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Point mutations in BHV-1 Us3 gene abolish its ability to induce cytoskeletal changes in various cell types

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ABSTRACT

The Us3 gene is conserved among alphaherpesviruses and codes for a protein kinase, a multifunctional protein involved in many phases of virus infection, like nuclear egress, modulation of apoptosis and modification of the cellular cytoskeleton. Bovine herpesvirus (BHV-1), a member of the *Alphaherpesvirinae*, contains an open reading frame homologous to Us3 of other herpesviruses, which has been identified as a serine/threonine kinase (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). To study the activity of BHV-1 Us3, we have cloned its sequence under control of the human cytomegalovirus (HCMV) promoter/enhancer and introduced it into a recombinant baculovirus (Bac Us3). Confocal microscopy analysis showed profound cytoskeletal modifications in various BHV-1-permissive and non-permissive cells transduced with BacUs3. We observed that Us3 expression changed cellular shape and induced formation of long microtubule-containing cell projections, a phenomenon which had also been observed in cells expressing pseudorabies virus Us3. The intracellular localization of Us3 was mostly nuclear but when the protein accumulated it could be detected in the cytoplasm, cell membranes and projections. Mutated forms of BHV-1 Us3 with point mutations near or within the kinase catalytic domain did not affect cell morphology indicating that kinase activity of BHV-1 Us3 is required for its cytoskeleton remodelling function.

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1. Introduction

Bovine herpesvirus 1 (BHV-1), a member of alphaherpesvirus subfamily, is a pathogen of cattle causing respiratory and genital infections (Muylkens et al., 2007). Like other herpesviruses, BHV-1 exploits and extensively modifies the internal environment of infected cells. One of the viral proteins produced by herpesviruses for interaction with the host cell is a protein kinase (PK), encoded by the Us3 gene. The Us3 gene is highly conserved among alphaherpesviruses. It codes for a serine/threonine kinase, a multifunctional protein which was reported to play a role in the nuclear egress of capsids, prevention of apoptosis, modulation of host immune response and modifications of the cytoskeleton (Klupp et al., 2001; Mou et al., 2008; Benetti and Roizman, 2004; Murata et al., 2002; Liang and Roizman, 2008). The Us3 protein can induce extensive cytoskeletal alterations including the formation of long, actin-containing cellular projections which was for the first time observed for pseudorabies virus (PRV) (Van Minnebruggen et al., 2003; Favoreel et al., 2005). It has been demonstrated that the kinase activity of PRV Us3 is required for its actin modulating capability (Van den Broeke et al., 2009a). Recent findings indicate that group A p21-activated kinases are involved in the Us3-mediated effects on the actin cytoskeleton (Van den Broeke et al., 2009b).

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Table 1
Primers used for amplification of BHV-1 Us3 and mutant construction.

Primer name	Sequence (5′ → 3′)	Restriction site
Us3 wt F	AAAATTCGAAATGGAGCGCGCGGCGGAGCGGCTGG	AsuII
Us3 wt R	AAAATTCGAACTA**cagatcttcttcagaaataagttttttgttc**CCCCGAGGCCGCACCGAA	AsuII
Us3L265A F	AAAAAACGCGTGTTTGAGGCAACGGGGCCTGCTC	MluI
Us3L265A R1	GGAGTGCAGGTACGCGAGGCCCCG**TGC**CAC	
Us3L265A R2	AAAAAGACGTCCCGGTGAGCGATCCGGCGGGAGTGCAGGTA	AatII
Us3K282A F	AAAAAGACGTC**GC**AACGGAAAACGTCTTCCTCAACGGCCCA	AatII
Us3K282A R	AAAAACGTACGCGACGACGCCCGCGCTCCA	SplI

The underlined sequence indicates the restriction sites used in cloning, bold letters indicate bases modified for mutant construction and lower case letters define the c-Myc-tag sequence. The letters F and R indicate a forward or reverse primer.

BHV-1 Us3 gene encodes a protein exhibiting high amino acid similarity to the Us3 of PRV. It has earlier been identified as a 58 kD protein which posses PK activity (Takashima et al., 1999). Similarly to Us3 genes of other alphaherpesviruses, BHV-1 Us3 is non-essential for the growth of virus in cell culture. Contrary to HSV-1, an inhibition of apoptosis was not detected for BHV-1 Us3 (Takashima et al., 1999).

In the present report we characterized the effect of BHV-1 Us3 expression on the architecture of the cell. Our experiments show that BHV-1 Us3 induces dramatic cytoskeletal reorganization. We were also able to demonstrate that point mutations in or near the predicted kinase active site impair this activity.

2. Materials and methods

2.1. Cells

Bovine EBTr (embryonic bovine trachea, Institute for Animal Science and Health Lelystad, The Netherlands), and KOP (foetal bovine esophagus cells, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany) swine SK 6 (swine kidney) and Vero (African green monkey kidney) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 8% fetal bovine serum (FBS), 2 mM L-glutamine and antibiotics, at 37 °C in 5% CO_2 atmosphere.

Spodoptera frugiperda (Sf-9) cells were maintained in HyQ SFX-Insect MP (HyClone) when used for transfection or Grace's Insect medium (Sigma) mixed 1:1 (v/v) with TC-100 Insect Medium (Invitrogen, USA) supplemented with 4% FBS and antibiotics (for baculovirus amplification and titration).

2.2. Antibodies

Rabbit monoclonal antibody against c-Myc-tag (Santa Cruz Biotechnology) was used at a dilution of 1:100. Anti-gE mouse monoclonal antibody MAb3 was obtained from The Institute for Animal Science and Health, Lelystad, The Netherlands, and used at a dilution of 1:10 000. Mouse monoclonal anti-tubulin antibody (Sigma) was used at 1:2000 dilution. F-actin was stained with phalloidin–TRITC conjugate (Sigma) at a dilution of 1:2000. Secondary goat anti-rabbit IgG Alexa 488 and goat anti-mouse IgG Alexa 546 conjugates (Molecular Probes) were used at a dilution of 1:3000.

2.3. Generation of recombinant baculovirus vectors

Baculovirus recombinants were constructed using the Bac-to-Bac system (Invitrogen) according to the manufacturer's protocol. Modified FastBacDual plasmid, pFBD, in which the polyhedrin promoter was replaced by the HCMV immediate-early promoter/enhancer (described in Grabowska et al., 2009) was used as a transfer plasmid. To facilitate propagation of recombinant baculoviruses in insect cells, the GFP gene was cloned into XhoI–BpiI restriction sites under the p10 baculovirus promoter. All recombinants were constructed by PCR using the primers listed in Table 1. Genomic DNA from BHV-1 strain Lam (Metzler et al., 1985) was used as a template. In all constructs the Us3 gene was amplified in fusion with a c-Myc-tag. The purified PCR product was inserted into the AsuII restriction site downstream of the HCMV promoter of pFBD. To construct Us3L265A (leucine substituted by alanine), a 311 bp fragment from nucleotide 532–842 of the Us3 sequence was amplified by PCR and cloned between the AatII and MluI sites. To construct Us3K282A (lysine substituted by alanine), a 189 bp fragment of Us3 gene from nucleotide 843–1031 was amplified by PCR and cloned between the AatII and SpII restriction sites.

2.4. Transduction of mammalian cells with baculoviruses

Mammalian cells were seeded onto 12-well plates and cultured overnight. Before transduction cells were washed three times with PBS, and baculovirus inocula in D-PBS (phosphate-buffered saline containing Ca^{2+} and Mg^{2+}, Sigma) were added at a multiplicity of infection (MOI) of 4. After incubation for 1 h at 27 °C and 1 h at 37 °C, the cells were washed, fresh complete medium was added, and the cells were cultured further for the time required for next analysis.

2.5. Immunofluorescence and confocal microscopy analysis

For confocal microscopy analysis, cells were grown and transduced on glass coverslips. After two washes with PBS, cells were fixed with cold 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.2% Triton X-100 in PBS for 5 min and washed three times with PBS. Cells were incubated for 1 h with primary antibodies, washed three times with PBS and incubated for 1 h with respective secondary antibodies. All antibody

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