Evaluation of the anti-apoptotic activity of bovine alphaherpesvirus type 5 US3 protein kinase in insect cells using a recombinant baculovirus

Alice M. Silva¹ · Fabrício S. Morgado² · Leonardo A. Silva² · José R. J. Borges³ · Simone Perecmanis¹ · Daniel M. P. Ardisson-Araújo⁴ · Bergmann M. Ribeiro² · Fabrício S. Campos⁵

Received: 24 August 2019 / Accepted: 14 December 2019 / Published online: 7 January 2020
© Sociedade Brasileira de Microbiologia 2020

Abstract

Bovine alphaherpesvirus type 5 (BoHV-5) is one of the main agents responsible for meningoencephalitis in cattle in Brazil, causing significant economic losses. It is known that other viruses of the *Herpesviridae* family such as *Bovine alphaherpesvirus* type 1, *Swine alphaherpesvirus* type 1, and the *Human alphaherpesvirus* types 1 and 2 encode genes homologous to BoHV-5, with recognized action in the control of apoptosis. The objective of this work was to express the BoHV-5 US3 gene in a baculovirus-based expression system for the production of the serine/threonine kinase protein and to evaluate its activity in the control of apoptosis in vitro. A recombinant baculovirus derived from the *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) containing the US3 gene and a deletion in the baculovirus anti-apoptotic *p35* gene was constructed using the Bac-to-Bac™ system. This recombinant baculovirus was used to evaluate the anti-apoptotic activity of the recombinant US3 protein in insect cells comparing with two other AcMNPV recombinants, one containing a functional copy of the AcMNPV anti-apoptotic *p35* gene and an AcMNPV *p35* knockout virus with the anti-apoptotic *iap-3* gene from *Anticarsia gemmatilis* multiple nucleopolyhedrovirus (AgMNPV). We found that the caspase level was higher in insect cells infected with the US3-containing recombinant virus than in cells infected with the AcMNPV recombinants containing the *p35* and *iap-3* genes. These results indicate that the BoHV-5 US3 protein kinase gene is not able to block apoptosis in insect cells induced by the infection of a *p35* knockout AcMNPV.

Keywords Bovine alphaherpesvirus 5 · US3 · Apoptosis · Baculovirus

Responsible Editor: Giliane Souza Trindade.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s42770-019-00215-x>) contains supplementary material, which is available to authorized users.

✉ Fabrício S. Campos
camposvet@gmail.com

Alice M. Silva
martins.as.am@gmail.com

Fabrício S. Morgado
fabsmorga@gmail.com

Leonardo A. Silva
leocbq@gmail.com

José R. J. Borges
jrbborges@unb.br

Simone Perecmanis
perecmaniss@unb.br

Daniel M. P. Ardisson-Araújo
daniel.araujo@ufsm.br

Bergmann M. Ribeiro
bergmann.ribeiro@gmail.com

- ¹ Laboratory of Veterinary Microbiology, Faculty of Veterinary Medicine and Agronomy, University of Brasília, Brasília, DF, Brazil
- ² Laboratory of Baculovirus, Cell Biology Department, University of Brasília, Brasília, DF, Brazil
- ³ Veterinary Hospital, Faculty of Veterinary Medicine and Agronomy, University of Brasília, Brasília, DF, Brazil
- ⁴ Department of Biochemistry and Molecular Biology, Federal University of Santa Maria, Santa Maria, RS, Brazil
- ⁵ Laboratory of Bioinformatics and Biotechnology, Federal University of Tocantins, Campus of Gurupi, Gurupi, TO, Brazil

Introduction

Bovine alphaherpesvirus 5 (BoHV-5) is a pathogen causing mainly meningoencephalitis in young cattle, and occasionally respiratory and reproductive disease. BoHV-5 belongs to family *Herpesviridae* and is very similar to *Bovine alphaherpesvirus 1* (BoHV-1) [1, 2]. Like other herpesviruses, BoHV-5 has evolved strategies to escape from the immune system and remain in the host for its entire lifespan [3]. These viruses efficiently invade the peripheral nervous system and establish lifelong latency in neurons present in peripheral ganglia [4, 5].

The serine/threonine kinase protein encoded by US3 gene has recognized anti-apoptotic activity and is produced by members of subfamily *Alphaherpesvirinae*, such as *Human alphaherpesvirus 1* (HHV-1), *Human alphaherpesvirus 2* (HHV-2), *Suid alphaherpesvirus 1*, and *Gallid alphaherpesvirus 2* [6–10]. Orthologs of US3 genes have different functions like infected cell cytoskeletal rearrangements, virion maturation and egress, evasion of the antiviral response, and others [6]. Virally encoded kinases are able to phosphorylate viral and cellular substrates. In HHV-1, some studies have shown that the protein encoded by the US3 gene is able to phosphorylate the pro-apoptotic proteins Bad (Bcl-2-antagonist of cell death) and Bid (BH3 interacting-domain death agonist) to block their function in promoting apoptosis [11–13]. These functionalities may be absent or reduced depending on the alphaherpesvirus species [6, 14].

A few studies have been carried out with respect to the BoHV-1 and BoHV-5 US3 genes [15–17]. Takashima et al. [15] in their work with BoHV-1 wild-type (wt) and BoHV-1 US3 mutants and did not observe apoptosis in any of the cells infected with the wt virus and with US3 mutants when these cells were treated with sorbitol, demonstrating that the protein kinase of US3 has no direct effect in apoptosis. In a more recent study involving the BoHV-1 US3 gene, Brzowska et al. [17] have demonstrated with more modern and sensitive techniques that the protein kinase of US3 is able to inhibit apoptosis triggered by viral infection and by external stimuli (sorbitol or staurosporine). They linked the presence of US3 protein with the phosphorylation of Bad, a pro-apoptotic protein. Similar data were observed for HHV-1 and SHV-1 [9, 12]. Ladelfa et al. [16] carried out a similar study with the BoHV-5 US3 gene, concluding that the protein encoded by US3 gene confers resistance to apoptosis and is capable of inducing modifications in the cellular cytoskeleton. The aim of the present work was to test the anti-apoptosis activity of the BoHV-5 US3 in insect cells using a recombinant insect virus (baculovirus), containing the US3 gene under the control of a baculovirus late promoter and with a deletion in the *p35* gene (a baculovirus anti-apoptotic gene). We have chosen a baculovirus expression system to test the anti-apoptotic activity of the BoHV-5 US3 protein since baculovirus has its own inhibitor of apoptosis and we wanted to know whether the

inhibition of apoptosis in bovine cells by US3 protein would work in insect cells. Besides, characterizing and determining gene activity in different cells is critical to an understanding of virus biology and gene-specific activity.

Materials and methods

Viruses and cells

CRIB cells [18] were cultured in Eagle's minimal essential medium supplemented with 5% fetal bovine serum (FBS) and antibiotics for BoHV-5 virus replication. The BoHV-5 strain EVI 88/95 [19] was used for virus multiplication in CRIB cells, with multiplicity of infection of 1 (MOI = 1). Viral DNA was extracted following standard phenol/chloroform protocol. *Trichoplusia ni* (TN5B) [20], *Spodoptera frugiperda* (Sf-21) [21], and *S. frugiperda* 9 Easy Titration (Sf9-ET) [22] insect cells were cultured for the replication of recombinant and control baculoviruses at 28 °C in TC-100 medium (Vitrocell, Campinas, SP, Brazil) supplemented with 10% FBS (ThermoFisher) (for details about cells and virus used in this study, see Table 1 in supplementary material).

BoHV-5 US3 gene cloning

BoHV-5 US3 gene was amplified by PCR from viral DNA (BoHV-5 EVI88/95 strain) and cloned into pFastBac™1 donor plasmid vector (ThermoFisher). First, amplification was carried out with forward primer 6xHis-US3F: 5'- GGAT CCA TGC ACC ATC ACC ATC ACC ATG GAG CGCGCGCGGAGCGGCTGGCC-3' and reverse primer US3R: 5'- AAGCTTTTACCCCAAGGCCGCGCTGA AGGCGG-3'. The primers were designed manually according to the virus BoHV-5 DNA genome sequence (NC_005261.3, strain SV507/99) and were introduced the recognition sites for *Bam*HI, 6xHis Tag, and *Hind*III, respectively. The BoHV-5 US3 PCR product of 1,335 bp in length was amplified with 5 µL of 10× PCR buffer, 1 mM MgCl₂, 3% DMSO, 10 µM of each primer, 200 µM dNTPs, and 5 units of Taq DNA polymerase (ThermoFisher), and was added ultrapure water to a final volume of 50 µL. The reaction was carried out for 35 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min 30 s and final extension for 10 min at 72 °C. The PCR product was visualized after electrophoresis by UV light on ethidium bromide-stained 0.8% agarose gel and purified with the GFX PCR Kit and Gel Band Purification Kit (GE Healthcare, Chicago, Illinois, USA). Following PCR, the purified product and the donor plasmid vector were digested with the restriction enzymes described above, ligated, and transformed into DH10β competent cells to generate pFastBac1.US3 (Fig. 1). The correct orientation of the US3

Fig. 1 Construction of AcMNPV Δ p35.US3 recombinant. The US3 gene is fused with 6xHis Tag and cloned into pFastBacTM1.US3 donor plasmid via the BamHI/HindIII linker under the regulation of polyhedrin promoter. The transposition occurs between the mini-Tn7 to the mini attTn7 on the bacmid to generate a recombinant bacmid (for details, see the manual Bac-to-Bac®). Adapted from Muyilkens et al. [23]

BoHV-5 gene into this plasmid was checked by plasmid DNA sequencing at Macrogen (South Korea).

Recombinant virus

The pFastBac1.US3 was then used to produce a recombinant virus via transposition in *E. coli* DH10Bac cells containing the *Autographa californica nucleopolyhedrovirus* (AcMNPV) genome with a deletion in the baculovirus anti-apoptotic *p35* gene [24] in a plasmid form called bacmid, according to the Bac-to-BacTM system manual. The AcMNPV *p35* knockout with BoHV-5 US3 gene (AcMNPV Δ p35.US3) bacmid DNA was extracted and then was used for transfection in Sf-21 and TN5B cells. Virus titrations were done in 96-well microtiter plates [25] using Sf9-ET insect cells [22] in biological triplicates.

BoHV-5 US3 protein expression analysis

Western blot was used to detect the expression of BoHV-5 US3 in Sf-21 cells at 72 hours post-infection (h.p.i.). Sf-21 (10^6 cells) were infected at a MOI=1 in biological triplicates, with the following control viruses: (i) AcPG (AcMNPV with GFP) [26], (ii) AcMNPV Δ p35 (AcMNPV *p35* knockout) [24], (iii) AcMNPV Δ p35.IAP3 [AcMNPV *p35* knockout with the *Anticarsia gemmatilis multiple nucleopolyhedrovirus iap-3* gene] (unpublished data), and (iv) AcMNPV Δ p35.US3 (AcMNPV *p35* knockout with BoHV-5 US3 gene) (this study), (see Table 1 in supplementary material) and mock inoculated. The

supernatant was removed; cells were washed with $1 \times$ PBS (phosphate-buffered saline) and centrifuged at $5000 \times g/5$ min. The precipitate was separated by electrophoresis on 12% polyacrylamide denaturing gel (SDS-PAGE) and transferred to a PVDF (polyvinylidene difluoride) membrane with a semi-dry transfer cell (BioRad) following the manufacturer's instructions. The membrane was blocked in $1 \times$ PBS Buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.4) containing 3% skimmed milk powder for 16 h at 4 °C, washed three times with PBS tween (0.05%) and incubated with mouse monoclonal anti-hexa-histidine (anti-6xHis) antibody (Sigma), followed by incubation with the alkaline phosphatase-conjugated anti-mouse/rat or anti-rabbit secondary antibodies (Sigma). In order to detect the recombinant BoHV-5 US3 polypeptides expressed in the Sf-21 cells, blots were developed using the NBT/BCIP (Sigma) substrate dissolved in alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl_2 , and 100 mM Tris-HCl pH 9.0).

Morphological and immunofluorescence assay analysis

Sf-21 (10^6 cells) cells were infected at a MOI=1 in biological triplicates with (i) AcPG, (ii) AcMNPV Δ p35, (iii) AcMNPV Δ p35.IAP3, (iv) AcMNPV Δ p35.US3, and mock inoculated. At 48 h.p.i., cells were visualized and photographed under a light microscope. Cells infected with recombinant AcMNPV Δ p35.US3 and mock control were fixed by incubation with 3.7% formaldehyde at

room temperature for 30 min, washed with $1\times$ PBS and permeabilized by incubation with 0.1% Triton-X at room temperature for 10 min. After blocking with 3% BSA solution, the anti-6xHis Tag primary antibody was added at a dilution of 1:1000 at 4 °C for 24 h. Cells were washed with $1\times$ PBS and incubated with secondary antibodies containing goat anti-rabbit IgG-Alexa (ThermoFisher) at 37 °C in the dark for 1 h to detect cellular localization of BoHV-5 US3. Cells were counter-stained with Hoechst (0.2 μ g/mL) (Invitrogen) solution according to the manufacturer instructions in $1\times$ PBS for 10 min at room temperature to label the nuclei and the microscopy images were analyzed by ImageJ software.

Assay for caspase 3/7 activity

Sf-21 cells (10^6 cells) were infected at a MOI = 1 in biological triplicates with (i) AcPG, (ii) AcMNPV Δ p35, (iii) AcMNPV Δ p35.IAP3, and (iv) AcMNPV Δ p35.US3, mock-inoculated, and tested for caspase-3/7 activity at 12, 24, and 48 h.p.i. using a substrate-specific, luminescence-based commercial kit (ApoLive-Glo™ Multiplex Assay Kit, Promega). Total luminescent light was measured in the same time points using GloMax Microplate Reader® (Promega).

Statistical analysis

Data were analyzed using GraphPad Prism Software version 7 (GraphPad Software, Inc., San Diego, CA, USA) and presented as mean $\pm$ standard deviation (SD).

Results

Virus replication and BoHV-5 US3 protein expression analysis in insect cells

BoHV-5 US3 protein was expressed in the cultured Sf-21 insect cells infected with AcMNPV Δ p35.US3 and detected (72 h.p.i.) by SDS–PAGE and Western blot. The recombinant full-length serine/threonine kinase protein (US3) was detected as a single protein band between 55 and 70 kDa (Fig. 2), which is the expected theoretical molecular mass of the recombinant protein (Genbank accession number AAR86171.1). All viruses used in this work replicated in Sf21 cells and no significant difference was observed in the viral copy number during infection (see [supplementary data](#)).

Morphological and immunofluorescence assay analysis

Sf-21 cells exhibited various responses against baculovirus recombinants. AcPG showed few morphological changes at 24 h.p.i. (Fig. 3A). At 48 h.p.i., the cells showed the presence of occlusion bodies (also called polyhedra) in the nucleus of the cells, which is a typical cytopathic effect of a wild-type baculovirus infection, and did not show morphological signs of apoptosis (Fig. 3B). The AcMNPV Δ p35-infected cells showed apoptotic bodies, cell shrinkage, and disorganization of the plasma membrane at 24 h.p.i. (Fig. 3C) and multiple apoptotic bodies and polyhedra production at 48 h.p.i. (Fig. 3D). The AcMNPV Δ p35.IAP3 virus induced few morphological changes by 24 h.p.i. (Fig. 3E) and at 48 h.p.i., it

Fig. 2 SDS–PAGE and Western blot analysis with different recombinant AcMNPV baculoviruses expressed in Sf-21 cells (72 h.p.i.). **a** SDS–PAGE 12% of insect cells extracts from recombinant virus-infected cells showing different proteins. 1, Broad Range Protein Molecular Weight Marker (Promega); 2, AcPG; 3, AcMNPV Δ p35; 4, AcMNPV Δ p35.IAP3; 5, AcMNPV Δ p35.US3; 6, mock-infected cells.

b Western blot with 6xHis Tag antibody of cellular extracts infected with recombinant virus. 1, Page Ruler™ Prestained Protein Ladder (ThermoFisher); 2, AcPg; 3, AcMNPV Δ p35; 4, AcMNPV Δ p35.IAP3; 5, AcMNPV Δ p35.US3-infected insect cell extracts showing the detection of the US3 protein; 6, mock-infected cells

Fig. 3 Morphological analysis with Sf-21 insect cells infected with different recombinant AcMNPV baculoviruses (MOI = 1) and mock-infected cells. Cells were visualized under light microscope at 24 and 48 h.p.i. **A–B** Cells infected with AcPg. Arrow and inset: cells presenting occlusion bodies (polyhedral) in their nuclei. **C–D** Cells infected with AcMNPV Δ p35. Arrow and inset: cells presenting surface blebbing and presence of apoptotic bodies. **E–F** Cells infected with AcMNPV Δ p35.IAP3. Arrow and inset: cells presenting occlusion bodies (polyhedral) in their nuclei. **G–H** Cells infected with AcMNPV Δ p35.US3. Arrows on inset: cells presenting surface blebbing and presence of apoptotic bodies. At 48 h.p.i.: presence of cytoplasm disorganization and vacuoles. **I–J** Sf-21 mock-infected cells. Black bars correspond to 50 μ m and white bars correspond to 25 μ m

was possible to see the presence of polyhedra inside the cells and absence of morphological signs of apoptosis (Fig. 3F). The AcMNPV Δ p35.US3 induced the same morphological changes as the AcMNPV Δ p35 at 24 h.p.i. (Fig. 3G). At 48 h.p.i., it was possible to observe an increase in the number of apoptotic cells. Additionally, more elongated cells were noted, with formation of multiple and large cytoplasmic vacuoles and cells varying in size (Fig. 3H). Mock-infected cells showed the same pattern at 24 h.p.i. (Fig. 3I) and after 48 h.p.i. (Fig. 3J). To localize the US3 protein in Sf-21 cells infected with the AcMNPV Δ p35.US3, the infected cells were prepared for immunostaining labeling. The 6xHis Tagged BoHV-5 US3 was identified by indirect immunofluorescence using the 6xHis Tag monoclonal antibody and was found in the cytoplasm, surrounding the nucleus and the vacuoles (Fig. 4a). Additionally, Hoechst-stained nuclei showed nuclear fragmentation (Fig. 4a).

Assay for caspase 3/7 activity

Low or no caspase activity was detected overtime in mock-infected and AcPg and AcMNPV Δ p35.IAP3-infected Sf-21 cells (Fig. 5). In AcMNPV Δ p35-infected cells, the caspase activity increased between 12 and 24 h.p.i and decreased from 24 to 48 h.p.i. However, in AcMNPV Δ p35.US3, there was an increase of caspase activity overtime (Fig. 5).

Discussion

In this study, we used the baculovirus expression system to express the BoHV-5 US3 protein kinase in Sf-21 insect cells. Western blot of AcMNPV Δ p35.US3-infected Sf21 cells (72 h.p.i.) revealed the expression of a band between

Fig. 3 (continued)

55 and 70 kDa. A similar size band of US3 proteins has been observed in other studies [15, 17, 27, 28]. In addition, we compared the replication of three different

recombinants baculoviruses (AcPG, AcMNPVΔp35.US3, and AcMNPVΔp35.IAP3) carrying different anti-apoptotic genes. The BoHV-5 US3 protein showed poor

Fig. 4 Morphological analysis of Sf-21 insect cells infected with AcΔp35MNPV.US3 at 48 h.p.i. **(a)** and control cells without infection **(b)**. **a** Subcellular localization of US3 protein kinase by immunostaining and presence of nuclear fragmentation (white arrow). **b** Immunostaining

of control cells without infection. Green fluorescence corresponds to secondary antibody Alexa 488™ against 6xHis Tag. Blue fluorescence corresponds to nuclear staining, performed with Hoechst stained. White bars correspond to 25 μm

Fig. 5 Caspase 3/7 activity assay in Sf-21 insect cell after 12, 24, and 48 h.p.i. with the recombinant AcMNPV baculoviruses (MOI = 1) and mock-infected cells. Data represents the mean ($\pm$ standard deviation, SD) of independent experiments, each performed in triplicate. Error bars indicate SDs. RLU, relative light units. Acpg, AcPG virus; P35, AcMNPV Δ p35 virus; Iap3, AcMNPV Δ p35.IAP3; US3, AcMNPV Δ p35.US3; MOCK, mock-infected cells

apoptosis inhibition during infection of Sf-21 cells with the recombinant AcMNPV Δ p35.US3.

Unlike other US3 protein kinases of other alphaherpesviruses, our findings related to apoptosis differs from the data of US3 ortholog genes [9, 12, 17]. Here, Sf-21 insect cells exhibited various responses against the recombinant baculoviruses. During the infection, it was possible to visualize by light microscopy the evident phenotypes of apoptosis in the AcMNPV Δ p35-infected cells and with less intensity in the AcMNPV Δ p35.US3-infected cells. These data are in agreement with the phenotype presented in other studies that used the mutant AcMNPV Δ p35 as a tool for the study of apoptosis [29, 30]. AcPG and AcMNPV Δ p35.IAP3 viruses induced few significant morphological changes up to 48 h.p.i., and no apoptotic cells or cell death were observed, which is probably due to the effect of the expression of their apoptosis suppressor proteins (P35 and IAP-3) [24, 31].

The quantification of caspases 3/7 during infection of Sf21 cells by the different recombinant viruses were in agreement with the observed phenotypes shown by light microscopy. AcMNPV Δ p35 and AcMNPV Δ p35.US3 showed high levels of caspase activity during infection of Sf21 cells. On the other hand, AcPG and AcMNPV Δ p35.IAP3 showed low or no caspase activity, which was similar to those of mock cells. The delay in caspase activation in AcMNPV Δ p35.US3-infected Sf21 cells compared with AcMNPV Δ p35-infected cells before 24 h.p.i. could be due to an initial inhibition of apoptosis by the low expression of the US3 protein at early times post-infection and as the viral DNA increases its replication; the US3 protein was not able to efficiently block apoptosis due to the massive replication of the baculovirus DNA.

At late stages, there is an increase in US3 production, since the gene is under the command of a late baculovirus promoter, but since apoptosis has been already activated, the protein is not able to block its progression. A possibility to test this hypothesis is to clone the US3 gene under an early baculovirus promoter to see if an increase in the early expression of the US3 protein would be able to block apoptosis. In the studies performed with the wild-type SHV-1 US3 gene, 24 h.p.i showed low effector caspase 3 and low percentage of apoptosis-positive swine testicle (ST) cells by the TUNEL assay, compared with their deletion mutants in the US3 gene when induced to apoptosis by either staurosporine or sorbitol [9, 32]. Takashima et al. [15] demonstrated that BoHV-1 US3 does not have activity in blocking apoptosis in bovine MDBK cells, but no caspase activity was measured and apoptosis effects were only evaluated by DNA degradation, a technique with poor sensitivity. More recently, Brzozowska et al. [17] showed, by TUNEL assay and flow cytometry, that BoHV-1 US3 is capable to block apoptosis in bovine cells (MDBK and KOP cells) induced either by external stimuli or during infection. In addition, Ladelfa et al. [16] observed that the BoHV-5 US3 gene is able to neutralize apoptosis induced by external stimuli (staurosporine) in Vero- and ST-transfected cells, presenting 60% of effector caspase 3-labeled cells, while mock cells showed 80% of cells positive for caspase 3 labeling. These data indicate that there is activation of caspase 3 during infection of these cells, demonstrating the activation of the apoptotic pathway, and that the US3 gene of BoHV-5 was not able to inhibit or drastically reduce cellular apoptosis as demonstrated for SHV-1 or BoHV-1 [9, 17]. Moreover, Duroelle et al. [6] performed a comparison of the amino acid identity of the serine/threonine kinases proteins of some alphaherpesviruses with the BoHV-5 protein kinases and found only 31% identity to HHV-1 and HHV-2, 42% with SHV-1 and 81% identity to BoHV-1. These low identities between protein kinases possibly reflect the different efficacies in the control of apoptosis.

The labeling of the nucleus with Hoechst staining also demonstrates the apoptosis activity by fragmented DNA, a signal of apoptosis, similar to the cell nuclei observed by Murata et al. [8] in cells induced to apoptosis by sorbitol. After 48 h.p.i., the localization of serine/threonine kinases was visualized mainly in the cell cytoplasm like described for BoHV-5 and BoHV-1 after 24 h.p.i. [16, 33]. Probably the cellular disorganization and the vacuoles in the cytoplasm could be caused by cytoskeleton disorganization due to the expression of this protein. However, more experiments like the ones carried out by Brozowska et al. and Ladelfa et al. [16, 33] should be performed to confirm this hypothesis. To summarize, we showed that BoHV-5 US3 can be successfully expressed in insect cells using the baculovirus expression system. We have also showed that the US3 protein is not able to block

apoptosis in insect cells, contrasting with the baculoviruses P35 and IAP-3 proteins which efficiently block apoptosis in insect cells. The anti-apoptotic activity of BoHV-1 and BoHV-5 proteins, including US-3 kinases, may vary by cell type and viral strain as seen by Ladelfa et al. [16], Marin et al. [34], and Rensetti et al. [35] and since herpesvirus is a mammal virus, the anti-apoptotic effect of viral encoded genes could be restricted to the host tissues. Therefore, the lack of US3 apoptotic blockage function in insect cells could also be due to the lack of the proper substrate for the US3 kinase activity.

Funding information This work was supported by Fundação de Apoio à Pesquisa do Distrito Federal- FAPDF (process number: 0193.001235/2016, FAPDF number: 1063/2016) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior –CAPES.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- French EL (1962) A specific virus encephalitis in calves: isolation and characterization of the causal agent. *Aust Vet J* 38:216–221
- Del Médico Zajac MP, Ladelfa MF, Kotsias F, Muylken B, Thiry J, Thiry E, Romera SA (2010) Biology of bovine herpesvirus 5. *Vet J* 184:138–145 Elsevier
- Griffin BD, Verweij MC, Wiertz EJ (2010) Herpesvirus and immunity: the art of evasion. *Vet Microbiol* 143:89–100 Elsevier
- Jones C (1998) Alphaherpesvirus latency: its role in disease and survival of the virus in the nature. *Adv Virus Res* 51:47–99
- Ciacchi-Zanella J, Stone M, Henderson G, Jones C (1999) The latency-related gene of bovine herpesvirus 1 inhibits programmed cell death. *J Virol* 73:9734–9740
- Deruelle MJ, Favoreel HW (2011) Keep it in the subfamily: the conserved alphaherpesvirus US3 protein kinase. *J. Gen. Virol.* 92: 18–30
- Kato A, Yamamoto M, Ohno T, Kodaira H, Nishiyama Y, Kawaguchi Y (2005) Identification of proteins phosphorylated directly by the Us3 protein kinase encoded by herpes simplex virus 1. *J Virol* 79(14):9325–9331
- Murata T, Goshima F, Yamauchi Y, Koshizuka T, Takakuwa H, Nishiyama Y (2002) Herpes simplex virus type 2 US3 blocks apoptosis induced by sorbitol treatment. *Microbes Infect* 4:707–712
- Deruelle MJ, Geenen K, Nauwynck HJ, Favoreel HW (2007) A point mutation in the putative ATP binding site of the pseudorabiesvirus US3 protein kinase prevents Bad phosphorylation and cell survival following apoptosis induction. *Virus Res* 128: 65–70
- Schumacher D, McKinney C, Kaufer BB, Osterrieder N (2008) Enzymatically inactive US3 protein kinase of Marek's disease virus (MDV) is capable of depolymerizing F-actin but results in accumulation of virions in perinuclear invaginations and reduced virus growth. *Virology* 375:37–47
- Munger J, Roizman B (2001) The US3 protein kinase of herpes simplex virus 1 mediates the posttranslational modification of BAD and prevents BAD-induced programmed cell death in the absence of other viral proteins. *Proc Natl Acad Sci* 98:10410–10415
- Cartier A, Komai T, Masucci MG (2003a) The Us3 protein kinase of herpes simplex virus 1 blocks apoptosis and induces phosphorylation of the Bcl-2 family member Bad. *Exp Cell Res* 291:242–250
- Cartier A, Broberg E, Komai T, Henriksson M, Masucci MG (2003b) The herpes simplex virus-1 Us3 protein kinase blocks CD8 T cell lysis by preventing the cleavage of Bid by granzyme B. *Cell Death Differ* 10:1320–1328
- Kato A, Kawaguchi Y (2018) Us3 protein kinase encoded by HSV: the precise function and mechanism on viral life cycle. In: Kawaguchi Y, Mori Y, Kimura H (eds) *Human Herpesviruses. Advances in experimental medicine and biology*, vol 1045. Springer, Berlin
- Takashima Y, Tamura H, Xuan X, Otsuka H (1999) Identification of the US3 gene product of BHV-1 as a protein kinase and characterization of BHV-1 mutants of the US3 gene. *Virus Res* 59:23–34
- Ladelfa MF, Kotsias F, Del Médico Zajac MP, Broeke CV, Favoreel H, Romera SA, Calamante G (2011) Effect of the US3 protein of bovine herpesvirus 5 on the actin cytoskeleton and apoptosis. *Vet Microbiol* 153:361–366
- Brzozowska A, Lipińska AD, Derewońko N, Lesiak D, Rychłowski M, Rąbalski L, Bieńkowska-Szewczyk K (2018) Inhibition of apoptosis in BHV-1-infected cells depends on Us3 serine/threonine kinase and its enzymatic activity. *Virology* 513: 136–145
- Flores EF, Donis R (1995) Isolation of a mutant MDBK cell line resistant to bovine virus diarrhoea virus (BVDV) due to a block in viral entry. *Virology* 208:565–575
- Salvador SC, Lemos RAA, Riet-Correa F, Roehle PM, Osório ALA (1998) Meningoencefalite em bovinos causada por herpesvírus bovino 5 no Mato Grosso do Sul e São Paulo. *Pesqui Vet Bras* 18(2):75–82
- Granados RR, Guoxun L, Derksen ACG, McKenna KA (1994) A new insect cell line from *Trichoplusia ni* (BTI-Tn-5B1-4). Susceptible to *Trichoplusia ni* single enveloped nuclear Polyhedrosis virus. *J Invertebr Pathol* 64:7
- Vaughn JL, Goodwin RH, Tompkins GJ, McCawley P (1997) The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Vitro* 13(4):213–217
- Hopkins R, Esposito D (2009) A rapid method for titrating baculovirus stocks using the Sf-9 easy titer cell line. *Biotechniques* 47:785–788
- Muylken B, Thiry J, Kirten FS, Thiry E (2007) Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. *Vet Res., France* 38:181–209
- Clem RJ, Miller LK (1994) Control of programmed cell death by the baculovirus genes p35 and iap. *Mol Cell Biol* 14:5212–5222
- O'Reilly D, Miller LK, Luckow VA (1992) *Baculovirus expression vectors: a laboratory manual*. Freeman and Company, New York
- Ardissou-Araújo DMP, Melo FL, Clem RJ, Wolff JLC, Ribeiro BM (2015) A betabaculovirus-encoded *gp64* homolog is a functional envelope fusion protein. *J Virol* 90:JVI. 02491–JVI. 02415
- Purves FC, Longnecker RM, Leader DP, Roizman B (1987) Herpes simplex virus 1 protein kinase is encoded by open reading frame US3 which is not essential for virus growth in cell culture. *J Virol* 61:2896–2901
- Daikoku T, Yamashita Y, Tsurumi T, Maeno K, Nishiyama Y (1993) Purification and biochemical characterization of the protein kinase encoded by the US3 gene of herpes simplex virus type 2. *Virology* 197(2):685–694
- Chejanovsky N (2016) Using the Baculovirus/insect cell system to study apoptosis. *Methods Mol Biol* 1350:477–489

30. Yan F, Deng X, Yan J, Wang J, Yao L, Iv S, Qi Y, Xu H (2010) Functional analysis of the inhibitor of apoptosis genes in *Antheraea pernyi* nucleopolyhedrovirus. *J Microbiol* 48:199–205
31. Carpes MP, de Castro MEB, Soares EF, Villela AG, Pinedo FJR, Ribeiro BM (2005) The inhibitor of apoptosis gene (IAP-3) of *Anticarsa gemmatilis* multicap-sid nucleopolyhedrovirus (AgMNPV) encodes a functional IAP. *Arch Virol* 150:1549–1562
32. Geenen K, Favoreel HW, Olsen L, Enquist LW, Nauwynck HJ (2005) The pseudorabies virus US3 protein kinase possesses anti-apoptotic activity that protects cells from apoptosis during infection and after treatment with sorbitol or staurosporine. *Virology* 331: 144–150
33. Brzozowska A, Rychlowski M, Lipinska AD, Bienkowska-Szewczyk K (2010) Point mutations in BHV-1 Us3 gene abolish its ability to induce cytoskeletal changes in various cell types. *Vet Microbiol* 143:8–13
34. Marin MS, Leunda MR, Verna AE, Faverín C, Pérez SE, Odeón AC (2012) In vitro replication of bovine herpesvirus types 1 and 5. *J Virol Methods* 181(1):80–85
35. Rensetti DE, Marin MS, Morán PE, Odeón AC, Verna AE, Pérez SE (2018) Bovine herpesvirus type 5 replication and induction of apoptosis in vitro and in the trigeminal ganglion of experimentally-infected cattle. *Comp Immunol Microbiol Infect Dis* 57:8–14

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.