3B).

p21 IS UPREGULATED DURING BoHV-4 REPLICATION

p21 originally identified as an inhibitor of cyclin-dependent kinases (CDKs), is a key mediator of the growth arrest induced by the tumor

suppressor protein p53 in response to DNA damage and has a pivotal role both in apoptosis and autophagy. Taking into account that BoHV-4 replication is dependent on the S-phase of the cell cycle and p21 is a rate-limiting regulator of the G1/S-phase transition [Capocefalo et al., 2009], we analyzed p21 expression in BoHV-4 infected cells. As expected, p21 was detected like a faster-migrating band, of 21 kDa increasing in a time-dependent manner starting from 24 h post-infection (Fig. 4A,B).

DISCUSSION

Over the course of evolution, viruses have developed various strategies that modulate a variety of host cell signaling pathways to establish a moderate environment favorable for their survival or infection. In particular, modulation of autophagy affords great advantages to the virus, facilitating virus replication, the spreading of progeny virus to neighboring cells and providing the protection for the progeny virus against cellular enzymes [Deretic and Levine, 2009; Cavignac and Esclatine, 2010].

Autophagy protects host cells against viral attack by degrading the viruses in autolysosomes, or by activating the innate immunity of the cells by loading viral components onto endosomal sensors such as TLRs [Talloczy et al., 2006; Delgado et al., 2008]. It has been suggested that autophagy may have a deleterious effect on viral pathogenesis in the case of the neurotropic Sindbis virus [Liang et al., 1998], and the role of autophagy has been clearly demonstrated to limit the replication of the tobacco mosaic virus [Liu et al., 2005]. Autophagy is also involved in the adaptative immune responses to microorganisms infection, for example by providing viral endogenous antigens for loading onto major histocompatibility complex (MHC) class I and class II in order to activate the adaptive immunity [English et al., 2009; Gannage and Munz, 2009]. Some viruses have been shown to develop diverse tactics to antagonize the host cell's autophagic defence, inhibition of autophagy has also been reported in HIV-infected CD4 T-lymphocytes [Espert et al., 2009]. Moreover, HIV has been shown to inhibit the fusion of autophagosomes with lysosomes in macrophages through interaction of the viral protein Nef with Beclin 1, leading to an accumulation of autophagosomes in the cytoplasm [Espert et al., 2009; Kyei et al., 2009]. In some situations autophagy is also able to act as a pro-viral pathway that helps viruses to replicate or exit from cells [Kirkegaard, 2009]. For example, stimulation of autophagy has been reported to increase the yields of poliovirus, hepatitis C virus (HCV), Dengue virus and Coxsackie B virus [Jackson et al., 2005; Dreux et al., 2009]. It has also been suggested that autophagy may help poliovirus particles to exit from the cell during the late stages of infection [Taylor et al., 2009]. HCV induces the accumulation of autophagosomes in infected cells, but inhibits their fusion with lysosomes [Sir et al., 2008], and seems to require autophagy at an early stage of the infection [Dreux et al., 2009].

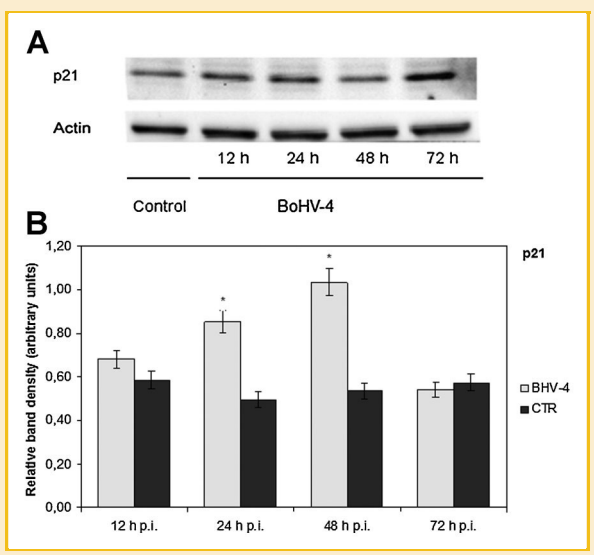

Fig. 4. p21 is upregulated during BoHV-4 replication. A: Replicate cultures of MDBK cells were mock infected or infected with BoHV-4 at MOI 1. At different time points after infection, cells were scraped, harvested, lysed and subjected to Western blot analysis with antibodies against actin and p21. Actin protein levels were detected to ensure equal protein loading. B: Densitometric analysis of blots relative to p21. Results are expressed as the mean ± SD of three independent experiments.

Then, despite the ability of autophagy to function as an anti-viral mechanism, some viruses appear to up-regulate the process and/or subvert autophagic genes in order to enhance their replication.

The experiments presented in this article were initiated to enhance our understanding of the interplay between BoHV-4 and the host cell. The most important finding of this report is that in BoHV-4 infected cells we observed an accumulation of the autophagosome-associated form of LC3, a marker of induction of autophagy. The modification of LC3 correlated with increased formation of autophagosomes, with cytoplasmic depletion and with degradation of the autophagy marker p62/SQSTM1 polyubiquitin-binding protein.

Our result agree with the results of other studies about herpesviruses, for example, it has been demonstrated that VZV infections induces autophagy in permissive cells line at late stage of infection with depletion of p62 [Takahashi et al., 2009], HSV-1 infection induces autophagy in macrophages [English et al., 2009] and a latency associated protein (LMP1) of Epstein–Barr virus, induces autophagy in B-cells [Lee and Sugden, 2008]. Conversely, it has been demonstrated that other γ-herpesvirus like human herpes virus 8 (HHV-8) and murine herpesvirus 68 (MHV-68) may inhibit autophagy. In contrast to other γ-herpesvirus like herpesvirus saimiri (HVS), HHV-8, and MHV-68, BoHV-4 has a reduced set of ORFs homologs to cellular genes. The genome structure, the gene arrangement, and the biological properties confirm that BoHV-4 belongs to the genus Rhadinovirus, and HVS appears to be its closest relative, as proposed in earlier studies [Lomonte et al., 1995]. Several γ-herpesviruses are associated with lymphoproliferative diseases and tumor development. These diseases seem to be associated with immunosuppression or a late-in-life infection of the natural host as well as with infection of a related unnatural host. For BoHV-4, no link to such diseases has been identified with evidence [Moreno-Lopez et al., 1989]. They may either have been overlooked or be

absent due to the lack of transforming genes and cellular homologs in the BoHV-4 genome. The set of such genes in BoHV-4 is reduced compared to other γ-herpesviruses with known transforming capacity. The differences, in the modulation of the autophagy pathway, between BoHV-4 and the other γ-herpesviruses could be explained by the small size of the genome and with the absence of oncogene [Zimmermann et al., 2001].

In BoHV-4 infected cells, induction of autophagy was bound to increased levels of Beclin-1, PI3 kinase, and Akt1/2 expression. Beclin1 is an interacting partner for the mammalian class III PI3 kinase Vps34 [Furuya et al., 2005; Funderburk et al., 2010], and it forms complex with Vps34, Vps15, and Atg14L to initiate autophagosome formation [Furuya et al., 2005; Zeng et al., 2006]. We observed an increased expression of Beclin1 protein at late stage of BoHV-4 infection and activation of PI3 kinase and Akt pathways in MDBK cells. Recent works indicated that several viruses exploit host cell signaling pathways to facilitate various steps of virus infection. For example, the PI3K/Akt pathway was proved to be critical in regulating vesicular trafficking for Ebola virus at the entry step. In addition, blocking phosphorylation of Akt at the early step nearly aborted virus replication [Saeed et al., 2008]. Influenza A virus triggered PI3K/Akt pathway activation only at the late phase in infection of human lung carcinoma cells (A549), and the activation has been shown to be required for efficient virus replication [Shin et al., 2007]. BoHV-1 infection of MDBK cells led to biphasic activation of PI3K/Akt and MAPK/Erk1/2 pathways and inhibition of PI3K/Akt pathway greatly reduced the virus production [Zhu et al., 2011]. Among the complex regulation of autophagy, the protein kinase mTOR is part of an[Q7] important signaling pathway [Codogno and Meijer, 2005]. Activation of mTOR downregulates autophagy, and is responsible for the phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), and ribosomal S6 protein kinase (p70S6K), two proteins involved in protein synthesis. We monitored the activity of mTOR and phospho mTOR by Western blot assay (Fig. 3) in BoHV-4 infected cells. We observed an increase in the expression of mTOR and its phosphorylated form during BoHV-4 infection between 24 and 48 h post-infection (Fig. 3B). These findings show that infection with BoHV-4 stimulates the mTOR-signaling pathway. This unexpected result prompted us to hypothesize that mTOR could not be a negative regulator during BoHV-4 induced autophagy. It is possible that BoHV-4 mediated autophagy and mTOR signaling could act concurrently or that induction of autophagy occurs upstream of mTOR-signaling pathway in BoHV-4 infected cells. As a potential explanation of this paradox is likely that BoHV-4 infection is inducing autophagy for establishment of infection, while activating mTOR signaling for cell growth. A similar scenario has been described by Shrivastava et al. [2012] who reported that human hepatitis virus mediated upregulation of Beclin1 expression occurs at the transcriptional level, which in turn, may initiate autophagy induction and that HCV-mediated autophagy may act on upstream of mTOR-signaling pathway. Thus, it cannot be excluded that the activation of mTOR-signaling could reflect an BoHV-4 anti-autophagic potential. In fact, it has been demonstrated that γ-herpesvirus 68 M11 protein, which is an homologue of BoHV-4 encoded v-Bcl-2, aids to establish a life-long persistent infection inhibiting autophagy and apoptosis through a mechanism that involves the binding of the Beclin 1 BH3 domain in the M11 hydrophobic surface groove [Xiaofei et al., 2009].

Many aspects of the BoHV-4-mediated induction of autophagy that we have reported will need to be addressed with further experimentation. In particular, the mechanism responsible for induction of autophagy in BoHV-4 infection as well as the impact of the BoHV-4 anti-autophagic potential remains to be identified. These issues are currently under investigation in our laboratory.

In conclusion, our findings highlight a novel and potentially important difference in the properties of BoHV-4 compared to those of the related gamma-herpesvirus HHV-8 and MHV-68. Interestingly, BoHV-4 replication induces autophagy at the late stage of infection and this activity is bound to an increase of pro-autophagic protein like Beclin-1. Our findings advance the understanding of the complex virus-host interactions that occur during BoHV-4 infection.

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