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The X proteins of bornaviruses interfere with type I interferon signalling

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Borna disease virus (BDV) is a neurotropic, negative-stranded RNA virus causing persistent infection and progressive neurological disorders in a wide range of warm-blooded animals. The role of the small non-structural X protein in viral pathogenesis is not completely understood. Here we investigated whether the X protein of BDV and avian bornavirus (ABV) interferes with the type I interferon (IFN) system, similar to other non-structural proteins of negative-stranded RNA viruses. In luciferase reporter assays, we found that the X protein of various bornaviruses interfered with the type I IFN system at all checkpoints investigated, in contrast to previously reported findings, resulting in reduced type I IFN secretion.

INTRODUCTION

Viruses comprise the largest group of biological entities on earth (Koonin *et al.*, 2006). During evolution, host cells have evolved defence mechanisms against viral infections and viruses have, consequently, developed effective methods to escape host defence mechanisms (García-Sastre & Biron, 2006). First in line of the host defence mechanisms is the type I interferon (IFN)-mediated innate immune response, commonly activated upon RNA virus infection. IFNs are secreted cytokines that are able to stimulate expression of hundreds of different genes and may thus affect several steps of the viral life cycle (Sen, 2001; Stark *et al.*, 1998).

The IFN-mediated antiviral programme is initiated by viral activation of cellular sensors. Toll-like receptors (TLRs), associated chiefly with endosomes, recognize ssRNA and dsRNA, as well as viral DNA. Retinoid-inducible gene I (RIG-I), protein kinase R (PKR) and melanoma differentiation-associated gene 5 (MDA5) are cytoplasmic sensors that recognize dsRNA (Baum & García-Sastre, 2010; García-Sastre & Biron, 2006; Sen, 2001). The activation of viral sensors triggers downstream signalling molecules, culminating in activation of constitutively expressed transcription factors, such as activator protein 1 (AP-1), nuclear factor kappa B (NF-κB) and IFN regulatory factors 3 and 7 (IRF3/7). NF-κB and AP-1 strongly upregulate type I IFN expression (Haller *et al.*, 2006). The secreted type I IFNs sequentially activate the IFN-stimulated response element (ISRE) in an autocrine and/or paracrine manner. The activated ISRE leads to the transcription of hundreds of IFN-stimulated genes (ISGs) that collectively determine the antiviral state of the host cell.

Borna disease virus (BDV) is the causative agent of Borna disease, a fatal neurological disease mainly affecting horses and sheep (Ludwig & Bode, 2000). The virus is a non-segmented, negative-stranded RNA virus that belongs to the order *Mononegavirales* (Lipkin & Briese, 2006). Due to its nuclear site of replication (Briese *et al.*, 1992) and use of the cellular RNA splicing machinery for gene expression (Cubitt *et al.*, 1994; Schneider *et al.*, 1994), BDV is the solitary member of the family *Bornaviridae*. BDV was first discovered in horses and sheep, although natural infection has been reported in a wide range of animals, including cats (Wensman *et al.*, 2008). Most BDV strains have high genetic similarity (95–99 %), where the highest genetic divergence of around 15 % was found for a strain (No/98) that was isolated from an Austrian horse (Nowotny *et al.*, 2000). Recently, even more divergent viruses have been described in psittacine birds with proventricular dilatation

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disease (PDD), designated avian bornaviruses (ABVs) (Honkavuori *et al.*, 2008; Kistler *et al.*, 2008).

A number of previous studies have discussed the role of type I IFNs in BDV infection. Type I IFNs were shown to block BDV replication in Vero cells, but not in C6 cells (Hallensleben & Staeheli, 1999). The phosphoprotein of BDV (BDV P) was reported to act as a decoy substrate for phosphorylation by TBK-1 and, as such, to compete with endogenous substrates (Unterstab *et al.*, 2005). Hence, BDV P inhibits the IFN-β expression normally induced upon TBK-1 activation of downstream transcription factors.

Several RNA viruses have developed type I IFN-inhibiting capacities at different levels in the signalling cascade. For example, the non-structural protein 1 (NS1) of influenza viruses is a well-known and potent inhibitor of type I IFN gene expression, and influences the activity of several ISGs (Fernandez-Sesma, 2007). Viruses within the order *Mononegavirales* have a similar genome organization, including a small non-structural protein (Pringle, 2005). In many cases, this small non-structural protein has type I IFN-inhibiting properties, e.g. VP35 of Ebola virus (Basler & Amarasinghe, 2009) and the V protein of paramyxoviruses (Goodbourn & Randall, 2009). The non-structural protein of BDV, called p10 or X (Schwardt *et al.*, 2005; Wehner *et al.*, 1997), has so far been shown not to have type I IFN-inhibiting characteristics (Unterstab *et al.*, 2005); however, this protein has been shown to contribute to the resistance to apoptosis induced upon BDV infection (Poenisch *et al.*, 2009). As the organization of the BDV genome is similar to that of other members of the order *Mononegavirales*, we investigated whether the small non-structural X protein of BDV has type I IFN-inhibiting properties. Furthermore, the possibility of strain differences in type I IFN inhibition was studied.

RESULTS

The X protein of BDV He/80 inhibits dsRNA- and Sendai virus (SV)-induced type I IFN signalling in A549 cells

We used the human alveolar cell line A549 in combination with a luciferase reporter system (Munir *et al.*, 2011) to investigate whether different BDV proteins could inhibit the type I IFN system upon dsRNA induction and SV infection. A549 cells were co-transfected with expression plasmids for the P and X proteins of one of the most used BDV reference strains (He/80), together with a reporter plasmid indicating functional type I IFN signalling (pISRE-Luc). The individual X or P proteins or combinations of the proteins (X+P) were expressed to investigate possible synergistic effects. Whilst expression of the X protein of BDV He/80 either alone or in concert with P inhibited type I IFN signalling significantly (P values $\leqslant 0.001$) following induction by dsRNA [poly(I:C)] or SV infection, P alone had no effect (Fig. 1a, b). The specificity of this finding was confirmed in that expression levels of all proteins were similar, as indicated by Western blotting (Fig. 1c).

The X proteins of different bornavirus strains have similar type I IFN-inhibiting capacity

Next, we wanted to compare the type I IFN-inhibiting capacity of BDV X proteins isolated from different host species. Besides BDV X of the strains He/80 and No/98, derived from horses, an X protein isolated from a cat with staggering disease (X feBDV) and the X protein of an ABV strain (1322) isolated from a psittacine bird with PDD were included. The NS1 protein of H5N1 avian influenza virus was used as a positive control. All variants of BDV X inhibited the pISRE activity stimulated by poly(I:C) or SV infection in a similar manner, except that feBDV and ABV X had a slightly higher level of inhibition upon SV infection ($P<0.001$). The level of inhibition for the X proteins was lower than that for NS1 (Fig. 1a, b).

To investigate whether any amino acid residues were responsible for this slight difference in type I IFN inhibition, we aligned the amino acid sequences of the X proteins (Fig. 1d). As expected, the ABV X had a high sequence dissimilarity from the other strains (52.8 % compared with the feline BDV X; 19.5 % compared with X of BDV No/98), whereas the X proteins of feBDV and BDV He/80 only differed at one amino acid residue (aa 81). The X proteins of BDV No/98 and the ABV X had identical amino acids at three positions (marked by green boxes in Fig. 1d); however, in the other two X proteins, these positions differed from BDV No/98 and ABV.

To support the specificity of the findings, we investigated whether BDV X exhibits a dose-dependent inhibitory effect (data not shown). For strain No/98 and the ABV strain, increasing amounts of the X protein resulted in a higher level of inhibition of the ISRE activity (from 25 and 50 % at 250 ng, respectively, to 75 % at 1000 ng for both proteins). BDV X of strain He/80 did not show any inhibition at the lowest amount (250 ng), whereas at 500, 750 and 1000 ng, the level of inhibition was similar (around 50 %). For the feBDV X, the system seemed saturated already at 250 ng, as an equal level of inhibition (about 50–60 %) was seen throughout the experiment.

Both secreted and intracellular type I IFN protein is reduced in BDV X- and ABV X-transfected A549 cells

To characterize the effects of BDV X on the type I IFN system further, we performed an IFN bioassay to measure the level of type I IFN protein expression in cell supernatants. All X variants, except for the feBDV X, reduced the type I IFN protein secretion upon stimulation by poly(I:C) significantly ($P\leqslant 0.05$), whereas BDV P did not show any significant reduction (Fig. 2a). BDV P was therefore excluded from the following experiments, as it did not show any inhibition of pISRE activity or reduction

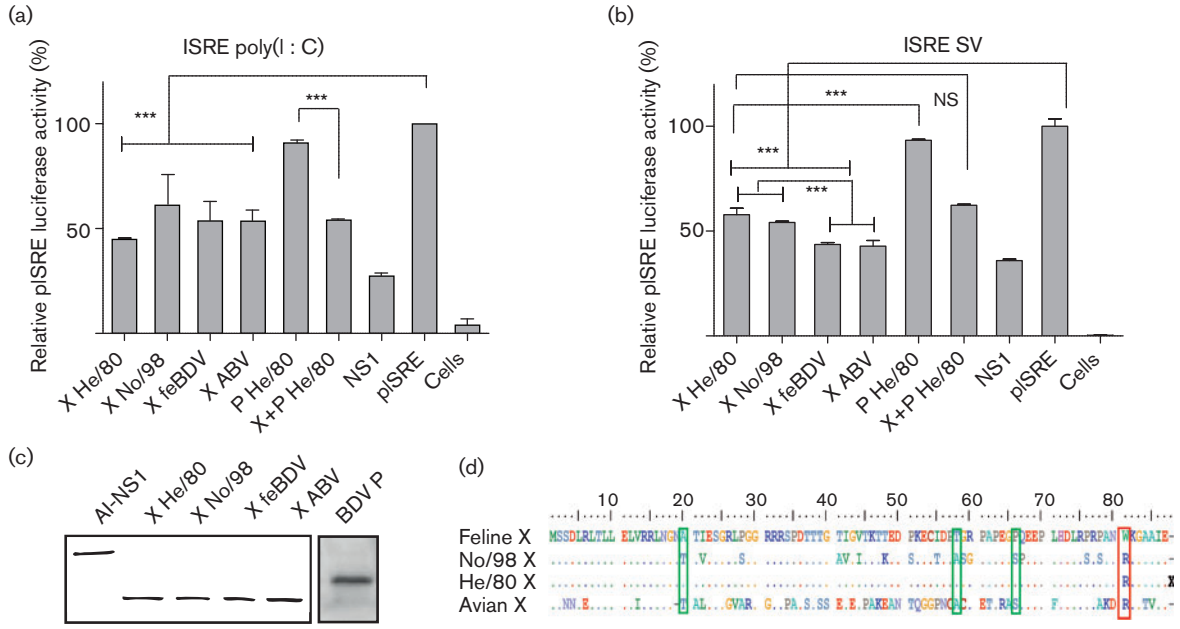

Fig. 1. BDV X interferes with type I IFN signalling induced by dsRNA and SV infection. A549 cells were co-transfected with 500 ng of the indicated constructs and a reporter plasmid (pISRE-Luc). Twenty-four hours post-transfection, cells were stimulated by either (a) 10 μg poly(I : C) ml^{-1} or (b) SV infection. After another 24 h, luminescence was measured and normalized to the luciferase activity of the control, transfected with an empty expression vector. Mean + SEM values of three (a) and two (b) independent experiments performed in duplicate are shown. ***$P \leqslant 0.001$; NS, non-significant using Student's *t*-test. Western blot analysis (c) was performed to assure comparable expression of the proteins. A549 cells were lysed 24 h post-transfection and processed as described in Methods. (d) Amino acid sequences of the different X proteins investigated were aligned. Green boxes indicate amino acid differences in common for X of BDV He/80 and the feline BDV in comparison to the other two proteins; the red box indicates a unique amino acid difference in the feline BDV X protein.

in type I IFN protein secretion (Figs 1 and 2a). The intracellular level of the type I IFN protein was also investigated by co-transfecting A549 cells with expression plasmids for BDV X and a reporter plasmid for p125 (p125-Luc), a promoter activated in the same way as intracellular IFN-β. All X proteins reduced p125 activity significantly ($P \leqslant 0.001$); however, ABV X seemed to have the highest potency ($P \leqslant 0.001$) (Fig. 2b).

BDV X and ABV X inhibit dsRNA-induced IRF3 promoter activation

As the X proteins of BDV and ABV inhibited ISRE-activation by interfering in type I IFN signalling (Fig. 1a, b) and expression of type I IFN protein (Fig. 2a, b), we continued our studies to explore the mechanism by which interference occurs. First, IRF3 promoter activation was studied by co-transfecting A549 cells with expression plasmids for the different X proteins and a reporter plasmid for the IRF3 promoter (4 × IRF3-Luc). All BDV X variants, including the ABV X, inhibited dsRNA-induced promoter activation to a similar extent; however, the level of inhibition was significantly lower compared with the NS1 protein of influenza virus ($P \leqslant 0.01$) (Fig. 3a).

dsRNA-induced MDA5 and RIG-I activity is reduced by the X protein of BDV and ABV

Two upstream host cellular sensors of intruding viruses are the RNA helicases MDA5 and RIG-I. Activation of either of these two proteins by viral dsRNA and 5′-triphosphate ssRNA, respectively, eventually leads to type I IFN induction. To investigate the capability of BDV X and ABV X to interfere at this level, A549 cells were co-transfected with expression plasmids for the different X variants and for MDA5 or RIG-I, respectively, together with a reporter plasmid (pISRE-Luc). All different X proteins of BDV and ABV reduced the dsRNA-induced activity of MDA5 (Fig. 3b) and RIG-I (Fig. 3c) to an extent similar to that seen with influenza virus NS1.

DISCUSSION

In this study, we explored whether the small non-structural X protein of BDV and ABV could interfere with type I IFN signalling, similarly to other non-structural proteins of negative-stranded RNA viruses, such as NS1 of influenza virus (Fernandez-Sesma, 2007), VP35 of Ebola virus (Basler & Amarasinghe, 2009) and the V proteins of paramyxoviruses

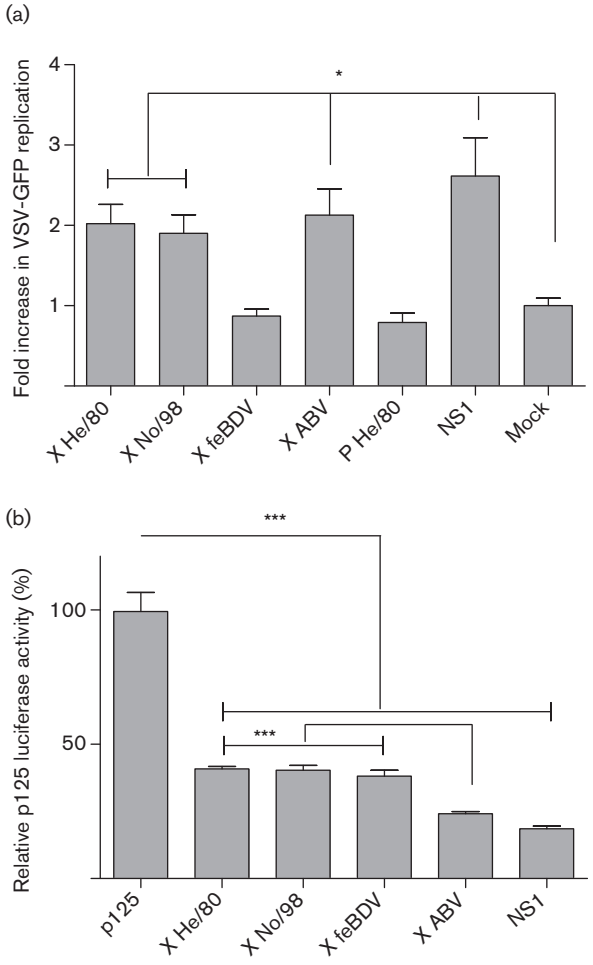

Fig. 2. IFN bioassay. Both secreted and intracellular type I IFN protein is reduced in BDV X- and ABV X-transfected A549 cells. (a) A549 cells were transfected with the indicated constructs or empty expression vector and stimulated by dsRNA for 24 h, followed by infection with VSV-GFP. In cells where type I IFN protein secretion is reduced, VSV-GFP can replicate, whereas in cells where type I IFN is not reduced, VSV-GFP replication is inhibited. The signals were quantified and normalized to mock-treated cells as indicated in Methods. (b) A549 cells were transfected with the indicated constructs and a reporter plasmid for p125, a promoter activated like intracellular IFN-β. Mean + SEM values of three independent experiments performed in duplicate are shown. *$P \leqslant 0.05$; ***$P \leqslant 0.001$ using Student's *t*-test.

(Goodbourn & Randall, 2009). In a series of independent experiments designed to test different checkpoints of the type I IFN signalling pathway, we confirmed that the X proteins of BDV and ABV also have type I IFN-inhibiting properties.

In the first experiment, where ISRE activity was investigated, we induced type I IFN using dsRNA and SV infection. These two type I IFN inducers have TLR3 as a common pattern-recognition receptor (PRR); however, SV infection also induces type I IFN production through activation of RIG-I (Baum & García-Sastre, 2011) and MDA5 (Gitlin *et al.*, 2010). During BDV replication, the triphosphate group at the 5′ end of the genomic RNA is replaced by a monophosphate group (Schneider *et al.*, 2005). This allows RIG-I evasion when genomic RNA is transfected into cells (Habjan *et al.*, 2008; Reuter *et al.*, 2010) and presumably facilitates BDV and ABV evasion of type I IFN in wild-type infection. Our data indicate that the X protein can interfere with the PRRs MDA5 and RIG-I, although the exact mechanism of interference has not been determined.

BDV P, which interferes with the IFN-β mRNA expression by acting as a decoy substrate for phosphorylation by TBK-1 (Unterstab *et al.*, 2005), has also been implicated in the evasion of innate immunity. We did not find significant type I IFN inhibition by BDV P upon stimulation by exogenous dsRNA or in SV-infected cells (Fig. 1). Interestingly, in a study by Unterstab *et al.* (2005), BDV P did not interfere with type I IFN signalling upon exogenous stimulation of IFN-α, but this was demonstrated in cells infected with an influenza virus lacking the NS1 protein (delNS1 influenza virus). Moreover, BDV X was shown not to interfere with type I IFN signalling in cells infected with delNS1 influenza virus (Unterstab *et al.*, 2005). Hence, BDV P does not seem to be able to interfere with type I IFN signalling upon exogenous stimulation (dsRNA and IFN-α), but is fully capable of interfering with type I IFN signalling through interaction with TBK-1 in cells infected with delNS1 influenza virus (Unterstab *et al.*, 2005). On the other hand, BDV X interferes with type I IFN signalling upon exogenous (dsRNA) stimulation, as well as in SV-infected cells (Fig. 1), but not in cells infected with delNS1 influenza virus (Unterstab *et al.*, 2005). Obviously, the induction pathways are important for the outcome, and in this regard, influenza virus and SV probably induce different pathways. Influenza virus usually induces type I IFN by TRIM25 and RIG-I interaction (Munir, 2010), whereas SV is sensed by TLR3, RIG-I and MDA5 (Baum & García-Sastre, 2011; Gitlin *et al.*, 2010).

Another possible explanation could be differences in cell line, as Unterstab *et al.* (2005) used MDCK and 293T cells, whereas we used A549 cells. Preliminary data show that persistently BDV-infected C6 cells have a lower ISRE activity than non-infected C6 cells (K. Hörnaeus, M. Berg & J. J. Wensman, unpublished data), in line with the results presented here, and that type I IFNs are induced early upon BDV infection of A549 cells (M. Munir, M. Rizwan & J. J. Wensman, unpublished data). Cell line differences in type I IFN functionality have been reported, where Vero cells, but not C6 cells, become less susceptible to BDV replication and infection following treatment with type I IFN (Hallensleben & Staeheli, 1999).

One consequence of the type I IFN host immune response is the induction of apoptosis in virus-infected cells (Chawla-Sarkar *et al.*, 2003). BDV can, through the X protein, interfere with this host defence, although the exact

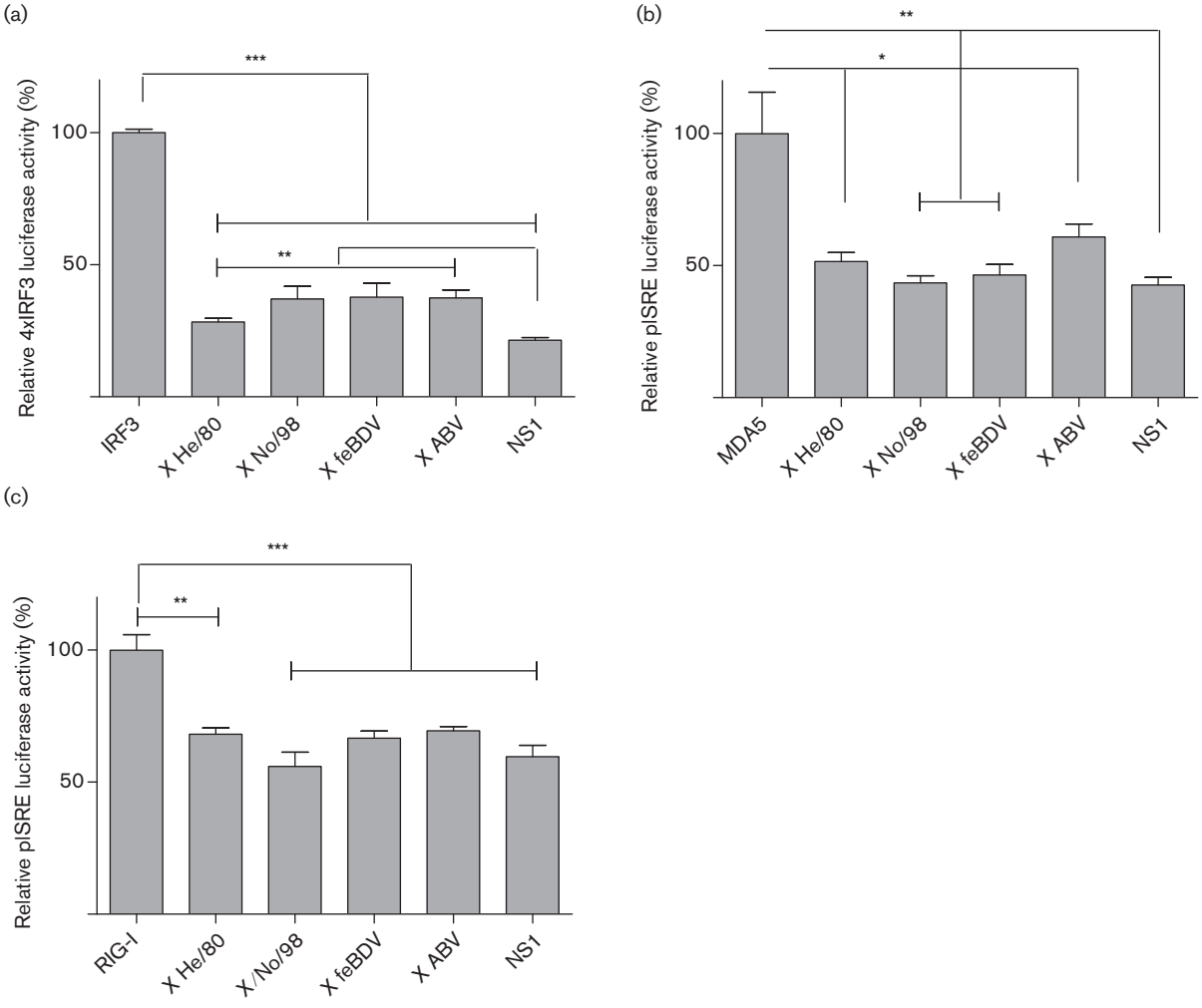

Fig. 3. BDV X inhibits dsRNA-induced (a) IRF3 promoter activation, (b) MDA5 activity and (c) RIG-I activity. A549 cells were transfected with 500 ng of the indicated constructs and a reporter plasmid for IRF3 (a), or expression plasmids for MDA5 (b) and RIG-I (c), together with a reporter plasmid for pISRE (b, c). Mean + SEM values of at least three independent experiments performed in duplicate are shown. *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.001$ using Student's *t*-test.

mechanism is not yet known (Poenisch *et al.*, 2009). We speculate that the type I IFN interference by BDV X observed in this study may be connected to the apoptosis resistance of BDV X, although the apoptosis-inhibiting effect of X was shown to depend on direct association of X with mitochondria (Poenisch *et al.*, 2009).

Conclusions

The presented data are the first, to our knowledge, to ascribe type I IFN-inhibiting properties to the small non-structural X protein of BDV and ABV, similar to those found for non-structural proteins of other negative-stranded RNA viruses. The molecular mechanisms and the significance of these findings in infected animals, as well as a comprehensive assessment of the differences in IFN inhibitory potential between different strains, warrant future investigations.

METHODS

Expression and reporter plasmids. The ORFs for the X protein of BDV (strains He/80, GenBank accession no. L27077, and No/98, accession no. AJ311524; a feline isolate derived from a cat with staggering disease, accession no. JX477137), and one strain of ABV (1322, accession no. FJ169441), were amplified using the primers X-*Xba*I-F (5′-ATCTCTAGAAAGCAA-3′) and X-*Kpn*I-R (5′-ATAGGT-ACCTCATTC-3′; DNA Technology A/S). The amplified PCR products were digested with *Xba*I and *Kpn*I and cloned into the mammalian expression vector pCMV-Flag (Sigma-Aldrich). The ORF for the P protein of BDV strain He/80 was amplified and cloned into the pcDNA3.1+ vector as described previously (Berg *et al.*, 1998). The orientation of the constructs of all plasmids was verified by sequencing.

Expression plasmids for RIG-I and MDA-5 were kindly provided by Dr S. Goodbourn, St Georges, University of London, UK (Andrejeva *et al.*, 2004; Childs *et al.*, 2007).

Reporter plasmids, using a firefly luciferase construct, for several checkpoints in the type I IFN signalling pathway were used: pISRE-Luc

(Clontech), p125-Luc (Sato *et al.*, 2000; kindly provided by Dr H. Li, WuHan Institute of Virology, Chinese Academy of Sciences, PR China), and 4 × IRF3-Luc (Ehrhardt *et al.*, 2004; kindly provided by Dr S. Ludwig, Institute of Molecular Virology, Westfälische Wilhelms-Universiteit Münster, Germany).

Cells, transfections and luciferase assays. A549 cells, a type II alveolar epithelial cell line derived from human adenocarcinoma (ATCC, CCL 185) were grown in folate-deficient Dulbecco's modified Eagles medium (FDMEM), supplemented with 10 % FBS, 2 mM L-glutamine, penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37 °C and 5 % CO_2 in a humidified atmosphere.

Transfection of A549 cells was performed using FuGENE6 reagent (Roche) in 24-well plates as instructed by the manufacturer. The day before transfection, 24-well plates were seeded with approximately 2.5×10^4 cells per well to attain 80 % confluence on the next day for transfection. Two hours before transfection of the cells, cell-culture medium was changed to one containing 1 % FBS. Transfection complexes containing 0.5 μg reporter plasmid and either expression plasmid or empty pcDNA3.1+ or pCMV were transfected at a ratio of 1 : 3 per well. Twenty-four hours post-transfection, the cell cultures were stimulated by addition of 10 μg poly(I : C) ml^{-1} (Invivogen) in 100 μl FDMEM without serum. For stimulation with SV, a titre of 1 : 512 (1 HA unit) was used. Twenty-four hours post-stimulation, the cells were lysed using the ONE-Glo luciferase assay system (Promega) and luminescence was measured in a Glomax-Multi+ Detection system with Instinct software (Promega), according to the manufacturer's recommended protocol.

IFN bioassay. A549 cells were transfected with 300 μg of the indicated expression plasmids using FuGENE 6 (Roche) or left untreated. Eighteen hours post-transfection, cells were stimulated with 5 μg dsRNA ml^{-1} for 24 h and then infected with vesicular stomatitis virus (VSV) expressing GFP (VSV-GFP) at an m.o.i. of 2. Fluorescence intensity was measured at 18 h post-infection: images were taken using an immunofluorescence microscope (Nikon), then the number of signals was quantified by visual counting, performed by two independent examiners, and normalized to mock-treated cells. VSV-GFP was a kind gift of Dr J. Rose (Yale University, New Haven, CT, USA).

Western blot analysis. The transfections for Western blot analysis were performed in six-well plates. At 90 % confluence, A549 cells were transfected with 1 μg of each of the plasmids. After 24 h, cells were washed and lysed using NP-40 lysis buffer. Concentration and quality of protein were measured using NanoDrop ND1000 (NanoDrop Tec.). A total of 50 μg lysate was separated by SDS-PAGE in Ready Gel J 7.5 % (Bio-Rad) and then transferred to PVDF membranes (GE Healthcare). The membranes were incubated in blocking buffer [PBS, 2 % (w/v) BSA] at room temperature for 1 h with slow agitation, followed by incubation in Anti-Flag primary antibodies (Sigma-Aldrich) diluted 1 : 200 or rabbit polyclonal anti-BDV P antibodies diluted 1 : 3000 (Johansson *et al.*, 2002) in 2 % BSA at 4 °C overnight. After intensive washing with PBST (PBS, 0.2 % Tween 20), membranes were incubated with HRP-conjugated anti-rabbit secondary antibodies for 2 h at room temperature with continuous agitation. The blots were developed by an ECL advance kit from GE Healthcare, and visualized by the ChemDoc XRS system from Bio-Rad with Quantity One software.

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